Pharmacokinetics of Codeine in Mitragynine-pretreated Rats

Hawanee Saca

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Pharmacology
Prince of Songkla University
2019

Pharmacokinetics of Codeine in Mitragynine-pretreated Rats

Hawanee Saca

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology

Prince of Songkla University

2019

Thesis Title Pharmacokinetics of Codeine in Mitragynine-pretreated Rats

Author Miss Hawanee Saca

Major Program Pharmacology

Major Advisor

…….……
(Assoc. Prof. Somsmorn Chittrakan)

Co-advisor:

…….
(Asst. Prof. Dr. Somchai Sriwiriyajan)

…….
(Assoc. Prof. Dr. Juraithip Wungsintaweekul)

Examining Committee:

……...…. Chairperson
(Assoc. Prof. Dr. Verawan Uchaipichat)

…….. Committee
(Assoc. Prof. Somsmorn Chittrakan)

…….. Committee
(Asst. Prof. Dr. Somchai Sriwiriyajan)

……...Committee
(Assoc. Prof. Dr. Juraithip Wungsintaweekul)

…….. Committee
(Dr.Supattra Limsuwanchote)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Pharmacology

….……..
(Prof. Dr.Damrongsak Faroongsarng)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

……Signature
(Assoc. Prof. Somsmorn Chittrakan)
Major Advisor

……Signature
(Miss Hawanee Saca)
Candidate

I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

……Signature
(Miss Hawanee Saca)
Candidate

ชื่อวิทยานิพนธ์	เภสัชจลนศาสตร์ของโคเดอีนในหนูขาวใหญ่ที่ได้รับมิตรากัยนีน
ผู้เขียน	นางสาวฮาวานี สาคอ
สาขาวิชา	เภสัชวิทยา
ปีการศึกษา	2561

บทคัดย่อ

พืชกระท่อม (*Mitragyna speciosa* (Korth.) Havil.) วงศ์ Rubiaceae เป็นพืชท้องถิ่นที่พบได้มากทางภาคใต้ของประเทศไทย มีสารกลุ่มอัลคาลอยด์ที่สำคัญได้แก่ สารมิตรากัยนีน มีฤทธิ์ทางเภสัชวิทยาในการระงับปวด แก้ท้องเสีย และยับยั้งการอักเสบ เป็นต้น แต่อย่างไรก็ตาม สารมิตรากัยนีนในใบกระท่อม ยังมีฤทธิ์ในการยับยั้งการทำงานของเอนไซม์ไซโทโครม พี450 โดยเฉพาะอย่างยิ่ง CYP2D6 ซึ่งเป็นเอนไซม์ที่มีบทบาทในการเมแทบอลิซึมยาโคเดอีน เป็นมอร์ฟีน การได้รับพืชกระท่อมร่วมกับยาโคเดอีน อาจทำให้เกิดการเปลี่ยนแปลงเภสัชจลนศาสตร์ของยาโคเดอีนได้ ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์ เพื่อศึกษาเภสัชจลนศาสตร์ของโคเดอีนในหนูขาวใหญ่ ภายหลังการได้รับมิตรากัยนีน ทำการศึกษาโดยแบ่งหนูขาวออกเป็นสองกลุ่ม กลุ่มละ 13 ตัว หนูกลุ่มควบคุม จะได้รับการเหนี่ยวนำด้วยตัวทำละลาย และหนูกลุ่มทดลองจะได้รับการเหนี่ยวนำด้วยมิตรากัยนีน ขนาด 15 mg/kg โดยการป้อนทางปาก วันละหนึ่งครั้ง ต่อเนื่องเป็นระยะเวลา 7 วัน ภายหลังจากนั้น หนูขาวทั้งสองกลุ่มจะได้รับยาโคเดอีน โดยการป้อนทางปาก ขนาด 25 mg/kg ทำการเก็บเลือดจากเส้นเลือดดำบริเวณปลายหาง ที่เวลา 0 , 5, 10, 15, 30, 45, 60, 90, 120, 180 และ 240 นาที วิเคราะห์พารามิเตอร์ทางเภสัชจลนศาสตร์ด้วยแบบจำลองแบบไม่แบ่งส่วน ผลการศึกษาพบว่าหนูขาวกลุ่มที่ได้รับการเหนี่ยวนำด้วยมิตรากัยนีน มีความเข้มข้นของโคเดอีนในเลือดสูงกว่ากลุ่มควบคุม อย่างมีนัยสำคัญทางสถิติ โดยมีค่า C_{max} เพิ่มขึ้นจาก 1.76 เป็น 8.44 µg/mL ค่า AUC_{4h} และ $AUC_{0\rightarrow\infty}$ เพิ่มขึ้นจาก 193.74 เป็น 774.87 µg·min/mL และ 306.08 เป็น 993.93 µg·min/mL ตามลำดับ ในขณะที่ความเข้มข้นของมอร์ฟีนกลุ่มทดลองต่ำกว่ากลุ่มควบคุม อย่างมีนัยสำคัญทางสถิติ (p<0.05) โดยมีค่า C_{max} ลดลงจาก 2.32 เป็น 0.45 µg/mL และ AUC_{4h} ลดลงจาก 415.42 เป็น 58.03 µg·min/mL ดังนั้นผลการทดลองนี้จึงสามารถสรุปได้ว่า สารมิตรากัยนีนสามารถเปลี่ยนแปลงเภสัชจลนศาสตร์ของโคเดอีนไปจากเดิม โดยเพิ่มการดูดซึมโคเดอีน และลดการแปรรูปเป็นมอร์ฟีน ซึ่งการเปลี่ยนแปลงสันนิษฐานว่าอาจผ่านการยับยั้งการทำงานของเอนไซม์ CYP2D6 ภายในตับ ดังนั้นจึงจำเป็นที่จะต้องทำการศึกษากลไกการยับยั้งต่อไปในอนาคต

Thesis Title	Pharmacokinetics of Codeine in Mitragynine-pretreated rats
Author	Miss Hawanee Saca
Major Program	Pharmacology
Academic	2018

Abstract

Kratom (*Mitragyna speciosa* (Korth.) Havil.) belonging to the family Rubiaceae is the native plant in Southern Thailand. Mitragynine is the major alkaloid from kratom leaves. It has many pharmacological activities such as analgesic, antidiarrheal as well as anti-inflammatory effects. However, it has been reported as CYP450 enzymes inhibitors, especially CYP2D6. CYP2D6 is an enzyme which plays a role in the codeine metabolism to morphine. The combination use of kratom leaves and codeine may alter the pharmacokinetics of codeine. The objective of this study is to investigate the effect of mitragynine on the pharmacokinetics of codeine in mitragynine-pretreated rats. Rats were divided into two groups (n=13 per group). The first was the control group, rats were orally pretreated with the vehicle and another group was pretreated once a day with 15 mg/kg of mitragynine for seven consecutive days. After mitragynine or vehicle pretreatments, rats were administered single oral dose of codeine (25 mg/kg). Blood was collected from the tail vein at 0 before, and after 5, 10, 15, 30, 45, 60, 90, 120, 180, and 240 min of codeine administration. The plasma concentration versus time profile of codeine and morphine were analyzed by non-compartment pharmacokinetics model. Compared with the control group, the result showed that plasma concentration of codeine was significant increase in mitragynine pretreatment group. The relevant pharmacokinetic parameters were as follow: C_{max} from 1.76 to 8.44 μg/mL, AUC_{4h} from 193.74 to 774.87 μg·min/mL and $AUC_{0\rightarrow\infty}$ from 306.08 to 993.93 μg·min/mL (p<0.05). Whereas the plasma concentration of morphine was significant decrease with C_{max} from 2.32 to 0.45 μg/mL and AUC_{4h} from 415.42 to 58.03 μg·h/mL (p<0.05). This study suggested that mitragynine was altered the pharmacokinetics of codeine in rats by increase codeine absorption and decrease codeine

metabolism to morphine. The inhibition of CYP2D6 isoenzyme by mitragynine was postulated, which needs to further confirm this evidence.

ACKNOWLEDGEMENT

In the name of Allah, the Most Beneficent and the Most Merciful. All the praises and thanks be to Allah for guiding me to the straight way, pass during a difficult time and let me met the wonderful people to help me. Firstly, I would like to express my gratitude and sincere appreciation to Assoc. Prof. Somsmorn Chittrakan, my major advisor and Asst. Prof. Dr. Somchai Sriwiriyajan, who is the most important co-advisor, for the helpful suggestion, valuable guidance, and comment throughout this study. Sincere appreciation is extended to Assoc. Prof. Dr. Juraithip Wungsintaweekul, my co-advisor for valuable contribution and advice relation to this study.

Following, I would like to express my grateful thanks to Dr. Supattra Limsuwanchote for valuable assistance and guidance relation to this study. I would like to thanks Mrs. Khemjira Mana and the staff working from Central Lab Thailand for LC-MS/MS equipment supported and I would also like to thank all the participant who sacrificed time and help as part of this study.

Finally, I would like to thanks my beloved family for their support and always beside me. Then, I want to thanks all of my friends who are help, cheer and encouraging me. And, I also want to thank myself for not giving up. This valuable thesis would not be completed without help by the wonderful people as mentioned above.

Hawanee Saca

CONTENTS

LIST OF TABLES

LIST OF TABLES (cont.)

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS AND SYMBOLS

$ab_{1/2}$	Absorption of half-life
AE	Absolute matrix effect
AED	Animal equivalent dose
AUC	Area under the concentration-time curve
C_{max}	Maximum plasma concentration
Cod	Codeine
Cl_t/F	Clearance
cm	Centimeter
CV	Coefficient of variation
CYPs	Cytochromes P450
ESI	Electrospray ionization
ES+	Positive electrospray ionization
F	Bioavailability
g (1)	Earth's gravitational force
g (2)	Gram
h	Hour
h^{-1}	Per hour
HPLC	High performance liquid chromatography
HQC	High quality control
IC_{50}	The half maximal inhibitory concentration
IS	Internal standard
k_a	Absorption rate constant
K_{el} or λ_z	Elimination rate constant
kg	Kilogram
K_i	The inhibition constant
K_m	The correction factor

LIST OF ABBREVIATIONS AND SYMBOLS (cont.)

L	Liter
LC	Liquid chromatography
LD_{50}	Lethal dose, 50%
LLOQ	Lower limit of quantification
LQC	Low quality control
m	Meter
mg	Milligram
MG	Mitragynine
min	Minute
mL	Milliliter
mol	Mole
MQC	Median quality control
MS	Mass spectrometer
M-3-G	Morphine-3-glucuronide
M-6-G	Morphine-6-glucuronide
m/z	Mass to charge ratio
ng	Nanogram
nm	Nanometer
ppm	Part per million
QC	Quality control
RE	Relative matrix effect
r^2	The correlation coefficient
SD	Standard deviation
SPE	Solid phase extraction
T_{max}	Time to maximum concentration
TLC	Thin layer chromatography

LIST OF ABBREVIATIONS AND SYMBOLS (cont.)

$t_{1/2}$ or $t_{1/2\,\lambda z}$	Elimination half-life
UGT	Uridine 5'-diphospho-glucuronosyltransferase
ULOQ	Upper limit of quantitation
UV	Ultraviolet
V	Volt
V_z/F	Volume of distribution
v/v	Volume by volume
λ_z	Elimination rate constant
%	Percent
µg	Microgram
µL	Microliter
µM	Micromolar
µ-	Mu
δ-	Delta
κ-	Kappa
^oC	Degree celsius

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Kratom (*Mitragyna speciosa* (Korth.) Havil.) belongs to the family Rubiaceae. It is a native plant of Thailand and Malaysia, which can be found in tropical and sub-tropical regions of Southeast Asia. In Thai traditional medicine, the leaves have been used to treat intestinal infections, diarrhea, muscle pain, cough, and hypertension. The Thai natives widely used as a stimulant to strengthen and sun tolerant, especially in laborers by chewing fresh leaves. However, there are some side effects such as anorexia, dry-mouth, diuresis, and constipation. The withdrawal symptoms are found in users who consume kratom leaves for a long time. Those symptoms including hostility, aggression, aching of muscles and bones, jerky movements of the limbs, anorexia to weight loss, and insomnia (Suwanlert, 1975)

Kratom has been classified in Category V of the Thai Narcotics Act B.E. 2522. In 2016, the Office of the Narcotics Control Board (ONCB) surveyed a size estimation of the substance user population in southern Thailand. They found kratom is the most popular substance abuse, the second and third are boil kratom leaves and marijuana, respectively (Assanangkornchai et al., 2016). In the south of Thailand, kratom is not comprehended as illegal drugs, but it is also part of the way of life likewise traditionalism. They usually use by chewing fresh leaves, brewing with hot water and drinking as a tea. Nevertheless, now the teenagers drink kratom cocktail (4 x 100), which considers an addictive substance. The cocktail is a mixture of boiled kratom leaves with coke, antitussive syrup, coffee or codeine, etc. (Chittrakarn, Penjamras, & Keawpradub, 2012).

Over 40 alkaloids were isolated from kratom leaves, especially indole alkaloids. The major alkaloid is mitragynine and its minor alkaloids are speciogynine, paynantheine, and speciociliatine, etc. There are many pharmacological activities such as analgesic, antidiarrheal, anti-diabetes, anti-depressant as well as anti-inflammatory

effects. However, it has been reported as CYP450 enzyme inhibitors. The inhibition of this enzyme may contribute to drug interaction.

Drug interaction is a modification of drug effect by another combined drug, herbals or some foods. The combination probably alters the pharmacokinetics or pharmacodynamics of the main drug. This reaction may cause adverse effects or ineffective in clinical used. Many cases of drug interaction are caused by pharmacokinetic interaction, which can occur in several steps including absorption, distribution, metabolism, and/or excretion. According to the clinical pharmacology studies, most drug interactions were metabolism processes, particularly drug metabolism through CYP450 enzymes. These interactions can alter drug concentration in the plasma, either increase or decrease drug levels.

CYP450 is an enzyme system present in the human liver microsome. It is a very important role in the metabolism of endogenous and exogenous compounds especially drugs and xenobiotics. There are many isoforms present in human, only 15 isoforms involve drug and xenobiotic metabolism (Pelkonen et al., 2008). Almost of the metabolism become ineffective metabolite. However, some metabolites are effective more than the parent drugs, it is called prodrug. Among the important prodrugs is codeine.

Codeine is an opioid analgesic. It used for relieving mild to moderate degrees of pain. The potential analgesic activity of codeine is affected by morphine. Morphine is codeine CYP2D6 catalyzed O-demethylation. Although, O-demethylation present about 0-15% from codeine metabolism pathways but it becomes morphine which is higher affinity on μ-opioid receptor than codeine. The other metabolism pathways are N-demethylation (10-15%) through CYP3A4 become norcodeine and glucuronidation (50-70%) through UGT2B7 and UGT2B4 become inactive form as codeine-6-glucuronide (C-6-G), further excrete via kidney (Thorn, Klein, & Altman, 2009).

The most of CYP2D6 isoenzyme present in the liver microsome. The activity of this enzyme can be induced and inhibited by CYP2D6 substrates, further alter drug metabolism and result in drug-drug interaction. Quinidine is CYP2D6 inhibitor, it showed inhibits CYP2D6 isoenzyme in human liver microsome with an IC_{50} value was 3.6 μM and K_i value was 0.6 μM (Kobayashi et al., 1989). The combination of codeine and quinidine

has decreased the transformation of codeine to morphine by increase plasma codeine concentration and decrease plasma morphine concentration (Sindrup et al., 1992). Moreover, in 2008-2009 there are 68 cases death report related to the combination of codeine and CYP2D6 inhibitors in Toronto, Canada. The post-mortem blood was assessed and showed plasma concentration of codeine was higher than morphine (Lam et al., 20014).

Since the previous study demonstrated that kratom leaves extracts exerted an inhibitory effect on CYP2D6 isoenzyme activity *in vitro* model. The methanolic extract, alkaloid extract as well as mitragynine showed the inhibition on CYP2D6 isoenzyme with an IC_{50} value was 3.6 µg/mL, 0.636 µg/mL, and 0.45 µM, respectively (Hanapi, Azizi, Ismail, & Mansor, 2010; Kong et al., 2011; Ismail, Mansor, & Hanapi, 2013). Mitragynine is the major compound and showed the strongest inhibitor on CYP2D6 isoenzyme. The combination of kratom with the CYP2D6 substrate such as codeine may decrease the concentration of morphine in plasma and lead to low analgesic activity. However, the pharmacokinetic interaction between mitragynine and codeine has not been reported *in vivo* model. Therefore, this study was performed by orally administered mitragynine, repeated for 7 days. Then orally administered codeine as a single oral dose. The pharmacokinetics of codeine and its metabolite as morphine were analyzed using non-compartment model. The result of this study can be useful data for the prevention of drug-drug interaction between mitragynine and codeine in clinical use.

1.2 LITERATURE REVIEWS

1.2.1 *Mitragyna speciosa* (Korth.) Havil.

1.2.1.1 Botanical characteristics

M. speciosa (Korth.) Havil. (kratom) belongs to the family Rubiaceae. It can grow in the tropical and sub-tropical regions of Southeast Asia. In Thailand, it can be found throughout the country but mostly found in the southern part. Kratom is a medium-sized tree that can reach a height of 10-15 m and 5 m wide. The stem is straight

and branching. The simple leaves are dark green and glossy, arranged in an opposite pattern with interpetiolar stipules. The leaves an ovate-acuminate shape, obtuse base, entire edge, a length of 8-14 cm and a wide of 5-10 cm. Two kinds of the leaf have been provided by the vein colors including red-vein and green-vein. The red-vein assumed to have stronger biological activities than the green one. The flowers are yellow and its seeds are winged (Chittrakarn, Sawangjaroen, Prasettho, Janchawee, & Keawpradub, 2008; Keawpradub, 1990; Shellard, 1974; Shellard & Lees, 1965) (Fig. 1.1).

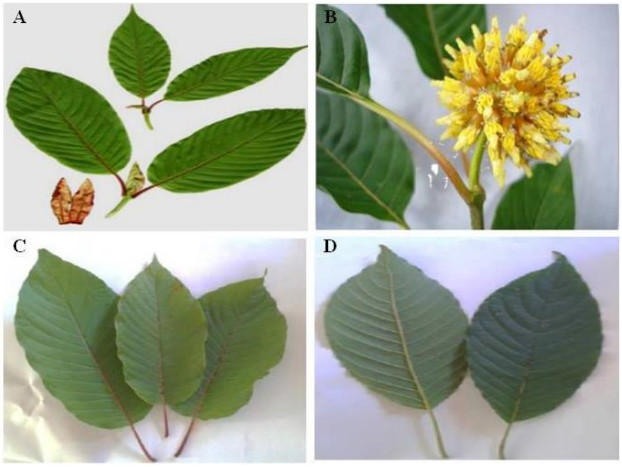


Fig. 1.1 Botanical characteristics of *Mitragyna speciosa* (Korth.) Havil. A) ovate-acuminate leaves shape with interpetiolar stipules, B) inflorescences, C) red-veined leaves, D) green-veined leaves. (Assanangkornchai et al., 2013; Wungsintaweekul, 2017)

1.2.1.2 Traditional use

The leaf of kratom is a traditional Thai herbal medicine. The Thai native commonly used to treat intestinal infections, diarrhea, muscle pain, caught, and hypertension (Suwanlert, 1975). Regularly, it also used as a stimulant for strengthening and sun tolerance in hard workers (Grewal, 1932). In the original, it was used by chewing fresh leaves alone with free veins or adding bitter salt ($MgSO_4$) to prevent constipation and also used by smoking, boiling as well as drinking as a tea. (Hassan et al., 2013). Furthermore, kratom has been reported as opium-like effects, but it is weaker

and shorter duration of action than opium. So, it was used to opium substitution during drug withdrawal (Norakanphadung, 1966). However, there are side effects including anorexia, dry-mouth, diuresis, and constipation. The withdrawal symptoms acquired in chronic users with high dose, it was consisted of aggression, hostility, aching of muscles and bones, jerky movements of the limbs, anorexia to weight loss, and insomnia (Suwanlert, 1975)

1.2.1.3 Chemical constituents

Over 40 alkaloids were isolated from kratom leaves, namely mitragy nine, dihydrocorynantheidine, corynantheidine, mitraphylline, speciogynine, paynan theine, isomitraphylline, ajmalicine, speciociliatine, speciophylline, speciofoline, rhyn chophylline, 3-dehydromitragynine, mitragynalinic acid, corynantheidinalinic acid, 7α-hydroxy mitragynine, (+)-pinoresinol, 3,4,5,6 dehy-dromitragynine, mitralactonal, mi trasulgynine, mitragynaline, corynantheidaline, 9-methoxymitralactonine, mitralac tonine, 7-hydroxyspeciociliatine, mitraciliatine, 3-isopaynantheine, corynoxine, cory noxeine, isocorynoxeine, isospeciofoline, mitrafoline, isomitrafoline, specionoxeine, isospecionoxeine, ciliaphylline, isorhycho-phylline, mitragynine oxindole B, rhyncho ciline, 3-isoajmalicine, mitrajavine, javaphylline, akuammigine and mitragy nine oxindole A. (Beckett, Shellard, Phillipson, & Lee, 1966b, 1966a; Houghton, Latiff, & Said, 1991; Shellard, Houghton, & Resha, 1978b, 1978a; Shellard & Lees, 1965; Takayama, Kurihara, Kitajima, Said, & Aimi, 1998). The major alkaloid is mitragynine (66%), the second is paynantheine (9%), speciogynine (7%), 7-hydroxy mitragynine (2%) and speciociliatine (1%), respectively (Hassan et al., 2013). The alkaloids content varies on the source, season and age (Adkins, Boyer, & McCurdy, 2011). About 66.2% of mitragynine in alkaloid extract was found from Thai species, while only 12% was found in Malaysian species (Ponglux et al., 1994; Takayama, 2004). The chemical name of mitragynine is 9-methoxy-corynantheidine. Its molecular formula is $C_{23}H_{30}O_4N_2$ and its molecular weight is 398. 503 g/mol. Its character is white amorphous powder and soluble in alcohol, chloroform as well as acetic acid (Hassan et al., 2013) (Fig. 1.2).

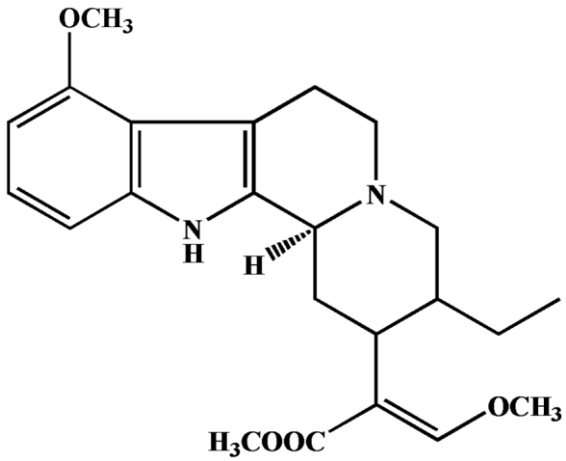


Fig. 1.2 Chemical structure of mitragynine

1.2.1.4 Pharmacological activities

Several studies have demonstrated that kratom leaves extract possessed various pharmacological activities. Those studies showed both *in vitro* and *in vivo* models, including antinociceptive effects, gastrointestinal effects, anti-inflammatory effects as well as anti-psychotic effects.

Antinociceptive effects

There are a number of studies exhibited that kratom leaves extract as well as mitragynine and its derivatives had the antinociceptive activity. Mitragynine displayed as opioid receptor agonists like morphine. There is a high affinity on μ-opioid receptors, but low affinity on δ and κ-opioid receptors. The potential mechanism of action could explain by mitragynine blocked neuronal Ca^{2+} channels, which is present at the supraspinal (Matsumoto et al., 1996a; Thongpradichote et al., 1998; Matsumoto et al., 2005). Its analogue, 7-hydroxymitragynine exhibited stronger analgesic potency than mitragynine (Matsumoto et al., 2006). The molecular docking study showed mitragynine and 7-hydroxymitragynine are partial agonists on human μ-opioid receptors. Whereas, they were a competitive antagonist on δ and κ-opioid receptors (Kruegel et al., 2016). Moreover, the analgesic effects of mitragynine also resulted from

descending noradrenergic and serotonergic pathways, which are different mechanism pathways from morphine (Matsumoto et al., 1996b).

Gastrointestinal effects

Acute and chronic administration of kratom leaf extract become low weight gaining in rats. They decreased food and water intake (Kumarnsit, Keawpradub, & Nuankaew, 2006). According to previous studies, it had been demonstrated that mitragynine inhibited gastric acid secretion via the inhibition of the κ-opioid receptor (Tsuchiya et al., 2002). Whereas, the methanolic extract of kratom exhibited antidiarrheal in castor oil-induced diarrhea in rats by decrease intestinal transit. This mechanism not related to opioid receptors and also cholecystokinin (Chittrakarn et al., 2008). In addition, the extraction of kratom leaves increased glucose uptake in muscle cells by increased GLUT1 protein content, which is the protein level of glucose transporter. This effect may result in anti-diabetes (Purintrapiban et al., 2011).

