PHARMACOKINETIC STUDY OF CUCURBITACIN B FROM *TRICHOSANTHES CUCUMERINA* L. IN RATS

Mr. Natthaphon Hunsakunachai

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Pharmacology and Toxicology
Department of Pharmacology and Physiology
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Academic Year 2018

การศึกษาเภสัชจลนศาสตร์ของคิวเคอร์บิทาซิน บี จาก *Trichosanthes cucumerina* L. ในหนูแรท

นายนัฏฐพล หรรษคุณาชัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชวิทยาและพิษวิทยา ภาควิชาเภสัชวิทยาและสรีรวิทยา
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2561

Thesis Title	PHARMACOKINETIC STUDY OF CUCURBITACIN B FROM *TRICHOSANTHES CUCUMERINA* L. IN RATS
By	Mr. Natthaphon Hunsakunachai
Field of Study	Pharmacology and Toxicology
Thesis Advisor	Associate Professor PHISIT KHEMAWOOT, Ph.D.
Thesis Co Advisor	Associate Professor Weena Jiratchariyakul, Dr.rer.nat.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirement for the Doctor of Philosophy

..... Dean of the Faculty of Pharmaceutical Sciences

(Assistant Professor Rungpetch Sakulbumrungsil, Ph.D.)

DISSERTATION COMMITTEE

..... Chairman

(Associate Professor Thongchai Sooksawate, Ph.D.)

..... Thesis Advisor

(Associate Professor PHISIT KHEMAWOOT, Ph.D.)

..... Thesis Co-Advisor

(Associate Professor Weena Jiratchariyakul, Dr.rer.nat.)

..... Examiner

(Assistant Professor Flying Officer Pasarapa Towiwat, Ph.D.)

..... Examiner

(Assistant Professor Pornpimol Kijsanayotin, Ph.D.)

..... External Examiner

(Professor Kesara Na-Bangchang, Ph.D.)

นัฏฐพล หรรษคุณาชัย : การศึกษาเภสัชจลนศาสตร์ของคิวเคอร์บิทาซิน บี จาก *Trichosanthes cucumerina* L. ในหนูแรท. (PHARMACOKINETIC STUDY OF CUCURBITACIN B FROM *TRICHOSANTHES CUCUMERINA* L. IN RATS) อ.ที่ปรึกษาหลัก : รศ. ภก. ดร.พิสิฐ เขมาวุฒฆ์, อ.ที่ปรึกษาร่วม : รศ. ภญ. ดร.วีณา จิรัจฉริยากูล

คิวเคอร์บิทาซิน บี เป็นสารสำคัญในกลุ่มไตรเทอร์พีนอยด์ที่สามารถพบได้พืชวงศ์ Cucurbitaceae โดยเฉพาะในผลของต้น *Trichosanthes cucumerina* L. ซึ่งสารคิวเคอร์บิทาซิน บี นี้มีการศึกษาฤทธิ์ทางเภสัชวิทยามาอย่างยาวนาน โดยเฉพาะอย่างยิ่ง ฤทธิ์ในการต้านเซลล์มะเร็ง และฤทธิ์ต้านการอักเสบ อย่างไรก็ดี การศึกษาทางด้านเภสัชจลนศาสตร์ของคิวเคอร์บิทาซิน บี ยังคงมีอยู่อย่างจำกัด ซึ่งข้อมูลดังกล่าวมีความสำคัญมากต่อการพัฒนาคิวเคอร์บิทาซิน บี เพื่อเป็นยารักษาโรค ดังนั้นการศึกษาปัจจุบัน จึงมีวัตถุประสงค์เพื่อศึกษารูปแบบทางเภสัชจลนศาสตร์ของสารคิวเคอร์บิทาซิน บี หลังจากที่ให้สารคิวเคอร์บิทาซิน บี ในหนูแรท ด้วยขนาดยา และวิธีบริหารยาที่แตกต่างกัน โดยในการศึกษา เลือกใช้หนูแรท สายพันธุ์ Wistar ที่มีน้ำหนักประมาณ 400-500 กรัม จากนั้นแบ่งกลุ่มของสัตว์ทดลองออกเป็นทั้งหมด 4 กลุ่มด้วยกัน ได้แก่ กลุ่มที่ได้รับคิวเคอร์บิทาซิน บี ทางหลอดเลือดดำ ในขนาด 0.1 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัว กลุ่มที่ได้รับคิวเคอร์บิทาซิน บี โดยการป้อน ในขนาด 1 2 และ 4 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัว จากนั้นทำการเก็บตัวอย่างเลือด ปัสสาวะ อุจจาระ และ อวัยวะภายใน ตามเวลาที่กำหนด แล้วนำตัวอย่างไปตรวจวิเคราะห์ระดับคิวเคอร์บิทาซิน บี ด้วยเครื่อง Liquid chromatography tandem mass spectrometry จากผลการศึกษาพบว่า ค่าชีวประสิทธิผลของสารคิวเคอร์บิทาซิน บี มีค่าอยู่ที่ประมาณ 10% จากขนาดยาทั้งหมดที่ให้ และระดับยาสูงสุดที่พบในพลาสมามีค่าอยู่ในช่วง 4.85-7.81 ไมโครกรัมต่อลิตร โดยตรวจพบอยู่ที่ช่วงเวลาประมาณ 30 นาที หลังจากป้อนสารทดสอบ นอกจากนี้พบว่าเมื่อเพิ่มขนาดของคิวเคอร์บิทาซิน บี ในสัตว์ทดลอง จะมีการเพิ่มขึ้นของ AUC ในพลาสมา ที่เป็นสัดส่วนโดยตรงกับขนาดยาที่เพิ่มขึ้น จากนั้นคิวเคอร์บิทาซิน บี สามารถแพร่ไปยังส่วนต่าง ๆ ของร่างกายสัตว์ทดลองอย่างรวดเร็ว ซึ่งมีค่าปริมาตรการกระจายอยู่ที่ 51.65 ลิตรต่อกิโลกรัมของน้ำหนักตัว และมีค่าสัดส่วนของระดับยาในอวัยวะต่อพลาสมา ที่ประมาณ 60 ถึง 280 เท่า ในหลายอวัยวะ ส่วนในกระบวนการขจัดออก พบว่ามีสัดส่วนการขับออกในรูปที่ไม่เปลี่ยนแปลงน้อยว่า 1% ทั้งในตัวอย่างปัสสาวะ และอุจจาระ โดยสรุปแล้วสารคิวเคอร์บิทาซิน บี เป็นสารที่มีค่าชีวประสิทธิผลต่ำ แต่สามารถแพร่กระจายไปยังส่วนต่าง ๆ ของร่างกายได้ดี แต่อย่างไรก็ตาม การขจัดออกในรูปไม่เปลี่ยนแปลงทั้งในตัวอย่างปัสสาวะ และอุจจาระมีสัดส่วนที่น้อยมาก จึงสามารถสรุปได้ว่า สารคิวเคอร์บิตาซิน บี นั้น น่าจะถูกเปลี่ยนแปลงโดยกระบวนการเปลี่ยนแปลงยา ก่อนที่จะถูกขับออกจากร่างกาย ดังนั้นในอนาคตจึงจำเป็นต้องมีการศึกษาเพิ่มเติมเกี่ยวกับวิถีการเปลี่ยนแปลง และขจัดออกของสารดังกล่าว เพื่อนำไปพัฒนา และพัฒนาไปสู่การศึกษาในชั้นคลินิกต่อไป

สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อนิสิต .....
ปีการศึกษา	2561	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
		ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5876453333 : MAJOR PHARMACOLOGY AND TOXICOLOGY

KEYWORD: Cucurbitacin B, Trichosanthes cucumerina, Cucurbitaceae, Pharmacokinetics, Bioavailability

Natthaphon Hunsakunachai : PHARMACOKINETIC STUDY OF CUCURBITACIN B FROM *TRICHOSANTHES CUCUMERINA* L. IN RATS. Advisor: Assoc. Prof. PHISIT KHEMAWOOT, Ph.D. Co-advisor: Assoc. Prof. Weena Jiratchariyakul, Dr.rer.nat.

Cucurbitacin B is a triterpenoid, majorly found in Cucurbitaceae family, particularly in the *Trichosanthes cucumerina* L. fruits. The pharmacological properties of cucurbitacin B have been studied for decades, particularly an anti-tumor and anti-inflammatory activity. However, the pharmacokinetic profile of this compound is still limited and the investigation is needed for further pharmaceutical product development. This study aimed to investigate the pharmacokinetic profile of cucurbitacin B after administering the compound at different doses and routes to the rats. Male Wistar rats, weighed approximately 400-500 g, were treated with cucurbitacin B extracted from *Trichosanthes cucumerina* L. The cucurbitacin B was administered at 0.1 mg/kg intravenously or at 1, 2, and 4 mg/kg orally. Blood, urine, feces, and internal organs were collected after administration at a designated time. The level of cucurbitacin B in biological samples was determined by liquid chromatography-tandem mass spectrometry. The absolute oral bioavailability of cucurbitacin B was approximately 10%. The maximum concentration in plasma after normalization by dose ranged from 4.85-7.81 μg/L and the time to reach maximum value was approximately within 30 min after oral dosing. The level of cucurbitacin B in plasma increased proportionally to the given dose. After intravenous administration, cucurbitacin B had a large volume of distribution of about 51.65 L/kg and exhibited a high tissue to plasma concentration ratio, approximately 60 to 280-fold in several organs. A negligible amount of unchanged cucurbitacin B could be detected in urine and feces and accounted for less than 1% of the administered dose. Cucurbitacin B had low oral bioavailability but could be distributed extensively into internal organs with a high volume of distribution and tissue to plasma ratio. Only negligible amounts of unchanged cucurbitacin B were excreted via urine and feces suggesting that the compound might be biotransformed before undergoing an excretion. Further studies of the metabolic pathway and tissue uptake mechanism are required to strategize the future development of cucurbitacin B into clinical studies.

Field of Study:	Pharmacology and Toxicology	Student's Signature .....
Academic Year:	2018	Advisor's Signature .....
		Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Associate Professor Dr. Phisit Khemawoot for his unwavering support not only the valuable knowledge that he gave but also an immersive experience when I work under his expertise. His guidance always leads to the right way in a research career. He never takes his student for granted and always treats every student equally. He always provides an opportunity for his students to experience the world outside, not just an academic perspective in the university. I would never make it to this point without his support.

I also would like to express my gratitude to all teachers and staff from the Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their tireless effort in the process of making one person be worthy of the Ph.D. title.

I would like to thank my family including my father, mother, younger sister, and whoever involved for their unconditional love and support. I could not go this far from the beginning without their positive force that pushes me through the hardship and barricade.

I would like to thank my friends who always support each other in my Ph.D. journey, particularly Mr. Tossapol Anukunwithaya, Miss Pilaslak Poo, Miss Patcharaporn Hengjumrut, Mr. Narudol Teerapattarakan, Miss Hattaya Benya-aphikul, Miss Surassawadee Treesuwan, and Mr. Dhirarin Jansaeng. This thesis could not be completed without their support.

Lastly, I am grateful to the 100th Anniversary Chulalongkorn University fund for doctoral scholarship and this work was also supported in part by the Graduate School, Chulalongkorn University; Ratchadapiseksomphot Endowment fund of Chulalongkorn University; and Agricultural Research Development Agency. I could never complete this work without their support.

Natthaphon Hunsakunachai

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LISTS OF ABBREVIATION

ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
$AUC_{0\text{-}inf}$	=	area under the curve from time zero to infinity
$AUC_{0\text{-}t}$	=	area under the curve from time zero to last sampling time point
CL	=	clearance
Cr	=	creatinine
C_{max}	=	maximum plasma concentration
CuB	=	cucurbitacin B
CV	=	coefficient of variation
DMSO	=	dimethylsulfoxide
F	=	absolute oral bioavailability
HQC	=	high concentration quality control
IFCC	=	The International Federation of Clinical Chemistry and Laboratory Medicine
K_{app}	=	tissue to plasma concentration ratio
LC-MS/MS	=	liquid chromatography tandem mass spectrometry
LLOQ	=	lower limit of quantitation
LQC	=	low concentration quality control
MRM	=	multiple reaction monitoring
MRT	=	mean residence time
MQC	=	medium concentration quality control
m/z	=	mass-to-charge ratio
QC	=	quality control
R^2	=	correlation coefficient
RE	=	relative error
SD	=	standard deviation

SULT	=	Sulfotransferase
$t_{1/2}$	=	elimination half-life
T_{max}	=	time to maximum plasma concentration
UGT	=	UDP-glucuronosyltransferase
V_d	=	volume of distribution
XlogP	=	calculated partition coefficient

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Cancer is one of the most problematic diseases worldwide nowadays, considered to be the second leading cause of death globally in 2018[1]. Cancer can influence on the overall public health and economic status, particularly in low to middle income countries because of the complicated treatment and poor prognosis[2]. Loss of life years in premature aged patients and increase in costs associated with illness and therapy can result in lower productivity in entire population[3]. From WHO reports in 2008, around 12.7 million new patients were diagnosed and about half of them were anticipated to die from cancer worldwide[4]. The cancer situation in Thailand has been altered since 1999. In the past, cardiovascular disease and fatal accident were the first two leading causes of death, but cancer surpassed them and took the first priority in 1999. The incidence of new cancer patient increases every year and this increasing pattern seems similar to that happened in global population[5]. Currently, as of 2014, the mortality rate from cancer had been declined over the past two decades by reason of early diagnosis and advanced treatment[6]. However, the decrease in responsiveness to the current chemotherapy had also been reported in clinical practice[7]. In case of Thailand, most of the chemotherapy have been imported from other countries because there were no production values for antineoplastics since 2012. As a consequence, investigation and development for new chemical entities to deal with this healthcare problem is crucial and need to take consideration in the present[5].

Thailand is located in the tropical zone, which possesses a vast variety of herbs. *Trichosanthes cucumerina* L. (*T. cucumerina*) is one of the most promising plants for antineoplastic purpose, which can be found in most area of Thailand[8]. The supporting information for *T. cucumerina* and its pharmacological activities were widely established. Fruits of *T. cucumerina* contain a large amount of triterpenes and

sterols, especially cucurbitacin E and cucurbitacin B (CuB). The latter compounds account for more than 1.7-3.7 mg per 100 g of fruits, which are the highest contents among triterpenes[9]. Cucurbitacin B gains some interests from the researchers because it exerts numerous pharmacological activities, particularly anti-inflammation and antitumor activity[10, 11]. In the drug development process, pharmacokinetic data is prerequisite to support their use in a clinical setting. Generally, the pharmacokinetics will be tested primarily in an animal model to help researchers understanding the fate and disposition of the drug. Moreover, the data obtained from the animal model are useful for dose extrapolation guidance in human. In the present, preclinical pharmacokinetic study of cucurbitacin B is very limited due to lack of dose escalation pattern and organ disposition. The completed preclinical pharmacokinetic profile of cucurbitacin B is necessary for further drug development.

1.2 Objectives

- To investigate the pharmacokinetic profiles of cucurbitacin B given as intravenous and oral preparation at different therapeutic doses.
- To explore the pharmacokinetic profiles of possible cucurbitacin B glucuronide metabolite after intravenous and oral administration of cucurbitacin B.

1.3 Hypothesis

- Cucurbitacin B has low oral bioavailability with F < 10%.
- Cucurbitacin B has high tissue distribution with K_{app} > 1.
- Cucurbitacin B is metabolized by phase II enzyme, UGT, more than 50% of the dose.

1.4 Expected benefits from the study

This study could provide more information about the fate and drug disposition of cucurbitacin B in the rat model, which is helpful to evaluate the drug concentration in various organs. Besides the evaluation of pharmacological activity related to the cucurbitacin B concentration, a prediction for possible toxicity can also be performed by comparing the concentration at each target to the known toxicity level.

1.5 Scope of the research

This study focuses mainly on profiling the pharmacokinetic data of cucurbitacin B and also the possible metabolite when cucurbitacin B is given to rats at different doses and routes. Wistar rats are used as the animal model for this pharmacokinetic study. Pharmacokinetic profiles of cucurbitacin B and its metabolite are investigated in each aspect, including absorption, distribution, metabolism, and excretion phase, respectively. Plasma concentration-time profiles are examined by collecting blood specimen serially after test compound administration. Tissue distribution is studied by collecting internal organs involving drug elimination and being the targets for drug therapy. Route of excretion is also evaluated by collecting urine and feces. Samples received from each experiment are quantified by liquid chromatography tandem mass spectrometry. All experiments are conducted in the Animal Research Unit, Faculty of Pharmaceutical Sciences, Chulalongkorn University (Protocol No. 16-33-002, Approval date 9th June 2017).

1.6 Experimental design

This study is an experimental study with control. The brief detail of experimental design is shown in Figure 1.

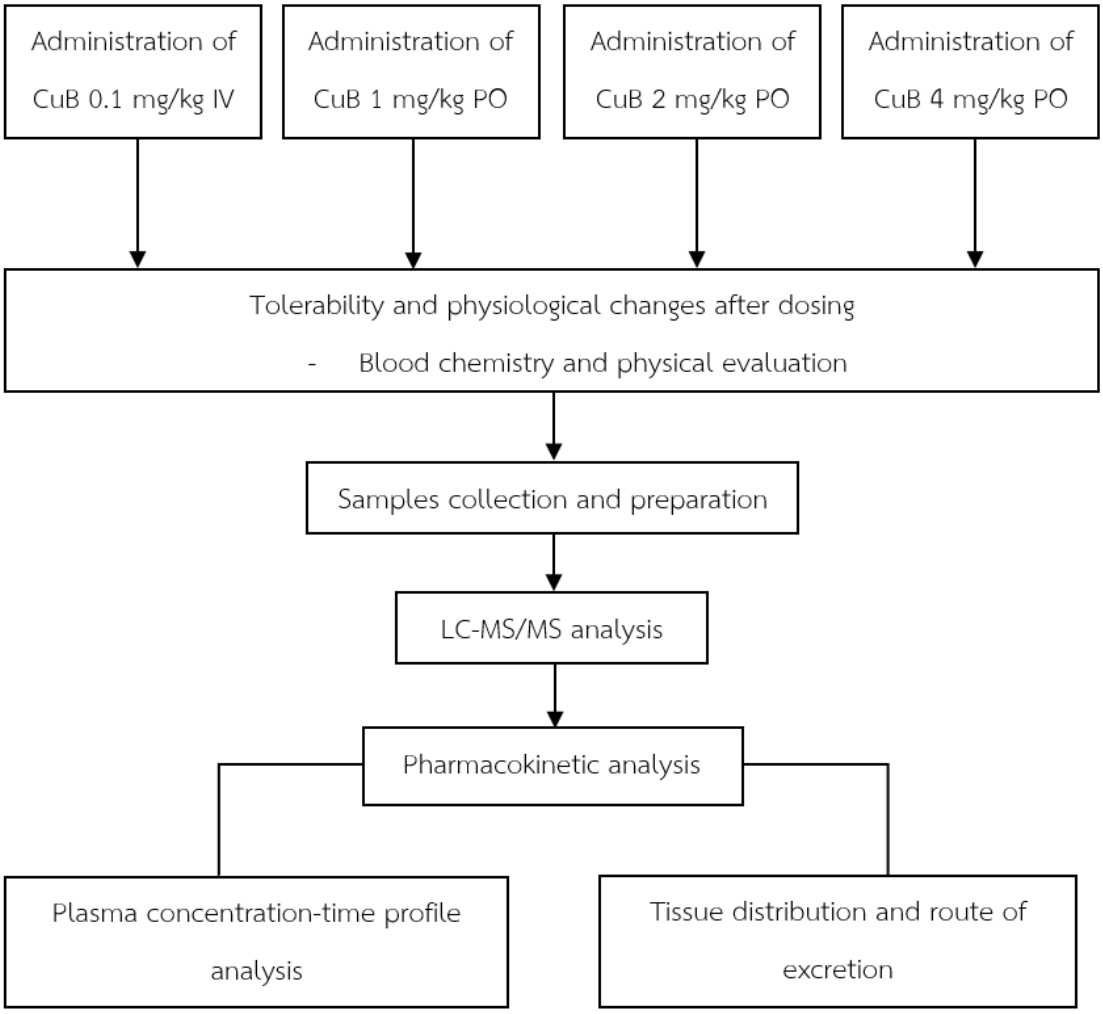

Figure 1 Schematic diagram of the experimental design.

1.7 Conceptual framework

The conceptual framework of this study is shown in Figure 2.

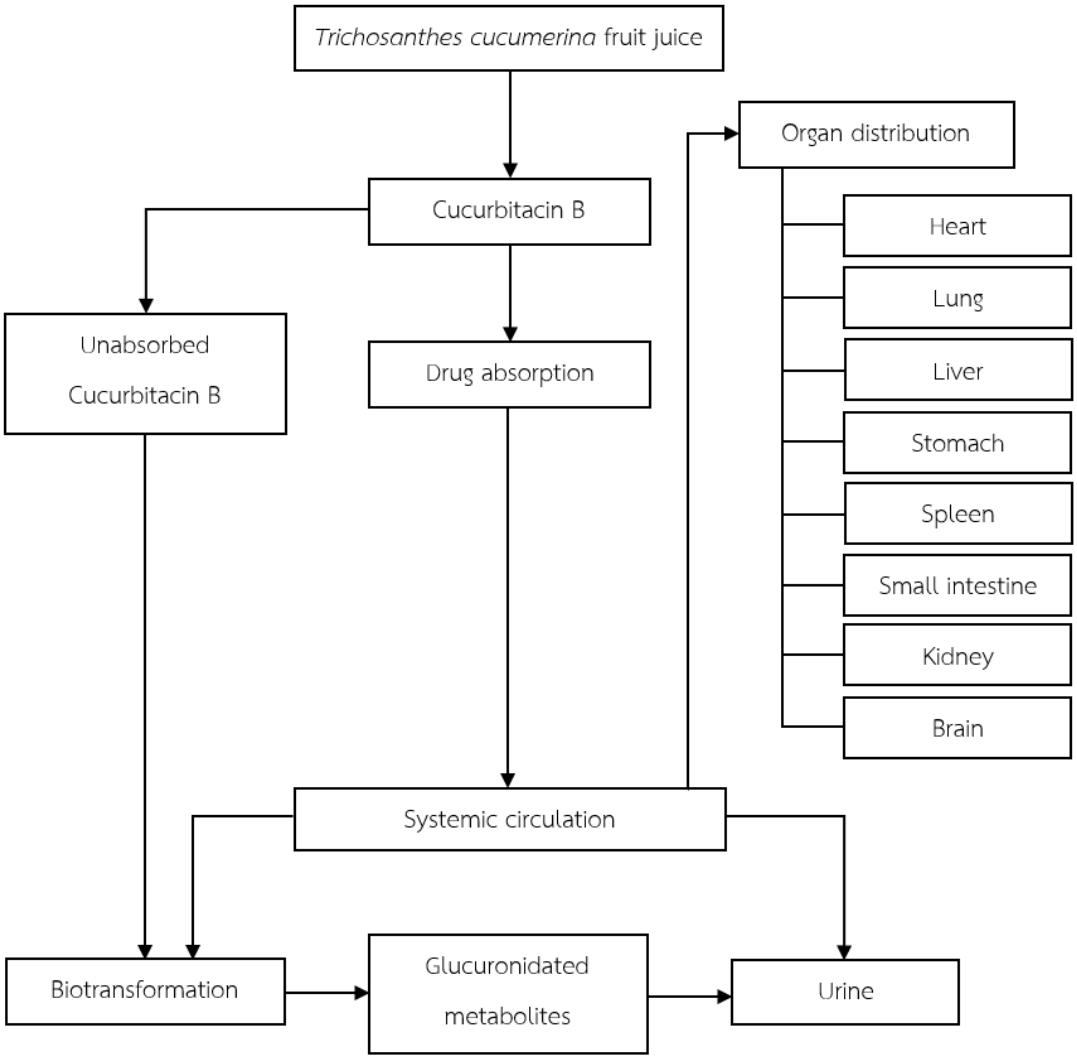

Figure 2 Schematic diagram of conceptual framework

CHAPTER II

LITERATURE REVIEW

2.1 Definition and epidemiology of cancer

The terminology of cancer was first described by the Greek physician, Hippocrates in approximately 460-370 BC. Primarily, both ulcer and non-ulcer forming tumors were defined by using term carcinos and carcinoma. These words refer to crab in Greek because the swollen veins spreading around tumors resemble the limbs of a crab[6]. Cancer is considered as one of the most fearsome diseases troubling the entire population around the world. This disease is characterized by uncontrollable growth combined with invasion to other tissue in the body[12]. The incidence of cancer mostly occurs in the aging population but somehow there are several reports suggesting some types of cancer can also dominate specifically in children[13]. The advancement of cancer can be induced by both internal and external factors, for example, inherited genetic mutations, hormones, smoking, and overweight etc[13-16]. To prevent cancer development, avoidance of the associated factors is necessary to implement. A previous report suggested that around 1/4 of cancer patients died from excessive tobacco smoking. Moreover, about 20% of patient deaths are associated with overweight, sedentary behavior, and over alcohol consumption[12]. The incidence of cancer in 2008 by WHO demonstrated that around 12.7 million patients were diagnosed as new cancer patients and they account for 13% of overall causes of death worldwide[4]. For Thailand, cancer has been considered as the first leading cause of death since 1996. According to the National Cancer Control Programmes in 2009, the top five priorities of cancer type that found in Thailand are different, depending on gender as shown in Figure 3. Liver and bile duct cancers were found predominantly in both genders and considered as the highest priority in male, which was accounted for 42.8% of overall cancers. However, breast cancer accounted for the most proportion among cancer types in women, which was approximately 25.6%[5].

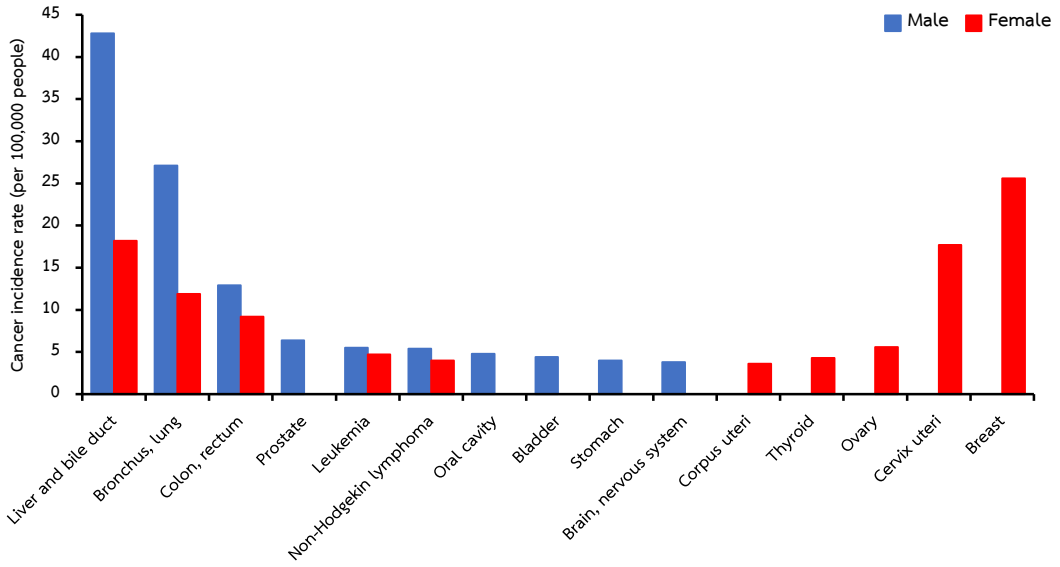

Figure 3 Incidence of each cancer type in Thai patients in 2005 both in male and female population (per 100,000 people)[5]

The incidence of cancer has been increasing every year as shown in Figure 4. The trend of disease has changed since 1996 from the most anticipated cause of death like cardiovascular diseases and fatal accidents were shifted to malignancy in 1999. This change is still ongoing and need to take a consideration onward.

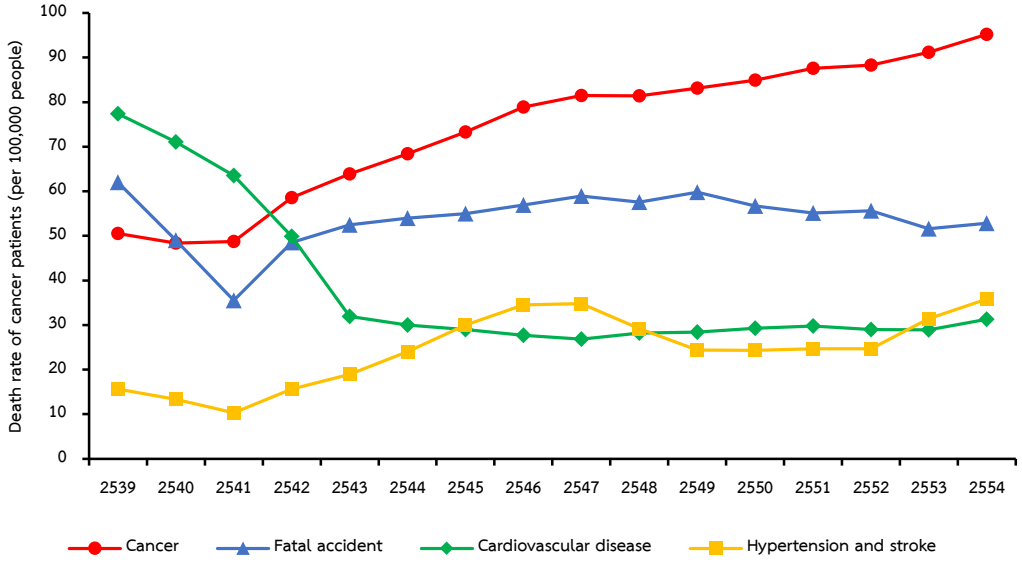

Figure 4 Amount and death rate of patients from overall causes per 100,000 people in Thai patients[5]

The overall cancer status in Thailand possesses a similar pattern to the global, which the incidence rate increases every year. This could be explained by the reduction of mortality rate during childbirth and a longer lifespan in the entire population. Cancer is the disease, which takes a long time for development and progression, so the increase of death rate from cancer is anticipated in the aging population. In the present, population structure in Thailand is starting to shift to aging society (> 60 years old), resulting in the numbers of newly diagnosed patient are accumulated every year[5]. Therefore, investigation for new chemical entities needs to be conducted, especially in Thailand that most of medicines in cancer therapy are imported from Europe and America. According to the Thai FDA report, Thailand had no production value for antineoplastic drugs since 2012[17] while the imported amount of this category has been increased every year. The total cost of anticancer medicine accounted for 18% of total imported value in 2015, which was considered as the highest expense among other pharmacological categories[18].

Development of new lead compounds, which are effective against cancer cells, from natural sources is an interesting target for pharmacologists and researchers, especially in tropical and sub-tropical zones. Thailand is located in a tropical area, which possesses a vast variety of tropical herbs; however, they still lack of systematically pharmacological evaluation. Investigation on the pharmacological activity of certain herbs to promote the use in a clinical setting could provide a beneficial impact not only to the patients but also the overall economic status. Because the major income of Thailand comes from the export values of agricultural products, the promotion of medicinal plant use could support both the national incomes and cultivator's finances.

2.2 Natural products and cancer therapy

From previous animal experiment and epidemiological studies, they firmly suggested that there was a strong association between an increase in consumption of natural product such as fruits, vegetables, and certain herbs and a decrease in cancer risk. Unarguably, these natural products contain bioactive molecules, which can prevent and counteract against malignancy[19]. Secondary metabolites from natural

sources have been proved as a magnificent reservoir of new medicinal lead compounds. In the past, some antineoplastics have been isolated from plant origins, for example, *Taxus brevifolia*, *Catharanthus roseus*, *Camptotheca acuminate*, *Erythroxylum pervillei, Centaurea schischkinii,* and *Podophyllum* species etc[20].

According to the high diversity of natural compounds derived from natural resources, each compound possesses the mechanism against cancer cell in its distinctive way, for example, curcumin, soy isoflavones, sulforaphane, and resveratrol can influence on the cancer stem cell and self-renewal pathways. There are pieces of evidence, supporting the activities of plant metabolites against cancer cells. They demonstrated that most of the natural compounds exerted the pro-apoptotic properties by activation of both extrinsic and intrinsic apoptotic pathways[20, 21]. The summary of plant derived compounds, used in cancer therapy is demonstrated in Table 1 and Table 2.

Table 1 List of plant derived compounds used in cancer therapy[20]

Derived compounds	Species and Genus	Experimental model	Mechanism of action	Indications
Vindesine and Vinorelbine	- *Catharanthus roseus*	- Leukemias - Lymphomas - Advanced testicular cancer - Breast cancer - Lung cancer - Kaposi's sarcoma	Inhibit mitosis	- Acute lymphotic leukemia - Hodgekin lymphoma - Breast cancer - Ovarian cancer - Chorionic cancer
Paclitaxel	- *Taxus brevifolia* Nutt - *Taxus baccata*	- Metastatic cancer - Breast cancer - Ovarian cancer - Lung cancer - Prostate cancer - Lymphoid malignancy	Inhibit mitosis (specific to the late G_2- and M-phase)	- Melanoma - Ovarian cancer - Breast cancer - Lung cancer
Topotecan	- *Camptotheca acuminata*	- Epithelial ovarian cancer - Small cell lung cancer	Inhibit DNA topoisomerase I (specific to the S/G_2-phase)	- Small cell lung cancer - Ovarian cancer - Cervical cancer

Table 2 List of plant derived compounds, which is studied for anti-cancer property[20]

Compounds	Species and Genus	Experimental model	Mechanism of action
Berbamine	- *Berberis amarensis*	- Chronic myeloid leukemia	Caspase-3-dependent apoptosis
Betulinic acid	- *Betula alba*	- Exhibits anti-cancer activity in humans	Trigger mitochondrial pathway of apoptosis
Cucurbitacin	- Cucurbitaceae species	- Various cancer cell lines	Inhibit signal transducer/JAK 2 activity and activates STAT3 pathway
Curcumin	- *Curcuma longa*	- Colorectal cancer - Multiple myeloma - Pancreatic cancer.	Inhibit cancer stem cells and self-renewal pathways
Daidzein and Genistein	- *Lupinus* species - *Vicia faba* - *Glycine max* - *Psoralea corylifolia*	- Ovarian cancer - Breast cancer - Stomach cancer - Prostate cancer	Inhibit 3A 4-mediated metabolism and oxidative metabolism
Ellipticine	- *Ochrosia borbonica* - *Excavatia coccinea,* - *Ochrosia elliptica*	- Various cancer cell lines	Interfere by DNA intercalation and inhibit the topoisomerase II
Flavopiridol	- *Amoora rohituka* - *Dysoxylum binectariferum*	- Colorectal - Non-small cell lung cancer - Renal cell carcinoma - Non-Hodgkin's lymphoma	Inhibit cell cycle progression at G1 or G2 phase
Harringtonine and Homoharringtonine	- *Cephalotaxus harrintonia* - *Cephalotaxus hainanensis* - *Cephalotaxus qinensis*	- Acute myeloid leukemia - Chronic myeloid leukemia	Inhibit of protein synthesis and chain elongation during translation

Table 2 List of plant derived compounds, which is studied for anti-cancer property (Continued)[20]

Compounds	Species and Genus	Experimental model	Mechanism of action
Ingenol 3-oangelate	- *Euphorbia peplus* L.	- Actinic keratosis - Basal cell carcinoma	Cause necrosis of tumor by the activation of PKC
Irisquinone	- *Iridaceaelatea pallasii* - *Iris kumaoensis*	- Good activity in transplantable rodent tumors	Act as a chemosensitizer
Silvestrol	- *Aglaia foveolata*	- Prostate cancer - Breast cancer - Lung cancer	Involve the apoptosome and mitochondrial pathway triggering extrinsic pathway of programmed cell death of tumor cells
Salvicine	- *Salvia prionitis*	- Malignant tumors	Inhibition of topoisomerase II

The natural compounds from plants demonstrated the antineoplastic activity by various mechanisms. Interestingly, cucurbitacin is a group of compounds that shows effectiveness against various cancer cell lines[22, 23]. Moreover, they also exerted the activity in an animal model by reducing the tumor size injected into the rodents. The cucurbitaceous plant is the major source of cucurbitacin, which grows abundantly in Southeast Asia and surrounding. *Trichosanthes spp*. is considered as the majority in this family, which provides a high quantity of cucurbitacin derivatives, particularly in *T. cucumerina*. Thus, *T. cucumerina* is one of the promising targets for anti-cancer drug development.

2.3 *Trichosanthes cucumerina* L.

2.3.1 Origin and distribution

T. cucumerina belongs to the family of Cucurbitaceae, which is the same family of cucumbers, melons, and pumpkins. Common names of *T. cucumerina* can be diverse, depending on each region. This plant originates from Southeast Asia, Australia, and some areas of the Western Pacific[24]. It is generally called as snake gourd, viper gourd, or long tomato. Only in some exceptional areas such as Bengali, Tamil, and Thailand, *T. cucumerina* is called Chichinga/Chichinge, Pudalankaai, and Buap Khom/Buap Nguu/Ma noi, respectively[25]. This plant is a well-known plant because its fruit is mainly consumed as a vegetable in Africa, which is imported from India origin. The cultivation of this plant gains some importance in many areas of Africa since it can be used as a consumable product or medicinal herb. *Trichosanthes spp.* has around 100 species, which only a few have been dominated in Asia. *T. cucumerina* is one of the most distinguished species, found in Thailand. Two varieties are notable among *T. cucumerina*, including the wild var., distributing from India, China, Southeast Asia, and Northern Australia; and the variety of anguina, presenting in Africa and Southeast Asia[24]. In Thailand, the variety of anguina is widespread in cultivation, which can be found in Chiang Mai, Phrae, Phitsanulok, Trat, Chon Buri, Phra Nakhon Si Ayutthaya, Ang Thong, Saraburi, and Bangkok[8].

2.3.2 Description

T. cucumerina is a monoecious annual or subperennial herb that possesses 2 to 3 branched tendrils up to approximately 5 to 6 m high. The stems are slender, greenish, 4-angled, and furrowed with somewhat hairy. Their roots are characterized as whitish tone and tuberous shape, which have the function of nutrient storage. The leaves are alternate and palmately 5 to 7 lobed, sized 7 to 25 cm by 8 to 20 cm without stipules. The lobes are broad, rounded or obtuse. The sinuses are also broad and rounded. The base is broadly heart-shaped and the scabrid hair can be found on both sides of the leaves. The flowers are unisexual, which male and female flowers are completely separated. The male flowers are packed on staminate inflorescences,

which have approximately 6 to 15 flowers with axillary racemes on 10 to 30 cm long peduncles. Their petal layer or corolla is a tubular shape with white color and hair-like outgrowth. The female flower is solitary, compared to the male opposite, which is inflorescence. Female flowers have the inferior type of ovary that contains a single cell ovule. In contrast, the male flowers possess long peduncles with three stamens for each flower. The fruits are ovoid or narrowly elliptic, narrowed towards apex. The size of berry is approximately 2.5 to 5 cm by 1.5 to 4 cm. The colors of berry can appear different, depending on ages. The white to greenish berry could be found in immature age while the dark red is observed when it is ripen. The soft pulp inside contains the seeds and has a color characteristic for each sub-species. The whitish pulp is rather sweet while the green-black pulp is quite bitter. The seeds are edge undulated form with greyish-brown color[8, 24, 26].

2.3.3 Traditional uses

T. cucumerina has been used in traditional medicine for a decade, especially in India. It was reported that *T. cucumerina* could be used in different ailments with part dependent manner. The list of the traditional application of *T. cucumerina* is shown in Table 3.