Anti-inflammatory effect

Kratom extract suppressed inflammation in carrageenan-induced paw edema in rats with extreme inhibition during three h after dosing. It could inhibit pro-inflammatory and vascular permeability via the arachidonic pathway. This mechanism associated with the immune system and the tissue healing process (Mossadeq et al., 2009). Accordingly, a recent study showed mitragynine inhibits COX-2 mRNA and protein expression as well as prostaglandin E_2 (PGE_2) formation in RAW 264.7 macrophage cell (Utar, Majid, Adenan, Jamil, & Lan, 2011).

Neurophysiological effects

Since 1932, Grewal mentioned kratom leaves extract effect on the central and autonomic nervous system (Grewal, 1932). Mitragynine showed anti-depressant properties by the restoration of monoamine neurotransmitters and reducing corticosterone concentration. The concentration of corticosterone decreased via the interaction with the neuroendocrine hypothalamic-pituitary-adrenal axis (Idayu et al., 2011). Chronic intraperitoneal administration mitragynine could reduce cognitive function by decrease locomotors activity and object recognition (Apryani, Hidayat,

Moklas, Fakurazi, & Idayu, 2010; Hazim, Mustapha, & Mansor, 2011). Whereas acute oral methanolic extracts of kratom improved short-term memory, but no benefit on long-term memory (Senik, Mansor, KJ, & Abdullah, 2012).

Other effects

Kratom leave extract exhibited antioxidant and antimicrobial activity. For the antioxidant activity, the methanolic extract showed stronger activity than water and alkaloid extract. On the other hand, the alkaloid extracts had the most effective for antimicrobial activity. The organisms that resisted the compose of *Salmonella typhi* and *Bacillus subtilis* with the minimum inhibitory concentrations (MICs) range of 3.12 - 6.25 mg/mL (Parthasarathy et al., 2009).

In addition, methanolic extract of kratom and its pure compound mitragynine also produced muscle relaxant. Its mechanism could explain by blockage nerve action potential, such as blocked nerve conduction, amplitude and duration of action at the neuromuscular junction (Chittrakarn, Keawpradub, Sawangjaroen, Kansenalak, & Janchawee, 2010).

1.2.1.5 Toxicological effects

Acute oral toxicity studies of methanolic and alkaloids extract from Thai kratom leaves have been investigated in mice model. They found the LD_{50} value of methanolic extract was 4.90 g/kg and alkaloid extract was 173.20 mg/kg (Reanmongkol, Keawpradub, & Sawangjaroen, 2007). While, Sabetghadam and colleagues (2013) reported that the LD_{50} values of alkaloids extract and mitragynine from Malaysian kratom leaves were 591.6 mg/kg and 477.1 mg/kg, respectively (Sabetghadam, Navaratnam, & Mansor, 2013). The lethal dose of alkaloids and mitragynine was 200 mg/kg (Hassan et al., 2013).

The single oral dose of standardized methanolic extracts from kratom leaves at 100, 500, and 1,000 mg/kg were observed in rats for 14 days. The results showed no mortality, normal behavior and normal food and water consumption. However, they found an increase in systolic blood pressure, as well as biochemical parameters such as ALT, AST, albumin, triglyceride, and cholesterol but the creatinine

value was increased only at the highest dose. So, this study indicated that kratom leaves extracts were hepatotoxicity and nephrotoxicity (Harizal, Mansor, Hasnan, Tharakan, & Abdullah, 2010).

Sub-chronic toxicity was reported by oral administered mitragynine at 1, 10, and 100 mg/kg once per day for 28 days. The results showed no mortality at all and no toxic effects in low and intermediate doses groups. Whereas the highest dose, they found an increased liver weight in both male and female groups, while body weight loss was found only females groups (Sabetghadam, Ramanathan, Sasidharan, & Mansor, 2013).

1.2.1.6 Pharmacokinetics

The Pharmacokinetics studies of mitragynine were demonstrated both *in vitro* and *in vivo* models. *In vitro* model, mitragynine showed intermediate intestinal absorption with low bioavailability and unstable in the simulated gastric fluid. It has a lipophilic property and can penetrate through the blood-brain barrier to CNS. There is high plasma protein binding (>90%), which associated with a high volume of distribution. The metabolism is stable in human liver microsome, which could describe long elimination half-life ($t_{1/2}$). Moreover, mitragynine showed the inhibition on P-glycoprotein. The inhibition may become drug interaction when co-administered with P-glycoprotein substrates (Manda et al., 2014).

According to *in vivo* model, there are three studies related to the pharmacokinetics of mitragynine in the rat model. Firstly, Janchawee and colleagues have demonstrated the pharmacokinetics of mitragynine after a single oral dose in rats (40 mg/kg) using HPLC-UV detector. The pharmacokinetic parameters of mitragynine showed the maximum plasma concentration (C_{max}) of 0.63 ± 0.18 μg/mL at the time to reach maximum plasma concentration (T_{max}) of 1.83 ± 1.25 h with an absorption rate constant (k_a) of 1.43 ± 0.90 h^{-1}. The volume of distribution (V_z/F) of 89.50 ± 30.3 L/kg. The elimination was slow with an elimination rate constant (λ_z) of 0.07 ± 0.01 h^{-1}, elimination half-life ($t_{1/2}$) was 9.43 ± 1.74 h, total clearance (Cl_t/F) was 1.60 ± 0.58 L/h and the area under the plasma concentration versus time curve from time zero to infinity ($AUC_{0\rightarrow\infty}$) was 6.99 ± 2.93 μg·h/mL (Janchawee et al., 2007). Secondly, de Moraes and

colleagues demonstrated the pharmacokinetics of mitragynine after a single oral dose of 20 mg/kg in rats using LC-MS/MS analysis. The pharmacokinetic parameters showed C_{max} of 423.68 ± 61.79 ng/mL at T_{max} of 1.26 ± 0.20 h with k_a value of 0.04 ± 0.01 min^{-1}. The value of $AUC_{0\rightarrow\infty}$ was 188.98 ± 19.40 μg·min/mL and the value of V_z/F, λ_z, $t_{1/2}$ as well as Cl_t/F were 37.90 ± 5.41 L/kg, 0.18 ± 0.02 h^{-1}, 3.85 ± 0.51h, 6.35 ± 0.43 L/h/kg, respectively (de Moraes, Moretti, Furr, McCurdy, & Lanchote, 2009). Finally, Parthasarathy and colleagues determined mitragynine level in rat plasma after oral and intravenous administration using rapid HPLC-UV analysis. After 1.5 mg/kg of intravenous administration showed, C_{max} was 2.3 ± 1.2 μg/mL at T_{max} was 1.26 ± 1.1 h, $AUC_{0\rightarrow\infty}$ value was 9.2 ± 6.5 μg h/mL. The values of V_d, $t_{1/2}$ and CL were 0.79 ± 0.42 L/kg, 2.9 ± 2.1 h and 0.29 ± 0.27 L/h/kg, respectively. After oral administration of 50 mg/kg of mitragynine showed C_{max} was 0.70 ± 0.21 μg /mL at T_{max} 4.5 ± 3.6 h. The $AUC_{0\rightarrow\infty}$ was 8.2 ± 3.0 μg h/mL. The values of V_z/F, $t_{1/2}$ and Cl_t/F were 64 ± 23 L/kg, 6.6 ± 1.3 h and 7.0 ± 3.0 L/h kg, respectively. The bioavailability was 3.03 ± 1.47 % (Parthasarathy et al., 2010).

Recently, the pharmacokinetics of mitragynine in humans has been investigated in chronic, regular and healthy users. The results were different from animal studies. The pharmacokinetic parameters showed T_{max} was 0.83 ± 0.35 h, the value of $t_{1/2}$, V_z/F and CL were 23.24 ± 16.07 h, 38.04 ± 24.32 h, and 98.1 ± 51.34 L/kg, respectively (Wananukul et al., 2015).

1.2.1.7 Metabolism

Mitragynine metabolized through phase I and II metabolism. Phase I, it was metabolized by hydrolysis of the methylester in position C16 and O-demethylation in position 9 and 17of methoxy group. Following, the carboxylic acids and alcohol were formed from intermediate aldehydes by oxidation and reduction reaction. Further, they conjugated with glucuronides and sulfate groups. The metabolites were different between human and rat. Three glucuronides and sulfates were presented in humans, while four glucuronides and one sulfate appeared in the rat. The postulated metabolism

pathways were shown in Fig 1.3 Afterward, the metabolites excreted via urine (Philipp et al., 2009).

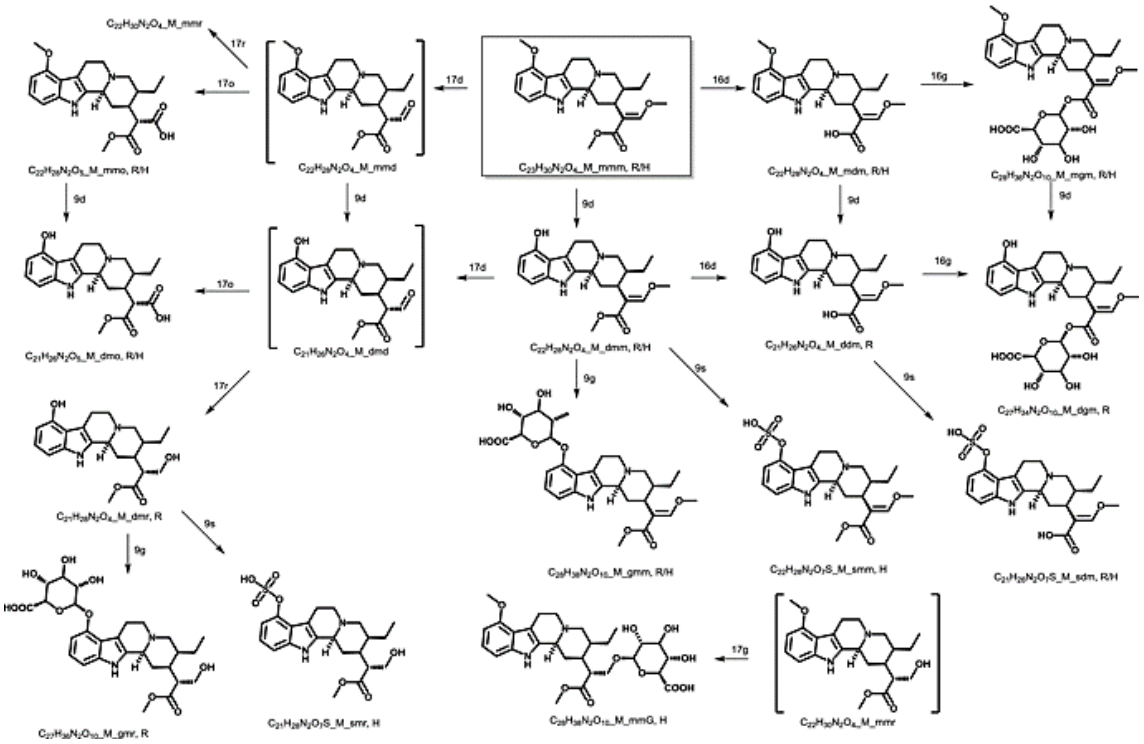


Fig 1.3 The postulated metabolic pathways of mitragynine in rat (R) and human (H) liver microsome using LC-MS/MS method. The assumed intermediate metabolite present in square brackets (Philipp et al., 2009). The abbreviated composed of M (mitragynine), m (methyl group), d (demethylation), g (glucuronidation after d), s (sulfation), o (oxidation), r (reduction), and G (glucuronidation after d) (modified from Limpanuparb, Noorat, & Tantirungrotechai, 2019)

1.2.1.7 Potential interaction on CYP450

There are a number of studies reported that kratom extracts inhibited human recombinant CYP450. The potency of inhibition was clarified by half-maximal inhibitory concentration (IC_{50}) values. The methanol, alkaloids extracts as well as mitragynine showed the potent inhibitory activity against CYP2D6 isoenzyme with IC_{50} values were 3.6 ± 0.1 μg/mL, 0.636 μg/mL, and 0.45 ± 0.33 μM, respectively. Alkaloid extracts exhibited competitive inhibition, while mitragynine showed non-

competitive inhibition on CYP2D6 isoenzyme. In addition, kratom extracts also inhibit other CYP450 isoenzymes besides CYP2D6 as shown in Table 1.1 (Hanapi et al., 2010; Kong et al., 2011; Ismail et al., 2013). Therefore, kratom may alter the pharmacokinetics of drugs that catalyzed through CYP450 isoenzymes, such as the substrate of CYP2D6, CYP3A4, and CYP2C9, when co-administered and might lead to drug interaction.

Table 1.1 Inhibitory effect of kratom extracts on CYP450

Kratom extracts	**CYP450 isoenzymes**	**IC_{50} values**	**K_i values**	**Reference**
Methanol extract	CYP2D6	3.6 ± 0.1 µg/mL	NA	Hanapi et al., 2010
	CYP3A4	142.8 ± 13.8 µg/mL		
	CYP2C9	NA		
Alkaloid extract	CYP2D6	0.636 µg/mL	2.6 µg/mL	Kong et al., 2011
	CYP3A4	0.78 µg/mL	1.526 µg/mL	
	CYP1A2	39 µg/mL	18.57 µg/mL	
	CYP2C19	NA	84.88 µg/mL	
Mitragynine	CYP2D6	0.45 ± 0.33 µM	12.86 µM	Ismail et al., 2013
	CYP3A4	41.32 ± 6.74 µM	379.18 µM	
	CYP2C9	9.70 ± 4.80µM	155.80 µM	

NA: not applicable

1.2.1.9 Pharmacokinetic interactions

There are many studies that reported the pharmacokinetic interactions between kratom extract and other drugs. Caffeine increased the elimination of alkaloids extract from kratom leaves by increasing elimination rate constant (K_{el}) and clearance (CL) while decreasing elimination half-life ($t_{1/2}$). Whereas, codeine was expedited kratom absorption by decreasing absorption half-life ($ab_{1/2}$) (Botpiboon O, Prutipanlai S, Janchawee B, & Thainchaiwattana S, 2009). In addition, mitragynine and also alkaloid extract exhibited toxicokinetic interaction with permethrin, which is a

pyrethroid insecticide. Permethrin was detoxicated and eliminated by hydrolysis via carboxylesterase to inactive form as phenoxybenzylalcohol (PBALc). The result showed that mitragynine and alkaloid extracts delayed elimination of permethrin by decreasing elimination rate constant (K_{el}) and produced long elimination half-life. Whereas, enhancing elimination of phenoxybenzylalcohol (PBALc) by increasing elimination rate constant (K_{el}) and decreasing elimination half-life. So, the inhibition effects of mitragynine and alkaloid extract in permethrin metabolism and elimination might cause permethrin toxicity. (Srichana, Janchawee, Prutipanlai, Raungrut, & Keawpradub, 2015).

In the clinical case report, there are nine unintentional death cases related to Krypton intake. Krypton is a mixture of kratom powder with another μ-opioid receptor agonist. Mitragynine and O-desmethyltramadol were detected in the postmortem blood samples (Kronstand, Roman, Thelander, & Eriksson, 2011). O-desmethyltramadol is the active metabolized of tramadol which is a strong μ-opioid receptor agonist and responsible for analgesic effect. O-desmethyltramadol is detoxicated by glucuronidation through UGT2B7 and UGT1A8. The alkaloid from kratom leaves, 7-hydroxymitragynine (7-OH) showed inhibits UGT2B7 in recombinant human liver microsome with an IC_{50} value of 26.44 ± 1.33 μM (Haron & Ismail, 2015). The Krypton may produce drug interaction by the inhibition effect of 7-OH on UGT2B7 further increase O-desmethyltramadol concentration and synergistic effect on μ-opioid receptor agonist.

1.2.2 Codeine

Codeine belongs to the class of opiate used as a narcotic analgesic, antitussive and antidiarrheal drug. It usually uses in combination with other drugs such as paracetamol, aspirin, diphenhydramine, and NSAIDs for the treatment of mild to moderate degrees of pain. It is available in many pharmaceutical products presented around the world. The rout administration of this drug composes of per oral (PO), subcutaneously (SC), intramuscularly (IM), and also per rectal (PR). Whereas, it cannot

administer through intravascular (IV) because it might cause facial swelling, pulmonary edema, and various cardiovascular effects (Parke, Nandi, Bird, & Jewkes, 1992).

1.2.2.1 Chemistry of codeine

Codeine (Fig 1.4) is the natural alkaloid was isolated from opium, which occurred in the poppy plant (*Papaver somniferum*) or derivatives. It was synthesized by 3-O-methylation of morphine. The structure name is (5α, 6α)-7, 8-didehydro-4, 5-epoxy-3-methoxy-17-methylmorphinan-6-ol. The molecular formula is $C_{18}H_{21}NO_3$ and the molecular weight is 299. 37 g/ mol. The alternative chemical names include codeinum, methylmorphine, morphine 3-methyl ether, and morphine monomethyl ether. Codeine appears as colorless crystals or white crystalline powder. It is a weak base, slightly soluble in water and freely soluble in alcohol. The drug is available as the phosphate or sulfate salt. The phosphate salt is the most common ingredient in the pharmaceutical product than sulfate salt (Paolino, 1990).

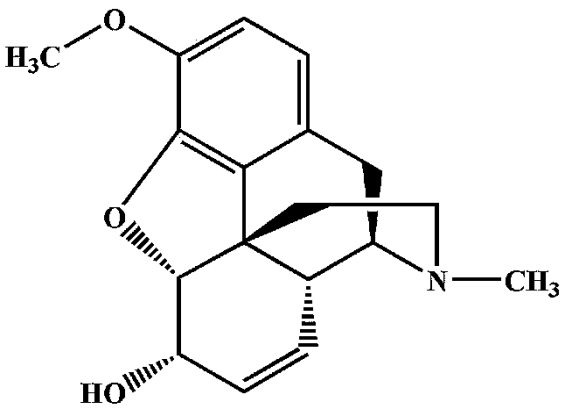


Fig. 1.4 Chemical structure of codeine

1.2.2.2 Mechanism of action

Codeine is a partial opioids agonist act on the μ-opioid receptor. The analgesic property of codeine is produced by its conversion form as morphine. Morphine binds to the opioid receptors which locate throughout the central and

peripheral nervous system. The general mechanism of opioid agonists was explained by the inhibition of primary afferent neurons in the periphery, where it serves the pain signals to the dorsal horn of the spinal cord. Opioids were inhibited by blockage calcium ion channel, result to reduce neurotransmitter released from the primary to secondary neuron e. g. glutamate and neuropeptide. In addition, opioids also inhibit the postsynaptic neuron. They act as neuropeptide antagonists present on tachykinin (NK1) receptor and others as shown in Fig 1.5 and presumption site of action as shown in Fig 1.6 (Katzung, 2018).

Opioid receptors are G-protein coupled receptor available on the transmembrane portion. At the molecular level, following the opioid agonists bind the opioid receptor, the receptors were phosphorylated and changed from inactive form (guanosine diphosphate; GDP) to active form (guanosine triphosphate; GTP), caused the α-GTP complex dissociated away from the βγ complex and then interacted with their target proteins. Following, the Gα subunit inhibited adenylyl cyclase caused low in intracellular cyclic adenosine monophosphate (cAMP) levels. The low of cAMP activated potassium ion (K^+) influx into the cells. It caused hyperpolarization and also blocked sodium (Na^+) and calcium (Ca^{2+}) ion channel. Finally, the results showed inhibit action potential, further reduced neurotransmitter release in neuronal cells (Pathan & Williams, 2012).

Moreover, opioids also indirectly inhibit pain through the activation of descending inhibitory pathways, which occurred in the brain stem and served the process to the spinal cord. This mechanism could inhibit pain transmission neuron as shown in Fig 1.7. and the site of action as shown in Fig 1.8 (Katzung, 2018).

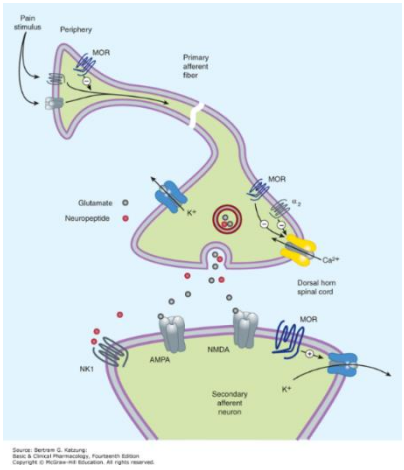


Fig. 1.5 The potential mechanism of opioid agonist on analgesic activity. Opioids act on the μ-opioid receptors, which presented at the periphery cause decrease pain signals to the dorsal horn. The μ-opioid receptors also present at pre-and post-synaptic neuron in the brain and spinal cord. The binding of opioid agonists at pre-synaptic neuron inhibits excitatory neurotransmitter release and the binding at post-synaptic neuron prolong repolarization. (https://accessmedicine.mhmedical.com).

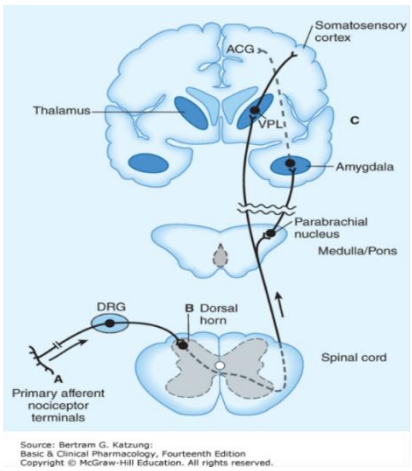


Fig. 1.6 The sites of action of opioid analgesics in the afferent pain transmission pathway. A: Peripheral tissues. B: Dorsal horn of spinal cord. C: Brain stem, possible in the amygdala, anterior cingulate gyrus (ACG), and ventral posterolateral nucleus of the thalamus (VPL) (https://accessmedicine.mhmedical.com).

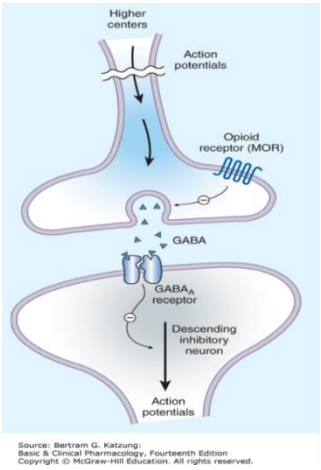


Fig. 1.7 Indirectly inhibitory pathway of the opioid agonist. Opioids agonists are activated descending inhibitory pathway through the inhibition of GABAergic interneuron releasing. (https://accessmedicine.mhmedical.com).

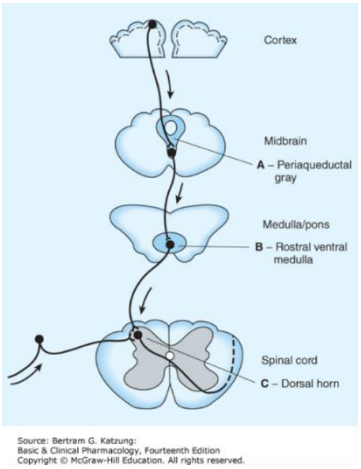


Fig. 1.8 Sites of action of opioids on descending inhibitory pathway in the midbrain and medulla including, periaqueductal gray area (A), rostral ventral medulla (B) and the locus coeruleus. These indirectly control the pain transmission pathway by increasing descending inhibition to the dorsal horn (C) (https://accessmedicine.mhmedical.com).

1.2.2.3 Pharmacological effects

Codeine is an opioid receptor agonist. It has been demonstrated that produced several pharmacological effects, for example antinociceptive, antitussive as well as antidiarrheal effects.

Antinociceptive effects

There are several studies exhibited codeine produced antinociceptive effects in animals by using various tests. Codeine showed an antinociceptive effect in guinea pigs by using the toe- pinch test and antagonized by nalorphine (Adcock, Schneider, & Smith, 1988). In rats models, It has been showed that effects in various tests, such as tail withdrawal test for acute thermal nociception, a formalin test for chemically induced inflammatory pain and a Von Frey test for mechanical hypersensitivity (Meert & Vermeirsch, 2005)

The main mechanism of opioid induced- analgesia was occurred in the midbrain by indirectly stimulated descending inhibitory pathways. Opioid agonists act on μ- opioid receptor upon periaqueductal gray (PAG) and nucleus reticularis paragigantocellularis (NRPG) caused greater neuronal traffic through the nucleus raphe magnus (NRM). The interneuron was stimulated by 5-hydroxytryptamine (5-HT) and enkephalin, which is connected with the substantia gelatinosa of the dorsal horn. Moreover, the opioid agonists could directly inhibit substantia gelatinosa and peripheral nociceptive afferent neurons. The result showed that they reduced nociceptive transmission from the periphery to the thalamus (Pathan & Williams, 2012).

Antitussive effects

Codeine was shown an antitussive effect in anesthetized cats, guinea pig, and mice by using various stimulating cough reflex. Its effects revealed in several routes such as oral, systemic, and inhalation administrations. All routes showed that codeine produced antitussive effects by itself and its metabolite as morphine (Chau & Harris, 1980; Karlsson, Lanner, & Persson, 1990; Kotzer et al., 2000). In addition, the antitussive effect of codeine is mediated by a directly stimulated receptor in the cough center of medulla. The cough suppression was completed by a lower dose than analgesic effects (Sindrup & Brøsen, 1995).

Antidiarrheal effects

The constipation activity of codeine was utilized by relieving diarrhea. There are studies showed therapeutic doses of codeine (3-60 mg/kg, IM) can be reduced stool volume in diarrhea induced by rapid intragastric infusion of a balanced electrolyte

solution (Schiller, Davis, Ana, Morawski, & Fordtran, 1982). The subcutaneous administration of codeine inhibits gastrointestinal propulsive activity. The low dose of codeine of 0.63 mg/kg can inhibits diarrheal in castor oil-induced diarrhea in rats model (Meert & Vermeirsch, 2005)

1.2.2.4 Adverse drug reaction

The most frequently observed adverse drug reactions of codeine including itching, sweating, dry mouth, drowsiness, dizziness, nausea, vomiting, miosis, orthostatic hypotension, urinary retention, and constipation (Eckhardt et al., 1998). The serious adverse drug reactions associated with overdose compose of respiratory depression, circulatory depression, respiratory arrest, shock, and cardiac arrest. The slow rate of metabolism was possibly caused by liver damage, it might be led to prolonging respiratory depression (Huffman & Ferguson, 1975).

1.2.2.5 Toxicology

Toxicity studies of codeine in the animal model have been shown in several route administrations. The median lethal dose (LD_{50}) value of peroral was 427 mg/kg, intravenous was 75 mg/kg and subcutaneous was 229 mg/kg (Paolino, 1990). The chronic toxicity studies of codeine were observed in rat and mice models, they received codeine dosing 390 to 6250 ppm per oral (PO) once per day for 13 weeks. The results showed no dose-related mortality, histopathologic lesions as well as clinical signs of toxicity in both of them. Although in the highest dose showed low food consumption during the first 3 weeks, after that return to a normal level as control groups. However, in the rat model, the body weight was decreased at the dose of 3125 and 6250 ppm, while in mice found a small body weight gain only at the dose of 6250 ppm (Dunnick & Elwell, 1989).

Recently, there are case studies of codeine-related deaths, which associated with drug interaction in Toronto, Canada. Sixty-eight cases investigated over 3 years (2006-2008). This study assessed the ratio of morphine and codeine

concentration in post-mortem blood. They found approximately 51% of the deaths involved a combination of codeine and CYP2D6 inhibitor. The common CYP2D6 inhibitor composes of an antidepressant drug such as bupropion, fluoxetine, paroxetine, citalopram, diphenhydramine as well as sertraline. The concomitant use of CYP2D6 inhibitor associated with a significant decrease in morphine/codeine ratio. These results suggested that the combination of codeine with CYP2D6 inhibitors was more toxic than codeine alone (Lam et al., 2014).

1.2.2.6 Pharmacokinetics

Oral administration of codeine rapidly and well absorbed through the digestive system. In rat model, a single oral dose of codeine (5mg/ kg) become C_{max} of 101.3 ng/mL at time to reach C_{max} (T_{max}) of 6.4 min. The $AUC_{0\rightarrow last}$ and $AUC_{0\rightarrow\infty}$ were 3936 and 4074 ng·min/mL, respectively. The Cl_t/F of 6.3 L/kg/h, $t_{1/2}$ of 39.6 min. However, there is approximately 50% first-pass metabolism in the gut and liver, bioavailability (F) was estimated of 8.3 %. Codeine rapidly metabolized to morphine with the ratio of 1.5-fold. The pharmacokinetic parameters of morphine showed C_{max} of 68.7 ng/mL at T_{max} of 6.5 min, and $AUC_{0\rightarrow last}$ of 3656 ng·min/mL. The volume of distribution of codeine observed after intravenous injection, it found a high volume of distribution (V_d) of 5.1 L/kg, The CL of 6.2 L/kg/h and $t_{1/2}$ of 34.1 ± 5.7 min. The $AUC_{0\rightarrow last}$ and $AUC_{0\rightarrow\infty}$ were 29732 and 30279 ng·min/mL, respectively. The conversion form as morphine showed C_{max} of 24.8 ng/mL at T_{max} of 4.2 min (Shah and Mason, 1990). Almost all of the codeine excreted through the renal either unchanged form and a conjugated form (Vree & Wissen, 1992).

Codeine is a prodrug and weak analgesic activity. It is necessary to metabolize to active form as morphine. The major metabolism pathways of codeine presented in the liver and slightly in the intestine and brain. The most of codeine, about 50-70% were metabolized to codeine-6-glucuronide (C-6-G) by UGT2B7 and possibly UGT2B4 (Court et al., 2003). Approximately 10-15% of codeine were metabolized to norcodeine through N-demethylation by CYP3A4 and about 0-15% of codeine were metabolized to morphine through O-demethylated by CYP2D6. Morphine is an active

metabolite of codeine. It is a stronger affinity for the μ-opioid receptors than codeine about 200-fold and it is analgesics properties of codeine. Continually, about 60% of morphine glucuronidated to morphine-3-glucuronide (M-3-G) and about 5-10% converted to morphine-6-glucuronide (M-6-G). Both of them were mainly catalyzed by UGT2B7 and a minor by UGT1A1 (Thorn et al., 2009) (Fig. 1.9). However, M-6-G is an active metabolite. It is stronger analgesic potency than morphine about four to six times, but it is a polar property that has less ability to pass the blood-brain barrier and might be no significant effects on the CNS (Katzung, 2018).

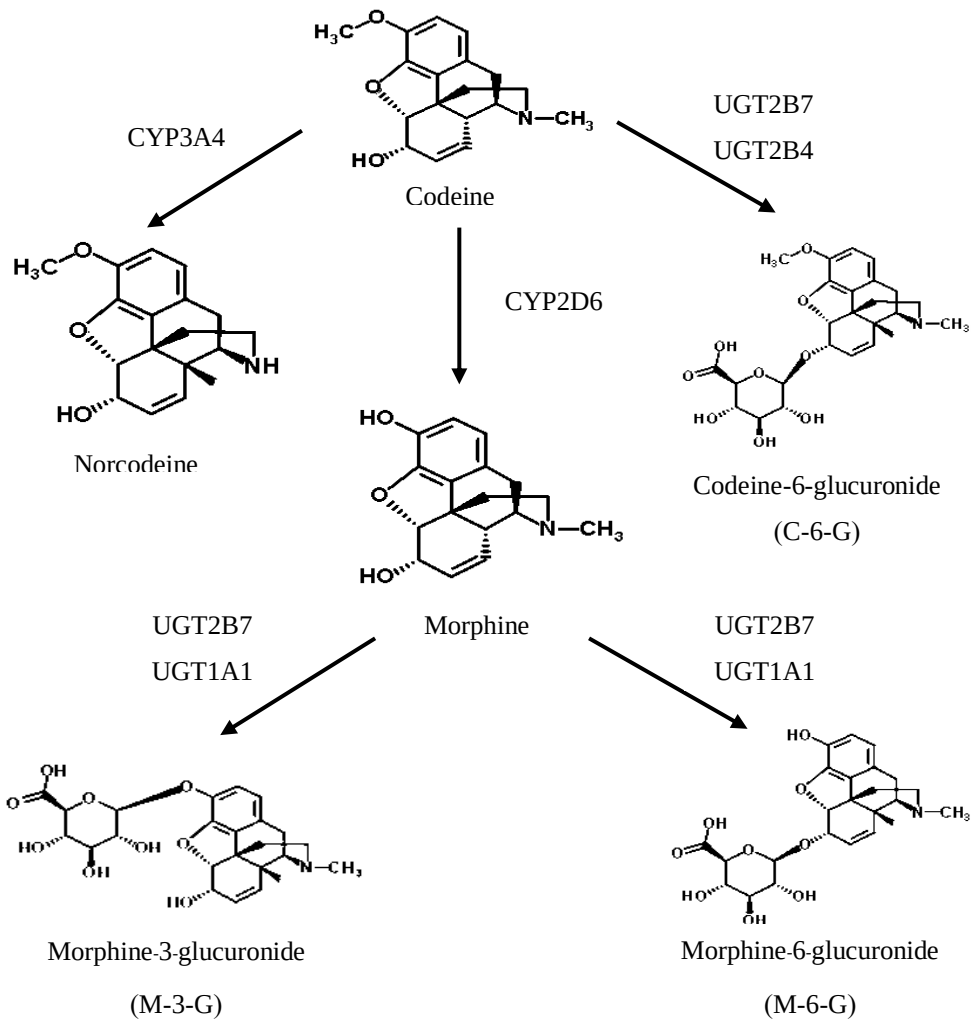


Fig. 1.9 The metabolism pathways of codeine and its metabolite norcodeine, codeine-6-glucuronide (C-6-G), morphine, morphine-3-glucuronide (M-3-G) and morphine-6-glucuronide (M-6-G)

1.2.3 Drug metabolism

Drug metabolism defines as the chemical modification of one chemical form, such as medicine or xenobiotics to the others form. This process results in pharmacological active, inactive, or toxic metabolite. Commonly, it associated with the detoxification process that converts lipophilic form (non-polar) to hydrophilic form (highly polar) for easily excrete. A transformation to active metabolites required for the pro-drug. (Taxak & Bharatam, 2014).

The metabolism is functioned by special enzymatic systems in the various organs. Approximately 70% of drugs were catalyzed by enzymes in the smooth endoplasmic reticulum of the liver cell, they are known as hepatic metabolism. The others site than liver called extrahepatic metabolism, including lungs, kidney, epithelial cells of the gastrointestinal tract, adrenals, skin, and placenta. The process of metabolism divided into two reaction phases. Phase I is functionalization and phase II is conjugation. These reactions occurred sequentially, independently or simultaneously. The phase I reaction composed of oxidation, reduction, hydrolysis, cyclization, and decyclization reactions. These reactions commonly catalyzed through the CYP450 enzyme, which found abundant in the liver. The most of phase I metabolites cannot directly be excreted until undergoing phase II. The phase II reaction as the real detoxification pathways that conjugated with a suitable moiety such as glucuronic acid, glutathione, sulfate, and glycine, etc. These reactions are catalyzed by a variety of transferase enzymes including uridine diphosphate (UDP)-glucuronosyltransferases (UGT), sulfotransferases (SULT), glutathione-S-transferases (GST) and *N*-acetyl-transferase (NAT). Following the conjugation led to increasing the size and strong polar metabolites, which is hydrophilicity and to further excreted via bile and/or urine (Hinson & Forkert, 1995; Taxak & Bharatam, 2014)

Accordingly, the enzymes' metabolism systems could perform both of detoxification for avoiding some adverse effects and bioactivation form is required by pro-drug. These reactions mentioned was regulated by several gene expressions. It is possibly impacted in the pharmacokinetics/dynamics or toxicokinetic/dynamics as well as the efficacy of therapeutic agents.

1.2.3.1 CYP450

Cytochrome P450 enzymes or CYP450 are the microsomal protein that is an important role in drugs, chemical, vitamin, and endogenous compound metabolism. It knew as microsomal mixed-function oxidases. Most of the CYP enzymes exposed in the smooth endoplasmic reticulum of the liver and slightly in other tissues. The CYP enzymes system consisted of a superfamily of heme- containing monooxygenase. The heme is the iron- porphyrin unit, where is the center of the oxidizing site and responsible for the oxidation of lipophilic compound to hydrophilic metabolites for further excretions (Taxak & Bharatam, 2014). Beside of CYP enzymes, the oxidation mentioned based on the mutual reaction of nicotinamide adenine dinucleotide phosphate (NADPH) as well as oxygen, as shown in the equation below.

$$R\text{-}H + O_2 + NADPH + H^{\cdot} \xrightarrow{CYP450} R\text{-}O\text{-}H + H_2O + NADP^{+}$$

1.2.3.2 Mechanism of metabolism by CYP450

The mechanism of CYP450 enzymes metabolism was shown in Fig. 1.10. The oxidation process started by the substrate (RH) bound with CYP-Fe^{3+} as its oxidized form producing a substrate-enzyme complex (CYP-Fe^{3+}-RH). The first, one-electron reduction from NADPH transferred into its complex, reduced to CYP-Fe^{2+}-RH. The ferrous form capability bound with molecular O_2, following transferred second one-electron reduction from NADPH, the one atom of O_2 removed as H_2O and formed the active form. Then, the atomic O insert into the substrate and finally its product dissociated from CYP450 enzyme (Liston, Markowitz, & DeVane, 2001; Taxak & Bharatam, 2010).

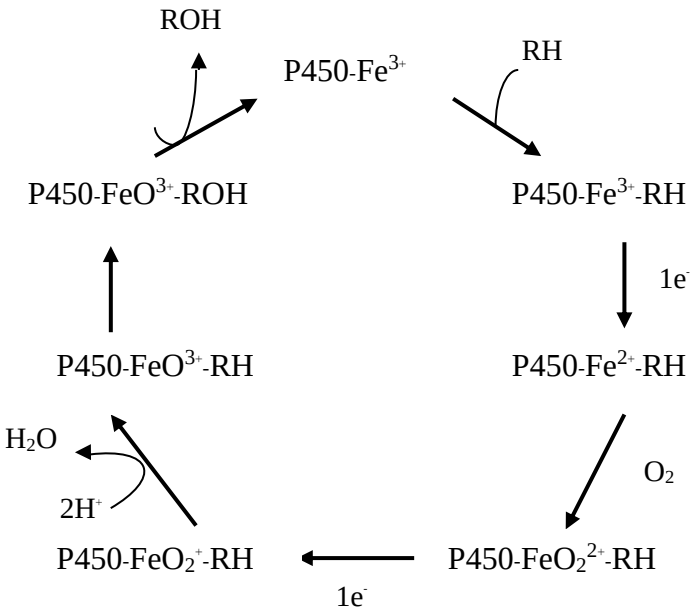


Fig. 1.10 The metabolism cycle of CYP450, leading to the oxidation of the substrate (modified cycle from Taxak & Bharatam, 2014)

1.2.3.3 CYP450 in organisms

The CYP450 was regulated by gene expressions, which is difference in any species and led to the biodiversity. The nomenclature of the CYP enzymes systems has been established by the nomenclature committee. The emblem of cytochrome P450 gene as "CYP" for human and "*Cyp*" for mouse and Drosophila. The Arabic numeral displayed CYP family, the alphabet (A, B, C) displayed subfamily and the last letter indicated the individual gene as isoform/isozyme/isoenzyme (Nelson et al., 1996).

The CYP450 superfamily consisted of more than 7,000 names sequence presented in the living organism. The human CYP450 enzymes reported 57 isoforms. Fifteen isoforms from CYP1, CYP2, and CYP3 families responsible for drug and/or xenobiotics metabolism. It including CYP1A1, CYP1A2, CYP2A6, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP3A4, CYP3A5, and CYP3A7. The most abundance form presented in the hepatic metabolism was CYP3A4 and it also metabolized a very large number of drugs and xenobiotics. Although CYP2D6 isoform is very low abundance, while it metabolizes

numerous drugs. The abundance of CYP450 isoenzyme represents in the liver as shown in Fig. 1.11. (Pelkonen et al., 2008).

The other species, rat and mice considered to be the CYP450 model. The rats presented 89 isoforms while mice presented 103 isoforms. Each isoform might identical or different to the human, as represented in Table 1.2.

Table 1. 2 The comparison of CYP450 isoenzyme between human, rat, and mice (modified from Löfgren et al., 2004; Mikus, Somogyi, Bochner& Eichelbaum, 1991).

Family	**Subfamily**	**Human**	**Mice**	**Rats**
CYP1	A	1A1, 1A2	1A1, 1A2	1A1, 1A2
	B	1B1	1B1	1B1
CYP2	A	2A6, 2A7, 2A13, 2A18	2A4, 2A5, 2A12, 2A22	2A2, 2A3
	B	2B6, 2B7	2B9, 2B10, 2B13, 2B19, 2B20x, 2B23	2B1, 2B2, 2B3
	C	2C8, 2C9, 2C18, 2C19	2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50,2C51x, 2C54, 2C55, 2C65, 2C66, 2C67, 2C68, 2C69, 2C70	2C6, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23
	D	2D6	2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40	2D1, 2D2, 2D3, 2D4, 2D5, 2D18
	E	2E1	2E1	2E1
CYP3	A	3A4X, 3A4, 3A5, 3A7, 3A43	3A11, 3A13, 3A16, 3A25, 3A41, 3A44, 3A57, 3A59	3A1, 3A2, 3A9, 3A18, 3A23

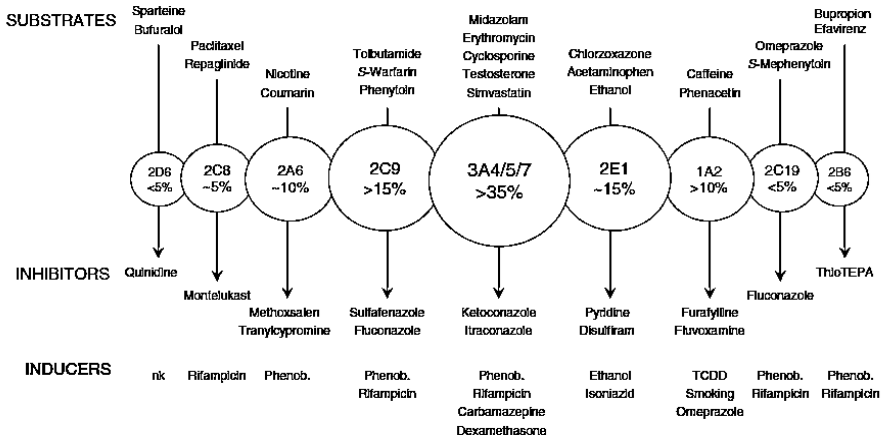


Fig. 1.11 Relative abundance of individual CYP forms in the liver and some examples of substrates, inhibitors, and inducers (Pelkonen et al., 2008).

1.2.3.4 CYP2D6

Although CYP2D6 isoenzyme has a relatively low expression within the liver, they are the most important role in drugs metabolism. Approximately 25% of clinical drug used was catalyzed by CYP2D6 isoenzymes. It composed β-adrenergic receptor antagonists such as propranolol and metoprolol, antidepressants such as fluoxetine and paroxetine as well as atypical antipsychotics such as aripiprazole and risperidone. The commonly substrates used for study this isoenzyme included dextromethorphan, bufuralol, debrisoquine, codeine, desipramine, atomoxetine, and thioridazine. The activity of CYP2D6 isoenzyme produced individuals because of genetic polymorphism. The polymorphism of *CYP2D6* gene included poor, ultra-rapid, intermediate, and extensive metabolizers. The Arabian, Eastern African and approximately 5.5% of Caucasians were ultra-rapid metabolizers, which is greater metabolism than normal and might cause insufficient therapy. Whereas, approximately 7% of Caucasians and a small number of Oriental populations were poor metabolizers, which is a loss function of isoenzymes leading to increase drug concentration in plasma and might cause toxicity. On the other hand, several drugs are prodrug such as codeine. This drug has become to the active form by CYP2D6. If the loss of this enzyme functioned might lead to sufficient analgesic effect. In addition, there are many of drug

effects on this enzyme activity known as enzyme inhibitors or/and inducers as represented in Table 1.3 (Monte et al., 2014; Pelkonen et al., 2008).

Table 1.3 Substrates, inhibitors and inducers of the CYP2D6 enzyme (modified from Pelkonen et al., 2008).

Substrate	**Inhibitors**	**Inducers**
Dextromethorphan	Quinidine	Glutethimide
Bufuralo	Terbinafine	Dexamethasone
Debrisoquine	Paroxetine	Rifampicin
Codeine	Fluoxetine	Haloperidol
Desipramine	Norfluoxetine	
Atomoxetine	Sertraline	
Thioridazine		

1.2.3.5 CYP2D1

Since debrisoquine and bufuralol were CYP2D6 substrate models (Pelkonen et al., 2008). *In vivo* study has been shown that Dark-agouti rat (DA) impaired metabolism of debrisoquine to 4-hydroxydebrisoquine and bufuralol to 1' hydroxybufuralol. So, in 1989 Matsunaga and colleagues conducted *CYP2D* gene expression between two strains of rats, included Dark-agouti rat (DA) and Sprague Dawley rat (SD). This study found DA rats lack *CYP2D1* mRNAs expression, they only expressed *2D2, 2D3* and *2D5* mRNAs, while SD rats expressed *2D1*, *2D2*, *2D3,* and *2D5* mRNAs. These results verified that the oxidation reaction of debrisoquine and bufuralol were mediated by *CYP2D1 gene* in the rat model. (Matsunaga et al., 1989). Accordingly, with Soucek and Gut (1992), they compared sequential homology between rat and human CYP450 forms. This study found *CYP2D6* gene of human have sequence similarity with *CYP2D1* gene of rats were 71% (Souček & Gut, 1992).

1.2.4. Drug interaction

Drug interaction is a change in the properties of a drug by the influence of another drug, herbals or some foods that co-administered. This action could be antagonistic or synergistic, caused increase or decrease its efficacy, toxicity and also producing a new effect that neither produces on its own. The interaction could occur between drug-drug, food-drug as well as herb-drug interaction. The alteration of pharmacokinetics was the main processed that induced drug interaction, such as altered the absorption, distribution, metabolism, and excretion. Alternatively, it possibly disturbed pharmacodynamic properties of the drugs, such as a direct effect on receptor function, interfered in biological or physiological control processes and cumulative or denied the pharmacological effect (Palleria et al., 2013).

1.2.4.1 Pharmacokinetic interactions

Pharmacokinetic interactions are the main cause of drug interactions. It was obtained by the change of absorption, distribution, metabolism or excretion of co-administered drugs. Generally, these alterations could change the concentration of drug in plasma. It was measured by a change of pharmacokinetic parameters such as maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}), area under the plasma concentration versus time curve (AUC), volume of distribution (V_d), elimination half-life ($t_{1/2}$) and elimination rate constant (λ_z), etc.

1.2.4.1.1 Absorption interactions

The absorption of drugs passed the gastrointestinal tract. It was influenced by a variety of factors. The changed in gastric pH was the first factor, basically, a drug required to dissolves and absorbed in a gastric pH between 2.5 to 3. Hence, drugs affected to change gastric pH could alter the kinetics of other concomitant drugs. For examples the antacid, PPI and H_2 antagonists, they capable increased the gastric pH leading to reduced cefpodoxime solubility and absorption, finally diminished the bioavailability of this drug. The second, the drugs were formed non-absorbable complexes by the combination with the metal ion, protein or bile acid. The

third, the drugs were changed in the intestinal motility. A drug that quickens gastric emptying, it will reduce time to reach between drug and gastrointestinal mucosa, which is the main absorption area and results in decrease the absorption rate. The P-glycoprotein (P-gp) intestinal is another factor that controls the absorption rate. It was protected drug diffusion by controlled drug absorption or promote drug excretion. The drugs that inhibit P-gp activity possibly increase the bioavailability of the co-administered drug. On the other hand, it might increase the toxicity of drugs that have a low therapeutic index.

1.2.4.1.2 Distribution interactions

The potential of distribution interaction was caused by plasma protein binding. Many kinds of plasma protein can interact with drugs such as albumin, α_1-acid glycoprotein, and lipoproteins. Only unbound drug capable diffused to extravascular and interacted with tissues site of action. The drugs that have a high affinity on the plasma protein (>90%) replaced the binding of the others and caused toxic effects.

1.2.4.1.3 Metabolism interactions

The majority of clinical drug interactions were caused by the metabolism interaction, especially drugs that metabolized via the CYP450 enzymes in the liver. The CYP450 enzyme plays an important role in the metabolism of a large number of drugs. There are 6 isoforms belong to the families 1-3 that involved in drug hepatic metabolism, these including CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP2E1. Many drugs metabolized via these enzymes, called substrate. The metabolism interaction of these drugs functioned the inhibition or induction of CYP450 enzymes.

Enzymes inhibitions

The interaction involved in enzyme inhibitions were occurred by directly inhibit enzyme activity. This reaction could change the drug level in the plasma. Most of them had reduced drug metabolism and elimination rate, while increased plasma drug concentration and might be led to adverse drug reactions. On the other hand, in the case of pro-drugs, enzyme inhibitors could reduce the amount of active form. The

result showed low therapeutic effect of this drug. Basically, the metabolic inhibition is caused by two mechanisms, included reversible and irreversible inhibition.

Reversible inhibitions are the substrate bound enzyme with non-covalent interactions. There are three types: competitive, metabolic-intermediate complex, and non-competitive inhibitions. Competitive inhibition is the competition of substrate and inhibitor to access at the same binding site of the enzyme. Usually, the inhibitor having more affinity than the substrate but it was rapidly reversible by sufficiently increased the concentration of substrate. Another one is metabolic-intermediate complexes. This complex produced from the inhibitors bound with the substrate-enzyme complex, which the inhibitors have an N-alkyl substituent. This reaction was found in CYP3A4. After the binding, CYP3A4 oxidized the inhibitor led inhibitor oxidize species and remained the complex. At the same time, they reduced heme group of CYP3A4 and formed a slowly reversible complex. The others are the non-competitive inhibitor. It was occurred by the inhibitor bound with allosteric site enzyme, then change the conformation of the active site and led decreased in the efficiency of enzyme-substrate binding. The results showed reduced enzyme activity.

Irreversible inhibition was presented by the inhibitors bound enzymes then modified the covalent bond. The crucial factor of this reaction could be explained by the total amount rather than the concentration of the inhibitor that was bound with CYP isoenzyme. Approximately 30-40% of drug metabolism has the potential to cause inhibition interaction and they were catalyzed by only one isoenzyme in the metabolic pathway (Levy, 1995). The results of inhibitory interaction showed decreased the metabolism of the substrate, while increased its effect and might be contributed to drug toxicity. If the drug is a pro-drug, the result is the opposite.