Table 3 Traditional uses of *Trichosanthes cucumerina* L.[24, 27]

Traditional uses of *Trichosanthes cucumerina* L.		
Roots	- Purgative action	- Anti-convulsant
	- Diabetes	- Abortifacient
	- Skin swelling	- Alexiteric
	- Anthelmintic	- Anti-septic
	- Astringent	- Anti-septic
Leaves	- Antipyretic	- Emetic
	- Antispasmodic	- Alexiteric
	- Diuretic	- Astringent
Fruits	- Anthelmintic	- Purgative action
	- Assist digestion	- Emetic
	- Skin swelling	
Seeds	- Purgative action	- Anti-diarrhea
	- Anti-spasmodic	- Anti-bacteria

2.3.4 Major bioactive components of *Trichosanthes cucumerina* L.

Previously, *T. cucumerina* had been prepared in the form of crude extract or as a component in traditional medicine recipe. The composition of the extract may differ, depending on each extraction. Typically, different parts of *T. cucumerina* will be dried, grounded and sometimes mixed with other botanicals. Juice from their fruits is also used for various ailments therapy. Nowadays, industrial and pharmacognosy technologies have been improved drastically, so the extraction process is also improved. According to the number of bioactive components is varied among each *T. cucumerina* preparation, the standardized extracts are preferred over the conventional preparation. Currently, the development of *T. cucumerina* standardized extracts is still ongoing. It is important to identify the major bioactive compound in *T. cucumerina* before further advancement, which is necessary to use cucurbitacin B as a marker in a quality control process.

According to various marked indications of *T. cucumerina* used in Ayuravedic and traditional medicine, searching for major bioactive constituents from this plant is

quite important for further drug development. At least 12 compounds were found in fruit juice and can be categorized into 2 main groups, which are shown in Table 4.

Table 4 Major chemical components from fruits of *Trichosanthes cucumerina* L.[28, 29]

Compounds	Chemicals
Triterpenes	- 23, 24-dihydrocucurbitacin D
	- 3, 24-dihydrocucurbitacin B
	- Cucurbitacin B
	- 3β-hydroxyolean-13(18)-en-28-oic acid
	- 3-oxoolean-13(18)-en-30-oic acid
	- Isocucurbitacin B
	- 23,24-dihydrocucurbitacin E
Sterols	- 3-O-β-D-glucopyranosyl-24-ethylcholest-7,22-dien
	- β-sitosterol
	- Stigmasterol

Apart from the cucurbitacins and sterols that are found in fruit juice, other triterpenes, including bryonolic acid, bryononic acid are detected specifically in the root extract. The latter compounds have been introduced for the first time in 2009 and recently they gained some interest from the researchers due to their cytotoxic activity against various tumor cells *in vitro*[30]. There is no specific report that suggests the amount of each bioactive compound in *T. cucumerina*. Devendra and colleagues reported only the total amounts of cucurbitacins. They found that the cucurbitacins are the most abundant in various part of *T. cucumerina,* which total cucurbitacins account around 1.7-3.7 mg/100 g of fruits. While the amounts of cucurbitacin are lower in other parts, which are about 0.5-1.7 mg/100 g and 0.17-0.56 mg/100 g in stems and leaves, respectively[9].

2.3.5 Physicochemical properties of cucurbitacin B

Cucurbitacin is an oxygen-rich containing compound that can be found mostly in cucurbitaceous plants[31]. Nowadays, there are at least 19 members that belong to cucurbitacin group. Each member has a slight difference in the chemical

structure, which can be identified as cucurbitacin A to cucurbitacin T[32]. Cucurbitacin B is one of the most studied bioactive components from *T. cucumerina*[10]. Chemical structure of cucurbitacin B is demonstrated in Figure 5. Cucurbitacin B belongs to the triterpene group, which has high lipophilic property, resulting in this compound can dissolve hardly in water or high polar solvent[31]. For physicochemical properties of cucurbitacin B, they are demonstrated in Table 5.

Table 5 Physicochemical properties of cucurbitacin B[33]

Properties	Value
Molecular formula	$C_{32}H_{46}O_8$
Molecular mass	558.712 g/mol
XLogP	2.6
Solubility in water	Slightly soluble
Solubility in ethanol	50 mg/mL at 25 °C
Solubility in DMSO	100 mg/mL at 25 °C

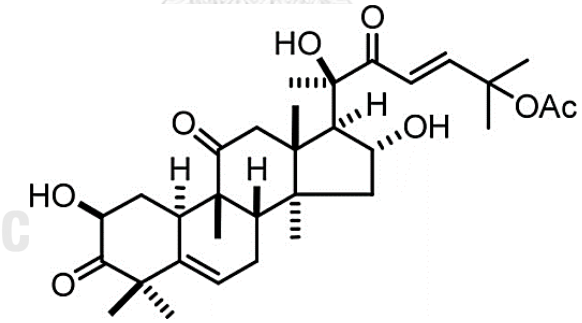

Figure 5 Chemical structure of cucurbitacin B

2.3.6 Pharmacological activity of cucurbitacin B

Cucurbitacin B is the major bioactive chemical, found mainly in *T. cucumerina.* Various reports have confirmed the pharmacological activity of cucurbitacin B. It has been widely used in the treatment of cancer, inflammation and atherosclerotic-related disease. It also exerts hepatoprotective effects in an animal model. The mechanisms of action of cucurbitacin B have been studied for a decade, which detailed information will be described by indication.

2.3.6.1 Anticancer activity

Anticancer activities of cucurbitacin B have been examined on a vast variety of cancer cell lines. Cucurbitacin B has the potential to inhibit cancer cell growth via diverse mechanisms. Cucurbitacin B exhibits anticancer properties, involving the proapoptosis, cell cycle arrest, and inhibition of cancer invasion, and migration[31, 32]. Moreover, cucurbitacin B also regulates multiple intracellular molecules in cell signaling pathways. The details about each anticancer mechanism are described below and the schematic picture of cucurbitacin B's mechanism is shown in Figure 6.

Apoptosis is one of the most essential mechanisms in the body to maintain and regulate the cellular balance in normal cell population. Improper control or dysfunction of this process may lead to the progression of certain diseases such as neoplasm[34]. Cucurbitacin B can promote the apoptosis of the cancer cells in various mechanisms. Most of studies suggested that cucurbitacin B could enhance cancer cell apoptosis by STAT3 pathway inhibition[35]. STAT3 plays an important role as a transcription factor that regulates gene expression. The regulation of STAT3 involves with other transcription factors such as β-catenin, NF-kB etc[36]. Liu and colleagues suggested the mechanism of cucurbitacin B in Hep-2 cells by inhibition of STAT3 phosphorylation and also attenuate the Bcl-2 expression[37]. In other cell lines such as SHSY5Y cells, it was demonstrated that cucurbitacin B exerted an anti-proliferative effect via JAK2/ STAT3 and MAPKs pathway[38]. On the other hand, cucurbitacin B exhibited the cell apoptosis induction by mediating a reactive oxygen species (ROS), which was independent to the JAK2/ STAT3 pathway in SW480 cell line[39]. In conclusion, cucurbitacin B possesses apoptosis promoting activity with cell line dependent manner.

Cucurbitacin B can promote cell cycle arrest by mediating multiple signaling pathways. cucurbitacin B can induce G2/M cell cycle arrest in a different type of cell, for example, non-small cell lung cancer (H1299, A549, HCC-827, H661) [40] ; osteosarcoma cells (U2OS, G292, MG-63)[41]; and breast cancer (MDA-MB-231, MCF-7)[42]. The inhibition of S phase was also reported in hepatocellular carcinoma cells by modulating the cyclin D1 and CDC2 levels, but there was no change in cyclin B1 level[43]. Moreover, cucurbitacin B also inhibits the telomerase by down regulation of

human telomerase reverse transcriptase together with c-Myc in breast cancer cells[44]. From this information, it indicates that the mechanisms of cucurbitacin B upon cell cycle arrest are different, depending on each cell type.

Metastasis is one of the most important characteristics of cancer cell and it can be the reason of treatment difficulty. Metastasis consists of the different processes, including cell migration, adhesion, and proteolysis of the basement membrane and extracellular matrix. Cucurbitacin B can interfere with the cancer cell migration by inhibiting the phosphorylation of Akt, p38, and ERK1/ 2, and down-regulation of MMP-9 in human hepatoma cells[45].

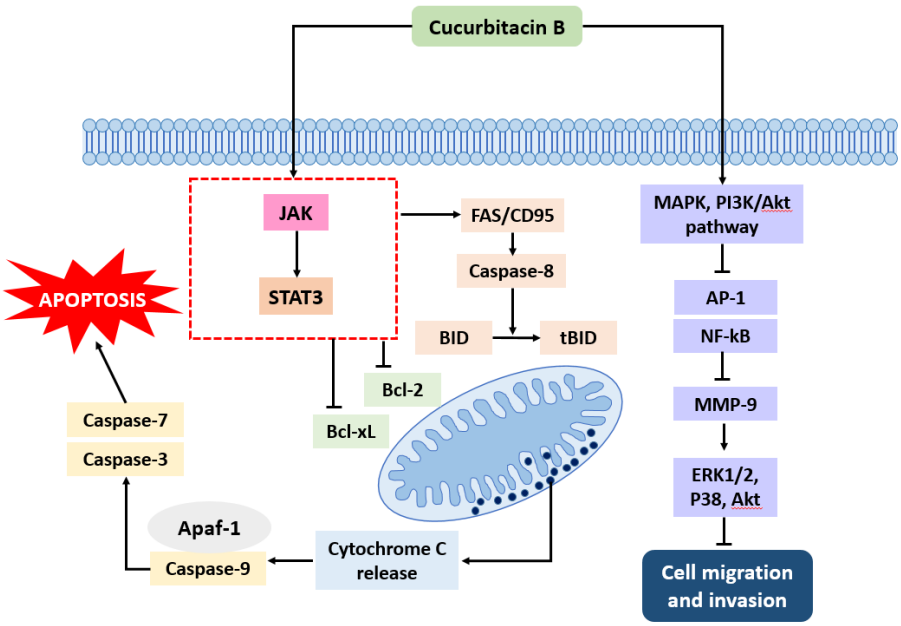

Figure 6 Schematic mechanism of cucurbitacin B for anticancer activity[32]

In addition to its anticancer property, it also possesses the synergistic effect when using in combination with current medications. Liu and colleagues demonstrated the antitumor effects after treatment with cucurbitacin B and cisplatin combination in Hep-2 cells. The results showed that cucurbitacin B/ cisplatin combination exerts the synergistic effects on apoptosis promotion, cell cycle arrest, and cell growth attenuation[37]. Other medications such as gemcitabine[46], methotrexate[41], and doxorubicin[47] were also tested when treated simultaneously in different cell lines and the results displayed synergistic effects in every combination.

Additionally, cucurbitacin also exerts antitumor activity in animal models. Wakimoto and colleagues displayed the effects of cucurbitacin B by reducing the tumor size in orthotopically placed human breast cancer cells in nude mice. The results showed that the relative tumor size was decreased significantly after the mice received 1 mg/kg of cucurbitacin B intraperitoneally for 6 weeks[48].

2.3.6.2 Anti-inflammatory activities

Some pieces of evidence have confirmed the anti-inflammatory activity of cucurbitacin B both *in vivo* and *in vitro*. The first study that showed the pharmacological activity of cucurbitacin B as an anti-inflammatory agent is Yesilada *et al.* in 1988. They discovered an anti-inflammatory effect of lyophilized *Ecballium elaterium* juice in male mice (dd strain) when tested by modified Whittle method. The $CHCL_3$ fraction isolated from *E. elaterium,* which later characterized as cucurbitacin B exerted a significant anti-inflammatory activity with dose-dependent manner. Based on the dose-response curves and probit analysis, the ED_{50} and LD_{50} of cucurbitacin B were calculated as 6.1 and 10.9 mg/kg, respectively[49]. This effect of cucurbitacin B was later confirmed in an animal model by carrageenan-induced paw-edema test. Cucurbitacin B isolated from *Wilbrandia ebracteata* showed anti-inflammatory activity by reducing the edema volume in rat paw by 30 minutes after administration. The dose of cucurbitacin B given as 1 mg/kg intraperitoneally exhibits the %edema inhibition at 60.8%[50, 51]. The mechanism of cucurbitacin B, involving this activity was also investigated. Jayaprakasam and colleagues demonstrated that purified cucurbitacin B from *Cucurbita andreana* possessed the inhibition activity of the COX-2 enzyme by 32% after being treated with the concentration at 100 mg/mL. On the contrary, cucurbitacin B did not show any COX-1 inhibition property at this concentration, which has a similar profile as rofecoxib, specific COX-2 inhibitor that used in the study[52]. It can be concluded that cucurbitacin B exerts the anti-inflammatory property in the animal models by inhibiting functional COX-2 enzymes specifically.

2.3.6.3 Anti-atherosclerotic activity

There is a report that suggests the activity of cucurbitacin B and E in glycosidic form against lipid peroxidation products. Malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE) are the products from lipid peroxidation process and they can be the cause of atherosclerosis involving the modification of lipoproteins[22].

2.3.6.4 Hepatoprotective activity

In Chinese traditional medicine, the *Cucumis melo,* which contains large amounts of cucurbitacin B has been used for hepatitis treatment. Cucurbitacin B exhibits the effect against chronic hepatitis by normalizing hepatic protein levels. Moreover, it also promoted the cellular immunity function together with increase the AMPc/GMPc ratio in the plasma of experimental animals. Cucurbitacin B at a dose of 0.2 mg/kg IP exerted hepatoprotective effect by reducing the GPT, collagen, and β-lipoprotein level in fatty liver induced rats. These effects are all beneficial in the prevention or cure of hepatitis[11].

2.3.7 Toxicological evaluation of cucurbitacin B

A toxicity evaluation for cucurbitacin B is very limited. Most of the reports demonstrated the toxicity that was occurred when given as total cucurbitacins. Cucurbitacin was identified as highly toxic compounds that can cause severe poisoning. Consumption of cucurbitacin containing plants such as *Cucumis* spp. and *Cucurbita* spp. may result in extensive poisoning and lead to death[53]. The toxicity range of cucurbitacins was reported between 2-12.5 mg/kg in rats. However, the toxicity dose range may differ between each variant. For example, cucurbitacin R can be given at 375 mg/kg PO without any toxicity in animal model[54]. The presence of a double bond at C-23 and acetyl group at C-25 have been shown to enhance the toxicity of these compounds which cucurbitacin B possesses that kind of structure[55]. Cucurbitacin B may exert potent pharmacological activity, but the therapeutic dose seems very close to the toxic dose. Precise dose selection of cucurbitacin B is crucial for clinical application.

The study of Gupta and colleagues in 2014 showed the effects of cucurbitacin B on the liver function when it was given at the dose of 1 mg/ kg intraperitoneally for 28 days. The hepatic enzymes including AST, ALT, and LDH were used to determine the liver function after treatment with cucurbitacin B. The results demonstrated that cucurbitacin B did not affect the liver enzyme levels when compared to the control. Moreover, it seems like the level of AST and LDH trend to decrease after cucurbitacin B administration for 28 days[56].

Another study was performed by Iwanski and colleagues to see the toxic effect of cucurbitacin B on the hematologic system. The colony-forming unit assay (CFU-C Assay) was selected to determine an impaired function of hematopoietic stem together with progenitor cells. The experiment was performed by collecting the bone marrow mononuclear cells from the mice after cucurbitacin B administration. The isolated cells were then plated in triplicate in a 6-well plate. The results showed that the colony counts of blast-forming unit-erythroid, colony forming unit granulocyte/ erythrocyte/ monocyte/ megakaryocyte, and colony forming unit granulocyte/macrophage were declined significantly compared to the control group both in doses of 0.5 and 1 mg/kg of cucurbitacin B[46].

2.3.8 Pharmacokinetics of cucurbitacin B

There is only one report that conducted the pharmacokinetic study of cucurbitacin B in an animal model. This study was performed by Zhao and colleagues who developed the method for LC-MS/ MS system to measure the amounts of cucurbitacin B in biological samples. Additionally, they also did the animal experiment for pharmacokinetic application by giving cucurbitacin B at the dose of 20 mg/ kg in 0.5% CMC. The six male Wistar rats were used in their experiment and then collected the blood serially at 0.083, 0.17, 0.33, 0.5, 1, 2, 3, 4, 6, 8 and 10 h post-dose. The pharmacokinetic parameters were calculated based on non-compartmental analysis and they are shown in Table 6.

Table 6 Pharmacokinetic parameters of cucurbitacin B after oral administration at 20 mg/kg[57]

Pharmacokinetic parameters	Mean ± SD
C_{max} (ng/mL)	5.90 ± 1.01
T_{max} (h)	1.75 ± 0.88
$t_{1/2}$ (h)	2.50 ± 0.58
AUC_{last} (μg·h/L)	22.71 ± 5.65
AUC_{inf} (μg·h/L)	24.67 ± 5.84
Cl (L/h/kg)	845.99 ± 183.70

The AUC_{inf} of cucurbitacin B in rat plasma was considered as 24.67 μg h/L while the T_{max} and C_{max} have the values of 1.75 h and 5.90 ng/mL, respectively. This compound has $t_{1/2}$ around 2.5 h, which is considered as short half-life. The clearance of this compound has a value of 845.99 L/h/kg[57]. The information about drug disposition or tissue distribution is still insufficient. There is a report that demonstrates the V_d value of dihydrocucurbitacin F, which has the value of 6.72 L/kg[58]. This value may be a good extrapolation for the V_d of cucurbitacin B due to their similarity in chemical structure. This value of V_d is quite high and it can be used to explain why only a little amount of cucurbitacin B could be found in the plasma. Currently, there is no proposed metabolic pathway of cucurbitacin B. Only the information supporting the metabolism of cucurbitacin D and cucurbitacin I is available. It is explained that the cucurbitacin I is the metabolite results from cucurbitacin E and then it can be further metabolized by phase II enzyme like UDP-glucuronosyl transferases (UGT) and sulfotransferases (SULT) enzymes together with cucurbitacin D[59]. The data that support the route of excretion of cucurbitacin B is not available, but it is possible that this cucurbitacin B may undergo phase II metabolism and later excrete via urine. Nowadays, the ratio of each phase II metabolite of cucurbitacin B in an animal model is still not available. However, the formation of glucuronide conjugates is the most important detoxification pathway in phase II metabolism in all vertebrates, which accounts for 40-70 % of all chemicals used in

human. Thus, the investigators plan to measure only the glucuronide metabolites for cucurbitacin B.

2.3.9 Dose justification

The dose selection of cucurbitacin B was based on previous reports. The LD_{50} of cucurbitacin B was demonstrated as 10.9 mg/kg in rats, so the dose selection of cucurbitacin B should be quite lower than 10.9 mg/kg to avoid the significant toxicity[49]. The therapeutic dose of cucurbitacin B that showed the most anti-tumor effect in the animal model was around 0.1 mg/kg when given as intravenous injection[37]. Thus, the intravenous dose of Cucurbitacin B for the pharmacokinetic study was selected as 0.1 mg/kg. Most of the bioactive components from herbs have the absolute oral bioavailability around 10% , which can help to estimate the dose used in oral administration as 1 mg/kg. The preliminary study of Jiratchariyakul and colleagues showed that cucurbitacin B could be given orally to the rats within the range of 1-4 mg/kg without significant clinical changes. The oral dose selection for this study is 1, 2, and 4 mg/kg for dose escalation pattern and expected to be safe in the animal model.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental animals

The 24 adult male Wistar rats (8 weeks) were purchased and delivered from the National Laboratory Animal Center, Mahidol University. Then, they were moved to the Laboratory Animal Research Facility, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Every rat was housed under controlled conditions, including regulated temperature at 24 ± 2 °C, and humidity at 40-60 % . A lighting system in the animal housing room was set at a 12-h light/dark cycle, and rats were allowed to access food and water *ad libitum*. Before the experiment, Wistar rats were divided into 4 groups randomly and separated into the designated cages. The weight of each rat was measured on the first day that they arrived, and recorded continuously every week until it reached the pre-specified range (400-500 g). The animal experiment was conducted under the protocol approved by the ethical committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University. The approved protocol review number is 16-33-002. This protocol was approved on 9th June 2017.