Enzymes inducers

Many factors were capable of induced CYP enzyme activity such as drugs, environmental pollutants as well as natural products. The mechanism of induction enzyme activity was completely different from the inhibitions. Commonly mechanism was described by the activation of the transcription factors resulted in increased CYP enzyme protein synthesis. These transcription factors were included in the pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl

hydrocarbon receptor (AhR), etc. In addition, there is an alternative mechanism, which involved stabilized compound translation or inhibited the protein degradation pathways (Tompkins & Wallace, 2007). The inductions of CYP enzyme resulted increased the amount of CYP enzyme and accelerated the oxidation process as well as induced drug metabolism.

1.2.4.1.4. Excretion interactions

Both kidney and liver were the major organs responsible for drug elimination. Likewise, the main sites were renal tubular and also biliary duct. Among the excretion site, renal tubule represents a considerable site for drug interactions (Kido, Matsson, & Giacomini, 2011). The mechanisms of interaction occurred by which one and the other drugs compete to excreted via the same transporter. Nevertheless, the competition of both drugs could apply to achieve therapeutic benefits, i.e. probenecid was increased the plasma concentration of penicillin by competitive inhibited an organic anion transporter in renal tubules, led to increasing the concentration of other substrates. Tubular reabsorption is another mechanism involved excretion interaction. The changes in urinary pH influenced the excretion of weak acids or weak bases, due to tubular reabsorption required an only unionized form. This mechanism can be used for the treatment when there is the overdose of weak acid and/or bases. The agent that alkalinized urine led to reducing the reabsorption and increases the excretion of the weak acid. Similarly, the agent that acidify urine, led to reducing the reabsorption and increases the excretion of the weak bases (Palleria et al., 2013).

1.2.4.2 Pharmacodynamic interaction

Pharmacodynamic interaction was another type of drug interaction. These interactions were occurred by one drug altered the effect of co-administered drugs at the active sites, such as receptor binding site, signaling site as well as effectors site. The mechanisms of reaction due to drug synergistic and antagonistic, further led to unexpected drug effects (Hinder, 2011).

Synergistic interactions

Synergistic or additive drug interactions presented in the combination of two drugs that have the same pharmacological properties. A basic example is a combination of ethanol with benzodiazepine, usually the overdose of benzodiazepine alone just caused prolonged sedation they are seldom fatal, but when combined with ethanol possible increased risk of death. The supra-additive effect was found in the combination of sulphonamide and trimethoprim. In general, they were bacteriostatic, while the combination had become bactericidal. Although the combination of two drugs that have different therapeutic indications but mutual pharmacological action, they could be led to adverse drug reactions. Severe side effect such as nephrotoxicity could be found in the combination between tobramycin and cephalothin, which is both are similarity side-effects.

Antagonistic interactions

The antagonistic interaction involved in the receptor binding site. It was occurred by another drug competed to bound at the same receptor of the main drug, but its effect becoming the opposite. Some of these are medicinal purposes, such as flumazenil reversed benzodiazepine-induced sedation and naloxone reversed opioid overdose. It also comprised functional antagonisms in two drugs that are contrary effects on different receptor systems. So, they physiologically resisted each other, such as glucocorticoids caused hyperglycemia and resisted the actions of hypoglycemic agents. The resisting effects of the two drugs are well known and the combination is seldom prescribed (Pleuvry, 2005).

1.3 Objective

To investigate the pharmacokinetics of codeine after mitragynine pretreated for seven days in the rat model.

CHAPTER 2

MATERIALS AND METHODS

2.1 Isolation of mitragynine

2.1.1 Plant material

Kratom leaves were collected from Nampu, Bannasarn, Surat Thani province, Thailand. The plant species were identified by Assoc. Prof. Dr. Juraithip Wungsintaweekul. The herbarium voucher specimens have been collected at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand, and recorded as (PSU herbarium No. 0012822).

2.1.2 Chemicals and reagents

The solvents used were commercial and analytical grades. The commercial grade solvents were distilled before used. Methanol, chloroform, and glacial acetic acid were purchased from Lab-scan Asia Co., Ltd (Bangkok, Thailand). TLC plate 60-F254 and 25% ammonia solution were purchased from Merck, Darmstadt, Germany. Petroleum ether and anhydrous sodium sulfate were purchased from J.T Baker® (PA, USA). Sodium chloride was purchased from Ajax Finechem Pty Ltd. (Australia). The authentic mitragynine was kindly provided from Assoc. Prof. Dr. Juraithip Wungsintaweekul, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

2.1.3 Extraction method

Mitragynine was isolated from dried kratom leaves. The extraction method was modified from the previous study (Kaewppradub, 1990). Firstly, the fresh leaves were cleaned, then dried in hot air oven at 50°C. Dry leaves weigh 1.23 kg were powdered. The powder was macerated with methanol (4 L) that was adjusted to pH 9.0 by 25% ammonia solution at room temperature for three days. After three days, the extract was filtered through the Whatman® filter paper and evaporated to dryness by the rotary evaporator. The residue was repeatedly extracted four times. All filtrates were pooled and concentrated to obtain the crude methanol extract (228.06 g).

The crude methanol extract was dissolved in 10% (v/v) glacial acetic acid, mixed and left to stand overnight. After that, the acidic part was filtered and partitioned with petroleum ether for 5 times. The acidic filter was then adjusted to pH 9.0 with 25% ammonia solution and extracted with chloroform for 5 times. The chloroform extract was washed with Brine's solution for 3 times. The excessed water in chloroform extract was removed by adding anhydrous sodium sulfate. Finally, the chloroform extract was evaporated to dryness and the crude alkaloid extract was obtained (9.82 g).

To purify mitragynine, the crude alkaloid extract was dissolved in a small volume of chloroform and loaded on to silica gel column chromatography (size 7 x 35 cm.) which holding in chloroform. The column was eluted with a gradient solution of chloroform:methanol from 99%:1% to 70%:30%. The volume of the collected fraction was 150 mL. The collected fractions were checked by TLC and detected using ultraviolet light at the wavelength of 254 and 366 nm and follow by Dragendorff reagent. The fractions with similar patterns on TLC were pooled and concentrated to obtain mitragynine (4.66 g). The yield of mitragynine was 0.37% based on dry leaves.

2.2 LC-MS/MS analysis

2.2.1 Chemical and reagents

Standard morphine and codeine were obtained from Faculty of Science, Prince of Songkla University, Thailand. Naloxone hydrochloride was purchased from Troikaa Pharmaceuticals Ltd., India. Methanol HPLC grade was purchased from Lab-scan Asia Co., Ltd (Bangkok, Thailand).

2.2.2 Instrument

Automatic pipette 20, 100, 200 1000 and 5000 µL (Gilson S. A. S., France), vortex mixer 2 genie (Scientific, USA), turbovap (Caliper LifeSciences, CA), analytical balance (Sartorius, Germany), pH meter (EUTECH, Thailand, magnetic stirrer (HL instrument, Thailand, freezer -20 °C (Panasonic, Japan), freezer -80 °C (Sanyo, Japan), centrifuge (Worldco Co Ltd., Thailand)

2.2.3 Chromatographic condition

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been optimized and used for the analysis of codeine, morphine, and mitragynine. The optimized conditions were shown in Table 2.1 and 2.2

Table 2.1 HPLC condition**s**

LC-system	Waters® Alliance 2695 HPLC pump
LC-column	UCT Selectra® DA HPLC column (5 µm, 50 × 2.1 mm i.d.)
Column temp (ºC)	20-25
Pressure (bar)	0-300
Mobile phase	A: 0.1% v/v formic acid in water B: 0.1% v/v formic acid in methanol

Gradient program	**Total run time (min)**	**A (%)**	**B (%)**
	0.00	95.0	5.0
	3.00	65.0	35.0
	5.00	55.0	45.0
	8.00	10.0	90.0
	10.10	95.0	5.0
	16.00	95.0	5.0
Flow rate (mL/min)	0.2		
Split column	1:1		
Run time (min)	16		
Injection volume (µL)	30		
Autosampler	Waters® Alliance 2695 Autosampler		
Temp control (ºC)	20		

Table 2.2 MS/MS conditions

Interphase	Electrospray ionization		
Source (ES+)	**Setting**	**Readbacks**	
Capillary (kV)	2.50	0.02	
Cone (V)	50	-1	
Source temp (ºC)	120	22	
Desolvation temp (ºC)	350		
Mass spectrometer	Waters® Quattro Ultima FS		
Mass analyzer	Triple quadrupole		
Mode	Multiple reaction monitoring mode (MRM)		
Mass to charge ratio	**Precusor ion**	**Product ion (1)**	**Product ion (2)**
Codeine	300.01	215.19	165.23
Morphine	285.98	201.2	165.27
Mitragynine	399.09	174.22	159.2

Analyser	Setting	Readbacks
LM1 Resolution	13.0	
HM1 Resolution	13.0	
Ion energy1	0.8	
Entrance	2	-1
Collision	34	0
Exit	2	-1
Automatic Gain Control		
LM2 Resolution	13.0	
HM2 Resolution	13.0	
Ion energy 2	1.0	
Multiplier (V)	650	3

2.3 Preparation of standard solutions

2.3.1 Stock standard solutions

The individual standard solution of codeine, morphine, and mitragynine at the concentration of 1 mg/ mL was prepared by dissolving standard powder in methanol. The standard powder 10 mg dissolved in methanol 10 mL. A stock solution of naloxone (IS) was prepared in methanol to obtain a concentration of 10 µg/mL. All stock solutions were stored in freezer at the temperature of -20°C.

2.3.2 Working standard solution

The working standard solutions of codeine, morphine, and mitragynine used for creating calibration curves and quality control samples (QC) were prepared by diluting the standard stock solution in plasma to make a concentration of 0.1, 1, and 10 µg/mL. An internal standard of naloxone was prepared by diluting the standard stock solutions in methanol to a concentration was 10 ng/mL. All working standard solutions were kept in the freezer at -20°C

2.4 Method validation

The method validation was performed according to the guidance for industry bioanalytical method validation of food and drug administration in the U.S. (USFDA, 2018). Blank human plasma was obtained from the Blood Bank of Songklanakarin Hospital, Prince of Songkla University, Thailand. The validation of parameters was described as follows.

2.4.1 Calibration curve

The calibration curves were determined by the preparation of seven-point concentrations of analytes in plasma (five replicates of each). They were prepared by spiking working standard solutions in blank plasma samples to make the final volume was 200 μL. The calibration curve of codeine was run with the concentration of 5, 10, 20, 40, 100, 200, and 400 ng/mL; of morphine were 5, 10, 20, 40, 75, 125, and 250 ng/mL; and of mitragynine were 5, 10, 20, 40, 80, 150, and 200 ng/mL. Then, 40 μL of naloxone (10 ng/mL) was spiked in each sample to make a concentration of 2.0 ng/mL. The calibration curves were constructed by plotting the relationship between the response (y), the peak area ratio of analytes to that IS versus the concentration of analytes (x). The regression analysis was performed to obtain the calibration equation and the correlation coefficient (r^2) should be more than 0.99. Acceptance calibrator value should be in the range of 85 to 115% of nominal concentrations, except the LLOQ which should be in the range of 80 to 120 %

2.4.2 Selectivity

The selectivity was estimated by analyzing blank plasma samples and zero calibrators from six different sources. Each sample was extracted and analyzed. The blank plasma should be absent from the interfere at the retention time of codeine, morphine, mitragynine, and IS.

2.4.3 Specificity

The specificity was determined by the absence of "cross-talk" between the channel of analytes and internal standard. They were prepared by spiking naloxone (IS) in samples as well as codeine morphine and mitragynine at the highest concentrations. All studies should be no "cross-talk" were observed.

2.4.4 Sensitivity

The sensitivity was demonstrated by the lowest standard concentration of the calibration curve (LLOQ). It was prepared by spiking the lowest concentration of codeine, morphine, and mitragynine at 5 ng/mL in blank plasma, then extracted and analyzed. They were performed in five replicates. The acceptance criteria were the response of the analytes should be more five times than the zero calibrators. The accuracy should be ± 20% of nominal concentration (80-120%) and the precision should be ± 20% CV.

2.4.5 Accuracy and precision

The accuracy and precision were estimated using the quality control (QC) samples. They were prepared by spiking each working solution in blank plasma to make the concentration of 15, 150, and 350 ng/mL for codeine; 15, 80, and 200 ng/mL for morphine; and 15, 75, and 180 ng/mL for mitragynine then extracted and analyzed. Each concentration was run in five replicates for intra-day and inter-day precision analysis. The acceptance criteria of accuracy should be ± 15% of nominal concentration (85-115%) and the precision should be ± 15% CV. The percentage coefficient of variation (CV) and accuracy was calculated according to the equation below.

$$\text{CV } (\%) = \frac{\text{Standard deviation (SD)}}{\text{Mean values}} \times 100$$

$$\text{Accuracy (\%)} = \frac{\text{Calculated concentration}}{\text{Nominal concentration}} \times 100$$

2.4.6 Recovery

The recovery was estimated by comparing the peak area of pre- and post-extraction of QC samples. It was prepared by an extracted plasma containing QC samples versus extracted blank plasma before spiked QC samples. Percent recovery should be >60%.

$$\text{Recovery (\%)} = \frac{\text{Pre-extracted QC sample in blank plasma}}{\text{Post-extracted QC sample in blank plasma}} \times 100$$

2.4.7 Dilution effects

The dilution effects were estimated by diluting codeine, morphine, and mitragynine at concentration 100 ng/mL to 10 ng/mL in plasma. , then extracted and analyzed. The samples were carried out in five replicates. The acceptance values of accuracy should be ± 15% of nominal concentration (85-115%) and the precision should be ± 15%CV.

2.4.8 Matrix effect

The matrix effect on the ionization of codeine, morphine, mitragynine and IS was estimated by comparing the mean concentration of standard in solution (prepared in methanol) with the mean concentration of post-extraction spiked samples in six different sources of blank plasma at codeine concentration of 15 and 350 ng/mL; morphine concentration of 15 and 200 ng/mL; and mitragynine concentration of 15 and 180 ng/mL. All samples were separated by LC and analyzed using ESI interfaces in the MS. Absolute matrix effect (AE) was calculated by comparing the mean concentration of the analytes in post-extraction spiked samples to the mean concentration of the same analyte in standard solutions, multiplied by 100. The acceptant criteria should be 85-

115 %. The relative matrix effects were evaluated by comparing the mean concentration of analytes among post-extraction spiked samples each source of blank plasma. The precision value was considered and the acceptance values should exceed 3-4% (Matuszewski, 2006).

2.4.9 Carryover

The potential impact of the accuracy of assay was investigated throughout the validation. A blank was sample injected following the injection of the upper limit of quantitation (ULOQ) of standard curve sample. The carryover should not exceed 20% of LLOQ.

2.4.10 Stability

The stability of codeine, morphine, and mitragynine in plasma was estimated under various storage conditions. The experiment was performed using 15 and 350 ng/mL of codeine, 15 and 200 ng/mL of morphine, 15 and 180 ng/mL of mitragynine, excepted extract (or processed sample) stability and stock solution stability (three replicated for each concentration). The accuracy was considered for this test by comparing with freshly prepared samples, acceptance criteria should be ± 15% accuracy (85-115%).

- **Autosampler stability**

Samples were spiked in blank plasma and extracted, then stored samples in autosampler at 25 ºC for 6 and 12 h.

- **Bench-top stability**

Samples were spiked in blank plasma and extracted, then left to stand at room temperature (25 ºC) for 6 and 12 h.

- **Extract (or processed sample) stability**

The calibration curves concentration were assessed the stability after processed for 6 and 12 h, which is the residence time in autosampler. The stability was compared with freshly prepared calibrators.

- **Freeze-thaw stability**

Samples were spiked in blank plasma, then stored in freezer (-20 ˚C). They were thawed every 24 h at room temperature (25 ºC) for three cycles before extracted.

- **Long-term stability**

Samples were spiked in blank plasma, then stored at -20 ºC for 30 and 60 days.

- **Stock solution stability**

Standard stock solution codeine and morphine at the concentration of 1 mg/mL, standard mitragynine at the concentration of 10 µg/mL were prepared in methanol and stored for 2 months at -20 ºC.

2.5 Animals

Male Sprague-Dawley rats (300-400 g) were provided from Nomura Siam LQC International Co., Ltd (Bangkok, Thailand) and they were housed under the controlled environment (temperature 22 ± 2 ºC and relative humidity 55 ± 10% with 12 h light and 12 h dark cycles) at The Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University, Thailand. They were free to access standard rodent diet and water *ad libitum*. They were acclimatized for 7 days and fasted for 12 h before the experiment. The animal experiment protocol was approved by the Animal Ethics Committee of Prince of Songkla University, Thailand (Ref. 3/2018; MOE 0521.11/072) as shown in Appendix A.

2.5.1 Sample size calculation

This study used the G*Power program to calculated sample size, as follows.

t tests - Correlation:	Point biserial model	
Analysis:	A priori:	Computer required sample size
Input:	Tail (s)	= One
	Effect size |r|	= 0.5
	α-err prob	= 0.05
	Power (1-β err prob)	= 0.8
Output:	Noncentrality parameter δ	= 2.645751
	Critical t	= 1.729133
	Df	= 19
	Total sample size	= 21
	Actual power	= 0.817228

The number of animals available for this study was 21 rats. However, it was necessary to increase the sample size to prevent the loss of animals during the experiment and/or offset the rejected from this study. Therefore, the total sample size used in this study was adjusted toward 26 rats.

2.2.2 Dose calculation

The animal equivalent dose (AED) was converted from human equivalent dose on the basis of body surface area. The data was shown in Appendix 2 with the equation below (Nair & Jacob, 2016).

$$\text{AED (mg/kg)} = \text{Human dose (mg/kg)} \times K_m \text{ ratio}$$

$$\text{Extend equation; } K_m = \text{Body weight (kg)} / \text{Body surface area } (m^2)$$

$$K_m \text{ ratio} = \text{Animal } K_m / \text{Human } K_m$$

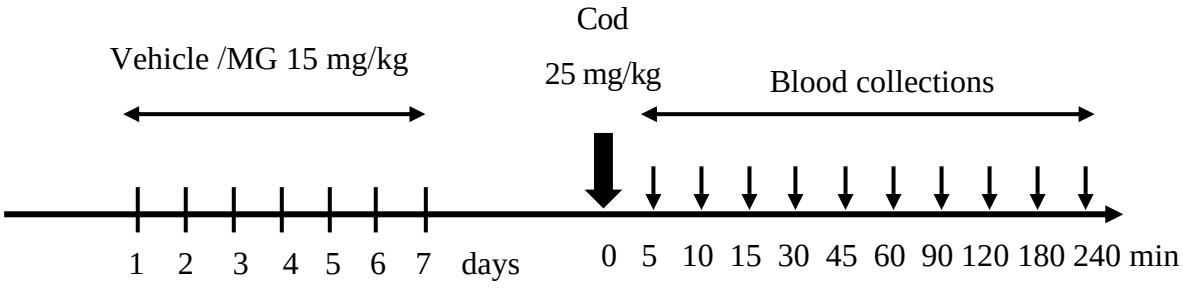
The recommended human equivalent dose (HED) of codeine for analgesia was 30-60 mg every four h, therefore, a daily maximum dosage was 240 mg. The AED of codeine was calculated by the equation above to obtain a dose of 25 mg/kg (24.8 mg/kg), according to the EC_{50} of codeine (Schellekens et al., 1986).

The HED of mitragynine was followed by Buasri and colleagues (2017). A glass of boil kratom leaves (50 mL) contains mitragynine 45 mg. The AED of mitragynine was calculated by the equation above to obtain a dose of 4.65 mg/kg. For the efficacy of inhibitor, the dose was increased toward 15 mg/kg (Buasri et al., 2017).

2.6 Pharmacokinetics study

2.6.1 Study design

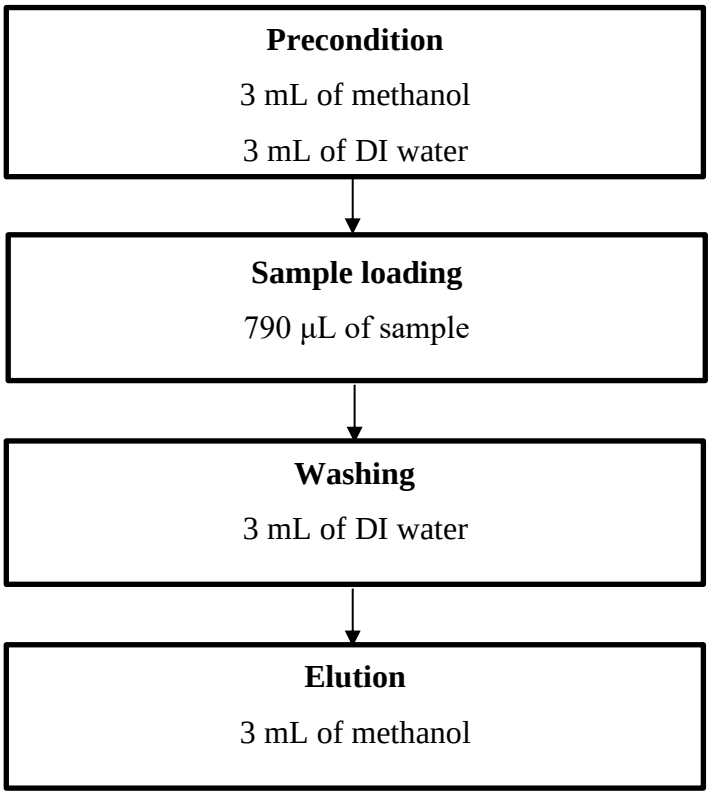
Twenty- six male Sprague-Dawley rats (250-300 g) were randomly divided into two groups (n=13 for each group). Group one as the control group, rats were pretreated with vehicle (propylene glycol, 2 mL/kg) once a day for seven days. Group two as experiment group, rats were orally pretreated with 15 mg/ kg of mitragynine dissolved in the vehicle once a day for seven days. Two h after the last dose of vehicle or mitragynine, rats were orally administered with 25 mg/kg of codeine dissolved in sterile water. Blood samples (500 µL) were collected from the animal tail vein and sampling at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180 and 240 mins after codeine administration. The blood samples were stored in micro- centrifuge tube containing 2 µL of heparin (5,000 unit/mL), slightly shaken, then centrifuged at 5000 g for 10 mins at 4 ºC, and kept the plasma samples at -80 ºC.



Scheme. 2.1 Study design

2.6.2 Sample preparation

The samples were extracted using the solid phase extraction method (SPE). Firstly, 200 μL of rat plasma samples were mixed with 40 μL of IS solution to final concentration 2.0 ng/mL, 50 μL of methanol:water (50:50, v/v) and 500 μL of water to bring the final volume of 790 μL. The samples were mixed for 20 sec by vortex mixer before extraction. The used SPE was Onepure HLB SPE cartridge (3 mL, 60 mg of sorbent) that were preconditioned with 3 mL of methanol and then 3 mL of DI water. 790 μL of the sample was slowly loaded on to the cartridge and washed with 3 mL of water. Finally, the SPE column was eluted with 3 mL of methanol. The eluent was evaporated to dryness under nitrogen steam (Caliper Life Sciences, CA, USA) at 50 ºC for 30 mins and reconstituted with 300 μL of methanol:0.04% formic acid solution (50:50, v/v). The extracted samples were analyzed by LC-MS/MS with an injection volume of 30 μL. The scheme of SPE method was shown below.



The scheme 2.2 SPE condition for plasma extraction

2.6.3 Pharmacokinetic analysis

The pharmacokinetic profiles of codeine and morphine were analyzed using non-compartmental model of software WinNonlin® version 1. 1 (Pharsight Corporation, Mountain View, CA, USA). The parameters consist of the area under the plasma concentration curve versus time profile from zero to the last time ($AUC_{0\rightarrow t}$). The $AUC_{0\rightarrow t}$ was calculated using the linear trapezoidal rule from 0 to 4 h and extrapolated to the area under the plasma concentration curve versus time profile from zero to infinity ($AUC_{0\rightarrow\infty}$). The maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) of codeine, morphine, and mitragynine were obtained from individual actual data. The elimination rate constant (λ_z), elimination half-life ($t_{1/2}$), clearance (CL/F) and volume of distribution (V_z/F) were estimated using equation as follow.

- **The elimination rate constant**

$$\lambda_z = \frac{\text{- (slope of the terminal phase of concentration - curve)}}{2.303}$$

- **Elimination half-life**

$$t_{1/2} = \frac{0.693}{\lambda_z}$$

- **Clearance**

$$Cl_t/F = \lambda_z \cdot V_z/F$$

- **Volume of distribution**

$$V_z/F = \frac{\text{Dose}}{\lambda_z \cdot AUC_{0\rightarrow\infty}}$$

2.7 Statistic analysis

All pharmacokinetic parameters were expressed as the mean ± standard deviation ($\bar{x} \pm SD$). Independent *t*-test was applied for comparisons. The significant level was set at *p*-value less than 0.05. The used software was R Statistical Software.