According to the submitted protocol to the ethical committee, all animal-related experiment was conducted with 3R consideration, including Replacement, Reduction, and Refinement. In term of Replacement, the *in vivo* pharmacokinetic study needs to be conducted only in the animal model since in order to determine the fate and disposition of compound requires the function of physiologically active organs in the animals. The computational simulation data like PBPK model might be applicable in current drug investigation; however, the pharmacokinetic study in the animal is still necessary for model validation. In the current study, a rat is selected as the first priority due to the similarity of various organ system and relatively small in size compared to the other species. *Rattus norvegicus*, strain Wistar, is selected for

pharmacokinetic experiment owing to convenience, inexpensiveness, and their similarity in genetic, biological and behavioral characteristics. Seeing that the pharmacokinetic study required a significant amount of plasma over 24 h, the rat is favorable over the mice. For Reduction, the sample size was calculated by G*power software with integration of standard deviation (SD) received from a preliminary study. In Refinement, pain and distress were alleviated by Isoflurane inhalation under a fume hood, especially when cucurbitacin B was administered or blood was collected via the lateral tail vein. Early endpoints are defined as significant damage around the tail area, which can be appraised by visual assessment. Severe inflammation, infections, necrosis, and tail ripped off are clarified as significant damage. Moreover, changes in behavior, or presentation of severe trauma including significant weight reduction (> 15% of body weight), ruffled fur, hunched over, and squinty eyes also fall within early endpoint criteria.

3.1.2 Chemicals

- Cucurbitacin B powder for the pharmacokinetic experiment is obtained from the Faculty of Pharmaceutical Sciences, Mahidol University. Cucurbitacin B was extracted from *T. cucumerina* juice by diethyl ether with a ratio of 1:1. The % purity of Cucurbitacin B was 90.7% when evaluated by HPLC-UV at a wavelength of 210 nm.
- Cucurbitacin B as analytical standard (Sigma-Aldrich, Corp., MO, USA)
- Glycyrrhizic acid as internal standard (Sigma-Aldrich, Corp., MO, USA)
- Isoflurane, Terrell (MINRAD, Inc., PA, USA)
- Dimethyl sulfoxide (Sigma-Aldrich, Corp., MO, USA)
- Heparin (LEO Pharma A/S, Denmark)
- Methanol, Supergradient for HPLC (RCI Labscan Ltd., Thailand)
- Water, HPLC grade (RCI Labscan Ltd., Thailand)
- Formic acid (Merck KGaA, Corp., Germany)
- 0.9 % Normal saline (General Hospital Products Public, Corp., Thailand)

- β-glucuronidase from *Escherichia coli* type VII-A (Sigma-Aldrich, Corp., MO, USA)

3.1.3 Equipment

Animal experiment

- Metabolic cage 3701M081 (Tecniplast, S.P.A., Italy)
- Insulin syringe, 1 mL (0.4x12 mm) (Nipro, Corp. Ltd., Thailand)
- Gavage needle, 13G, 3 inches (BiolascoThai Corp. Ltd., Thailand)
- Surgical instruments (FST GmbH, Germany)

Sample preparation

- Microliter centrifuge, model MIKRO 120 (Andreas Hettich, GmbH & Co. KG, Germany)
- Tabletop centrifuge, model EBA 20 (Andreas Hettich, GmbH & Co. KG, Germany)
- Micropipette (Labnet International, Inc., NJ, USA)
- Homogenizer, model Yellowline DI 18 Basic (IKA-Werke GmbH & Co. KG, Germany)
- Homogenizer, model WT-130 (Success Technic, Malaysia)
- Vortex mixer, model VX-200 (Labnet International, Inc., NJ, USA)
- Dry bath Incubator, model EL-02 Dual Block (Major Science Co., Ltd., CA, USA)
- Analytical balance, model AG135 (Mettler-Toledo International, Inc. , Switzerland)
- Analytical balance, model UMT2 (Mettler-Toledo International, Inc. , Switzerland)

LC-MS/MS analysis

- Shimadzu 8060 LC-MS/MS system (Shimadzu Corp., Kyoto, Japan)
- HPLC column C-18, Synergi Fusion-RP (Phenomenex, Inc., CA, USA)

- Guard column for C-18, SecurityGuard Fusion-RP (Phenomenex, Inc., CA, USA)

3.2 Methodology

3.2.1 Chemical preparation

Solubility and stability of cucurbitacin B were evaluated before the experimentation to obtain a proper ratio of dimethyl sulfoxide (DMSO) and water. Cucurbitacin B was dissolved in various proportion of DMSO from 10 - 40 % in sterile water for injection. The highest amount of cucurbitacin B, which is concordant with the pre-specified dose, is crucial for determining the proportion of DMSO (10 mg/mL). The solubility and stability test conditions are listed in Table 7. The solubility of cucurbitacin B was determined by visual assessment both under white and black screen. The appraisal was performed right after the addition of cucurbitacin B to the solvent system and 2 hours after that for stability confirmation. The solutions were later examined the %amount of Cucurbitacin B by LC-MS/MS system. The variation of cucurbitacin B found in preparation is limited within ±5% compared to the labeled amount.

Table 7 Solubility and stability test conditions of cucurbitacin B at 10 mg/mL

DMSO concentration	Evaluation time	Assessment condition
10% (v/v)	2 h after addition of CuB	Black and white screen
20% (v/v)	2 h after addition of CuB	Black and white screen
30% (v/v)	2 h after addition of CuB	Black and white screen
40% (v/v)	2 h after addition of CuB	Black and white screen

The DMSO proportion used in the experiment was 40% DMSO in sterile water for injection. The total volume of 40% DMSO, which gave to the animals is approximately 200-250 µL. The solubility of cucurbitacin B at 10 mg/mL in different ratio of DMSO at 2 h is illustrated in Figure 7. There are pieces of evidence that supported the use of DMSO at this concentration in various animal models.

Thackaberry and colleagues demonstrated the dose of DMSO, which could be administered intravenously in CD-1 mice, was up to 1650 mg/kg[60]. From the current study, 40% DMSO at 200-230 µL is equivalent to 220 mg/kg, which is quite lower compared to the toxic dose. Clinical changes could be observed after 2200 mg/kg of DMSO was injected in CD-1 mice via the lateral tail vein. Clinical alteration included rapid breathing, ataxia with whole or partial-body twisting. Moreover, DMSO was tested in rats and the results showed that the animals could tolerate well after intravenous injection at a dose of 200 mg/kg for 1 month. There was no significant change in physical appearance and behavior after the animals exposed to 200 mg/kg of DMSO for 1 month[61]. In this study, DMSO used in animals is equivalent to 220 mg/kg, which is slightly higher compared to the dose used in the previous report. The dose of 220 mg/kg DMSO is expected to be tolerable for the animals for it was given as a single dose compared to the dose of 200 mg/kg that was given as multiple doses.

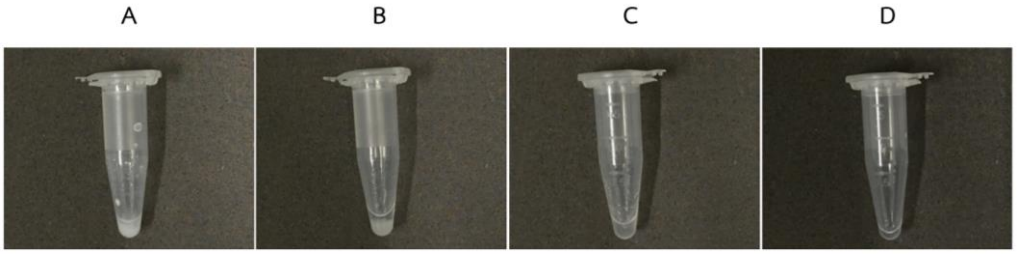

Figure 7 Solubility of cucurbitacin B at 10 mg/mLin A) DMSO 10%, B) DMSO 20%, C) DMSO 30%, and D) DMSO 40% at 2 h

3.2.2 Animal preparation

The 24 adult male Wistar rats were separated into the designated group as shown in Table 8. The numbers of rat used for each experimental group are 6, which is calculated by G*power software. The day before a pharmacokinetic study, each rat was isolated in the metabolic cage for blank urine and feces collection. Furthermore, access to food was prohibited for 10 h to prevent the interference of food interaction. Blood collection was also performed on that day for blood chemistry evaluation, including liver and kidney performance.

Table 8 Experimental group for Cucurbitacin B pharmacokinetic study

Group	Administration	Sample collection
1.	Cucurbitacin B at 0.1 mg/kg IV	Plasma, urine, feces, internal organs
2.	Cucurbitacin B at 1 mg/kg PO	Plasma, urine, feces
3.	Cucurbitacin B at 2 mg/kg PO	Plasma, urine, feces
4.	Cucurbitacin B at 4 mg/kg PO	Plasma, urine, feces

3.2.3 Chemical administration and blood collection

After 10 h fasting period, each rat was anesthetized by 10% v/v Isoflurane inhalation with chamber induction method. All anesthesia related procedures were performed under mobile fume hood and each rat's consciousness was confirmed by tail and toe pinch evaluation. An animal anesthesia required to be done 2-3 minutes before the designated sampling time to obtain the plasma at the designed time point. Cucurbitacin B solutions were prepared freshly prior to the pharmacokinetic study and then administered to the rats with the designated dose and route as described in the Table 8. For oral administration, gavage needle was used to give the pre-specified dose at 1, 2, and 4 mg/kg. In case of intravenous administration and blood collection, both approaches were executed via lateral tail vein. The dose of cucurbitacin B for injection was calculated based on the content of cucurbitacin B in the solution to provide the amount of compound equal to 0.1 mg/kg. Heparinized needles and syringes were used during the blood sampling process. The 300 µL of blood samples were collected serially after administration at different time points including 0.083 (5 min), 0.25 (15 min), 0.5 (30 min), 1, 2, 4, 8, and 24 hours. Collected blood samples were transferred to the 1.5 mL microcentrifuge tubes and kept in the ice bath while they were waiting for the further process. After finish each blood sampling session, the rat was placed in metabolic cage 3701M081 (Tecniplast, S.P.A., Italy) for urine and feces collection afterward. The feces found in the induction chamber during anesthesia procedure was also collected. For blood collection process, sampling time point and administration time are shown in Figure 8.

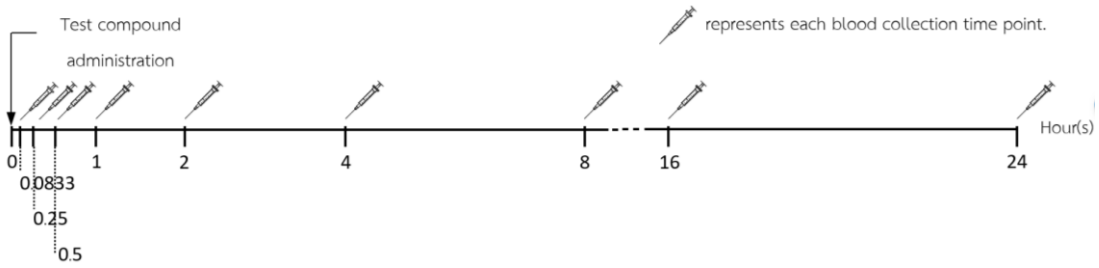

Figure 8 Blood collection timeline for pharmacokinetic study

3.2.4 Urine and feces collection

After Cucurbitacin B administration, urine and feces were collected separately during the whole process both within the metabolic cage and also the induction chamber. While the rats stayed in metabolic cages, urine and feces were segregated automatically by the cages' functionality. Alongside the sample collection, measurement of urine volume and feces weight were needed to be done at 24 hours, and 48 hours after administration. These measuring parameters will be essential to determine the level of cucurbitacin B and its metabolites. After that the feces samples were submerged with methanol to stop the possible catalytic reaction, which might be occur by the microflora activity. Both urine and feces sample were kept in the freezer at -20 °C until further analysis.

3.2.5 Collection of internal organs

Cucurbitacin B solution with the same dose as described in previous section was injected into the rat via lateral tail vein. Internal organs including heart, lung, liver, stomach, spleen, small intestine, kidney, and brain were collected right after euthanization by Isoflurane overdose. The animals were terminated at 1, 2, and 4 hours after administration, which death will be confirmed by exsanguination. The hepatic vein was cut at the designated time (1, 2, and 4 h) considering the distribution phase in animals was stopped. Before organ removal, 1 mL of blood was collected from heart by cardiac puncture and stored in a freezer at -20 °C until further analysis. Unnecessary connective tissue was removed from the isolated organs by surgical blade and scissor. Excessive blood within the removal organ was washed 2 times by iced 0.9% saline solution.

3.2.6 Sample pretreatment

The collected blood samples were thawed on an ice bath and then centrifuged at 3,000 x*g* for 10 minutes by using microliter centrifuge, model MIKRO 120 (Andreas Hettich, GmbH & Co. KG, Germany). The samples were later processed by collecting 50 µL of plasma and then treated with 150 µL of methanol plus 50 µL of glycyrrhizic acid (500 ng/mL) as the internal standard. The samples were mingled together by vortex mixer, model VX-200 (Labnet International, Inc., NJ, USA) for 10 minutes after that mixed samples were centrifuged again at 10,000 x*g* for 10 minutes. The 150 µL of supernatant from each sample was collected for further LC-MS/MS analysis.

For urine specimen, volume of each sample was measured and then mixed by vortex mixer, model VX-200 (Labnet International, Inc., NJ, USA) for 10 minutes. Thereafter urine samples were centrifuged at 3,000 x*g* for 10 minutes by using tabletop centrifuge, model EBA 20 (Andreas Hettich, GmbH & Co. KG, Germany) and each supernatant was collected. The 100 µL of supernatant was diluted by 900 µL of distilled water. The mixture was later treated with 150 µL of methanol plus 50 µL of glycyrrhizic acid (500 ng/mL) as the internal standard. The final mixture was centrifuged once again at 10,000 x*g* by using microliter centrifuge, model MIKRO 120 (Andreas Hettich, GmbH & Co. KG, Germany) for 10 minutes and 150 µL of supernatant from each sample was collected for further LC-MS/MS analysis.

In case of feces, weight of each sample was measured and then homogenized thoroughly in methanol by homogenizer, model Yellowline DI 18 Basic (IKA-Werke GmbH & Co. KG, Germany). When the feces homogenate was mixed completely, volume of the mixture was adjusted to 10 mL by methanol. The obtained mixtures were then centrifuged at 3,000 x*g* by using tabletop centrifuge, model EBA 20 (Andreas Hettich, GmbH & Co. KG, Germany) for 10 minutes. Supernatant was collected from each sample and then treated with 150 µL of methanol plus 50 µL of glycyrrhizic acid (500 ng/mL) as the internal standard. The final mixture was centrifuged at 10,000 x*g* by using microliter centrifuge, model

MIKRO 120 (Andreas Hettich, GmbH & Co. KG, Germany) for 10 minutes and 150 µL of supernatant from each sample was collected for further LC-MS/MS analysis.

Internal organs including heart, lung, liver, stomach, spleen, small intestine, kidney, and brain were weighed out 50 mg on analytical balance, model AG135 (Mettler-Toledo International, Inc., Switzerland) and then diluted with 150 µL of methanol. The mixtures were later homogenized in ice bath for 10 minutes by using homogenizer, model WT-130 (Success Technic, Malaysia). The 50 µL of glycyrrhizic acid (500 ng/mL) was added to the homogenate and then mingled together for 10 minutes by vortex mixer, model VX-200 (Labnet International, Inc., NJ, USA). After mixing, the final mixtures were centrifuged at 10,000 x*g* by using microliter centrifuge, model MIKRO 120 (Andreas Hettich, GmbH & Co. KG, Germany) for 10 minutes. 150 µL of supernatant from each sample was collected for further LC-MS/MS analysis.

3.2.7 Glucuronide metabolite quantification

Expected metabolites of cucurbitacin B are cucurbitacin B glucuronide. Measurement of these metabolites were done by detecting the level of cucurbitacin B after hydrolysis reaction. β-glucuronidase from *Escherichia coli* type VII-A (Sigma-Aldrich, Corp., MO, USA) was used for enzymatic hydrolysis of cucurbitacin B glucuronide to cucurbitacin B. The general β-glucuronidase hydrolysis reaction is demonstrated in Figure 9. β-glucuronidase, which have activity over 300,000 units/g solid, was used and later diluted with 75 mM phosphate buffer pH 6.8. Optimization of incubation time needs to be performed by varying the time from 15 to 90 minutes. After optimal condition was indicated, the 50 µL of plasma sample was mixed with 50 µL of β-glucuronidase enzyme and then incubated in dry bath Incubator, model EL-02 Dual Block (Major Science Co., Ltd., CA, USA) at 37 ^{o}C. The mixtures were then diluted with 300 µL of methanol plus 100 µL of glycyrrhizic acid (500 ng/mL) as the internal standard. The 150 µL of supernatant from each sample was then collected for further LC-MS/MS analysis.

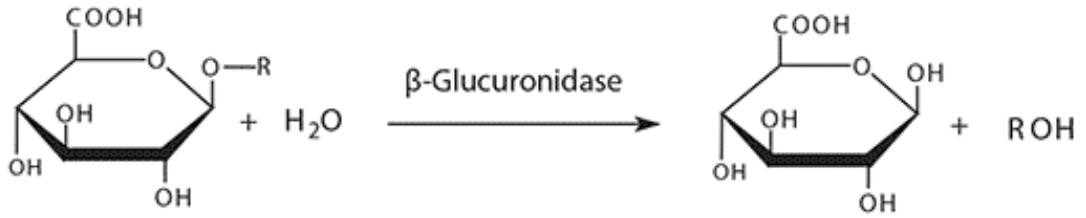

Figure 9 General β-glucuronidase hydrolysis reaction

In case of urine, feces, and internal organs, 50 µL of supernatant from each sample was diluted with 150 µL of 75 mM phosphate buffer pH 6.8 and then mixed with 50 µL of β-glucuronidase enzyme solution at optimal incubation time. The mixtures were then diluted with 300 µL of methanol plus 100 µL of glycyrrhizic acid (500 ng/mL) as the internal standard. 150 µL of supernatant from each sample was then collected for further LC-MS/MS analysis.

3.2.8 Blood chemistry

Blood chemical parameters were evaluated to observe the influences of chemicals and vehicle (40% DMSO) on physiological functions. The major organs involving drug metabolism and excretion are liver and kidney, respectively, thus evaluation of these organ functions needs to be performed before and after treatment (24 hours). Liver function was determined by measuring the level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). For kidney function, it was evaluated by detecting the level of creatinine (Cr). The acquired results will be reported as mean ± standard deviation of the means and compared the significant difference by paired t-test (significant level = 0.05).

3.2.9 LC-MS/MS system

To determine the cucurbitacin B concentration in biological samples, including plasma, internal organs, and excreta, LC-MS/MS is the preferred system for this kind of analysis since it provides high sensitivity, reproducibility, and accuracy. The LC-MS/MS system used in current study is composed of Nexera UHPLC (Kyoto, Japan), a liquid chromatography compartment and Shimadzu 8060 LCMS (Kyoto, Japan), a mass spectrometry compartment. Nexera UHPLC (Kyoto, Japan) consists of a vacuum degasser, a binary pump, an autosampler, and a column thermostat. To operate both of liquid chromatography and mass spectrometry, LabSolution version 5.86 software (Kyoto, Japan) is currently used to control the system. The mobile phase is comprised of 0.2% v/v formic acid in water (pH 2.5), and methanol. The flow of mobile phase through the column was set as gradient phase elution, which the proportion of mobile phase was altered during the running process. The summary of changing in ratio of organic and inorganic phase is demonstrated in Table 9. The flow rate was set at 0.3 mL/min with an injection volume of 15 µL. The chromatography column using for compound separation is C18 reversed phase column, model Phenomenex Synergi Fusion-RP (Torrance, United States) equipped with Guard C18 column, model Phenomenex SecurityGuard Fusion-RP (Torrance, United States). The temperature in column oven was set at 40 °C.