CHAPTER 3

RESULTS

3.1 Isolation of mitragynine

Mitragynine, the major active compound was isolated from kratom leaves (*Mitragyna speciosa* (Korth.) Havil.). Dry kratom leaves weigh 1.23 were macerated in methanol that was adjusted to pH 9.0 (repeated for 4 times) to obtain crude methanol extract 228.06 g. The crude methanol extract was separated by acid-base extraction method to obtain the alkaloid extract 9.82 g. The crude alkaloid extract was separated on silica gel column chromatography and obtained a major compound wich defined as mitragynine 4.66 g. The characteristic of mitragynine was yellowish-brown amorphous solid. The percent yield was 0.37% based on dry leaves. The isolated compound was checked by thin-layer chromatography (TLC) detected under ultraviolet light at the wavelength of 254 and 366 nm and sprayed with Dragendorff reagent. It was compared with the authentic mitragynine in three suitable solvent systems including hexane:ethyl acetate (8:2), chloroform:ethyl acetate (9:1), and chloroform : methanol (7:3). The R*f* values were 0.3, 0.5, and 0.7, respectively. Mitragynine was further confirmed by ^{1}H-NMR spectroscopy. The spectra data of mitragynine (appendix D) was compared with the published data as shown in Table 3.1 and the molecular formula was identified as $C_{23}H_{30}N_2O_4$ (Kaewpradub, 1990).

Table 3.1 The ^{1}H-NMR spectral data of mitragynine in deuterated chloroform ($CDCl_3$, 500 MHz) compared with the published chemical shift assignment (Kaewpradub, 1990).

Position	**δ_H (ppm) (J Hz)** **Isolated mitragynine**	**δ_H (ppm) (J Hz)** **Mitragynine**
NH	7.68 (1H, br-s)	7.67 (br-s)
3	2.80-3.25 (1H, m)	3.12 (m)
5	2.80-3.25 (1H, m)	2.92 (m)
	2.44-2.52 (1H, br-d, *J*= 12.2 Hz)	2.42 (br-d, *J*=12.7 Hz)
6	2.80-3.25 (1H, m)	3.19 (m)
	2.80-3.25 (1H, m)	2.96 (m)
10	6.43 (1H, d, *J*=7.8Hz)	6.43 (d, *J*=7.78 Hz)
11	6.97 (1H, dd, *J*=8.1, 7.8Hz)	6.97 (t, *J*=8.03 Hz)
12	6.88 (1H, d, *J*=7.6 Hz)	6.88 (d, *J*=8.04 Hz)
14	2.49 (1H,m)	2.47 (m)
	1.77 (1H, m)	1.78 (m)
15	2.80-3.25 (1H, m)	3.00 (m)
17	7.41 (1H,s)	7.41 (s)
18	0.84 (3H, br-t)	0.83 (t, *J*=7.2 Hz)
19	1.77 (1H, m)	1.76 (m)
	1.17 (1H, m)	1.17 (m)
20	(1H, overlapped)	1.64 (m)
21	2.80-3.25 (1H, m)	3.02 (m)
	2.45 (1H, d, *J*=12.2 Hz)	2.42 (br-d, *J*=12.7 Hz)
9-OCH_3	3.85 (3H, s)	3.86 (s)
17- OCH_3	3.71 (3H, s)	3.72 (s)
22- OCH_3	3.68 (3H, s)	3.69 (s)

3.2 LC-MS/MS analysis

The predominant transitions of codeine, morphine, and mitragynine were quantified by mass to charge ratio (m/z). The precursor and product ions of the analytes were shown in Table 3.2.

Table 3.2 The precursor and product ions (m/z) of codeine, morphine, mitragynine, and IS

Analytes	**Precursors (m/z)**	**Primary products (m/z)**	**Secondary products (m/z)**
Codeine	300.01	215.19	165.23
Morphine	285.98	201.2	165.27
Mitragynine	399.09	174.22	159.2
IS	328.05	310.23	NA

NA: not applicable

3.3 Validation method

3.2.1 Calibration curve

The calibration curves of codeine were shown over the concentration range were 5-400 ng/mL with the coefficient of determination (r^2) of 0.9999. Each concentration was replicated five times. The accuracy and precision of codeine were within 93.55-106.72% and 0.29-7.06%, respectively. The linear regression equation of calibration curve was shown in Fig 3.1.

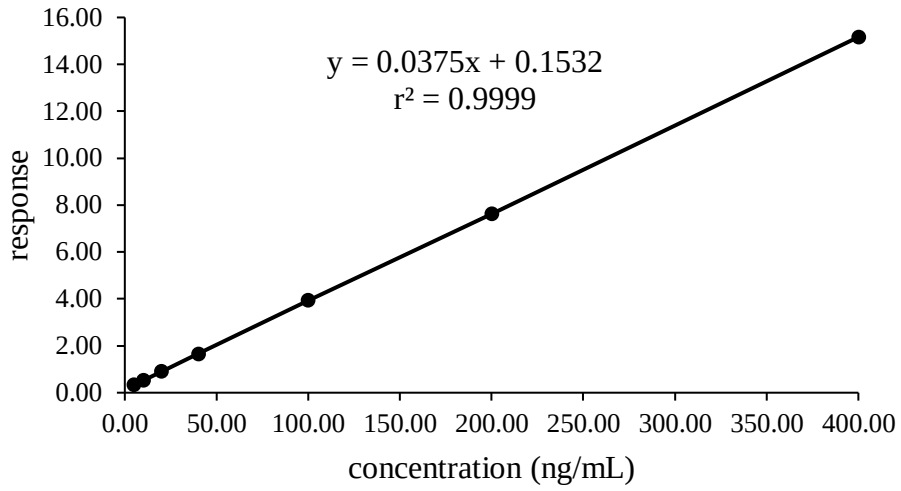


Fig 3.1 The calibration curve was plotted between response (y), the peak area ratio of codeine to that IS against the concentration (x) of codeine with $r^2 = 0.9999$.

The calibration curve of morphine was fitted over the concentration range of 5-250 ng/mL with r^2 of 0.9999. Each concentration was replicated five times. The accuracy and precision of morphine were within 98.34-107.16 and 0.84-5.48 %, respectively. The linear regression equation of the calibration curve was shown in Fig 3.2.

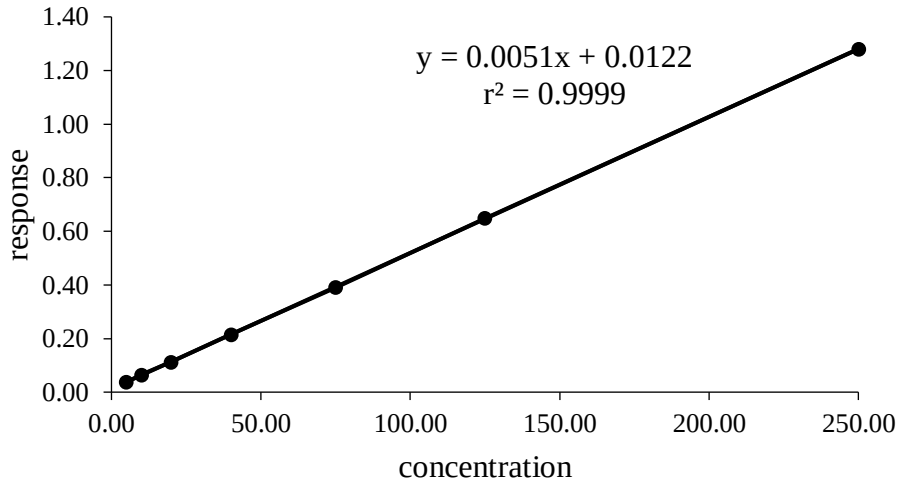


Fig 3.2 The calibration curve was plotted between response (y), the peak area ratio of morphine to that IS against concentration (x) of morphine with $r^2 = 0.9999$.

The calibration curve of mitragynine was fitted over the concentration range of 5-200 ng/mL with r^2 of 0.9998. Each concentration was replicated five times. The accuracy and precision of mitragynine were within 93.28-106.45 and 0.72-11.08 %, respectively. The linear regression equation of the standard curve was shown in Fig 3.3.

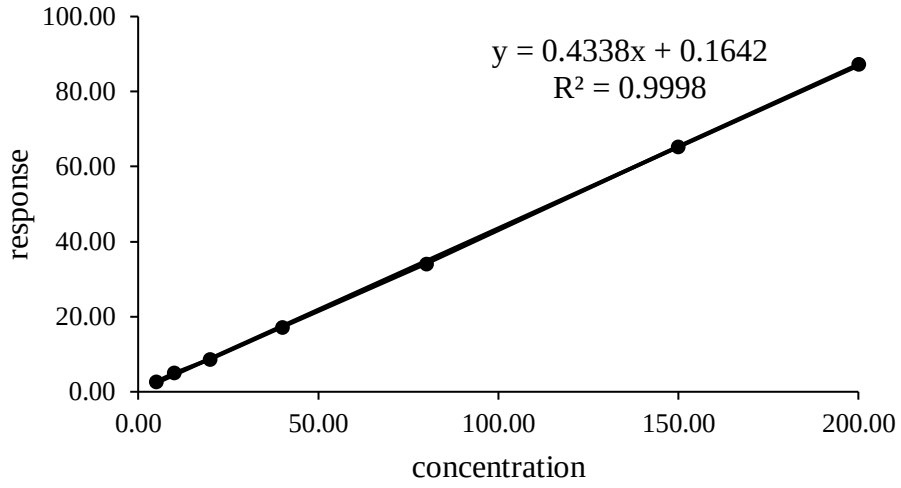


Fig 3.3 The calibration curve was plotted between response (y), the peak area ratio of mitragynine to that IS against concentration (x) of mitragynine with $r^2 = 0.9998$.

3.2.2 Selectivity

No interfering peaks were detected in blank plasma from six different sources at retention time for codeine of 8.5 min (Fig 3.4), morphine of 6.8 min (Fig. 3.5), mitragynine of (Fig. 3.6), and IS of 8.3 min (Fig. 3.7).

3.2.3 Specificity

The chromatogram of standard codeine (Fig 3.4), morphine (Fig. 3.5), mitragynine (Fig. 3.6), and IS (Fig. 3.7) spiked in blank plasma. They were detected at the individual retention time and not overlapped with each other. The retention times for codeine, morphine, mitragynine, and IS were 8.5, 6.8, 11.4, and 8.3, respectively. Although, the retention time of codeine close with IS but the m/z that considered was different.

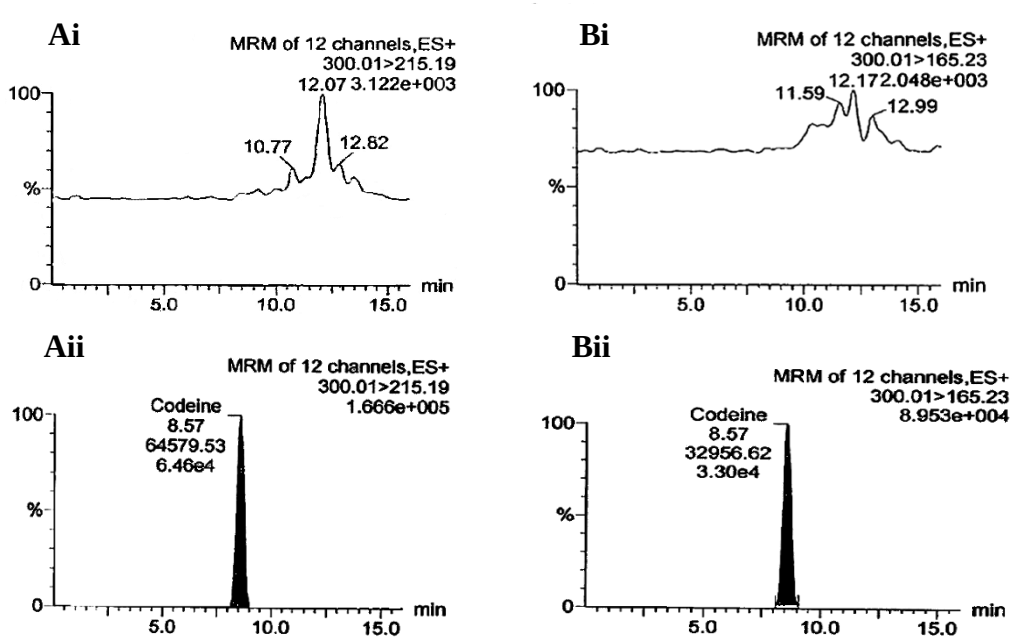


Fig. 3.4 LC-MS/MS chromatogram for codeine (A) m/z 300.01→215.19, (B) m/z 300.01→165.23: (i) blank plasma sample, (ii) blank plasma sample spiked standard codeine

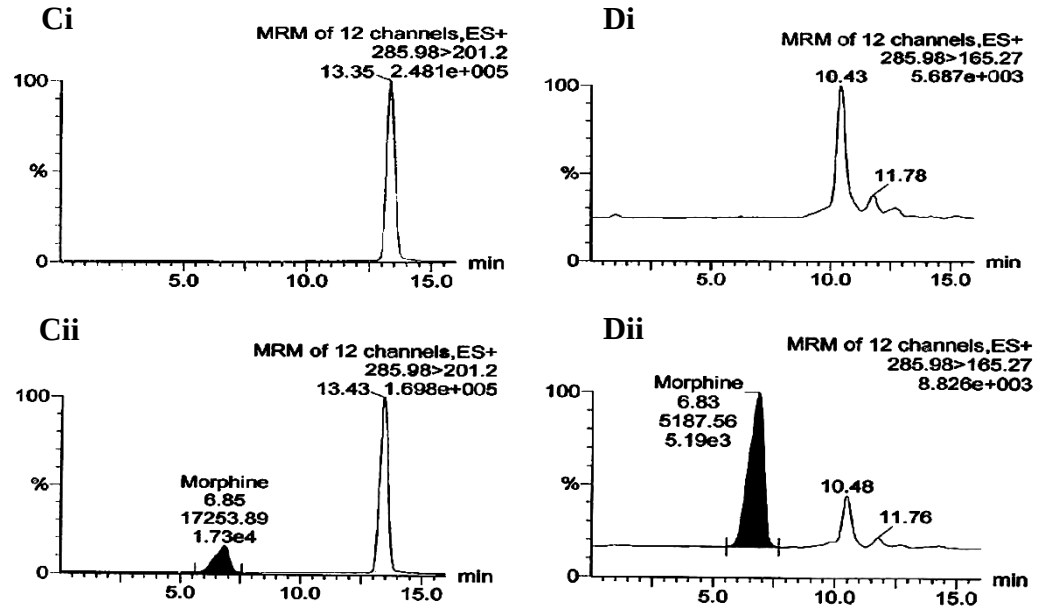


Fig. 3.5 LC-MS/MS chromatogram for morphine (C) m/z 285.98→201.2, (D) m/z 285.98→165.27: (i) blank plasma sample, (ii) blank plasma sample spiked standard morphine

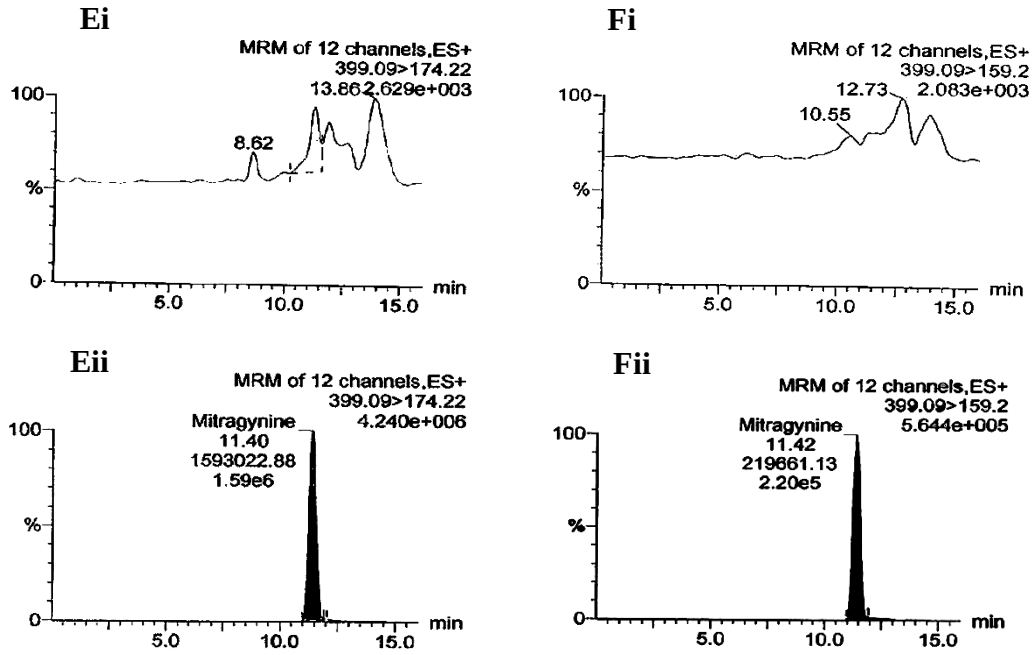


Fig. 3.6 LC-MS/MS chromatogram for mitragynine (E) m/z 399.09→174.22, (F) m/z 399.0→159.2: (i) blank plasma sample, (ii) blank plasma sample spiked standard mitragynine

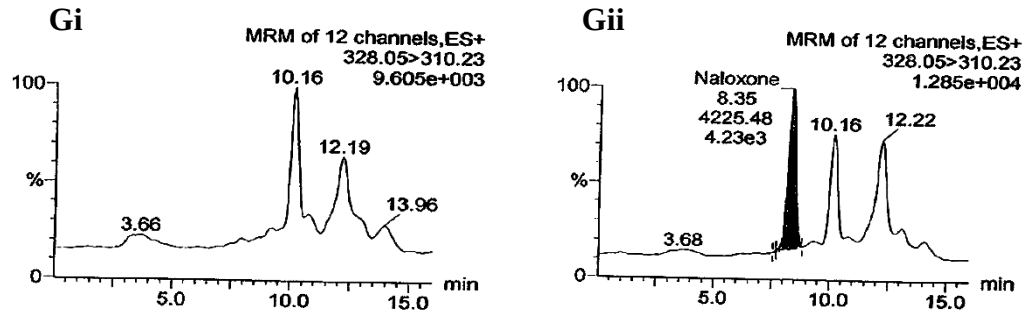


Fig. 3.7 LC-MS/MS chromatogram for IS (G) m/z 328.05→310.23: (i) blank plasma sample, (ii) blank plasma sample spiked IS

3.2.4 Carryover

No detected peak area of codeine, morphine, mitragynine and IS in blank plasma after the highest concentration in the calibration curve were injected. The chromatogram were shown (i) in Fig. 3.5, 6, 7, 8.

3.2.5 Sensitivity

The lower limit of quantification (LLOQ) of codeine, morphine, and mitragynine was 5 ng/mL with signal to noise ratio (S/N) more than five-time from blank plasma (five determination). The accuracy of codeine, morphine, and mitragynine were 98.51, 107.42, and 106.96%, the precision were 10.59, 5.70, and 6.24%, respectively as shown in Table 3.2.

3.2.6 Precision and accuracy

The intra-and inter-day precision, as well as accuracy of codeine and morphine, were evaluated by QC samples (replicated for five times). The intra- and inter-day precisions were 0.32-5.52 and 2.40-7.42%, respectively. The accuracy was 98.51-107.42%. The data was shown in Table 3.3.

Table 3.3 Sensitivity, intra- and inter-day precision and accuracy of codeine, morphine, and mitragynine (data showed mean ± SD, *n*=5).

Analyte	**Nominal conc. (ng/mL)**	**Intra-day precision**		**Inter-day precision**		**Accuracy (%)**
		Analytical conc. (ng/mL)	**CV (%)**	**Analytical conc. (ng/mL)**	**CV (%)**	
Codeine	5	NA	NA	4.93 ± 0.52	10.59	98.51
	15	16.06 ± 0.89	5.52	15.41 ± 1.09	7.08	102.73
	150	151.48 ± 1.53	1.01	151.49 ± 4.29	2.83	100.99
	350	328.39 ± 1.48	0.45	349.24 ± 12.58	3.60	99.78
Morphine	5	NA	NA	5.37 ± 0.31	5.70	107.42
	15	15.90 ± 0.26	1.62	15.02 ± 0.68	4.51	100.13
	80	77.36 ± 0.63	0.82	80.36 ± 1.93	2.40	100.45
	200	188.88 ± 0.61	0.32	202.55 ± 15.02	7.42	101.27

Analyte	Nominal conc. (ng/mL)	Intra-day precision		Inter-day precision		Accuracy (%)
		Analytical conc. (ng/mL)	CV (%)	Analytical conc. (ng/mL)	CV (%)	
Mitragy-nine	5	NA	NA	5.35 ± 0.33	6.24	106.96
	15	13.23 ± 0.27	2.02	14.87 ± 0.93	6.25	99.13
	75	71.85 ± 0.41	0.57	73.19 ± 3.95	5.40	97.58
	180	176.30 ± 0.38	0.22	175.61± 13.01	7.41	97.56

NA: not applicable

3.2.7 Recovery

The solid-phase extraction method provided percent recoveries at the concentration of QC samples more than 84.05% for codeine, 100.79% for morphine, and 91.07% for mitragynine (replicated for five times). The data was shown in Table 3.4.

Table 3.4 Recovery of codeine, morphine**,** and mitragynine (data showed mean pre and post-extraction ± SD, *n*=5).

Analytes	Concentration (ng/mL)			Recovery (%)
	Nominal conc.	pre-extraction	post-extraction	
Codeine	15	17.36 ± 0.27	16.05 ± 0.72	108.13
	150	142.74 ± 2.60	165.33 ± 5.61	86.33
	350	326.58 ± 3.61	388.57 ± 6.56	84.05
Morphine	15	16.28 ± 0.37	14.55 ± 0.35	111.85
	80	81.37 ± 0.40	80.73 ± 0.55	100.79
	200	205.68 ± 4.03	201.36 ± 1.69	102.15
Mitragynine	15	13.83 ± 0.19	13.52 ± 0.52	102.25
	75	71.40 ± 1.45	78.40 ± 1.23	91.07
	180	167.47 ± 5.07	182.16 ± 1.59	91.94

3.2.8 Matrix effect

The matrix effects were evaluated at LQC and HQC concentration. An absolute matrix effect (AE) of codeine, morphine, and mitragynine was 99. 65-103.37%. A relative matrix effect (RE) from six different sources of plasma was 1.03-2.97%. The data was shown in Table 3.5.

Table 3.5 Absolute (AE) and relative (RE) matrix effect of codeine, morphine, and mitragynine (data showed mean extracted and direct injection ± SD, *n*=6).

Analyte	**Nominal conc. (ng/mL)**	**AE**			**RE (%CV)**
		Mean conc. (ng/mL)		**AE (%)**	
		Extracted	**Direct inj.**		
Codeine	15	15.29 ± 0.16	15.35 ± 0.40	99.61	1.03
	350	355.97 ± 3.86	341.51 ± 9.73	104.23	1.09
Morphine	15	16.29 ± 0.48	15.75 ± 0.33	103.37	2.97
	200	206.73 ± 3.02	198.55 ± 4.95	104.12	1.46
Mitragynine	15	16.07 ± 0.37	15.71 ± 0.31	102.31	2.30
	180	167.59 ± 1.87	166.24 ± 4.95	100.81	1.11

3.2.8 Dilution effect

Codeine, morphine, and mitragynine were diluted from the concentration of 100 ng/mL to 10 ng/mL in plasma. The accuracy and precision were 99.83-106.52% and 0.77-8.35%, respectively (replicated for five times). The data was shown in Table 3.6.

Table 3.6 Dilution effect of codeine, morphine, and mitragynine (data showed mean ± SD, *n*=5)

Analyte	Nominal conc. (ng/mL)	Analytical conc. (ng/mL)	Accuracy (%)	Precision (%CV)
Codeine	10	10.01 ± 0.63	100.07	6.30
	100	106.42 ± 7.84	106.42	7.37
Morphine	10	10.38 ± 2.98	103.82	3.61
	100	99.83 ± 2.98	99.83	2.98
Mitragynine	10	10.65 ± 0.89	106.52	8.35
	100	100.61 ± 0.78	100.61	0.77

3.2.9 Stability

The stabilities test were performed using LQC and HQC concentration, except extract or processed sample stability. Codeine, morphine, and mitragynine were stable in all condition tests. Post-extraction sample was stable in an autosampler for 12 h at 20-25 ºC with an accuracy value range of 95.00-100.04%. The analytes in plasma were stable in room temperature for 12 h with accuracy value of 86.21-114.98%. The degradation of the analytes in freeze-thaw stability was not detected. The long term stability test after one-month storage at -20 ºC showed that all compounds remain stable. The accuracies were range of 91.82-112.00% and 97.94-114.09% for freeze-thaw and long term stability for one-month tests, respectively. However, codeine, morphine, and mitragynine were degraded after storage at -20 ºC for two months. Stock standard solutions in methanol at the concentration of 1 mg/mL of codeine and morphine and 0.01 mg/mL of mitragynine were stable in freezer at -20 ºC for three months with accuracy of 88.01-93.49 %. The data was shown in Table 3.7. The extract or processed sample stability was determined using the standard at the same concentration range of calibration curve preparation. The result showed that all concentrations studied were stable after storage in an autosampler (20-25 ºC) for 12 h. The accuracy was within 96.10-115.83%. The data was shown in Table 3.8.

Table 3. 7 Stability of codeine, morphine, and mitragynine which compose of autosampler, bench-top, freeze-thaw, long-term, and stock solution stability (data shown as %accuracy).

Analyte	**Conc. (ng/mL)**	**Auto sampler**		**Bench-top**		**Freeze-thaw (3 cycles)**	**Long-term (-20 ºC)**			**Stock solution (-20 ºC)**
		6 h	**12 h**	**6 h**	**12 h**		**Day 15**	**Day 30**	**Day 60**	
Codeine	15	100.13	96.29	103.89	95.72	112 .00	106.51	110.67	62.18	NA
	350	100.86	96.54	90.36	86.21	97.73	115.36	113.30	51.99	NA
	1×10^6	NA	NA	NA	NA	NA	NA	NA	NA	93.49
Morphine	15	94.78	98.14	90.96	96.27	99.65	100.94	97.94	96.92	NA
	200	99.33	97.20	93.02	89.72	98.11	110.91	111.36	65.83	NA
	1×10^6	NA	NA	NA	NA	NA	NA	NA	NA	89.99
Mitragynine	15	106.80	100.04	94.12	105.71	91.83	114.27	114.09	92.57	NA
	180	96.26	95.00	101.62	114.98	101.98	111.81	111.65	59.45	NA
	1×10^4	NA	NA	NA	NA	NA	NA	NA	NA	88.01

NA: not applicable

Table 3.8 The extract or processed sample stability of codeine, morphine, and mitragynine (data were shown %accuracy).

Analyte	**Nominal conc.**	**Time (h)**	
	(ng/mL)	**6 h**	**12 h**
Codeine	5	114.44	115.83
	10	106.36	114.10
	20	106.88	96.10
	40	91.94	96.61
	100	103.29	102.56
	200	98.25	97.96
	400	100.22	100.15
Morphine	5	94.77	105.42
	10	94.15	97.93
	20	98.10	105.91
	40	99.43	100.54
	75	99.66	97.05
	125	101.43	97.38
	250	99.21	99.46
Mitragynine	5	102.56	99.00
	10	110.53	100.78
	20	108.71	103.09
	40	96.31	99.34
	80	100.04	100.75
	150	97.05	98.30
	200	101.75	100.85

3.3 Effect of mitragynine on the pharmacokinetics of codeine

After pretreated rats with vehicle or mitragynine (15 mg/kg) for seven consecutive days (once per day), rats received a single oral dose of codeine (25 mg/kg). Whole blood was collected from the tail vein (550 μL). The plasma concentration versus time profiles of codeine was shown in Fig 3.8. The pharmacokinetic parameters were shown in Table 3.9. Compared with vehicle pretreatment group, the plasma concentration of codeine in MG pretreated group was significantly increased, the C_{max} increased from 1.76 μg/mL to 8.44 μg/mL, AUC_{4h} increased from 193.74 to 774.87 μg·min/mL and $AUC_{0\rightarrow\infty}$ increased from 306.08 to 993.93 μg·min/mL (p<0.05). The clearance of MG pretreated group was significant decrease, the V_z/F value decreased from 23.82 to 4.22 L/kg and Cl_t/F decreased from 0.30 to 0.03 L/kg/h (p<0.05). The half-life ($t_{1/2}$) in mitragynine pretreated group was not significantly prolonged from 71.21 to 98.71 min (p<0.095).

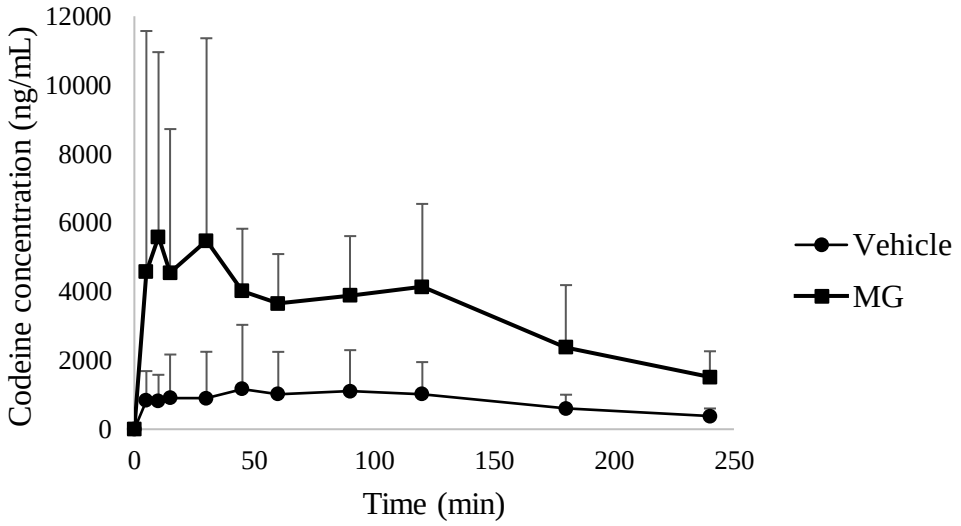


Fig 3.8 The plasma concentration versus time profile of codeine (mean ± SD) following administration of a single 25 mg/kg oral dose to vehicle-and MG-pretreated rats (n=13)

Table 3.9 Pharmacokinetic parameters of codeine (25 mg/kg) in vehicle- and MG- pre-treated rats pretreated rats (n=13)

Parameter (unit)	**Vehicle-pretreated**	**MG-pretreated**
T_{max} (min)	54.62 ± 46.21	41.15 ± 33.86
C_{max} (μg/mL)	1.76 ± 1.90	8.44 ± 7.66*
AUC_{4h} (μg·min/mL)	193.74 ± 177.44	774.87 ± 344.80*
$AUC_{0\rightarrow\infty}$ (μg·min/mL)	306.08 ± 248.96	993.93 ± 409.81*
λ_z (min^{-1})	0.0089 ± 0.0069	0.0081 ± 0.0034
$t_{1/2}$ (min)	71.21 ± 43.53	98.71 ± 36.64
Cl_t/F (L/kg/h)	0.30 ± 0.13	0.03 ± 0.01*
V_z/F (L/kg)	23.82 ± 22.33	4.22 ± 2.37*

* p<0.05, compared between vehicle pretreated and MG pretreated rats using the independent t-test.

The plasma concentration versus time profile of morphine was shown in Fig 3.9 and pharmacokinetic parameters were shown in Table 3.10. Compared with the vehicle pretreatment group, the plasma concentration of morphine in MG-pretreated group was significantly decreased, C_{max} decreased from 2.32 to 0.45 μg/mL and AUC_{4h} decreased from 415.42 to 58.03 μg·h/mL (p<0.05).

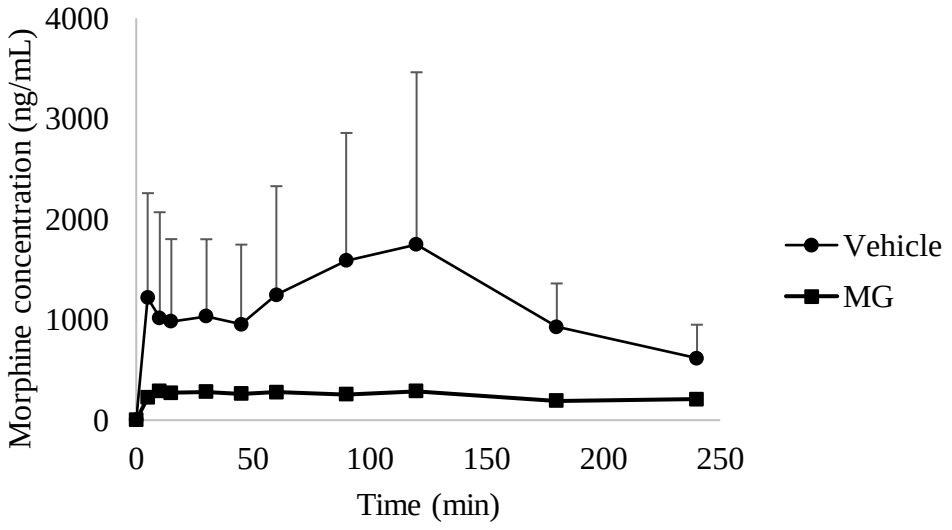


Fig 3.9 The plasma concentration versus time profile of morphine (mean ± SD) following a single oral dose of codeine 25 mg/kg to the vehicle- and MG-pretreated rats (n=13).

Table 3.10 Pharmacokinetic parameters of morphine following a single oral dose of codeine 25 mg/kg to the vehicle- and MG-pretreated rats (data showed mean ± SD, *n*=13).

Parameter (unit)	**Vehicle pretreated**	**MG pretreated**
T_{max} (min)	81.92 ± 62.80	69.23 ± 65.98
C_{max} (μg/mL)	2.32 ± 1.62	0.45 ± 0.23*
AUC_{4h} (μg·h/mL)	415.42 ± 479.48	58.03 ± 28.25*

* $p<0.05$, compared between vehicle pretreated and MG pretreated rats using the independent *t*-test

The plasma concentration versus time profile of mitragynine was shown in Fig 3.10. The pharmacokinetic parameters were shown in Table 3.11. The parameters, C_{max} was 0.25 μg/mL at T_{max} was 38.46 min. The AUC_{4h}, $AUC_{0\rightarrow\infty}$, $t_{1/2}$, and Vd values were 31.54 μg·min/mL, 59.58 μg·min/mL, 186.53 min, and 74.92 L/kg, respectively.

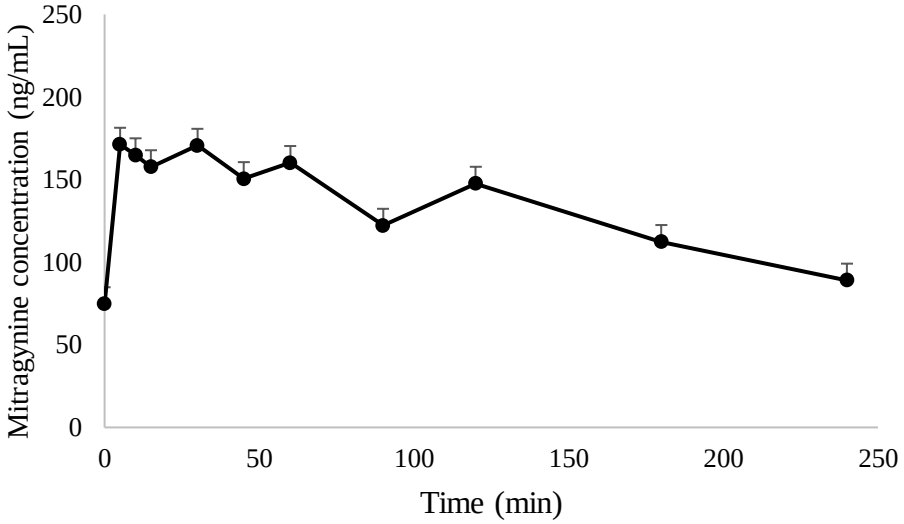


Fig 3.10 The plasma concentration versus time profile of mitragynine (mean ± SD) after receiving mitragynine for seven consecutive days (15 mg/kg, P.O.) and following by a single oral dose of codeine 25 mg/kg (*n*=13).

Table 3.11 Pharmacokinetic parameters of mitragynine after seven consecutive days (15 mg/kg, P.O.) and following a single oral dose of codeine 25 mg/kg (data showed mean ± SD, (*n*=13).

Parameter (unit)	**Mitragynine**
T_{max} (min)	38.46 ± 52.22
C_{max} (μg/mL)	0.25 ± 0.17
AUC_{4h} (μg·min/mL)	31.54 ± 20.18
$AUC_{0\rightarrow\infty}$ (μg·min/mL)	59.58 ± 40.28
λ_z (min^{-1})	0.0050 ± 0.0031
$t_{1/2}$ (min)	186.53 ± 108.91
Cl_t/F (L/kg/h)	0.38 ± 0.19
V_z/F (L/kg)	74.92 ± 60.16

CHAPTER 4

DISCUSSIONS

Kratom (*Mitragyna speciosa* (Korth.) Havil.) is a narcotic substance listed in Category V of the Thai Narcotics Act B.E. 2522. However, it is still widely used by Thai native people such as laborers and Thai Traditional medicine. The laborers use it for productive their work under the hot sun. In Thai Traditional medicine, kratom was used to treat intestinal infections, diarrhea, muscle pain, cough, and diabetes. In some native people of the Southern part of Thailand use kratom for social communication. Besides, it becomes substance abuse called "4 x 100", which is composed of many substances such as coke, antitussive drug, and codeine, etc. Either the usage or abuse, it is possible to combine kratom with other drugs.

Mitragynine is the major chemical constituent isolated from kratom leaves. Previously, it has been demonstrated that CYP2D6, CYP3A4, and CYP2C9 isoenzyme were inhibited by mitragynine. CYP2D6 is strongly inhibited by mitragynine with an IC_{50} value of 0.45 ± 0.33 µM and K_i value of 12.86 µM in human liver microsome (Ismail et al., 2013). The CYP2D6 isoenzyme inhibitor can cause pharmacokinetic drug interaction if combined with CYP2D6 substrates.

Mitragynine was isolated from dry kratom leaves. The dry leaves weigh 1.23 kg obtained pure compound mitragynine of 4.66g which estimates the percent yield of 0.37% base on dry leaves. The structure of mitragynine was confirmed by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR). The ^{1}H-NMR spectra data were confirmed by previous mitragynine published data and the molecular formula was identified as $C_{23}H_{30}N_2O_4$ (Kaewpradup, 1990; Limsuwanchote et al., 2014).

This study was performed in rat model. Sprague-Dawley (SD) rat is the suitable model for the human extensive metabolism of the CYP2D6 polymorphism. Since, SD rat expressed *CYP2D1* mRNAs which are similar sequence of gene expression with the CYP2D6 in human of 71% (Souček & Gut, 1992). The lack of *CYP2D1* expression in Dark-agouti rats (DA) caused CYP2D6 substrate metabolism deficiency (Mutsunaga et al., 1989). Dextromethorphan and propafenone are potent inhibitors of CYP2D6 isoenzyme in human liver microsome. According with the previous studied in

SD rats, quinine, methadone, propafenone, and dextromethorphan showed inhibit codeine catalyzed O-demethylation through the inhibition of CYP2D1 with K_i values of 0.07, 0.3, 0.58, and 2.53 μM, respectively (Mikus et al., 1991).

Codeine is an important CYP2D6 substrate related to kratom leaves because it is an ingredient of 4 x 100 and also is a narcotic analgesic drug. In clinical, codeine was used for pain relief and cough suppression. So, kratom users possible combine both substances and may cause drug interaction via CYP2D6 inhibition. In 2006-2008, there are 68 cases codeine relate death were reported in Toronto, Canada. About 51% of the deaths were determined CYP2D6 inhibitor in post-mortem blood test. The authors assumed the combination of codeine with CYP2D6 inhibitors may increase the risk of codeine toxicity (Lam et al., 2014).

However, the major metabolism pathway of codeine about 50-70% is glucuronidation through UGT2B7, but mitragynine could not inhibit UGT2B7 in rat and human liver microsome with no IC_{50} value could be determined (Haron & Ismail, 2015). Although, the O-demethylation by CYP2D6 isoenzyme is minor pathway of codeine metabolism (0-15%) while it becomes active metabolites for analgesic effects such as morphine and further M-6-G, which is the purpose effect of codeine administration (Thorn et al., 2009). Therefore, the combination of kratom leaves and codeine may decrease morphine level in plasma leading lower analgesic efficacy, while increase codeine level in plasma and may cause toxicity and drug tolerance.

This study performed to clarify the inhibition of mitragynine on CYP2D6 isoenzyme activity *in vivo* model. Twenty-six male SD rats were divided into two groups. Group one is the control group, rats were pretreated with vehicle and another group was pretreated by orally administered mitragynine 15 mg/kg for seven consecutive days (once/day). After two hours, rats were orally codeine 25 mg/kg. The dose was used following the therapeutic dose of codeine (240 mg/day). Blood were collected at 0 before and 5, 10, 15, 30, 45, 60, 90, 120, 180, and 240 min after receiving codeine.

The result of this study showed that the pharmacokinetic parameters of codeine and morphine were significant differences between vehicle-and mitragynine-pretreated groups. Rats pretreated mitragynine for seven consecutive days showed the plasma concentration of codeine was significant increase with the increase of absorption parameters. The C_{max} increased from 1.76 to 8.44 μg/mL. The AUC_{4h} and $AUC_{0\rightarrow\infty}$

increased from 193.73 to 774.87 µg·min/mL and 306.08 to 993.93 µg·min/mL, respectively (p<0.05). The clearance of codeine was decreased with presented by the decreased of Cl_t/F from 0.30 to 0.03 L/kg/h and V_z/F from 23.82 to 4.22 L/kg (p<0.05). However, the $t_{1/2}$ of codeine was longer than control group but there was no significant difference between the groups as well as λ_z and T_{max} values. Whereas, the plasma concentration of morphine and absorption parameters were significantly decreased. The C_{max}, decreased from 2.32 to 0.45 µg/mL and AUC_{4h} from 415.42 to 58.03 µg·min/mL (p<0.05).

The absorption parameters of codeine may increase by the pharmacological activity of mitragynine. The constipation was observed in mitragynine-pretreated rats which is consistent with the effect of mitragynine on the gastrointestinal system. Mitragynine is capable inhibiting gastric emptying (Tsuchiya et al., 2002) and decreasing intestinal transit (Chittrekarn et al., 2008). These may increase codeine absorption. The increase of absorption may improve bioavailability (F) leading to decrease clearance. Moreover, the high concentration and low clearance of codeine as well as low concentration of morphine in mitragynine-pretreated rats, may cause by the inhibition of CYP2D6 isoenzyme activity in the liver. According to the previous studies, mitragynine and also kratom extract exerted CYP2D6 isoenzyme inhibitor *in vitro* model. Methanol extract, alkaloid extract, and mitragynine from kratom leave showed strongly inhibit CYP2D6 in human liver microsome with the IC_{50} values were 3.6 µg/mL, 0.636 µg/mL, and 0.45 µM, respectively (Hanapi et al., 2010; Kong et al., 2011; Ismail et al., 2013). In addition, the inhibition of mitragynine on CYP3A4 isoenzyme activity which is secondary pathways of codeine metabolism may be another effect of this study. Mitragynine also showed inhibits CYP3A4 the in human liver microsome with IC_{50} value of 41.32 µM (Ismail et al., 2013).

Commonly, the extracts that have IC_{50} value less than 100 µg/mL or 10 µM explained potent inhibitors and IC_{50} value about 10-50 µM explained moderate inhibitors (Sevior et al., 2010; Ganzera, Schneider & Stuppner, 2006). Furthermore, the potential of drug interaction was categorized by IC_{50} value. The IC_{50} less than 1 µM showed high, 1-10 µM showed moderate, and more than 10 µM showed low potential drug interaction (Krippendorff, Lienau, Reichel & Huisinga, 2007).

On the other hand, the elimination rate constant (λ_z) of codeine was barely different in two groups. This effect may cause that mitragynine inhibits P-glycoprotein (P-gp) transporter at the intestinal barrier (Manda et al., 2014). Despite, the half-life ($t_{1/2}$) was increased but it was not significant because the $t_{1/2}$ is the parameter that extrapolated from λ_z value.

The AUC_{4h} and $AUC_{0\rightarrow\infty}$values were highly varied in this study. It may cause by the interindividual variability of rats. The genetic polymorphism was considered in CYP2D6 activity. Approximately 5.5% of Caucasians are ultra-rapid metabolizer and about 7% of Caucasians and less than 1% of Oriental are poor metabolizers. In addition, CYP2D6 isoenzyme activity was affected by enzyme inhibitors or enzyme inducers (Pelkonen et al., 2008). Since mitragynine was defined in enzyme inhibitor. It can inhibit the biotransformation from codeine to morphine leading low morphine level in plasma. This study was higher absorption parameters and lower clearance of codeine than codeine alone in rats. Rats were a single oral dose of codeine (5mg/ kg) become C_{max} of 101.3 ng/mL at time to reach C_{max} (T_{max}) of 6.4 min. The $AUC_{0\rightarrow last}$ and $AUC_{0\rightarrow\infty}$ were 3936 and 4074 ng·min/mL, respectively. The Cl_t/F of 6.3 L/kg/h and $t_{1/2}$ of 39.6 min. Codeine rapidly metabolized to morphine become C_{max} of 68.7 ng/mL at T_{max} of 6.5 min and $AUC_{0\rightarrow last}$ of 3656 ng·min/mL, no codeine detected 4 h after dosing (Shah & Mason., 1989). The differential of pharmacokinetic parameters of this study and Shah and Mason (1989) studied that mentioned above because of dose-dependent and the pretreatment of mitragynine which is CYP2D6 enzyme inhibitor. s

The inhibition of CYP2D6 isoenzyme in liver decrease morphine metabolite in plasma leads to low analgesic effects of codeine. Whereas, it improves the plasma concentration of codeine at the active sites. Since codeine is less polar, it penetrates the blood-brain barrier (BBB). Following codeine converts to morphine by CYP2D6 isoenzyme in the brain become the highest concentration of morphine at the active site and over interaction on μ-opioid receptor may lead to drug tolerance (Eckhardt et al., 1997).

The pharmacokinetic parameters of mitragynine in this study consistent with previous studied. Compared to Janchawee and colleagues (2007) studied, rats were a single oral dose of mitragynine (40 mg/kg) become T_{max}, C_{max}, $t_{1/2}$, and Cl_t/F more than

this study about 2.5-3 folds which is related with dose-dependent (Janchawee et al., 2007). In addition, over the times that increase of mitragynine concentration in plasma were showed potent inhibit codeine metabolism by high codeine and low morphine concentration were determined. Further assumed that the high concentration of mitragynine caused high inhibitor efficacy on CYP2D6 activity.

The result of this study suggested that mitragynine was altered the pharmacokinetics of codeine by increase codeine absorption. The determination showed plasma codeine concentration was increased while morphine was decreased. This interaction may cause by the inhibition of mitragynine through CYP2D6 O-demethylation or another codeine metabolism pathway such as CYP3A4 N-demethylation in the liver. In addition, this interaction may cause by pharmacological effect of mitragynine because it has the ability to inhibit gastric emptying and intestinal motility. Therefore, the mechanism of interaction needs to further confirm this evidence.

CHAPTER 5

CONCLUSION

This study was performed to investigate the effect of mitragynine on the pharmacokinetics of codeine in rat model. Since mitragynine was mentioned to that is potent CYP2D6 inhibitor in human liver microsome. The combination of codeine and mitragynine in rat showed alter pharmacokinetics of codeine. The absorption of codeine was significant increase whereas the clearances were significant decrease. Mitragynine impairs the metabolism of codeine to morphine which is low morphine concentration in plasma was determined. Now, the mechanism of interaction not yet to clear. This possible mechanism assumed that mitragynine may inhibit hepatic CYP2D6 metabolism which is metabolism pathway of codeine to that convert to morphine. Another assumption is mitragynine may increase the absorption through inhibits gastric emptying or intestinal motility. It needs to confirm this evidence study in the future. However, the combination of kratom leaves and codeine need to concern and awareness for the prevention of herb-drug interaction that may contribute to serious side effect.

BIBLIOGRAPHY

Adcock, J. J., Schneider, C., & Smith, T. W. (1988). Effects of codeine, morphine and a novel opioid pentapeptide BW443C, on cough, nociception and ventilation in the unanaesthetized guinea-pig. *British Journal of Pharmacology*, *93*(1), 93-100.

Apryani, E., Hidayat, M. T., Moklas, M. A. A., Fakurazi, S., & Idayu, N. F. (2010). Effects of mitragynine from *Mitragyna speciosa* Korth. leaves on working memory. *Journal of Ethnopharmacology*, *129*(3), 357-360.

Assanangkornchai, S., Chittrakarn, S., Tantirangsee, N., Saengow, U., Pruphetkaew, N., Sanphet, T., & Chukaew, W. (2016). Size estimation of substance user population in southern Thailand 2016. Office of the narcotics control board of Thailand.

Azizi, J., Ismail, S., Mordi, M. N., Ramanathan, S., Said, M. I. M., & Mansor, S. M. (2010). In vitro and in vivo effects of three different *Mitragyna speciosa* Korth. leaf extracts on phase II drug metabolizing enzymes-glutathione transferases (GSTs). *Molecules*, *15*(1), 432-441.

Babu, K. M., McCurdy, C. R., & Boyer, E. W. (2008). Opioid receptors and legal highs: *Salvia divinorum* and Kratom. *Clinical Toxicology*, *46*(2), 146-152.

Beckett, A. H., Shellard, E. J., Phillipson, J. D., & Lee, C. M. (1966a). THE MITRAGYNA SPECIES OF ASIA–Part. VI. Oxindole alkaloids from the leaves of *Mitragyna speciosa* Korth. 1, 2. *Planta Medica*, *14*(03), 266-276.

Beckett, A. H., Shellard, E. J., Phillipson, J. D., & Lee, C. M. (1966b). THE *MITRAGYNA* SPECIES OF ASIA–Part. VII. Indole Alkaloids from the leaves of *Mitragyna speciosa* Korth. 1, 2. *Planta Medica*, *14*(03), 277-288.

Botpiboon, O., Prutipanlai, S., Janchawee, B., & Thainchaiwattana, S. (2009). Effects of caffeine and codeine on antinociceptive activity of alkaloid extract from leaves

of kratom (*Mitragyna speciosa* Korth). (Master Thesis). Prince of Songkla University, Songkhla, Thailand.