Table 9 Gradient elution pattern used to determine cucurbitacin B concentration in biological samples

Time (min)	Methanol (%)	0.2% v/v Formic acid (%)
0.00	45	55
2.50	90	10
3.00	90	10
3.50	45	55
5.00	45	55

The mass spectrometry were optimized both in positive and negative ionization mode, which applied for cucurbitacin B and glycyrrhizic acid quantification, respectively. Multiple reaction monitoring (MRM) was used to perform mass spectrometric quantification of analyte and internal standard. MS/MS spectra of cucurbitacin B at m/z of 559.32 $[M+H]^+$ was not able to be detected and it displayed relatively low intensity compared to m/z of 581.25 $[M+Na]^+$. Accordingly, the $[M+Na]^+$ ion was chosen for the cucurbitacin B as the precursor ion. The ion transition of cucurbitacin B and glycyrrhizic acid was defined as m/z 581.25/521.20 and m/z 821.25/350.90, respectively. The MS/MS spectra of cucurbitacin B and glycyrrhizic acid were shown in Figure 10. Collision energy used in the system was -26.9 and 40.5 V applied for cucurbitacin B and glycyrrhizic acid, respectively. Interface condition was defined as follows: nebulizing gas flow: 3 L/min; heating gas flow: 10 L/min; interface temperature: 300 °C; heating block temperature: 400 °C; and drying gas flow: 10 L/min. Retention time of cucurbitacin B and glycyrrhizic acid was 3.01 and 3.65 min, respectively.

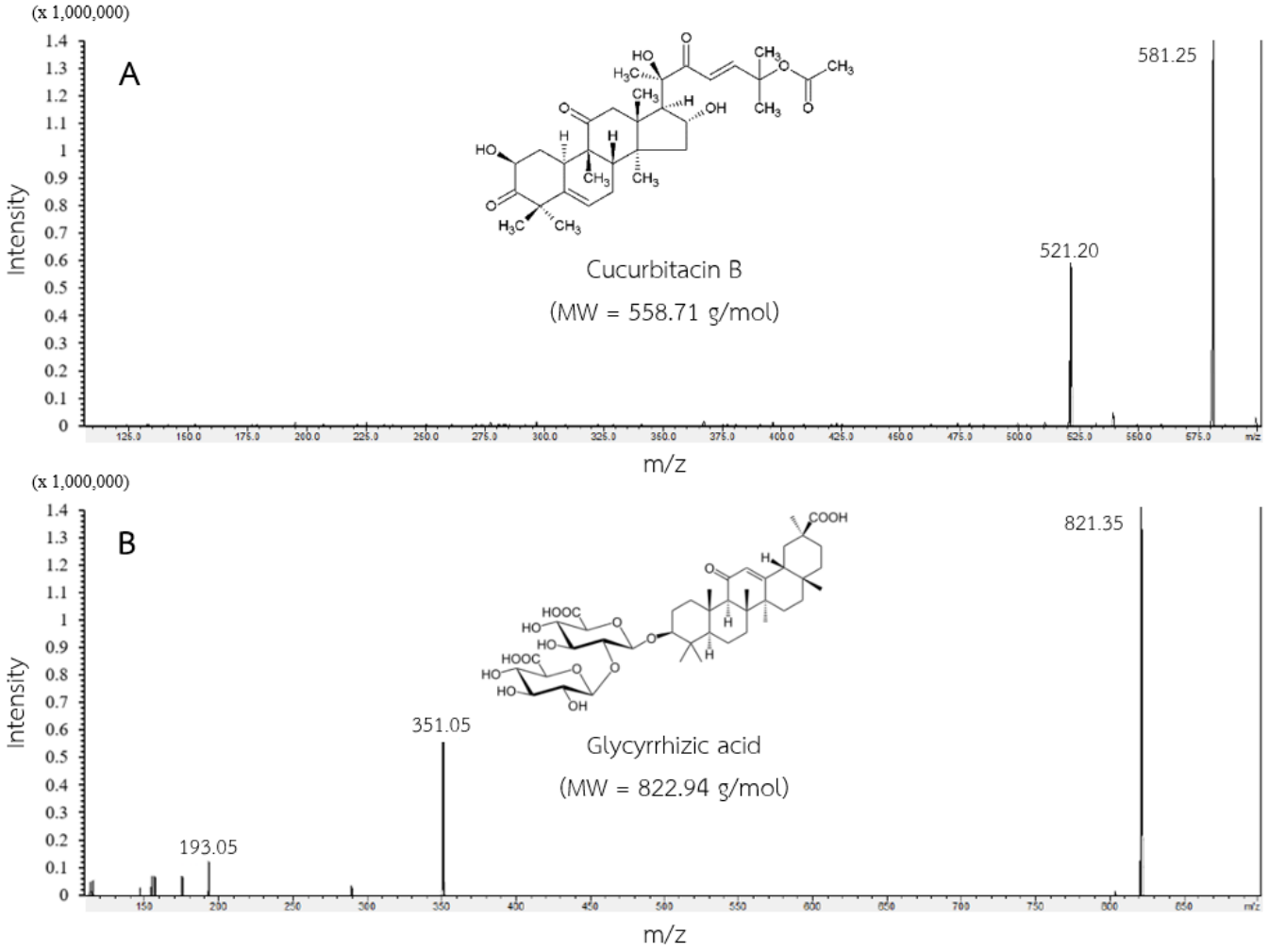

Figure 10 Product ion scanning of parent ionsfor cucurbitacin B (A) and glycyrrhizic acid, internal standard (B)

3.2.10 Method validation

The analytical method was performed following to the US FDA guidelines for industry for bioanalytical method validation[62]. The parameters were evaluated during the validation process, including selectivity, matrix effect and recovery, linearity, lower limit of detection, lower limit of quantification, accuracy, precision, and stability.

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma with the spiked plasma samples. Recovery and matrix effects were evaluated by comparing the peak area of cucurbitacin B and its metabolites spiked in post-extraction plasma at concentrations of quality control (QC) samples with peak area obtained from standard solutions.

Linearity was evaluated by constructing the calibration curve. Range of concentration in each analyte was determined by the results obtained from preliminary study. The numbers of calibration point were designed around 7 to 8 points. Each sample was quantified by calculating the peak area ratio (y) and concentration of each analyte (x). Calibration equation and correlation coefficient (R^2) were also calculated with least squares linear regression analysis. The lowest concentration of the calibration curve was defined as the lower limit of quantitation or LLOQ. At this point, LLOQ required to be less than or equal to 20% of both precision, and accuracy.

Accuracy could be calculated in term of relative error (% RE), which compared the value of concentration obtained from calibration equation with the actual analyte concentration. Intra-assay, and inter-assay precision were evaluated by comparing peak areas of six replicates of extracted QC samples on three different days, which were reported as coefficient variation (%CV). The equations for %RE and %CV calculation are listed below.

$$\text{Relative error (\%RE)} = \frac{\text{Measured value-Actual value}}{\text{Actual value}} \times 100$$

$$\text{Coefficient of variation (\%CV)} = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

The stability test consists of short term stability at room temperature, long term stability at storage condition, freeze-thaw stability, and autosampler stability. Short term stability was evaluated at room temperature for 24 hours. Long term stability was assessed at -20 °C for 3 months. The freeze–thaw stability of cucurbitacin B was tested by analyzing QC samples at three different concentrations subjected to three freeze (-20 °C) -thaw (25 °C) cycles. Lastly, autosampler stability was determined by measuring the peak area obtained from freshly prepared QC samples compared with samples that stored in autosampler compartment for 24 h.

3.2.11 Pharmacokinetic and statistical analysis

The plasma results obtained from LC-MS/MS analysis was further analyze by plotting between the calculated concentrations and blood sampling time points. Calculation of pharmacokinetic parameters were performed by PK solutions 2. 0 software (Summit Research Services, CO, USA) with non-compartmental analysis. The calculated pharmacokinetic parameters are listed below.

- Area under the curve from time zero to the last sampling time point (AUC_{0-t})
- Area under the curve from time zero to infinity ($AUC_{0-\infty}$)
- Maximum plasma drug concentration (C_{max})
- Time to reach maximum plasma drug concentration (T_{max})
- Volume of distribution (V_d)
- Mean residence time (MRT)
- Elimination half-life ($t_{1/2}$)
- Total Clearance (CL)

The acquired results were reported in mean ± standard deviation (SD). Statistical analysis used in this study is Mann-Whitney U test due to non-normal distribution of the pharmacokinetic parameters. The analyses were conducted to determine the difference between two oral dosing, which included 2 and 4 mg/kg.

The cucurbitacin B at 1 mg/kg oral dosing did not include into the analyses because only negligible amount of cucurbitacin B could be detected in the plasma. The significant difference was determined at p-value < 0.05.

In case of tissue distribution study, amount of cucurbitacin B and its metabolites were determined in each organ and then reported in ratio compared with the level in plasma at corresponding time point. The acquired ratio was determined and compared to 1 h from 2 h through 4 h after administration. Kruskal-Wallis test was used to examine the difference between each value and the significant level was determined at p-value < 0.05. % Recovery could be calculated by extraction of level found in urine and feces from amount of chemicals given to the rats.

CHAPTER IV
RESULTS

4.1 Method validation

Selectivity is the parameter to determine the possible interference, which co-eluted with cucurbitacin B and internal standard, glycyrrhizic acid. Generally, a chromatogram of the compound of interest should not overlay with other interference peaks, which might be eluted at the same retention time. The retention time of cucurbitacin B and glycyrrhizic acid are 3.01 and 3.65 min, respectively. As illustrated in Figure 11, there was no significant chromatographic peak, considering as an interference, which eluted at the same retention times as cucurbitacin B and glycyrrhizic acid. Thus, we concluded that the method used in current study provided a good selectivity to determine the cucurbitacin B concentration in biological samples.

The calibration curves were constructed in the range of 0.1-20 µg/L. Linearity of analytical method was determined by least square linear regression by using $1/x^2$ as a weighting factor. Before the measurement process, calibration curves were performed with triplicate set of standard solution at different days as demonstrated in Table 10. The results showed that all standard curves had good linearity with correlation coefficient (R^2) more than 0.995. Accuracy of each concentration used in the regression line is limited within the range of ±15% when analyzed by %RE, except for the LLOQ, which is acceptable within ±20%. The LLOQ was 0.1 µg/L for cucurbitacin B. The values obtained from 3 consecutive days showed a good precision when determined by %CV. The acceptable range for %CV is ±15%, except for the LLOQ that is ±20%.

Accuracy was analyzed by using three different concentrations including low concentration quality control (LQC) at 0.5 µg/L, medium concentration quality control (MQC) at 10 µg/L, and high concentration quality control (HQC) at 15 µg/L. %RE is a value used to determine the validity of measurement process, which is calculated by using the actual value obtained from the measurement compared with the known concentration. The accuracy was tested both within-batch and between-

batch of quality control samples. The results are summarized in Table 11, and Table 12, respectively. The results demonstrated that the %RE of three quality control concentrations were within the acceptable range of ±15%. Within-batch and between-batch precision were also examined for three consecutive days and the results were presented as %CV. The results confirmed that both intra- and inter-assay in three concentrations fell within an acceptable range of ±15%. These results allowed us to conclude that both accuracy and precision of this analytical method were suitable for cucurbitacin B determination.

Recovery is the parameter that describe how good of the extraction process for the compound of interest from biological sample. In current study, protein precipitation was used to extract the whole cucurbitacin B from the samples. The results are summarized in Table 13, and showed that the recoveries of cucurbitacin B were in the range of 73.14-79.73%. This suggested that there were some matrix effects affecting the cucurbitacin B measurement; however, the range was still acceptable. Because they provided similar recovery patterns with no significant difference (p-value > 0.05) between each concentration of quality control, the amount of cucurbitacin B expected to be isolated from biological samples with similar proportion in any studied doses.

Stability of cucurbitacin B was tested in various conditions and the results are shown in Table 14. The stability of this compound ranged from 81.30-109.50% depending on the test condition. The room temperature stability, when the quality control samples were left at 25 °C for 24 h, seemed to have the lowest recovery compared to the other test conditions (ranged from 81.30-86.10%). This suggested that the biological samples should be prepared freshly within 24 h. In other test conditions, they provided satisfactory recoveries, which ranged from 90.50-109.50%.

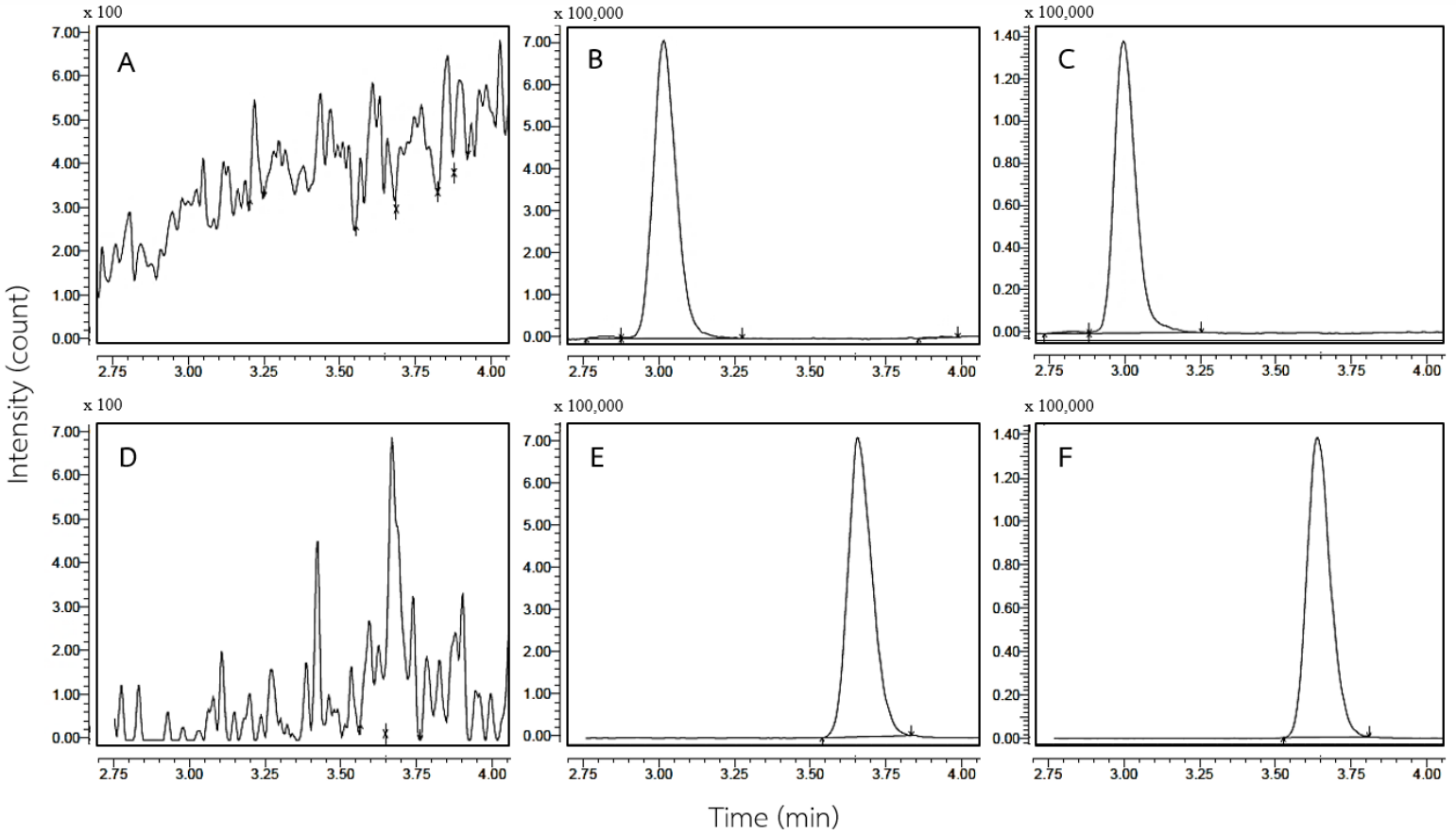

Figure 11 Representative chromatogramsof blank rat plasma after monitoring under cucurbitacin B condition (A); blank plasma with cucurbitacin B at 5 µg/L (B); cucurbitacin B in an unknown plasma sample (C); blank rat plasma after monitoring under glycyrrhizic acid condition (D); pre-spiked blank plasma with glycyrrhizic acid at 25 ng (E); glycyrrhizic acid added in an unknown plasma sample (F)

Table 10 Calibration curves of cucurbitacin B tested in 3 different days by LC-MS/MS

Nominal Concentration (ng/mL)	Experimental Concentration (ng/mL)			Mean	SD	%CV	%RE
	Day 1	Day 2	Day 3				
0.1	0.080	0.082	0.101	0.101	0.019	18.34	1.33
	0.108	0.107	0.13				
0.5	0.559	0.486	0.459	0.516	0.037	7.14	3.13
	0.538	0.533	0.519				
1	1.007	1.059	1.001	1.019	0.033	3.28	1.90
	0.968	1.046	1.033				
5	5.065	4.936	4.919	5.064	0.115	2.26	1.27
	5.155	5.108	5.198				
10	9.276	9.888	9.842	9.694	0.277	2.85	-3.06
	9.412	9.822	9.923				
15	14.697	14.381	14.074	14.511	0.260	1.79	-3.26
	14.549	14.817	14.545				
20	21.017	20.512	20.515	20.701	0.254	1.23	3.50
	20.767	20.424	20.968				
R^2	0.997	0.999	0.997				

CV, coefficient of variation; RE, relative error; R^2, correlation coefficient

Table 11 Within-batch accuracy and precision of cucurbitacin B analyzed by LC-MS/MS

Within-batch Accuracy and Precision (Day 1)						
Sample Number	LQC 0.5 ng/mL		MQC 10 ng/mL		HQC 15 ng/mL	
	Measured value	%RE	Measured value	%RE	Measured value	%RE
1	0.59	18.60	9.89	-1.14	14.15	-5.66
2	0.51	1.20	9.83	-1.70	13.93	-7.15
3	0.50	0.60	9.51	-4.93	13.88	-7.45
4	0.52	4.40	10.10	1.01	13.86	-7.63
5	0.52	4.20	9.70	-3.01	13.39	-10.73
6	0.52	3.40	10.02	0.16	13.26	-11.63
Mean	0.53	5.40	9.84	-1.60	13.74	-8.38
SD	0.03		0.22		0.34	
%CV	6.31		2.19		2.51	
Within-batch Accuracy and Precision (Day 2)						
Sample Number	LQC 0.5 ng/mL		MQC 10 ng/mL		HQC 15 ng/mL	
	Measured value	%RE	Measured value	%RE	Measured value	%RE
1	0.49	-2.60	10.06	0.62	13.75	-8.31
2	0.50	-0.20	9.70	-3.04	14.18	-5.47
3	0.49	-1.20	9.99	-0.13	13.65	-8.99
4	0.49	-1.80	9.76	-2.39	14.05	-6.37
5	0.52	3.40	9.58	-4.23	14.20	-5.34
6	0.49	-1.20	9.40	-6.05	13.30	-11.31
Mean	0.50	-0.60	9.75	-2.54	13.86	-7.63
SD	0.01		0.25		0.35	
%CV	2.13		2.56		2.53	

LQC, low concentration quality control; MQC, medium concentration quality control; HQC, high concentration quality control; CV, coefficient of variation.

Table 11 Within-batch accuracy and precision of cucurbitacin B analyzed by LC-MS/MS (Continued)

Within-batch Accuracy and Precision (Day 3)						
Sample Number	LQC 0.5 ng/mL		MQC 10 ng/mL		HQC 15 ng/mL	
	Measured value	%RE	Measured value	%RE	Measured value	%RE
1	0.52	3.00	9.15	-8.52	13.79	-8.09
2	0.54	8.00	8.93	-10.75	13.91	-7.25
3	0.51	1.60	9.31	-6.94	14.16	-5.57
4	0.51	2.40	9.23	-7.71	14.17	-5.54
5	0.45	-11.00	9.22	-7.84	13.39	-10.75
6	0.48	-3.20	9.22	-7.84	13.83	-7.81
Mean	0.50	0.13	9.17	-8.27	13.87	-7.50
SD	0.03		0.13		0.29	
%CV	6.51		1.44		2.09	

LQC, low concentration quality control; MQC, medium concentration quality control; HQC, high concentration quality control; CV, coefficient of variation.