Buasri, N., Sengnon, N., Sriwiriyajan, S., Wungsintaweekul, J., Udomaksorn, W., Limsuwanchote, S., …. Assanangkornchai, S. (2017). Pharmacokinetics of mitragynine from kratom leaves in Thai healthy male volunteers and its metabolites. (Master Thesis). Prince of Songkla University, Songkhla, Thailand.

Chau, T. T., & Harris, L. S. (1980). Comparative studies of the pharmacological effects of the d- and l-isomers of codeine. *Journal of Pharmacology and Experimental Therapeutics*, *215*(3), 668-672.

Chittrakarn, S., Keawpradub, N., Sawangjaroen, K., Kansenalak, S., & Janchawee, B. (2010). The neuromuscular blockade produced by pure alkaloid, mitragynine and methanol extract of kratom leaves (*Mitragyna speciosa* Korth.). *Journal of Ethnopharmacology*, *129*(3), 344-349.

Chittrakarn, S., Penjamras, P., & Keawpradub, N. (2012). Quantitative analysis of mitragynine, codeine, caffeine, chlorpheniramine and phenylephrine in a kratom (*Mitragyna speciosa* Korth.) cocktail using high-performance liquid chromatography. *Forensic Science International*, *217*(1-3), 81-86.

Chittrakarn, S., Sawangjaroen, K., Prasettho, S., Janchawee, B., & Keawpradub, N. (2008). Inhibitory effects of kratom leaf extract (*Mitragyna speciosa* Korth.) on the rat gastrointestinal tract. *Journal of Ethnopharmacology*, *116*(1), 173-178.

Court, M. H., Krishnaswamy, S., Hao, Q., Duan, S. X., Patten, C. J., Von Moltke, L. L., & Greenblatt, D. J. (2003). Evaluation of 3'-azido-3'-deoxythymidine, morphine, and codeine as probe substrates for UDP-glucuronosyltransferase 2B7 (UGT2B7) in human liver microsomes: specificity and influence of the UGT2B7*2 polymorphism. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *31*(9), 1125-1133.

de Moraes, N. V., Moretti, R. A. C., Furr, E. B., McCurdy, C. R., & Lanchote, V. L. (2009). Determination of mitragynine in rat plasma by LC– MS/MS: Application to pharmacokinetics. *Journal of Chromatography B, 877*(24), 2593-2597.

Dunnick, J. K., & Elwell, M. R. (1989). Toxicity studies of amphetamine sulfate, ampicillin trihydrate, codeine, 8- methoxypsoralen, alpha- methyldopa, penicillin VK and propantheline bromide in rats and mice. *Toxicology, 56*(2), 123-136.

E. Adkins, J., W. Boyer, E., & R. McCurdy, C. (2011). *Mitragyna speciosa*, A Psychoactive Tree from Southeast Asia with Opioid Activity. *Medicinal Chemistry, 11*(9), 1165-1175.

Eckhardt, K., Li, S., Ammon, S., Schänzle, G., Mikus, G., & Eichelbaum, M. (1998). Same incidence of adverse drug events after codeine administration irrespective of the genetically determined differences in morphine formation. *Pain, 76*(1-2), 27-33.

Ganzere, M., Schneider, P., & Stuppner, H. (2006). Inhibitory effects of the essential oil of chamomile (*Matricaria recutita* L.) and its major constituents on human cytochrome P450 enzymes. *Life Science Journal, 78, 856-861.*

Grewal, K. S. (1932). Observations on the Pharmacology of Mitragynine. *Journal of Pharmacology and Experimental Therapeutics*, *46*(3), 251-271.

Hanapi, N. A., Azizi, J., Ismail, S., & Mansor, S. M. (2010). Evaluation of Selected Malaysian Medicinal Plants on Phase I Drug Metabolizing Enzymes, CYP2C9, CYP2D6 and CYP3A4 Activities in vitro. *International Journal of Pharmacology*, *6*(4), 494-499.

Harizal, S. N., Mansor, S. M., Hasnan, J., Tharakan, J. K. J., & Abdullah, J. (2010). Acute toxicity study of the standardized methanolic extract of *Mitragyna speciosa* Korth. in Rodent. *Journal of Ethnopharmacology, 131*(2), 404-409.

Haron, M., & Ismail, S. (2015). Effects of mitragynine and 7-hydroxymitragynine (the alkaloids of *Mitragyna speciose* Korth) on 4-methylumbelliferone glucuronidation in rat and human liver microsomes and recombinant human

uridine 5'-diphospho-glucuronosyltransferase isoforms. *Pharmacognosy Research, 7(4)*, 341-349.

Hassan, Z., Muzaimi, M., Navaratnam, V., Yusoff, N. H. M., Suhaimi, F. W., Vadivelu, R., … Müller, C. P. (2013). From Kratom to mitragynine and its derivatives: Physiological and behavioural effects related to use, abuse, and addiction. *Neuroscience & Biobehavioral Reviews*, *37*(2), 138-151.

Hazim, A. I., Mustapha, M., & Mansor, S. M. (2011). The effects on motor behaviour and short-term memory tasks in mice following an acute administration of *Mitragyna speciosa* alkaloid extract and mitragynine. *Journal of Medicinal Plants Research*, *5*(24), 5810-5817.

Hinder, M. (2011). Pharmacodynamic Drug–Drug Interactions. In H. G. Vogel, J. Maas, & A. Gebauer (Eds.), *Drug Discovery and Evaluation: Methods in Clinical Pharmacology* (pp. 367-376). Berlin, Heidelberg: Springer Berlin Heidelberg.

Hinson, J. A., & Forkert, P. G. (1995). Phase II enzymes and bioactivation. *Canadian Journal of Physiology and Pharmacology*, *73*(10), 1407-1413.

Houghton, P. J., Latiff, A., & Said, I. M. (1991). Alkaloids from *Mitragyna speciosa*. *Phytochemistry*, *30*(1), 347-350.

Huffman, D. H., & Ferguson, R. L. (1975). Acute codeine overdose: correspondence between clinical course and codeine metabolism. *The Johns Hopkins Medical Journal*, *136*(4), 183-186.

Idayu, N. F., Hidayat, M. T., Moklas, M. A. M., Sharida, F., Raudzah, A. N., Shamima, A. R., & Apryani, E. (2011). Antidepressant-like effect of mitragynine isolated from *Mitragyna speciosa* Korth. in mice model of depression. *Phytomedicine*, *18*(5), 402–407.

Ismail, S., Mansor, S., & Hanapi, N. (2013). Inhibitory effect of mitragynine on human cytochrome P450 enzyme activities. *Pharmacognosy Research*, *5*(4), 241-247.

Janchawee, B., Keawpradub, N., Chittrakarn, S., Prasettho, S., Wararatananurak, P., & Sawangjareon, K. (2007). A high-performance liquid chromatographic method for determination of mitragynine in serum and its application to a pharmacokinetic study in rats. *Biomedical Chromatography*, *21*(2), 176-183.

Karlsson, J. A., Lanner, A. S., & Persson, C. G. (1990). Airway opioid receptors mediate inhibition of cough and reflex bronchoconstriction in guinea pigs. *Journal of Pharmacology and Experimental Therapeutics*, *252*(2), 863-868.

Katzung, B. G. (2018). *Basic & clinical pharmacology*. Retrieved from http://accessmedicine.mhmedical.com/book.aspx?bookid=2249.

Keawpradub, N. (1990). Alkaloids from the fresh leaves of *Mitragyna speciosa*. (Master Thesis). Chulalongkorn University, Bangkok, Thailand

Kido, Y., Matsson, P., & Giacomini, K. M. (2011). Profiling of a prescription drug library for potential renal drug-drug interactions mediated by the organic cation transporter 2. *Journal of Medicinal Chemistry*, *54*(13), 4548-4558.

Kobayashi, S., Murray, S., Watson, D., Sesadic, D., Davies, D. S., & Boobis, R. (1989). The specificity of inhibition of debrisoquine 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man. *Biochemical pharmacology*, *38*(17). 2795-2799.

Kong, W. M., Chik, Z., Ramachandra, M., Subramaniam, U., Aziddin, R. E. R., & Mohamed, Z. (2011). Evaluation of the Effects of *Mitragyna speciosa* Alkaloid Extract on Cytochrome P450 Enzymes Using a High Throughput Assay. *Molecules*, *16*(9), 7344-7356.

Kotzer, C. J., Hay, D. W., Dondio, G., Giardina, G., Petrillo, P., & Underwood, D. C. (2000). The antitussive activity of δ-opioid receptor stimulation in guinea pigs. *Journal of Pharmacology and Experimental Therapeutics*, *292*(2), 803-809.

Krippendorff, B., Lienau, P., Reichel, A., and Huisinga, W. (2007). Optimizing Classification of Drug-Drug Interaction Potential for CYP450 Isoenzyme

Inhibition Assays in Early Drug Discovery. *Journal of biomolecular screening*, *12*(1), 92-99.

Kronstrand, R., Roman, M., Thelander, G., & Eriksson, A. (2011). Unintentional Fatal Intoxications with Mitragynine and O-Desmethyltramadol from the Herbal Blend Krypton. *Journal of Analytical Toxicology,s 35*, 242-247.

Kruegel, A. C., Gassaway, M. M., Kapoor, A., Váradi, A., Majumdar, S., Filizola, M., … Sames, D. (2016). Synthetic and Receptor Signaling Explorations of the *Mitragyna* Alkaloids: Mitragynine as an Atypical Molecular Framework for Opioid Receptor Modulators. *Journal of the American Chemical Society*, *138*(21), 6754-6764.

Kumarnsit, E., Keawpradub, N., & Nuankaew, W. (2006). Acute and long-term effects of alkaloid extract of *Mitragyna speciosa* on food and water intake and body weight in rats. *Fitoterapia*, *77*(5), 339-345.

Lam, J., Woodall, K. L., Solbeck, P., Ross, C. J. D., Carleton, B. C., Hayden, M. R., … Madadi, P. (2014). Codeine-related deaths: The role of pharmacogenetics and drug interactions. *Forensic Science International*, *239*, 50-56.

Levy, R. H. (1995). Cytochrome P450 isozymes and antiepileptic drug interactions. *Epilepsia*, *36 Suppl 5*, S8-13.

Limpanuparb, T., Noorat, R., & Tantirungrotechai, Y. (2019). In silico investigation of mitragynine and 7-hydroxymitragynine metabolism. *BMC Research Notes, 12*(451), 1-5

Limsuwanchote, S., Wungsintaweekul, J., Keawpradub, N., Putalun, W., Morimoto, S., & Tanaka, H. (2014). Development of indirect competitive ELISA for quantification of mitragynine in Kratom (*Mitragyna speciosa* (Roxb.) Korth.). *Forensic Science International*, *244*, 70-77.

Liston, H. L., Markowitz, J. S., & DeVane, C. L. (2001). Drug glucuronidation in clinical psychopharmacology. *Journal of Clinical Psychopharmacology*, *21*(5), 500-515.

Löfgren, S., Hagbjörk, A.-L., Ekman, S., Fransson-Steen, R., & Terelius, Y. (2004). Metabolism of human cytochrome P450 marker substrates in mouse: a strain and gender comparison. *Xenobiotica*, *34*(9), 811-834.

Manda, V., Avula, B., Ali, Z., Khan, I., Walker, L., & Khan, S. (2014). Evaluation of In Vitro Absorption, Distribution, Metabolism, and Excretion (ADME) Properties of Mitragynine, 7-Hydroxymitragynine, and Mitraphylline. *Planta Medica*, *80*(07), 568-576.

Matsumoto, K., Hatori, Y., Murayama, T., Tashima, K., Wongseripipatana, S., Misawa, K., … Horie, S. (2006). Involvement of mu-opioid receptors in antinociception and inhibition of gastrointestinal transit induced by 7-hydroxymitragynine, isolated from Thai herbal medicine *Mitragyna speciosa*. *European Journal of Pharmacology*, *549*(1-3), 63-70.

Matsumoto, K., Yamamoto, L. T., Watanabe, K., Yano, S., Shan, J., Pang, P. K., … Horie, S. (2005). Inhibitory effect of mitragynine, an analgesic alkaloid from Thai herbal medicine, on neurogenic contraction of the vas deferens. *Life Sciences*, *78*(2), 187-194.

Matsumoto, K., Mizowaki, M., Suchitra, T., Murakami, Y., Takayama, H., Sakai, S., … Watanabe, H. (1996b). Central antinociceptive effects of mitragynine in mice: contribution of descending noradrenergic and serotonergic systems. *European Journal of Pharmacology*, *317*(1), 75-81.

Matsumoto, K., Mizowaki, M., Suchitra, T., Takayama, H., Sakai, S., Aimi, N., & Watanabe, H. (1996a). Antinociceptive action of mitragynine in mice: Evidence for the involvement of supraspinal opioid receptors. *Life Sciences*, *59*(14), 1149-1155.

Matsunaga, E., Zanger, U. M., Hardwick, J. P., Gelboin, H. V., Meyer, U. A., & Gonzalez, F. J. (1989). The CYP2D gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry*, *28*(18), 7349-7355.

Matuszewski, B.K. (2006). Standard line slopes as a measure of a relative matrix effect in quantitative HPLC–MS bioanalysis. *Journal of chromatography B, 830*, 293-300.

Meert, T. F., & Vermeirsch, H. A. (2005). A preclinical comparison between different opioids: antinociceptive versus adverse effects. *Pharmacology Biochemistry and Behavior*, *80*(2), 309-326.

Mikus, G., Somogyi, A. A., Bochner F., & Eichelbaum, M. (1991). Codeine O-demethylation: Rat strain differences and the effects of inhibitors. *Biochemical pharmacology. 41*(5). 757-762

Monte, A. A., Heard, K. J., Campbell, J., Hamamura, D., Weinshilboum, R. M., & Vasiliou, V. (2014). The Effect of CYP2D6 Drug- Drug Interactions on Hydrocodone Effectiveness. *Academic Emergency Medicine: Official Journal of the Society for Academic Emergency Medicine*, *21*(8), 879-885.

Mossadeq, W. S., Sulaiman, M. R., Mohamad, T. T., Chiong, H. S., Zakaria, Z. A., Jabit, M. L., … Israf, D. A. (2009). Anti-inflammatory and antinociceptive effects of *Mitragyna speciosa* Korth. methanolic extract. *Medical Principles and Practice*, *18*(5), 378-384.

Nair, AB., & Jacob, S., (2016). A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharma, 7*, 27-31.

Nelson, D. R., Koymans, L., Kamataki, T., Stegeman, J. J., Feyereisen, R., Waxman, D. J., … Nebert, D. W. (1996). P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*, *6*(1), 1-42.

Norakanphadung, P. (1966). Pramuan Khuamru Ruang Yaseptit Hai Thot. *Thanyarak Hospital, Bangkok*. 16-20.

Palleria, C., Di Paolo, A., Giofrè, C., Caglioti, C., Leuzzi, G., Siniscalchi, A., … Gallelli, L. (2013). Pharmacokinetic drug- drug interaction and their implication in clinical management. *Journal of Research in Medical Sciences: The Official Journal of Isfahan University of Medical Sciences*, *18*(7), 601-610.

Paolino,P.(1990).Codeine. Retrieved from http://www.inchem.org/documents/pims/pharm/codeine.htm.

Parke, T. J., Nandi, P. R., Bird, K. J., & Jewkes, D. A. (1992). Profound hypotension following intravenous codeine phosphate. Three case reports and some recommendations. *Anaesthesia*, *47*(10), 852-854.

Parthasarathy, S., Bin Azizi, J., Ramanathan, S., Ismail, S., Sasidharan, S., Said, M. I. M., & Mansor, S. M. (2009). Evaluation of antioxidant and antibacterial activities of aqueous, methanolic and alkaloid extracts from *Mitragyna speciosa* (Rubiaceae family) leaves. *Molecules*, *14*(10), 3964-3974.

Parthasarathy, S., Ramanathan, S., Ismail, S., Adenan, M. I., Mansor, S. M., & Murugaiyah, V. (2010). Determination of mitragynine in plasma with solid-phase extraction and rapid HPLC– UV analysis, and its application to a pharmacokinetic study in rat. *Analytical and Bioanalytical Chemistry*, *397*(5), 2023-2030.

Pathan, H., & Williams, J. (2012). Basic opioid pharmacology: an update. *British Journal of Pain*, *6*(1), 11-16.

Pelkonen, O., Turpeinen, M., Hakkola, J., Honkakoski, P., Hukkanen, J., & Raunio, H. (2008). Inhibition and induction of human cytochrome P450 enzymes: current status. *Archives of Toxicology*, *82*(10), 667–715.

Philipp, A. A., Wissenbach, D. K., Zoerntlein, S. W., Klein, O. N., Kanogsunthornrat, J., & Maurer, H. H. (2009). Studies on the metabolism of mitragynine, the main alkaloid of the herbal drug Kratom, in rat and human urine using liquid chromatography-linear ion trap mass spectrometry: Mitragynine metabolism by LC-MS. *Journal of Mass Spectrometry*, *44*(8), 1249-1261.

Pleuvry, B. J. (2005). Pharmacodynamic and pharmacokinetic drug interactions. *Anaesthesia and intensive care medicine*, *6*(4), 129-133.

Ponglux, D., Wongseripipatana, S., Takayama, H., Kikuchi, M., Kurihara, M., Kitajima, M., … Sakai, S. (1994). A new indole alkaloid, 7 α-hydroxy-7H-mitragynine,

from *Mitragyna speciosa* in Thailand. *Planta Medica*, *60*(06), 580-581.

Purintrapiban, J., Keawpradub, N., Kansenalak, S., Chittrakarn, S., Janchawee, B., & Sawangjaroen, K. (2011). Study on glucose transport in muscle cells by extracts from *Mitragyna speciosa* (Korth) and mitragynine. *Natural Product Research*, *25*(15), 1379-1387.

Reanmongkol, W., Keawpradub, N., & Sawangjaroen, K. (2007). Effects of the extracts from *Mitragyna speciosa* Korth. leaves on analgesic and behavioral activities in experimental animals. *Songklanakarin Journal Science and Technology*, *29*(1), 39-48.

Rodríguez-Fragoso, L., Martínez-Arismendi, J. L., Orozco-Bustos, D., Reyes-Esparza, J., Torres, E., & Burchiel, S. W. (2011). Potential risks resulting from fruit/vegetable-drug interactions: effects on drug-metabolizing enzymes and drug transporters. *Journal of Food Science*, *76*(4), R112-124.

Sabetghadam, A., Navaratnam, V., & Mansor, S. M. (2013a). Dose-Response Relationship, Acute Toxicity, and Therapeutic Index between the Alkaloid Extract of *Mitragyna speciosa* and Its Main Active Compound Mitragynine in Mice: Mitragynine Efficacy and Safety. *Drug Development Research*, *74*(1), 23-30.

Sabetghadam, A., Ramanathan, S., Sasidharan, S., & Mansor, S. M. (2013b). Subchronic exposure to mitragynine, the principal alkaloid of *Mitragyna speciosa*, in rats. *Journal of Ethnopharmacology*, *146*(3), 815-823.

Schellekens, K., Awouters, F., Artois, K., Frederickx, R., Hendrickx, H., Bruggen, W., & Nlemegeers, C. (1986). R 62 818, a New Analgesic: A Comparative Study with Codeine. *Drug Development Research, 8,* 353-360.

Schiller, L. R., Davis, G. R., Ana, C. A. S., Morawski, S. G., & Fordtran, J. S. (1982). Studies of the Mechanism of the Antidiarrheal Effect of Codeine. *Journal of Clinical Investigation*, *70*(5), 999-1008.

Senik, M. H., Mansor, S. M., KJ, J. T., & Abdullah, J. M. B. (2012). Effect of acute

administration of *Mitragyna speciosa* Korth. standardized methanol extract in animal model of learning and memory. *Journal of Medicinal Plants Research, 6*(6), 1007-1014.

Sevior, D. K., Hokkanen, J., Tolonen, A., Abass, K., Tursas, L., Pelkonen, O., Ahokas, J.T. (2010). Rapid screening of commercially available herbal products for the inhibition of major human hepatic cytochrome P450 enzymes using the N-in-one cocktail. *Xenobiotica, 40*(4), 245-254.

Shah, J., & Mason, W. D. (1990). Pharmacokinetics of codeine after parenteral and oral dosing in the rat. Drug Metabolism and Disposition: *The Biological Fate of Chemicals*, 18(5), 670-673.

Shellard, E. J. (1974). The alkaloids of Mitragyna with special reference to those of *Mitragyna speciosa*, Korth. *Bulletin on Narcotics, 26*(2), 41-55.

Shellard, E. J., Houghton, P. J., & Resha, M. (1978a). Mitragyna species of Asia. XXX. Oxidation products of mitragynine and speciociliatine. *Plant Med*. 34(5), 26-36.

Shellard, E. J., Houghton, P. J., & Resha, M. (1978b). Mitragyna species of Asia. XXXI. The alkaloids of *Mitragyna speciosa* Korth from Thailand. *Plant Med*. 34, 253-263.

Shellard, E. J., & Lees, M. D. (1965). The Mitragyna species of Asia. V. The anatomy of the leaves of Mitragyna speciosa Korth. *Planta Medica, 13*(3), 280-290.

Sindrup, S. H., Arendt-Nielsen, L., Brosen, K., Bjerring, P., Angelo, H.R., Eriksen, B., & Gram, L.F. (1992). The effect of quinidine on the analgesic effect of codeine. *European Journal of Clinical Pharmacology, 42*, 587-592.

Sindrup, S. H., & Brøsen, K. (1995). The pharmacogenetics of codeine hypoalgesia. *Pharmacogenetics, 5*(6), 335-346.

Souček, P., & Gut, I. (1992). Cytochromes P-450 in rats: structures, functions, properties and relevant human forms. *Xenobiotica, 22*(1), 83-103.

Srichana, K., Janchawee, B., Prutipanlai, S., Raungrut, P., & Keawpradub, N. (2015). Effects of Mitragynine and a Crude Alkaloid Extract Derived from *Mitragyna speciosa* Korth. on Permethrin Elimination in Rats. *Pharmaceutics, 7*(2), 10-26.

Suwanlert, S. (1975). A study of kratom eaters in Thailand. *Bulletin on Narcotics, 27*(3), 21-27.

Takayama, H. (2004). Chemistry and pharmacology of analgesic indole alkaloids from the rubiaceous plant, *Mitragyna speciosa*. *Chemical and Pharmaceutical Bulletin, 52*(8), 916-928.

Takayama, H., Kurihara, M., Kitajima, M., Said, I. M., & Aimi, N. (1998). New indole alkaloids from the leaves of Malaysian *Mitragyna speciosa*. *Tetrahedron, 54*(29), 8433-8440.

Taxak, N., & Bharatam, P. V. (2010). An insight into the concept and details of mechanism- based inhibition of CYP450. *Current Research Inform Pharmaceutical Science, 11*, 62-67.

Taxak, N., & Bharatam, P. V. (2014). Drug metabolism: A fascinating link between chemistry and biology. *Resonance, 19*(3), 259-282.

Thongpradichote, S., Matsumoto, K., Tohda, M., Takayama, H., Aimi, N., Sakai, S., & Watanabe, H. (1998). Identification of opioid receptor subtypes in antinociceptive actions of supraspinally-admintstered mitragynine in mice. *Life Sciences, 62*(16), 1371-1378.

Thorn, C. F., Klein, T. E., & Altman, R. B. (2009). Codeine and morphine pathway: *Pharmacogenetics and Genomics, 19*(7), 556-558.

Tompkins, L. M., & Wallace, A. D. (2007). Mechanisms of cytochrome P450 induction. *Journal of Biochemical and Molecular Toxicology, 21*(4), 176-181.

Tsuchiya, S., Miyashita, S., Yamamoto, M., Horie, S., Sakai, S.-I., Aimi, N., … Watanabe, K. (2002). Effect of mitragynine, derived from Thai folk medicine,

on gastric acid secretion through opioid receptor in anesthetized rats. *European Journal of Pharmacology*, *443*(1), 185-188.

USFDA. (2018). Bioanalytical method validation guidance for industry. Retrieved from https://www.fda.gov/media/70858/download.

Utar, Z., Majid, M. I. A., Adenan, M. I., Jamil, M. F. A., & Lan, T. M. (2011). Mitragynine inhibits the COX-2 mRNA expression and prostaglandin E2 production induced by lipopolysaccharide in RAW264.7 macrophage cells. *Journal of Ethnopharmacology*, *136*(1), 75-82.

Vree, T. B., & Wissen CP, V. V. W. (1992). Pharmacokinetics and metabolism of codeine in humans. *Biopharmaceutics & Drug Disposition*, *13*(6), 445-460.

Wananukul, W., Trakulsrichai, S., Sathirakul, K., Auparakkitanon, S., Krongvorakul, J., Sueajai, J., … Sukasem, C. (2015). Pharmacokinetics of mitragynine in man. *Drug Design, Development and Therapy*, *9*(4). 2421-2429.

Wu, X., Zhang, W., Bai, Y., Guo, T., & Gu, J. (2013). Simultaneous analysis of codeine and its active metabolites in human plasma using liquid chromatography–tandem mass spectrometry: Application to a pharmacokinetic study after oral administration of codeine. *Journal of Pharmaceutical and Biomedical Analysis*, *78*(79), 261-68.