Table 12 Between-batch accuracy and precision of cucurbitacin B analyzed by LC-MS/MS

Sample/Batch	LQC 0.5 ng/mL		MQC 10 ng/mL		HQC 15 ng/mL	
	Measured value	%RE	Measured value	%RE	Measured value	%RE
Day 1	0.59	18.60	9.89	-1.14	14.15	-5.66
	0.51	1.20	9.83	-1.70	13.93	-7.15
	0.50	0.60	9.51	-4.93	13.88	-7.45
	0.52	4.40	10.10	1.01	13.86	-7.63
	0.52	4.20	9.70	-3.01	13.39	-10.73
	0.52	3.40	10.02	0.16	13.26	-11.63
Day 2	0.49	-2.60	10.06	0.62	13.75	-8.31
	0.50	-0.20	9.70	-3.04	14.18	-5.47
	0.49	-1.20	9.99	-0.13	13.65	-8.99
	0.49	-1.80	9.76	-2.39	14.05	-6.37
	0.52	3.40	9.58	-4.23	14.20	-5.34
	0.49	-1.20	9.40	-6.05	13.30	-11.31
Day 3	0.52	3.00	9.15	-8.52	13.79	-8.09
	0.54	8.00	8.93	-10.75	13.91	-7.25
	0.51	1.60	9.31	-6.94	14.16	-5.57
	0.51	2.40	9.23	-7.71	14.17	-5.54
	0.45	-11.00	9.22	-7.84	13.39	-10.75
	0.48	-3.20	9.22	-7.84	13.83	-7.81
Mean	0.51	1.64	9.59	-4.14	13.82	-7.84
SD	0.03		0.36		0.32	
%CV	5.77		3.75		2.28	

LQC, low concentration quality control; MQC, medium concentration quality control; HQC, high concentration quality control; CV, coefficient of variation.

Table 13 The recovery of cucurbitacin B after extraction from rat plasma

Sample Number	Concentration of Cucurbitacin B (µg/L)					
	LQC 0.5 µg/L		MQC 10 µg/L		HQC 15 µg/L	
	Un-extract	Extract	Un-extract	Extract	Un-extract	Extract
1	0.79	0.59	11.72	9.57	18.77	14.15
2	0.63	0.51	12.69	9.83	18.71	13.93
3	0.67	0.50	12.48	9.51	18.77	13.88
4	0.61	0.52	12.52	10.10	18.69	13.86
5	0.64	0.52	12.39	9.70	18.92	13.39
6	0.62	0.52	11.96	10.02	18.89	13.26
Mean	0.66	0.53	12.29	9.79	18.79	13.74
SD	0.07	0.03	0.37	0.24	0.09	0.35
%CV	10.25	6.31	3.01	2.45	0.50	2.51
Absolute Recovery	79.73		79.61		73.14	

LQC, low concentration quality control; MQC, medium concentration quality control; HQC, high concentration quality control; CV, coefficient of variation.

Table 14 The stability of cucurbitacin B at different storage conditions

Conditions	QC concentration (μg/L)	Measured values (μg/L)	SD	%CV	%RE	%Recovery
Room temperature (12 h)	0.5	0.43	0.02	4.90	14.20	81.34
	10	8.20	0.19	2.34	18.01	83.32
	15	11.80	0.15	1.25	21.35	86.16
Storage at -20 °C (3 months)	0.5	0.48	0.03	5.66	4.60	90.54
	10	9.81	0.65	6.58	1.86	99.72
	15	13.46	0.42	3.10	10.29	97.93
Three freeze-thaw cycles	0.5	0.49	0.03	6.39	3.00	92.01
	10	9.79	0.22	2.21	2.13	99.50
	15	13.68	0.46	3.34	8.81	99.54
Autosampler (10 °C)	0.5	0.47	0.03	7.25	6.20	89.05
	10	10.78	0.76	7.01	-7.78	109.57
	15	13.84	0.39	2.82	7.71	100.75

QC, quality control; CV, coefficient of variation; RE, relative error.

4.2 Animal tolerability

All animals tested with cucurbitacin B in all studied dose range showed a good tolerability after administration for 24 h. The general appearance of animals was observed carefully during the experimentation. There was no significant alteration in behavior, be it eating, playing, or glooming. The change in physical appearance or organ dysfunction could not be detected in overall experiment. Only soft stool in some animals, which belonged to high dose administered group were detected at 0-24 h after administration; however, they returned to normal after 48 h. Liver and kidney function of animals were also evaluated with plasma biochemical markers as showed in Table 15. For liver function, AST and ALT were measured and compared between before and after administration in each intervention. The results demonstrated that after intervention, neither AST nor ALT altered significantly compared to before treatment. AST level was increased slightly in the group that received 0.1 mg/kg of intravenous cucurbitacin B and 4 mg/kg of oral dose. The minimal change in this enzyme is considered as insignificance in the clinical status. Likewise, creatinine level used to determine the kidney function was not altered after cucurbitacin B administration for 24 h.

Table 15 General appearance and biochemical markers indicating liver and kidney functions before and after cucurbitacin B treatment

Parameters	Cucurbitacin B 0.1 mg/kg IV		Cucurbitacin B 2 mg/kg PO		Cucurbitacin B 4 mg/kg PO	
	Pre-dosing (0 h)	Post-dosing (24 h)	Pre-dosing (0 h)	Post-dosing (24 h)	Pre-dosing (0 h)	Post-dosing (24 h)
General appearance	Normal	Normal	Normal	Normal	Normal	Normal
AST (U/L)	35.40 ± 4.15	39.20 ± 3.27	36.67 ± 1.53	32.33 ± 11.68	33.50 ± 8.10	40.25 ± 0.96
ALT (U/L)	11.40 ± 6.31	11.00 ± 3.94	11.00 ± 1.00	6.33 ± 1.53	16.75 ± 6.50	12.25 ± 5.62
Creatinine (mg/dL)	0.22 ± 0.03	0.23 ± 0.02	0.20 ± 0.02	0.19 ± 0.03	0.23 ± 0.02	0.22 ± 0.03

The data represent as mean ± SD (n=6). AST, aspartate aminotransferase; ALT, alanine aminotransferase.

4.3 Plasma concentration-time curve and pharmacokinetic parameters

Plasma concentration-time profile after cucurbitacin B intravenous injection is illustrated in Figure 12. The pharmacokinetic parameters of cucurbitacin B after administering at different doses and routes were calculated and displayed in Table 16. After intravenous administration, cucurbitacin B had a large volume of distribution (V_d = 51.65 ± 39.16 L/kg), but possessed a short half-life ($t_{1/2}$ = 5.08 ± 2.87 h). This indicated that cucurbitacin B was distributed thoroughly in the animal body and was cleared out of the plasma within a short period of time. It was concordant with the computed clearance (CL) in which the value was approximately 7.24 L/h/kg.

After rescaling the y axis of intravenous data to the logarithm scale, it was demonstrated that each concentration-time point seemed to align in a linear manner as shown in Figure 13. These data points were analyzed by using the least square linear regression. The result showed that the concentration-time points were arranged closely to the regression line with R^2 = 0.9509 as shown in Figure 14. The linear regression line is described by the equation $y = 2.3963e^{-0.19x}$.

The plasma concentration-time profile of orally administered cucurbitacin B had a similar pattern in a dose of 2 mg/kg and 4 mg/kg as illustrated in Figure 16. The results showed that a curve of cucurbitacin B in the 4 mg/kg dose was slightly higher than the dose of 2 mg/kg. The highest concentration of cucurbitacin B in plasma was detected within 1 h after oral administration. The maximum plasma concentration (C_{max}) was approximately 9.70 and 31.24 µg/L in 2 and 4 mg/kg dosing, respectively. The concentration-time profile of cucurbitacin B at 1 mg/kg did not show in current report because the concentrations found in plasma were unable to detect by LC-MS/MS (below LLOQ). The cucurbitacin B concentrations in both doses were gradually decreased to 1.09 and 2.66 µg/L within 8 h and could not be further detected at 16 h (below LLOQ). The calculated $AUC_{0\text{-inf}}$ of cucurbitacin B after 4 mg/kg oral dosing was 2-fold higher than that of 2 mg/kg. The plot between calculated $AUC_{0\text{-inf}}$ and the administered dose is illustrated in Figure 15. After applying the least square method to the data points, the result proved that the $AUC_{0\text{-inf}}$ was increased proportionally to the given doses with R^2 = 0.9996. According to the difference between doses, the $AUC_{0\text{-inf}}$, obtained from each dose were

adjusted by the dose amount to get the normalized values. Comparison of normalized $AUC_{0\text{-}inf}$ was performed and the result showed that there was no statistical difference between 2 and 4 mg/kg oral dosing.

The logarithm scale was applied to the concentration-time profile of oral dosing as demonstrated in Figure 17. In current study, non-compartmental analysis was used to analyze the pharmacokinetic characteristics of this compound, so the clearance and half-life in oral dosing were unable to interpret. However, we speculated that the higher dose of cucurbitacin B might not affect the elimination process of this compound as the slopes of elimination phase in both doses were similar.

The computed absolute bioavailability was approximately 10% in both oral doses. Comparison of the absolute bioavailability from each group was performed and no statistical difference between 2 mg/kg and 4 mg/kg oral dosing was detected. This suggested that different doses did not affect the extent of absorption of cucurbitacin B.

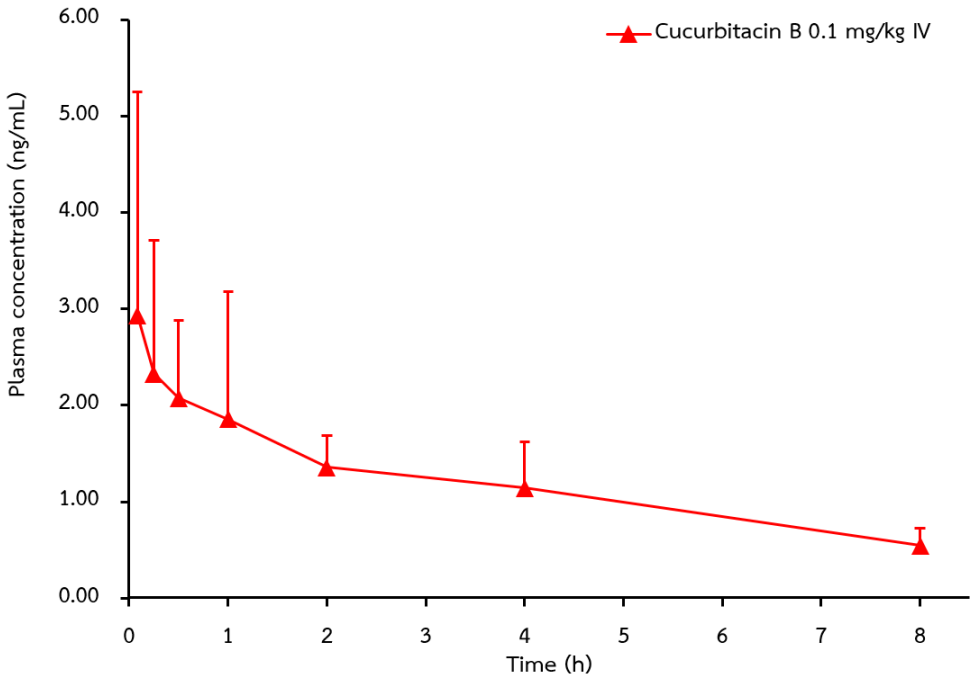

Figure 12 Mean plasma concentration-time curves of cucurbitacin B after intravenous administration at 0.1 mg/kg. Each data point represents as mean ± SD.

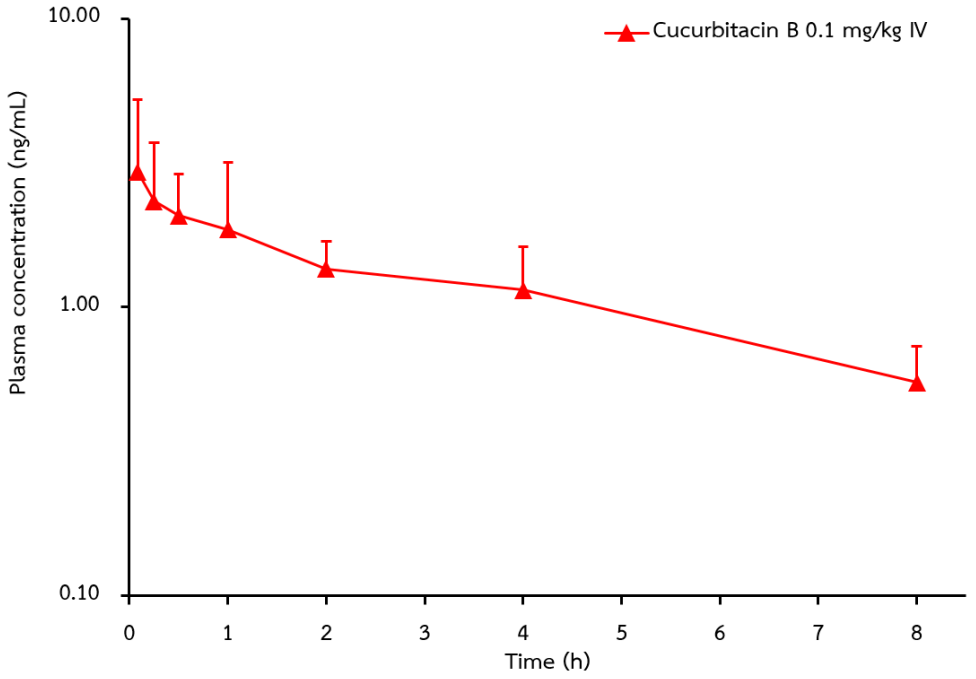

Figure 13 Mean plasma concentration-time curves of cucurbitacin B after intravenous administration at 0.1 mg/kg in logarithm scale. Each data point represents as mean ± SD.

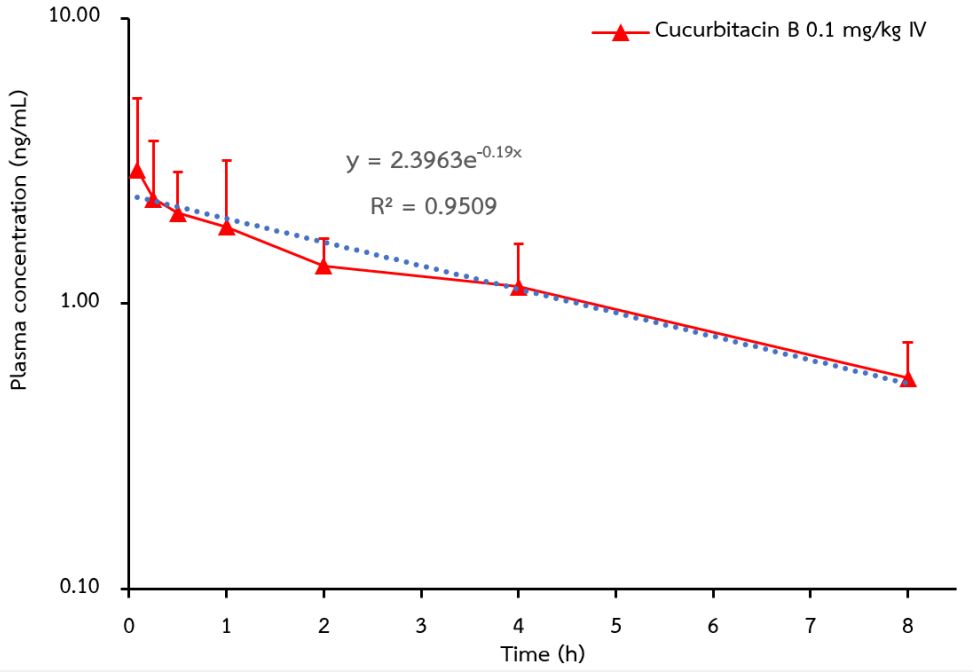

Figure 14 Least square linear regression analysis applying for mean plasma concentration-time curve of cucurbitacin B after intravenous administration at 0.1 mg/kg in logarithm scale. Each data point represents as mean ± SD.

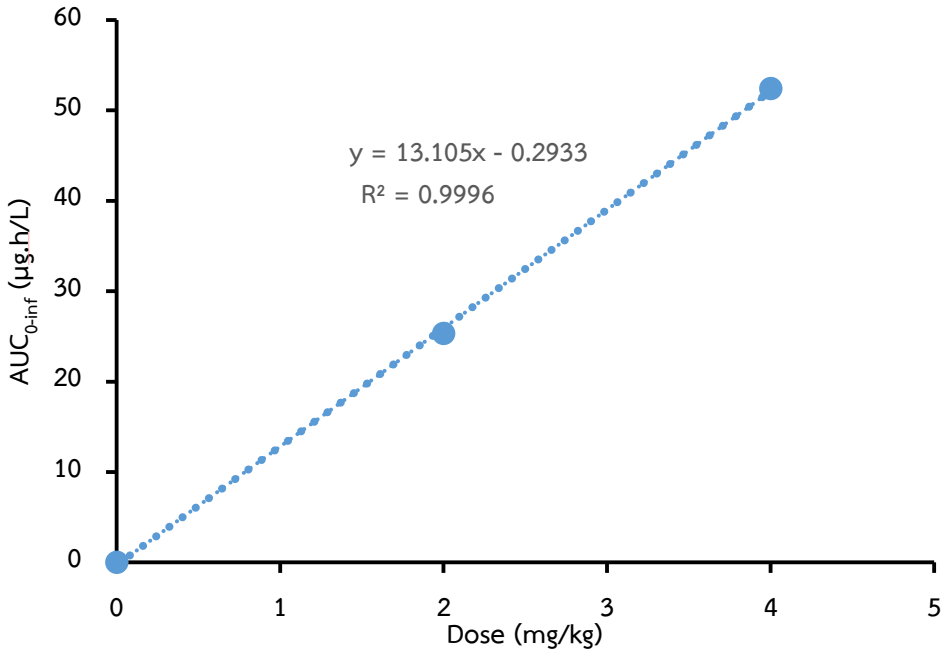

Figure 15 Relationship between oral cucurbitacin doses and their respective $AUC_{0\text{-}inf}$

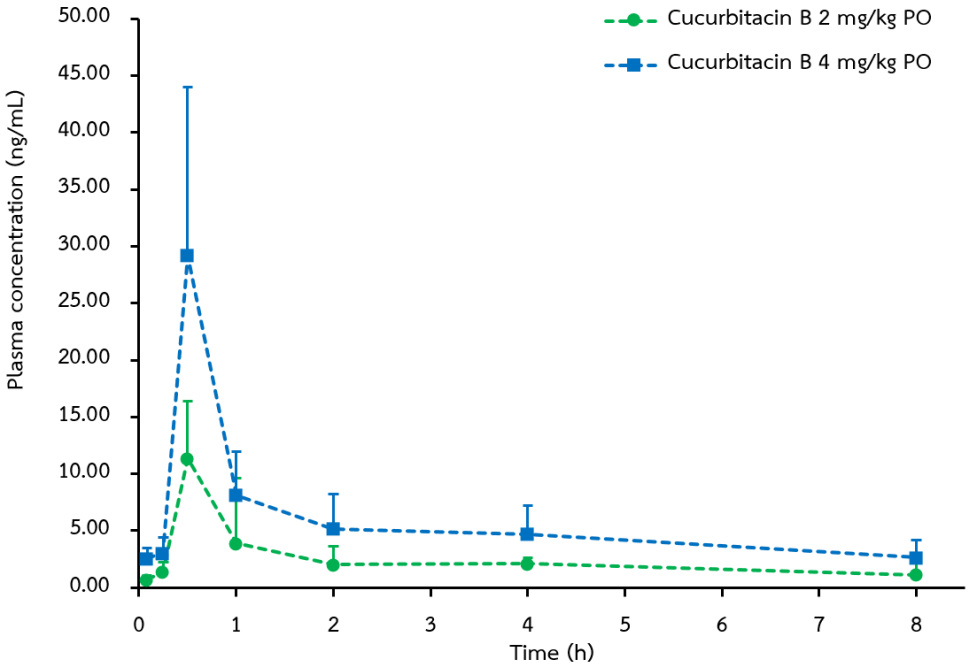

Figure 16 Mean plasma concentration-time curves of cucurbitacin B after oral administration at 2 mg/kg and 4 mg/kg. Each data point represents as mean ± SD.

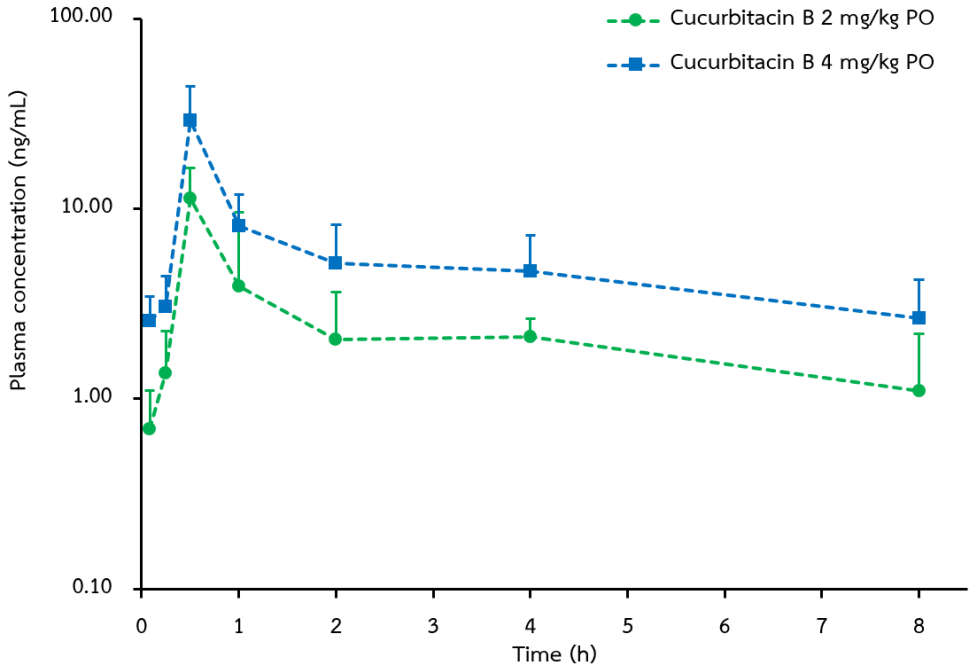

Figure 17 Mean plasma concentration-time curves of cucurbitacin B after oral administration at 2 mg/kg and 4 mg/kg in logarithm scale. Each data point represents as mean ± SD.

Table 16 Pharmacokinetic parameters of cucurbitacin B

Parameters	Cucurbitacin B 0.1 mg/kg IV	Cucurbitacin B 2 mg/kg PO	Cucurbitacin B 4 mg/kg PO
C_{max} (µg/L)	N/A	9.70 ± 3.95	31.24 ± 10.50
T_{max} (h)	N/A	0.50 ± 0.00	0.60 ± 0.22
AUC_{0-t} (µg·h/L)	13.92 ± 11.11	15.10 ± 3.57	45.22 ± 10.14
AUC_{0-inf} (µg·h/L)	17.95 ± 13.21	25.33 ± 12.13	52.42 ± 29.58
Normalized AUC_{0-inf} (µg·h/L)	179.50 ± 132.14	12.67 ± 6.07	13.11 ± 7.40
F (%)	N/A	10.25 ± 7.29	10.25 ± 5.63
V_d (L/kg)	51.65 ± 39.16		
CL (L/h/kg)	7.24 ± 2.92		
$t_{1/2}$ (h)	5.08 ± 2.87		
MRT (h)	6.03 ± 2.93	9.95 ± 12.27	5.50 ± 2.28

The values represent as mean ± SD (n=6). C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC_{0-t}, area under the curve from time zero to last sampling time point; AUC_{0-inf}, area under the curve from time zero to infinity; F, absolute oral bioavailability; V_d, volume of distribution; CL, clearance; $t_{1/2}$, elimination half-life; MRT, mean residence time.

4.4 Tissue distribution

Cucurbitacin B had a distribution potential in various organs as demonstrated in Figure 18. The tissue to plasma ratio (K_{app}) was calculated to compare the amount of cucurbitacin B in the organs with that presenting in plasma. The organs, including lung, spleen, and kidney had the highest K_{app}, respectively. At 1 h after intravenous administration, the levels of cucurbitacin B in lung and spleen were approximately 60 times higher than in the plasma and continually increase to approximately 240 times at 2 h. The K_{app} of cucurbitacin B in lung and spleen remained unchanged at 4 h post-injection compared to the 2 h, indicating that cucurbitacin B could deposit in these organs for a long period of time after intravenous administration. Kidney was another important organ that had a high distribution ratio in spite of a slightly lower K_{app} compared to lung and spleen. Other essential organs, including brain, heart, stomach, liver, and small intestine were also examined. Only trivial amounts of cucurbitacin B were detected in brain and heart. The exact amounts of cucurbitacin B in these organs were unable to measure since the concentration, found in the samples was lower than the LLOQ. Lesser ratios of cucurbitacin B in liver, stomach, and small intestine were investigated. Only 4-14 times K_{app} were observed at 1 h after administration. Interestingly, only the ratio found in liver could accumulate to 23.72 and 38.29 in 2 and 4 h, respectively. In contrast, the ratios in stomach and small intestine were not changed dependently with time. The K_{app} in stomach slightly increased, but unfortunately, the values between each time point were not significantly different.

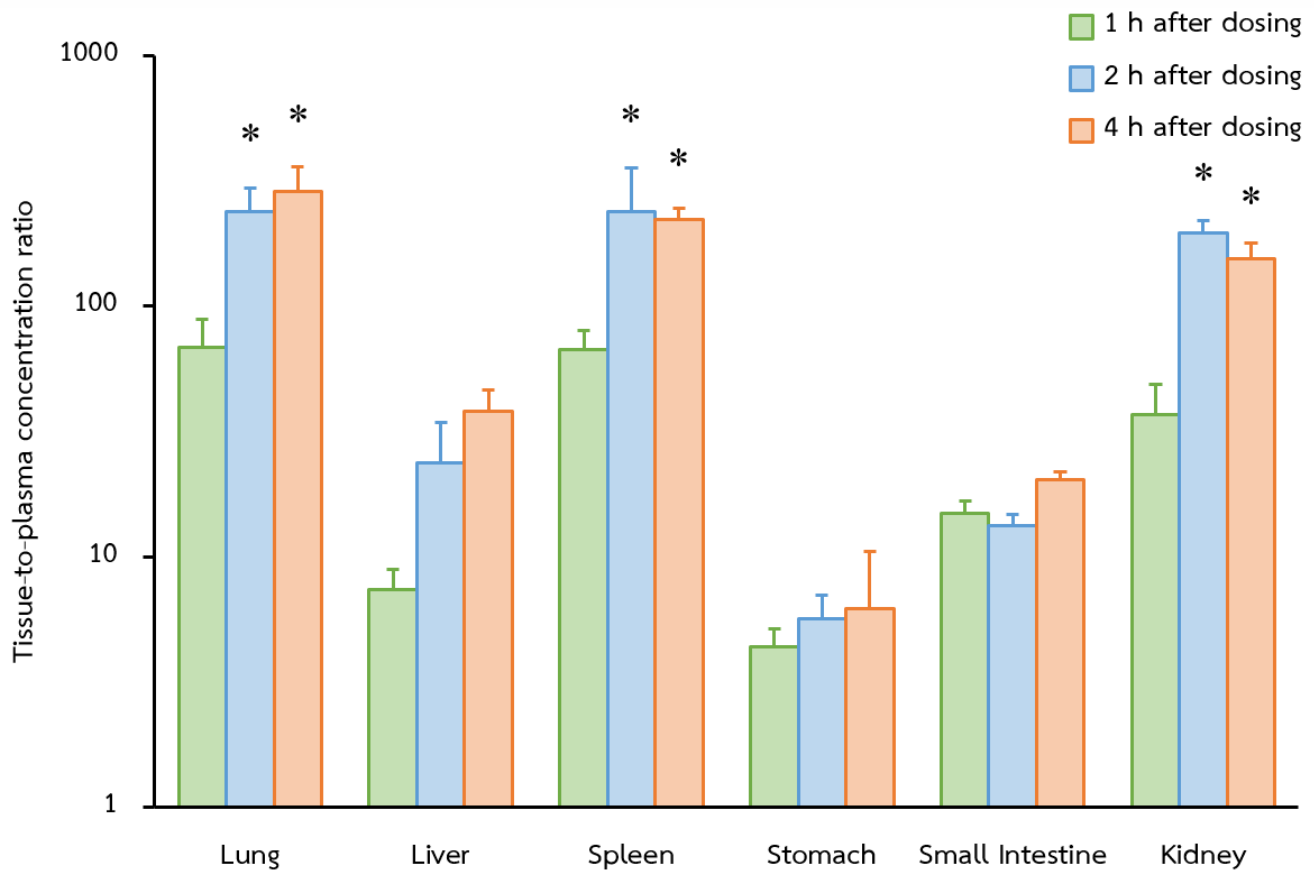

Figure 18 Mean tissue to plasma concentration ratio of cucurbitacin B after intravenous administration at 0.1 mg/kg.The data are represented as mean ± SD. * p-value < 0.05 compared to the mean tissue-to-plasma concentration ratio at 1 h after cucurbitacin B administration.

4.5 Metabolism study

A glucuronidated metabolite of cucurbitacin B was examined, using the β-glucuronidase as an enzyme to catalyze the hydrolysis reaction. The results were demonstrated in Figure 19 and Figure 20 as the glucuronide metabolite, presenting in plasma after intravenous and oral administration, respectively. After comparing the concentration at each time point both in intravenous and oral intervention, we found that there was no statistical difference between $AUC_{0\text{-}inf}$ of cucurbitacin B originally presenting in the plasma and the total cucurbitacin B after β-glucuronidase hydrolysis reaction in any dosing. The comparison between the AUC before and after reaction was demonstrated in Table 17. This suggested that only negligible amount of glucuronidated metabolite was detected in the plasma. In case of urine and feces, β-glucuronidase hydrolysis reaction was also conducted and the results were demonstrated in Table 18. The total glucuronidated metabolite in every excreta sample was less than 1% of the total administered dose, which was in accordance with the plasma profile. This concludes that the molecule of cucurbitacin B do not directly conjugate with the glucuronide moiety. The molecule possibly be biotransformed by other metabolizing step prior to the conjugation via phase II enzyme.

4.6 Excretion study

Urine and feces collected from the animals during the experiment were quantified for the cucurbitacin B level. Negligible amounts of unchanged cucurbitacin B were removed from the animals' bodies as shown in the Table 19. Less than 1% of unchanged cucurbitacin B could be detected in excreta after administration for 0-24 and 24-48 h.

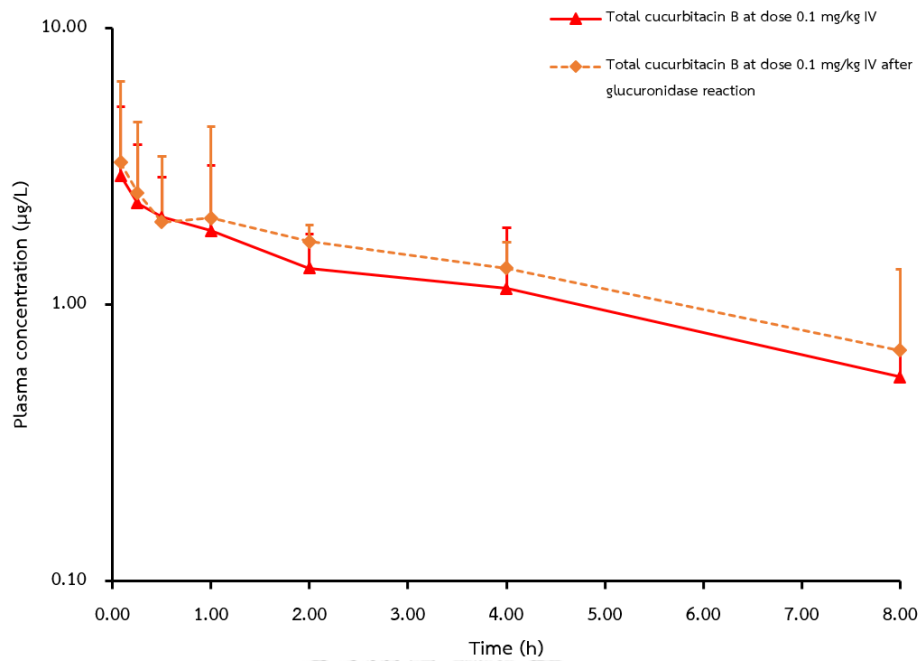

Figure 19 Mean plasma concentration-time curves of cucurbitacin B after intravenous administration at 0.1 mg/kg before and after β-glucuronidase reaction. Each data point represents as mean ± SD.

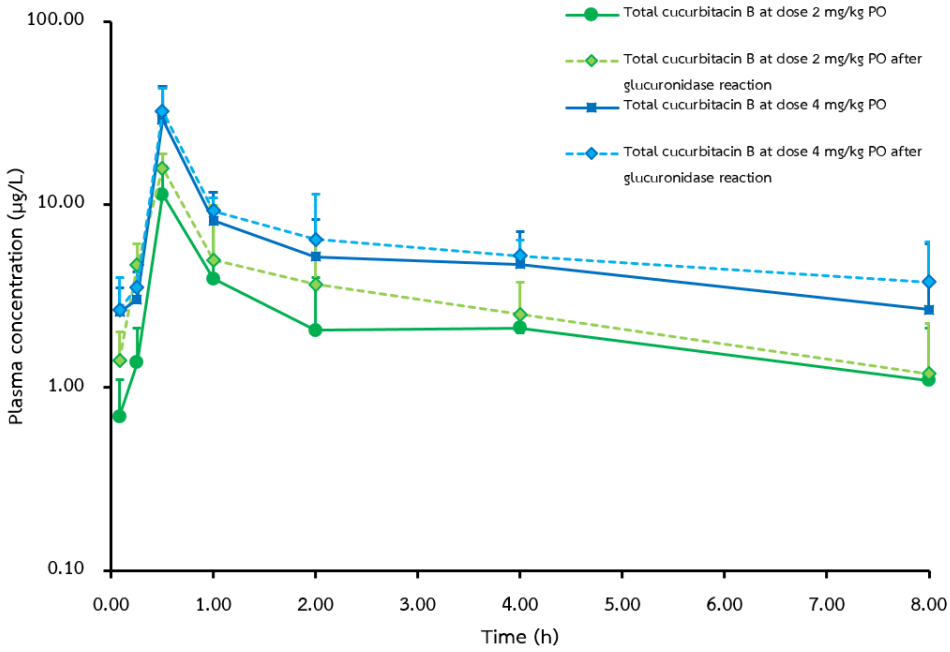

Figure 20 Mean plasma concentration-time curves of cucurbitacin B after oral administration at 2 and 4 mg/kg before and after β-glucuronidase reaction. Each data point represents as mean ± SD.

Table 17 The computed area under the curve of cucurbitacin B before and after β-glucuronidase reaction

Parameters	Cucurbitacin B 0.1 mg/kg IV		Cucurbitacin B 2 mg/kg PO		Cucurbitacin B 4 mg/kg PO	
	Before	After	Before	After	Before	After
AUC_{0-t} (μg·h/L)	13.92 ± 11.11	17.34 ± 13.55	15.10 ± 3.57	20.37 ± 6.41	45.22 ± 10.14	48.65 ± 12.59
AUC_{0-inf} (μg·h/L)	17.95 ± 13.21	21.41 ± 15.74	25.33 ± 12.13	29.14 ± 14.97	52.42 ± 29.58	55.71 ± 30.24

The values represent as mean ± SD (n=6). AUC_{0-t}, area under the curve from time zero to last sampling time point; AUC_{0-inf}, area under the curve from time zero to infinity

Table 18 The percent recovery of cucurbitacin B glucuronide in excreta compared to administered dose

Recovery (%)	Cucurbitacin B 0.1 mg/kg IV	Cucurbitacin B 2 mg/kg PO	Cucurbitacin B 4 mg/kg PO
Urine at 0-24h	< 1.00	< 1.00	< 1.00
Urine at 24-48h	< 1.00	< 1.00	< 1.00
Feces at 0-24h	< 1.00	< 1.00	< 1.00
Feces at 24-48h	< 1.00	< 1.00	< 1.00

Table 19 The percent recovery of unchanged cucurbitacin B in excreta compared to administered dose

Recovery (%)	Cucurbitacin B 0.1 mg/kg IV	Cucurbitacin B 2 mg/kg PO	Cucurbitacin B 4 mg/kg PO
Urine at 0-24h	< 1.00	< 1.00	< 1.00
Urine at 24-48h	< 1.00	< 1.00	< 1.00
Feces at 0-24h	< 1.00	< 1.00	< 1.00
Feces at 24-48h	< 1.00	< 1.00	< 1.00

CHAPTER V
DISCUSSION AND CONCLUSION

Cucurbitacin B was studied for a vast variety of pharmacological effects, which included anti-inflammatory, immunomodulatory, anti-atherosclerotic, hepatoprotective, and particularly anti-neoplastic activity[11]. Further study of pharmacokinetic properties of this compound could provide more information to understand the association between the amount of the compound, presenting in plasma or targeted organs and its therapeutic effects. Currently, the pharmacokinetic profile of cucurbitacin B was investigated, including the absorption, distribution, metabolism and excretion process. The dose of cucurbitacin B was selected on the basis of its anti-neoplastic activity. From the tolerability test, the results demonstrated that the animals could tolerate well with the administered doses. Neither behavioral nor physical change could be observed after administration for 24 h. The AST and ALT levels, which indicate the liver abnormalities were not affected when relatively measured with the baseline level. Even if the AST level was slightly higher in 0.1 mg/kg intravenous and 4 mg/kg oral dosing, it did not affect the clinical status of the animals as the values did not exceed the upper limit of the normal range. ALT level seemed to be a better marker in term of evaluation for liver performance due to the higher specificity[63]. Kidney function was evaluated, using creatinine as a marker and the results showed that there was no difference between pre- and post-dosing. From the results, we concluded that cucurbitacin B did not affect the liver or kidney function after single dose administration for 24 h. However, cucurbitacin B at 4 mg/kg orally showed the effects against gastrointestinal function since the soft stool could be observed at 0-24 h period. Fortunately, this effect was resolved within 48 h after administration.

Pharmacokinetics profile was initially analyzed by determining the plasma concentration of cucurbitacin B at collected time points. The plasma concentration-time profile of cucurbitacin B was constructed and plotted on a semi-log scale. In intravenous data, it was found that cucurbitacin B follows to the one compartment pharmacokinetic model. To prove this hypothesis, the least squares linear regression

analysis was applied to the data set and the result provided a good correlation coefficient with R^2 = 0.951. The elimination half-life was approximately 5.08 ± 2.87 h when calculated from intravenous data. The half-life, obtained from current study was higher than the value from previous reported, which was approximately 2.50-3.19 h[57, 64]. This could be explained by the different in ages and conditions of rodents used in the study. The rodents' age in current study was approximately 2-3 months, which was quite older than the rats, used in previous report. The renal and hepatic functions in rodents were decreased proportionally to the increased age, which might affect the ability to remove xenobiotics from the body[65, 66]. The volume of distribution was also calculated from the intravenous concentration-time curve. The value was accounted for 51.65 ± 39.16 L/kg, which could be expected that cucurbitacin B was highly distributed to several organs and tissue. This might be the reason why only a trivial amount of cucurbitacin B could be detected in plasma samples. For oral administration, cucurbitacin B reached the highest concentration within 30 min. This represented that cucurbitacin B could be absorbed promptly by the gastrointestinal tract. The previous studies reported the T_{max} values as approximately 1.75-2.41 h[57, 64], which were significantly slower than the current report. It is possible that the formulation, used in this study was 40% v/v dimethylsulfoxide solution, which could provide a clear solution for both intravenous and oral preparation. Compared to suspensions prepared by carboxymethylcellulose in other studies, the clear solution was readily absorbed[67], resulting in a shorter T_{max}. Despite the high absorption rate, the total amount of cucurbitacin B that could undergo to the systemic circulation was restricted. This is supported by the absolute oral bioavailability value, which was merely 10% compared to the intravenous dose. This might be explained by reason of incomplete absorption through intestinal lining. First pass metabolism by intestinal or hepatic enzymes before reaching the systemic circulation was another explanation for this phenomenon. Interestingly, the total exposure of cucurbitacin B in the plasma was increased proportionally to the given dose when determined by AUC. The normalized $AUC_{0\text{-}inf}$ of 2 and 4 mg/kg oral dosing were computed and the values were comparable between these two doses. This

pointed that cucurbitacin B possessed a linear pharmacokinetics at a pharmacologically active dose range.