Wungsintaweekul, J. (2017). Kratom. Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla university. Retrieved from http://ccpe.pharmacycouncil.org /showfile.php?file=251.

APPENDIX

APPENDIX A

PRINCE OF SONGKLA UNIVERSITY
15 Karnjanawanij Road, Hat Yai, Songkhla 90110, Thailand
Tel (66-74) 286940 Fax (66-74) 286961
Website : www.psu.ac.th

MOE 0521.11/ 012

Ref.3/2018

January 22 , 2018

This is to certify that the research project entitled "Pharmacokinetic study of codeine in rats received mitragynine from kratom" conducted by Assoc. Prof. Somsmorn Chittrakarn, Faculty of Science, Prince of Songkla University, has been approved by Institutional Animal Care and Use Committee, Prince of Songkla University.

Wantana Reanmongkol

Wantana Reanmongkol, Ph.D.
Vice Chairman,
Institutional Animal Care and Use Committee, Prince of Songkla University

APPENDIX B

Table B-1 Animal equivalent dose (AED) calculation based on body surface area (adapted and modified from FDA draft guideline, 2005).

Species	**Reference body weight (kg)**	**To convert dose in mg/kg to dose in mg/m2, divide by Km**	**To convert human dose in mg/kg to AED in mg/kg, either**	
			Multiply human dose by	**Divide human dose by**
Human	60.00	37		
Mouse	0.02	3	12.3	0.081
Hamster	0.08	5	7.4	0.135
Rat	0.15	6	6.2	0.162
Ferret	0.30	7	5.3	0.189
Guinea pig	0.40	8	4.6	0.216
Rabbit	1.80	12	3.1	0.324
Dog	10.00	20	1.8	0.541
Monkeys (rhesus)	3.00	12	3.1	0.324
Marmoset	0.35	6	6.2	0.162
Squirrel monkey	0.60	7	5.3	0.189
Baboon	12.00	20	1.8	0.541
Micro pig	12.00	27	1.4	0.730
Mini pig	40.00	35	1.1	0.946

APPENDIX C

Table C-1 The body weight of rat for dividing group base on pretreatment protocol.

n	Body weight (g)	
	Control	**Experiment**
1	400	375
2	395	375
3	390	375
4	385	370
5	385	365
6	380	365
7	380	365
8	380	360
9	380	360
10	380	355
11	375	355
12	375	350
13	375	345
Mean ±SD	383.08 ± 7.48	362.69 ± 9.71

APPENDIX D

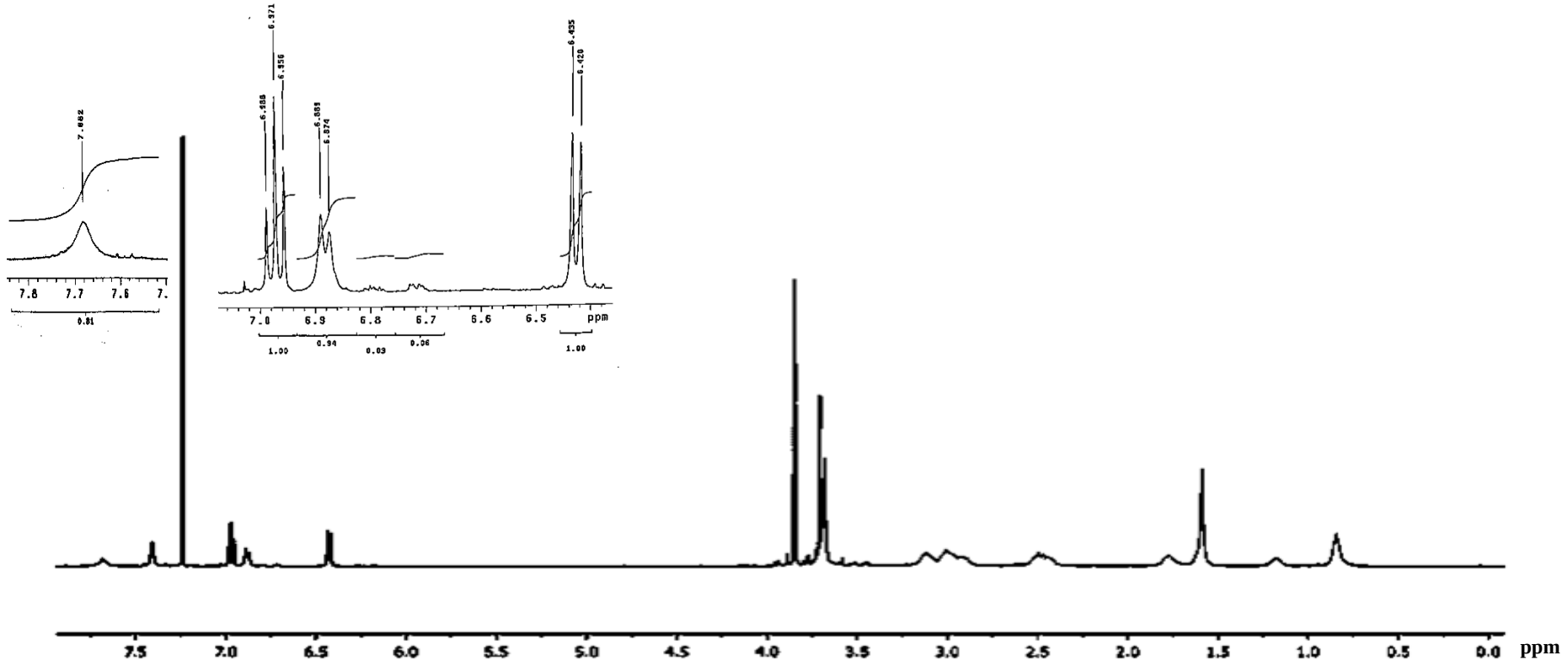


Fig. D-1 ^{1}H-NMR spectrum of mitragynine from *Mitragyna speciosa* (Korth.) Havil. leaves in deuterated chloroform ($CDCl_3$). The chemical shift (δ) of 7.68 represented NH and δ of 6.43, 6.88, and 6.97 represented aromatic ring of indole alkaloid.

APPENDIX E

Table E-1 Plasma concentration of codeine 25 mg/kg (P.O.) in rats pretreated with vehicle for seven days (control group).

Time (min)	Plasma codeine concentration (ng/mL) at each time of blood collected													maen
	No.1	No.2	No.3	No.4	No.5	No.6	No.7	No.8	No.9	No.10	No.11	No.12	No.13	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	401.50	1253.00	355.30	369.60	439.90	278.60	172.90	101.10	532.60	1600.00	2733.00	2258.00	291.70	829.78
10	294.10	487.50	803.40	749.00	1763.00	172.50	263.00	62.11	455.00	2813.00	869.70	1348.00	488.50	812.99
15	256.40	651.70	668.00	484.40	1887.00	271.00	166.80	60.58	248.30	4780.00	836.00	1046.00	508.90	912.70
30	249.50	356.20	569.80	519.40	2244.00	132.40	157.80	49.66	416.20	5015.00	556.50	823.90	537.90	894.48
45	329.50	412.90	656.60	583.70	3244.00	404.30	112.80	25.35	212.30	6703.00	429.70	1528.00	505.10	1165.17
60	125.80	375.90	692.10	554.20	2654.00	599.10	89.96	45.53	218.90	4293.00	1233.00	1634.00	766.10	1021.66
90	155.30	494.80	525.20	820.80	3287.00	1056.00	61.52	90.69	866.90	2048.00	634.50	3757.00	570.10	1105.22
120	152.20	513.30	586.70	501.70	3019.00	1405.00	44.98	44.67	634.00	1384.00	1006.00	2637.00	1281.00	1016.12
180	98.98	507.10	555.10	631.00	1292.00	321.80	129.60	80.03	490.50	909.10	611.60	1285.00	872.30	598.78
240	107.80	527.80	575.50	403.10	548.80	156.40	33.20	69.07	568.00	359.50	560.60	701.60	296.10	377.50

Table E-2 Plasma concentration of morphine after receiving codeine 25 mg/kg (P.O.) in rats pretreated with vehicle for seven days (control group).

Time	**Plasma morphine concentration (ng/mL) at each time of blood collected**													**mean**
(min)	**No.1**	**No.2**	**No.3**	**No.4**	**No.5**	**No.6**	**No.7**	**No.8**	**No.9**	**No.10**	**No.11**	**No.12**	**No.13**	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	2361.00	336.90	724.50	381.10	3136.00	2386.00	2671.00	631.60	273.40	246.90	875.20	1316.00	488.00	1217.51
10	2308.00	113.00	1429.00	1033.00	80.53	1493.00	3792.00	486.50	223.00	561.80	355.90	803.20	547.30	1017.40
15	1864.00	184.00	1044.00	359.90	1072.00	3009.00	1696.00	594.80	111.50	1148.00	276.70	848.20	558.90	982.08
30	1838.00	282.00	1091.00	641.90	2937.00	1481.00	1474.00	559.30	297.00	1200.00	220.90	612.70	789.10	1032.61
45	600.20	240.70	1224.00	905.50	2913.00	941.40	1179.00	475.00	121.80	2088.00	240.60	477.70	997.30	954.17
60	1306.00	288.20	975.50	1098.00	2436.00	4153.00	1410.00	428.60	184.50	1524.00	208.90	843.10	1359.00	1247.29
90	2296.00	380.30	1291.00	1407.00	1892.00	5184.00	1033.00	695.40	844.60	870.20	659.10	2692.00	1385.00	1586.89
120	2141.00	715.30	1039.00	884.70	2135.00	6881.00	793.90	775.10	534.30	739.20	991.60	2289.00	2798.00	1747.47
180	1356.00	775.90	759.10	589.00	809.10	1761.00	925.90	942.60	353.10	466.50	504.60	1388.00	1431.00	927.83
240	1487.00	468.80	737.50	30.49	561.00	837.30	583.00	694.80	575.30	323.30	402.70	627.00	686.20	616.49

Table E-3 Plasma concentration of codeine 25 mg/kg (P.O.) in rats were pretreated by MG for seven days (experiment group).

Time	**Plasma codeine concentration (ng/mL) at each time of blood collected**													**mean**
(min)	**No.1**	**No.2**	**No.3**	**No.4**	**No.5**	**No.6**	**No.7**	**No.8**	**No.9**	**No.10**	**No.11**	**No.12**	**No.13**	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	5378.00	27300.00	3380.00	836.30	3440.00	5543.00	2153.00	1322.00	439.90	2695.00	2880.00	2532.00	1496.00	4568.86
10	7864.00	21680.00	2013.00	1573.00	8389.00	2723.00	3252.00	4560.00	1763.00	3918.00	2824.00	4581.00	7543.00	5591.00
15	5350.00	17950.00	4677.00	1793.00	3867.00	2801.00	2328.00	3385.00	1887.00	2765.00	3381.00	4301.00	4590.00	4544.23
30	3767.00	12550.00	2194.00	2051.00	22820.00	3352.00	2593.00	3881.00	2244.00	5071.00	4619.00	2573.00	3483.00	5476.77
45	2534.00	7781.00	3896.00	1818.00	7595.00	3284.00	3449.00	4361.00	3244.00	4615.00	3579.00	2259.00	3784.00	4015.31
60	1904.00	3184.00	3523.00	1396.00	4381.00	5392.00	2069.00	3933.00	2654.00	4548.00	4360.00	6481.00	3643.00	3651.38
90	1564.00	5945.00	2907.00	1537.00	5189.00	7007.00	1703.00	5490.00	3287.00	3923.00	4383.00	4104.00	3483.00	3886.31
120	2078.00	2579.00	3600.00	1232.00	7202.00	8078.00	3018.00	9098.00	3019.00	3455.00	3228.00	3956.00	3279.00	4140.15
180	912.70	709.90	1807.00	785.70	6430.00	3026.00	2243.00	5843.00	1292.00	1666.00	2289.00	2466.00	1480.00	2380.79
240	723.10	770.90	1781.00	606.40	2642.00	2228.00	1303.00	2546.00	548.80	1247.00	2375.00	1611.00	1296.00	1513.71

Table E-4 Plasma concentration of morphine after receiving codeine 25 mg/kg (P.O.) in rats pretreated with MG for seven days (experiment group).

Time (min)	**Plasma morphine concentration (ng/mL) at each time of blood collected**													**mean**
	No.1	**No.2**	**No.3**	**No.4**	**No.5**	**No.6**	**No.7**	**No.8**	**No.9**	**No.10**	**No.11**	**No.12**	**No.13**	
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	1050.00	46.15	77.94	43.78	73.27	46.18	147.60	82.85	639.50	75.19	209.30	256.40	187.10	225.79
10	784.90	125.70	230.90	141.60	51.62	74.40	135.30	330.30	629.10	353.50	206.60	234.50	457.20	288.89
15	863.20	109.40	62.66	111.30	87.59	114.00	142.00	478.30	612.10	201.70	313.70	186.60	244.50	271.31
30	775.10	90.74	135.60	153.60	118.50	199.90	109.80	398.80	454.80	426.80	328.20	207.70	263.80	281.80
45	718.40	98.24	176.10	117.30	146.30	165.40	203.10	275.20	488.00	250.90	264.40	177.80	340.20	263.18
60	337.00	90.23	289.30	122.70	240.20	365.50	112.80	93.11	375.00	431.00	296.70	527.90	345.90	279.03
90	447.20	104.60	116.50	73.24	325.80	522.50	51.19	81.39	224.50	425.40	267.00	398.50	278.90	255.13
120	531.00	84.21	97.48	69.76	256.20	345.00	304.50	547.20	212.20	406.10	233.80	368.80	281.70	287.53
180	422.50	76.74	127.00	61.85	51.67	178.60	45.19	69.74	140.20	356.80	403.60	378.80	172.70	191.18
240	390.80	73.26	97.20	130.20	188.60	62.85	103.20	218.60	283.40	348.80	491.80	226.40	80.70	207.37

Table E-5 Plasma concentration of MG in after receiving for seven days (experiment group).

Time	**Plasma MG concentration (ng/mL) at each time of blood collected**													**mean**
(min)	**No.1**	**No.2**	**No.3**	**No.4**	**No.5**	**No.6**	**No.7**	**No.8**	**No.9**	**No.10**	**No.11**	**No.12**	**No.13**	
0	46.84	71.21	85.22	19.05	50.44	77.61	443.60	67.04	4.18	4.81	4.18	5.18	91.59	71.09
5	67.31	12.57	151.70	132.10	43.18	208.60	504.70	108.90	212.80	65.54	71.37	445.60	201.60	179.89
10	87.44	44.52	117.70	108.00	16.24	237.00	370.40	93.21	237.20	167.00	70.49	454.60	139.20	171.30
15	73.37	49.59	29.69	133.60	98.40	123.90	321.70	233.60	429.80	45.76	83.46	213.50	212.80	157.63
30	79.30	40.22	97.10	74.46	27.33	477.00	320.70	215.70	331.80	136.20	97.06	173.60	147.30	170.60
45	59.59	52.67	76.79	89.83	10.91	231.60	590.10	106.40	193.00	55.01	110.80	218.40	160.40	150.42
60	58.12	56.75	163.40	75.88	48.58	423.00	366.20	12.70	248.40	78.92	120.80	289.80	139.90	160.19
90	48.89	58.94	153.10	78.22	92.02	302.10	253.60	12.66	135.70	123.70	27.86	184.70	116.80	122.18
120	47.41	38.89	89.90	77.21	58.52	381.40	355.00	190.70	120.10	126.30	180.80	115.20	137.60	147.62
180	38.31	55.92	171.10	16.64	9.46	308.60	114.60	52.54	102.80	77.33	128.50	191.60	193.20	112.35
240	41.43	25.78	39.09	16.62	73.46	130.20	228.30	143.00	139.50	67.46	151.40	81.20	19.37	88.99

APPENDIX F

Table F-1 Pharmacokinetic parameter of codeine following administration of a single 25 mg/kg oral dose to vehicle- (phase 1) and MG-pretreated (15mg/kg) rats (phase 2) (*n*=13).

Rat No.	T_{max} (min)		C_{max} (µg/mL)		AUC_{4h} (µg·min/mL)		λ_z (min^{-1})		$t_{1/2}$ (min)		$AUC_{0\rightarrow\infty}$ (µg·min/mL)		V_z/F (L/kg)		Cl_t/F (L/kg/h)	
	Phase1	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**
1	5.00	10.00	0.40	7.86	38.24	474.23	0.01	0.01	96.67	100.34	53.27	580.20	65.40	6.20	0.47	0.04
2	120.00	120.00	1.40	8.08	145.40	1112.35	0.02	0.01	37.89	77.47	153.94	1361.34	8.90	2.10	0.16	0.02
3	10.00	45.00	0.26	3.45	22.57	548.06	0.02	0.01	30.54	98.99	24.04	734.10	45.80	4.90	1.04	0.03
4	5.00	60.00	0.10	6.48	15.18	794.25	0.02	0.01	29.36	92.57	18.10	1009.32	58.50	3.30	1.38	0.02
5	90.00	90.00	0.87	3.29	122.77	500.41	0.01	0.01	66.47	48.78	742.45	539.04	36.70	3.30	0.51	0.05
6	45.00	30.00	6.70	5.07	531.27	718.83	0.01	0.01	62.54	89.24	563.71	879.32	4.00	3.70	0.04	0.03
7	5.00	30.00	2.73	4.62	186.74	768.67	0.01	0.00	83.50	172.56	254.28	1359.82	11.80	4.60	0.10	0.02
8	90.00	60.00	3.76	6.48	430.04	794.25	0.01	0.01	61.63	92.57	492.41	1009.32	4.50	3.30	0.05	0.02
9	120.00	10.00	1.28	7.54	177.83	661.83	0.01	0.01	56.79	111.63	202.09	870.62	10.10	4.60	0.12	0.03
10	30.00	5.00	0.48	27.30	72.45	1161.23	0.01	0.02	77.37	44.04	86.98	1210.21	32.10	1.30	0.29	0.02
11	10.00	15.00	0.80	4.68	139.17	655.41	0.01	0.00	75.36	162.01	596.08	1071.62	0.00	5.50	0.00	0.02
12	90.00	30.00	0.82	2.02	136.60	286.29	0.00	0.01	199.53	110.17	252.65	382.68	28.50	10.40	0.10	0.07

Rat No.	T_{max} (min)		C_{max} (μg/mL)		AUC_{4h} (μg·min/mL)		λ_z (min^{-1})		$t_{1/2}$ (min)		$AUC_{0\rightarrow\infty}$ (μg·min/mL)		V_z/F (L/kg)		Cl_t/F (L/kg/h)	
	Phase1	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**
13	90.00	30.00	3.29	22.82	500.41	1597.45	0.01	0.01	48.78	82.92	539.04	1913.47	3.30	1.60	0.05	0.01
mean	54.62	41.15	1.76	8.44	193.74	774.87	0.01	0.01	71.26	98.71	306.08	993.93	23.82	4.22	0.30	0.03
SD	46.21	33.86	1.90	7.66	177.44	344.80	0.01	0.00	43.53	36.64	248.96	409.81	22.33	2.37	0.13	0.01

Table F-2 Pharmacokinetic parameter of morphine following a single oral dose of codeine 25 mg/kg to vehicle-(phase 1) and MG-pretreated (15mg/kg) rats (phase 2) (*n*=13).

Rat No.	**T_{max} (min)**		**C_{max} (μg/mL)**		**AUC_{4h} (μg·min/mL)**	
	Phase1	**Phase2**	**Phase1**	**Phase2**	**Phase1**	**Phase2**
1	5.00	5.00	2.36	1.05	399.16	122.18
2	120.00	90.00	6.88	0.52	775.20	59.25
3	10.00	120.00	3.79	0.30	260.56	31.11
4	180.00	120.00	0.94	0.55	169.83	56.86
5	90.00	5.00	0.84	0.64	101.86	68.24
6	45.00	60.00	2.09	0.43	1966.20	86.93
7	120.00	240.00	0.99	0.49	127.29	78.28
8	90.00	60.00	2.69	0.53	340.22	80.05
9	120.00	10.00	2.80	0.46	342.04	56.33
10	180.00	10.00	0.78	0.13	122.63	20.55
11	10.00	60.00	1.43	0.29	231.06	31.77
12	90.00	30.00	1.41	0.15	176.87	21.90
13	5.00	90.00	3.14	0.33	387.56	40.93
mean	81.92	69.23	2.32	0.45	415.42	58.03
SD	62.80	65.98	1.62	0.23	479.48	28.25

Table F-3 Pharmacokinetic parameter of MG after seven consecutive days (15 mg/kg, P.O.) and following a single oral dose of codeine 25 mg/kg (*n*=13).

Rat No.	**T_{max} (min)**	**C_{max} (μg/mL)**	**AUC_{4h} (μg·min/mL)**	**λ_z (min^{-1})**	**$t_{1/2}$ (min)**	**$AUC_{0\rightarrow\infty}$ (μg·min/mL)**	**V_z/F (L/kg)**	**Cl_t/F (L/kg/h)**
1	30.00	0.08	11.63	0.00	235.01	25.68	0.80	0.00
2	30.00	0.48	72.45	0.01	77.37	86.98	19.20	0.17
3	45.00	0.59	67.91	0.00	188.36	129.93	31.40	0.12
4	15.00	0.23	25.03	0.00	289.13	84.69	73.90	0.18
5	15.00	0.43	39.85	0.00	229.92	86.12	57.80	0.17
6	10.00	0.17	22.33	0.01	132.65	35.24	81.40	0.42
7	120.00	0.18	28.62	0.00	468.64	130.96	77.40	0.12
8	10.00	0.45	43.71	0.01	131.36	59.09	48.10	0.25
9	15.00	0.21	33.69	0.01	111.69	36.81	65.70	0.41
10	0.00	0.07	11.28	0.00	221.07	19.50	245.40	0.76
11	180.00	0.17	28.22	0.01	111.26	34.49	69.80	0.43
12	15.00	0.13	14.07	0.01	54.15	15.37	76.20	0.98
13	15.00	0.10	11.24	0.00	174.28	29.71	126.90	0.51
mean	38.46	0.25	31.54	0.01	186.53	59.58	74.92	0.38
SD	52.22	0.17	20.18	0.00	108.91	40.28	60.16	0.19

Appendix G

Fig. G-1 Certificate of oral proceeding in national academic conference

สถาบันการจัดการระบบสุขภาพภาคใต้ มหาวิทยาลัยสงขลานครินทร์

ขอมอบประกาศนียบัตรนี้ไว้เพื่อแสดงว่า

นางสาวฮาวานี สาคอ

ได้นำเสนอผลงานวิชาการแบบปากเปล่า (Oral Presentation) ในงานประชุมวิชาการระดับชาติ

เรื่อง "สานพลังการก้าวข้ามขีดจำกัดเพื่อภาคใต้แห่งความสุข"

วันที่ 5-7 สิงหาคม 2562

ณ โรงแรมนิภาการ์เด้น อำเภอเมือง จังหวัดสุราษฎร์ธานี

ผศ.ดร.ภก.พงศ์เทพ สุธีรวุฒิ

รองอธิการบดีฝ่ายวางแผนและนโยบายสาธารณะ

มหาวิทยาลัยสงขลานครินทร์

Fig. G-2 Certificate of award for academic oral proceeding in national academic conference

สถาบันการจัดการระบบสุขภาพภาคใต้ มหาวิทยาลัยสงขลานครินทร์

ขอมอบประกาศนียบัตรนี้ไว้เพื่อแสดงว่า

คุณ สาวน์ สาคอ

ได้รับรางวัลนำเสนอผลงานวิชาการแบบปากเปล่า (Oral Presentation) ดีเด่น
ในงานประชุมวิชาการระดับชาติ

เรื่อง "สานพลังการก้าวข้ามขีดจำกัดเพื่อภาคใต้แห่งความสุข"
วันที่ 5-7 สิงหาคม 2562
ณ โรงแรมนิภาการ์เด้น อำเภอเมือง จังหวัดสุราษฎร์ธานี

ผศ.ดร.ภก.พงศ์เทพ สุธีรวุฒิ
รองอธิการบดีฝ่ายวางแผนและนโยบายสาธารณะ
มหาวิทยาลัยสงขลานครินทร์

VITAE

Name Miss Hawanee Saca

Student ID 5910220081

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Thai Traditional Medicine (Second Class Honours)	Prince of Songkla University	2015

Scholarship and Awards during Enrolment

2017 The scholarship for Teacher Assistant from Faculty of Science, Prince of Songkla University

2019 Award for Academic Oral Presentation in National Academic Conference "สานพลังการก้าวข้ามขีดจำกัด เพื่อภาคใต้แห่งความสุข"

List of Publication and Proceeding

ฮาวานี สาคอ, สมสมร ชิตตระการ, สมชาย ศรีวิริยะจันทร์ และสุพัตรา ลิ่มสุวรรณโชติ. (2562). *การพัฒนาวิธีวิเคราะห์หา ปริมาณ โคเดอีน มอร์ฟีน และ มิตรากัยนีน พร้อมกันอย่างง่าย ด้วยเทคนิคลิควิดโครมาโทกราฟี แมสสเปกโทรเมทรีในพลาสมา.* การประชุมวิชาการระดับชาติ เรื่อง "สานพลังการก้าวข้ามขีดจำกัด เพื่อภาคใต้แห่งความสุข" (หน้า 23-30). สงขลา: สถาบันการจัดการระบบสุขภาพ มหาวิทยาลัยสงขลานครินทร์.