Tissue distribution was investigated by collecting the internal organs at different time points, including 1, 2, and 4 h after intravenous administration. Tissue to plasma concentration ratio (K_{app}) was computed to identify the amount of cucurbitacin B in each organ. The results demonstrated that cucurbitacin B could undergo extensively into lungs, spleen, and kidneys, respectively. These three organs have high blood perfusion rate, which allows the xenobiotics or drug molecules expose extensively and possibly accumulate in the tissue. Moreover, cucurbitacin B has a high lipophilic property (XlogP = 2.6), which also promote the penetration through the cell membrane or high lipid containing tissue. This result could support the *in vitro* study that tested its anti-cancer effect in kidney, and lung cancer cells. Kausar and colleagues reported that cucurbitacin B possessed an anti-proliferative activity by reducing the cell viability of HCC-827 to 25% after incubation at dose of 0.25 μM for 72 h[40]. The maximum concentration of cucurbitacin B, detected in plasma was approximately 29.21 μg/L, after 4 mg/kg oral administration; however, it was insufficient to exert an anti-cancer effect. Fortunately, the cucurbitacin B concentrations found in lungs were exceedingly higher than that presenting in the plasma. An intravenous dose of cucurbitacin B at 0.1 mg/kg showed a proper concentration for lung cancer cells inhibition. Nonetheless, the cucurbitacin B concentration, detected in our study was evaluated in healthy rodents. Physiological changes and microenvironments in cancerous condition might affect the pharmacokinetics of this cucurbitacin B in animal models. Lower K_{app} were observed in liver and small intestine, which might be explained by the biotransformation process occurring in these organs, resulting in a lower amount of cucurbitacin B. Only negligible amounts of cucurbitacin B could be detected in brains and hearts, which were lower than LLOQ. Nevertheless, cucurbitacin B could be remained in the organs at a much higher extent, and seemed to stay longer than the cucurbitacin B, which resided in the plasma. Consequently, the duration of action of cucurbitacin B could not be estimated alone by the elimination half-life. The concentration at the

targeted organs should be taken into consideration, as it might represent a better antineoplastic activity and its duration.

Excretion of unchanged cucurbitacin B was analyzed by calculating the %recovery relative to the administered dose. The results showed that less than 1% of unchanged cucurbitacin B could be detected in both urine and feces at 0-48 h after dosing. It concluded that cucurbitacin B was metabolized into the different form before undergoing to the excretion phase. At present, there was no available cucurbitacin B metabolic pathway, and the glucuronide conjugated cucurbitacin B was proposed as a major metabolite. Other reports suggested the metabolic pathway of other cucurbitacins, including cucurbitacin D and I. They were biotransformed by glucuronidation reactions[40, 68]. Therefore, we focused mainly on a glucuronidated metabolite because we believed that the similarity in chemical structure might undergo the same metabolic pathway. We tested the hypothesis by incubating the plasma and excreta samples with β-glucuronidase from *Escherichia coli* (1,000 units) for 15 min, the reaction was stopped by adding the methanol and then evaluated for unconjugated cucurbitacin B by LC-MS/MS. We found that the $AUC_{0\text{-}inf}$ of cucurbitacin B after β-glucuronidase reaction were not significantly different compared to without a reaction. This proved that cucurbitacin B did not majorly biotransformed by direct glucuronide conjugation. Screening for possible metabolites was also conducted by using Agilent 6540 UHD Accurate Mass TOF LC/MS (Santa Clara, United States) in positive ionization mode. There were some interesting molecules, which were detected in urine and fecal samples, including 835.2322, 319.1372, and 222.0799 Da. The precise matching between the indicated mass and the possible structure is not available at present. Characterization for the possible metabolites will require future study. From current knowledge, we concluded that cucurbitacin B was not metabolized by direct glucuronide conjugation. The molecule might be involved in the complex processes of phase I together with phase II enzymatic reactions.

In conclusion, cucurbitacin B had poor absolute oral bioavailability, which was approximately 10%. The compound could be distributed widely to several organs, which could be the site of action including lungs, spleen, and kidney after intravenous administration. The major metabolic pathway of cucurbitacin B still

remained unclear and the unchanged form was not found in excreta. The pharmacokinetic profile obtained from this study might be beneficial for researchers to strategize and design an appropriate dosage regimen of cucurbitacin B as an antineoplastic agent in future study.

REFERENCES

1. World Health Organization. Cancer 2018 [20 May 2019]. Available from: https://www.who.int/news-room/fact-sheets/detail/cancer.
2. Horton S, Gauvreau CL. Cancer in low- and middle-income countries: an economic overview. Cancer: Disease Control Priorities. 2015;3:263-80.
3. Miedema B, Easley J, Fortin P, Hamilton R, Mathews M. The economic impact on families when a child is diagnosed with cancer. Current Oncology. 2008;15:173.
4. Boyle P, Levin B. World cancer report 2008: IARC Press, International Agency for Research on Cancer; 2008.
5. National cancer control programme committee. National cancer control programme. Bangkok: The Agricultural Co-operative Federation of Thailand; 2013.
6. The American Cancer Society Medical and Editorial Content Team. Early History of Cancer 2018 [7 June 2019]. Available from: https://www.cancer.org/cancer/cancer-basics/history-of-cancer/what-is-cancer.html.
7. Luqmani Y. Mechanisms of drug resistance in cancer chemotherapy. Medical Principles and Practice. 2005;14:35-48.
8. Duyfjes BE, Pruesapan K. The genus Trichosanthes L. (Cucurbitaceae) in Thailand. Thai Forest Bulletin (Botany). 2004;32:76-109.
9. Devendra N, Attard E, Raghunandan D, Seetharam Y. Study on seasonal variation on the content of cucurbitacin of various vegetative parts of *Trichosanthes cucumerina* L. var. cucumerina. International Journal of Plant Research. 2011;1:25-8.
10. Lee DH, Iwanski GB, Thoennissen NH. Cucurbitacin: ancient compound shedding new light on cancer treatment. The Scientific World Journal. 2010;10:413-8.
11. Miro M. Cucurbitacins and their pharmacological effects. Phytotherapy Research. 1995;9:159-68.
12. American Cancer Society. Cancer facts and figures 2017. Atlanta: American Cancer Society; 2017.

13. Gurney JG, Severson RK, Davis S, Robison LL. Incidence of cancer in children in the United States. Sex-, race-, and 1-year age-specific rates by histologic type. Cancer. 1995;75:2186-95.
14. Dal Maso L, Bosetti C, La Vecchia C, Franceschi S. Risk factors for thyroid cancer: an epidemiological review focused on nutritional factors. Cancer Causes & Control. 2009;20:75-86.
15. Kaaks R, Lukanova A, Kurzer MS. Obesity, endogenous hormones, and endometrial cancer risk: a synthetic review. Cancer Epidemiology and Prevention Biomarkers. 2002;11:1531-43.
16. Kelsey JL. A review of the epidemiology of human breast cancer. Epidemiologic Reviews. 1979;1:74-109.
17. Bureau of Drug Control. Total values of drug production categorized by pharmacological class from 2012-2015 Bangkok: Thai FDA; 2015 [7 June 2019]. Available from: http://www.fda.moph.go.th/sites/drug/Shared%20Documents/Statistic/Value-P-ATC55-58.pdf.
18. Bureau of Drug Control. Total values of drug imports categorized by pharmacological class from 2012-2015 Bangkok: Thai FDA; 2015 [7 June 2019]. Available from: http://www.fda.moph.go.th/sites/drug/Shared%20Documents/Statistic/Value-I-ATC55-58.pdf.
19. Key T. Fruit and vegetables and cancer risk. British Journal of Cancer. 2011;104:6.
20. Nirmala MJ, Samundeeswari A, Sankar PD. Natural plant resources in anti-cancer therapy-a review. Research in Plant Biology. 2011;1.
21. Seca A, Pinto D. Plant secondary metabolites as anticancer agents: successes in clinical trials and therapeutic application. International Journal of Molecular Sciences. 2018;19:263.
22. Bernard SA, Olayinka OA. Search for a novel antioxidant, anti-inflammatory/analgesic or anti-proliferative drug: Cucurbitacins hold the ace. Journal of Medicinal Plants Research. 2010;4:2821-6.

23. Molavi O, Ma Z, Mahmud A, Alshamsan A, Samuel J, Lai R, et al. Polymeric micelles for the solubilization and delivery of STAT3 inhibitor cucurbitacins in solid tumors. International Journal of Pharmaceutics. 2008;347:118-27.
24. Sandhya S, Vinod K, Sekhar JC, Aradhana R, Nath VS. An updated review on *Tricosanthes cucumerina* L. International Journal of Pharmaceutical Sciences Review and Research. 2010;1:56-60.
25. Devi ND. Medicinal values of *Trichosanthus cucumerina* L. (snake gourd)-a review. British Journal Of Pharmaceutical Research. 2017;16.
26. Jiratchariyakul W. Anti-cancer agents from natural products. Bangkok: M&M Laserprint; 2558.
27. Nadkarni A. Indian Materia Medica. Bombay: Popular Prakashan; 2002. 371-72 p.
28. Buaprom M. The chemical constituents and biological activities of the fruit fibers of *Trichosanthes cucumerina*: M. Sc. thesis (Applied Chemistry), Ramkhamhaeng University, Bangkok; 2006.
29. Jiratchariyakul W, Frahm A. Cucurbitacin B and dihydrocucurbitacin B from *Trichosanthes cucumerina* L. Mahidol University Journal of Pharmaceutical Sciences. 1992;19:5-12.
30. Kongtun S, Jiratchariyakul W, Kummalue T, Tan-ariya P, Kunnachak S, Frahm AW. Cytotoxic properties of root extract and fruit juice of *Trichosanthes cucumerina*. Planta Medica. 2009;75:839-42.
31. Kaushik U, Aeri V, Mir SR. Cucurbitacins - an insight into medicinal leads from nature. Pharmacognosy Reviews. 2015;9:12.
32. Cai Y, Fang X, He C, Li P, Xiao F, Wang Y, et al. Cucurbitacins: a systematic review of the phytochemistry and anticancer activity. The American Journal of Chinese Medicine. 2015;43:1331-50.
33. National Institutes of Health (NIH). Compound Summary for CID 5281316 2019 [20 May 2019]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/5281316.
34. Xu G, Shi Y. Apoptosis signaling pathways and lymphocyte homeostasis. Cell Research. 2007;17:759.

35. Zhang M, Zhang H, Sun C, Shan X, Yang X, Li-Ling J, et al. Targeted constitutive activation of signal transducer and activator of transcription 3 in human hepatocellular carcinoma cells by cucurbitacin B. Cancer Chemotherapy and Pharmacology. 2009;63:635-42.
36. Aggarwa B, Sethi G, Ahn KS, Sandur S, Pandey MK, Kunnumakkara AB, et al. Targeting signal transducer and activator of transcription-3 for prevention and therapy of cancer. Annals of the New York Academy of Sciences. 2006;1091:151-69.
37. Liu T, Zhang M, Zhang H, Sun C, Deng Y. Inhibitory effects of cucurbitacin B on laryngeal squamous cell carcinoma. European Archives of Oto-rhino-laryngology. 2008;265:1225-32.
38. Zheng Q, Liu Y, Liu W, Ma F, Zhou Y, Chen M, et al. Cucurbitacin B inhibits growth and induces apoptosis through the JAK2/STAT3 and MAPK pathways in SH-SY5Y human neuroblastoma cells. Molecular Medicine Reports. 2014;10:89-94.
39. Yasuda S, Yogosawa S, Izutani Y, Nakamura Y, Watanabe H, Sakai T. Cucurbitacin B induces G2 arrest and apoptosis via a reactive oxygen species-dependent mechanism in human colon adenocarcinoma SW480 cells. Molecular Nutrition & Food Research. 2010;54:559-65.
40. Kausar H, Munagala R, Bansal SS, Aqil F, Vadhanam MV, Gupta RC. Cucurbitacin B potently suppresses non-small-cell lung cancer growth: identification of intracellular thiols as critical targets. Cancer Letters. 2013;332:35-45.
41. Lee DH, Thoennissen NH, Goff C, Iwanski GB, Forscher C, Doan NB, et al. Synergistic effect of low-dose cucurbitacin B and low-dose methotrexate for treatment of human osteosarcoma. Cancer letters. 2011;306(2):161-70.
42. Duangmano S, Dakeng S, Jiratchariyakul W, Suksamrarn A, Smith DR, Patmasiriwat P. Antiproliferative effects of cucurbitacin B in breast cancer cells: down-regulation of the c-Myc/hTERT/telomerase pathway and obstruction of the cell cycle. International Journal of Molecular Sciences. 2010;11:5323-38.

43. Chan KT, Meng FY, Li Q, Ho CY, Lam TS, To Y, et al. Cucurbitacin B induces apoptosis and S phase cell cycle arrest in BEL-7402 human hepatocellular carcinoma cells and is effective via oral administration. Cancer Letters. 2010;294:118-24.

44. Zhou X, Yang J, Wang Y, Li W, Li-Ling J, Deng Y, et al. Cucurbitacin B inhibits 12-O-tetradecanoylphorbol 13-acetate-induced invasion and migration of human hepatoma cells through inactivating mitogen-activated protein kinase and PI3K/Akt signal transduction pathways. Hepatology Research. 2012;42:401-11.

45. Liu T, Peng H, Zhang M, Deng Y, Wu Z. Cucurbitacin B, a small molecule inhibitor of the Stat3 signaling pathway, enhances the chemosensitivity of laryngeal squamous cell carcinoma cells to cisplatin. European Journal of Pharmacology. 2010;641:15-22.

46. Iwanski GB, Lee DH, En-Gal S, Doan NB, Castor B, Vogt M, et al. Cucurbitacin B, a novel in vivo potentiator of gemcitabine with low toxicity in the treatment of pancreatic cancer. British Journal of Pharmacology. 2010;160:998-1007.

47. Sadzuka Y, Hatakeyama H, Sonobe T. Enhancement of doxorubicin concentration in the M5076 ovarian sarcoma cells by cucurbitacin E co-treatment. International Journal of Pharmaceutics. 2010;383:186-91.

48. Wakimoto N, Yin D, O'Kelly J, Haritunians T, Karlan B, Said J, et al. Cucurbitacin B has a potent antiproliferative effect on breast cancer cells in vitro and in vivo. Cancer Science. 2008;99:1793-7.

49. Yesilada E, Tanaka S, Sezik E, Tabata M. Isolation of an anti-inflammatory principle from the fruit juice of *Ecballium elaterium*. Journal of Natural Products. 1988;51:504-8.

50. Siqueira Jr JM, Peters RR, Gazola AC, Krepsky PB, Farias MR, Rae GA, et al. Anti-inflammatory effects of a triterpenoid isolated from *Wilbrandia ebracteata* Cogn. Life Sciences. 2007;80:1382-7.

51. Peters RR, Farias MR, Ribeiro-do-Valle RM. Anti-inflammatory and analgesic effects of cucurbitacins from *Wilbrandia ebracteata*. Planta Medica. 1997;63:525-8.

52. Jayaprakasam B, Seeram NP, Nair MG. Anticancer and antiinflammatory activities of cucurbitacins from *Cucurbita andreana*. Cancer Letters. 2003;189:11-6.
53. Ríos JL, Escandell JM, Recio MC. New insights into the bioactivity of cucurbitacins. Studies in Natural Products Chemistry. 32: Elsevier; 2005. p. 429-69.
54. Musza LL, Speight P, McElhiney S, Barrow CJ, Gillum AM, Cooper R, et al. Cucurbitacins, cell adhesion inhibitors from Conobea scoparioides. Journal of Natural Products. 1994;57:1498-502.
55. Storrs A, Piearce G. " Don't eat these". A guide to some local poisonous plants and poisonous mushrooms. 1982.
56. Gupta P, Srivastava SK. Inhibition of HER2-integrin signaling by cucurbitacin B leads to in vitro and in vivo breast tumor growth suppression. Oncotarget. 2014;5:1812.
57. Zhao W, Xu D, Yan W, Wang Y, Zhang N. Development and validation of a UPLC-MS/MS method for the determination of cucurbitacin B in rat plasma and application to a pharmacokinetic study. Biomedical Chromatography. 2016;30:503-7.
58. Xu X, Deng Z, Fan H, Zhong H, Yao Q. Study on in vivo pharmacokinetics of cucurbitacin injection in rats. China Journal of Chinese Materia Medica. 2014;39:2126-30.
59. Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub. 2010;154:103-16.
60. Thackaberry EA, Wang X, Schweiger M, Messick K, Valle N, Dean B, et al. Solvent-based formulations for intravenous mouse pharmacokinetic studies: tolerability and recommended solvent dose limits. Xenobiotica. 2014;44:235-41.
61. Gad SC, Cassidy CD, Aubert N, Spainhour B, Robbe H. Nonclinical vehicle use in studies by multiple routes in multiple species. International Journal of Toxicology. 2006;25:499-521.

62. U.S. food and drug administration. Guidance for industry: bioanalytical method validation. Washington DC: U.S. Department of Health and Human Services; 2018.
63. Giannini EG, Testa R, Savarino V. Liver enzyme alteration: a guide for clinicians. Canadian Medical Association Journal. 2005;172:367-79.
64. Wang Z, Zhu W, Gao M, Wu C, Yang C, Yang J, et al. Simultaneous determination of cucurbitacin B and cucurbitacin E in rat plasma by UHPLC-MS/MS: a pharmacokinetics study after oral administration of cucurbitacin tablets. Journal of Chromatography B. 2017;1065:63-9.
65. Gregory JG, Barrows CH. The effect of age on renal functions of female rats. Journal of Gerontology. 1969;24:321-3.
66. Iwamoto K, Watanabe J, Araki K, Deguchi N, Sugiyama H. Effect of age on the hepatic clearance of propranolol in rats. Journal of Pharmacy and Pharmacology. 1985;37:466-70.
67. Faqi AS. A comprehensive guide to toxicology in preclinical drug development: Academic Press; 2012.
68. Abbas S, Vincourt J-B, Habib L, Netter P, Greige-Gerges H, Magdalou J. The cucurbitacins E, D and I: investigation of their cytotoxicity toward human chondrosarcoma SW 1353 cell line and their biotransformation in man liver. Toxicology Letters. 2013;216:189-99.

VITA

NAME	Natthaphon Hunsakunachai
DATE OF BIRTH	04 February 1991
PLACE OF BIRTH	Phitsanulok
INSTITUTIONS ATTENDED	Graduated from Bangkok Christian College, Mathematics and Sciences program; and completed the Bachelor's program from Chulalongkorn University, Faculty of Pharmaceutical Sciences, majored in Pharmaceutical Care (Pharm.D.).
HOME ADDRESS	99/1, Moo 2, Mookdautit Soi 1, Phothale, Phichit
PUBLICATION	Khemawoot, P., Hunsakunachai, N., Anukunwithaya, T., Bangphumi, K., Ongpipattanakul, B., Jiratchariyakul, W., et. al., Pharmacokinetics of Compound D, the Major Bioactive Component of Zingiber cassumunar, in Rats. Planta medica. 82, 1186-1191.