Effects of Caffeine and Codeine on Pharmacokinetics and Antinociceptive Activity of Alkaloid Extract from Leaves of Kratom (*Mitragyna speciosa* Korth.)

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ชื่อวิทยานิพนธ์ ผลของแคฟเฟอีนและโคเดอีนต่อเภสัชจลนศาสตร์และการออกฤทธิ์ลดปวดของสารแอลคาลอยด์จากใบกระท่อม
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บทคัดย่อ

พืชกระท่อม (*Mitragyna speciosa* Korth.) เป็นพืชสมุนไพรที่พบได้ทางภาคใต้ของประเทศไทย ชาวบ้านนิยมนำมาใช้เป็นยารักษาโรค นอกจากนี้พืชกระท่อมถูกนำมาใช้ในทางที่ผิดคือนำมาใช้ร่วมกับสารที่มีการกระตุ้นระบบประสาท เช่น เครื่องดื่มโคล่าที่มีส่วนผสมของแคฟเฟอีน ยาแก้ไอที่มีส่วนผสมของโคเดอีน การใช้ในลักษณะนี้รู้จักในหมู่ของผู้ใช้สารเสพติดว่า “สี่คูณร้อย” พืชกระท่อมมีสารมิทรากัยนีนเป็นส่วนประกอบหลักและพืชกระท่อมมีฤทธิ์ทางเภสัชวิทยามากมายแต่ยังไม่มีรายงานถึงผลจากการใช้แคฟเฟอีนและโคเดอีนร่วมกับพืชกระท่อม ดังนั้นในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาผลของแคฟเฟอีนและโคเดอีนต่อเภสัชจลนศาสตร์และการออกฤทธิ์ลดปวดของสารสกัดแอลคาลอยด์จากใบกระท่อม

การศึกษาทางเภสัชจลนศาสตร์ได้ทำการทดลองในหนูขาวใหญ่เพศผู้ โดยแบ่งหนูออกเป็น 4 กลุ่ม กลุ่มละ 6 ตัว หนูแต่ละกลุ่มได้รับสารที่แตกต่างกันทางปากเพียงครั้งเดียว ประกอบด้วยกลุ่มที่ได้รับสารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัม สารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัมร่วมกับแคฟเฟอีน 25 มิลลิกรัมต่อกิโลกรัม สารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัมร่วมกับโคเดอีน 3 มิลลิกรัมต่อกิโลกรัม และ กลุ่มที่ได้รับสารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัมร่วมกับแคฟเฟอีน 25 มิลลิกรัมต่อกิโลกรัมและโคเดอีน 3 มิลลิกรัมต่อกิโลกรัม หลังจากได้รับสารได้ทำการเก็บตัวอย่างซีรั่มและปัสสาวะเป็นระยะเวลา 24 ชั่วโมงเพื่อตรวจวิเคราะห์ความเข้มข้นของสารมิทรากัยนีนในตัวอย่างซีรั่มและปัสสาวะด้วยเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง ส่วนการศึกษาผลของแคฟเฟอีน โคเดอีน ต่อฤทธิ์ลดปวดของสารสกัดแอลคาลอยด์ที่สกัดจากใบกระท่อมในหนูขาวใหญ่ใช้วิธีกระตุ้นให้ปวดด้วยความร้อนและวัดระยะเวลาในการตอบสนองต่อความเจ็บปวดทุกๆ 30 นาทีเป็นเวลา 150 นาที โดยแบ่งหนูออกเป็น 6 กลุ่มๆ ละ 6 ตัว หนูแต่ละกลุ่มจะได้รับสารที่แตกต่างกันทางปากเพียงครั้งเดียว กลุ่มที่ 1 ได้รับน้ำ 5 มิลลิลิตรต่อกิโลกรัม กลุ่มที่ 2 ได้รับ propylene glycol 2 มิลลิลิตรต่อกิโลกรัม กลุ่มที่ 3 ได้รับสารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัม กลุ่มที่ 4 ได้รับสารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัมร่วมกับแคฟเฟอีน 25 มิลลิกรัมต่อกิโลกรัม หนูกลุ่มที่ 5 ได้รับสารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัมร่วมกับโคเดอีน 3 มิลลิกรัมต่อกิโลกรัม

และ หนูกลุ่มที่ 6 ได้รับสารสกัดแอลคาลอยด์ 100 มิลลิกรัมต่อกิโลกรัมร่วมกับแคฟเฟอีน 25 มิลลิกรัมต่อกิโลกรัมและโคเดอีน 3 มิลลิกรัมต่อกิโลกรัม

ค่าพารามิเตอร์ทางเภสัชจลนศาสตร์ของสารมิทรากัยนีนที่ถูกวิเคราะห์โดยใช้แบบจำลองแบบไม่แบ่งส่วนพบว่ามีการแตกต่างอย่างนัยสำคัญทางสถิติ โดยค่าความเข้มข้นสูงสุดของสารมิทรากัยนีนภายหลังที่ได้รับสารสกัดแอลคาลอยด์ร่วมกับแคฟเฟอีนทางปากมีความแตกต่างอย่างมีนัยสำคัญจากกลุ่มที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (1320.00±185.26 นาโนกรัมต่อมิลลิลิตร v.s. 835.00±123.52 นาโนกรัมต่อมิลลิลิตร; $p < 0.05$) แคฟเฟอีนทำให้ค่าเฉลี่ยของค่าคงที่ของอัตราการกำจัดสารมิทรากัยนีนของหนูที่ได้รับสารสกัดแอลคาลอยด์มีค่าสูงขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับกลุ่มที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (0.0308±0.0108 ต่อชั่วโมง v.s. 0.0067±0.0021 ต่อชั่วโมง; $p < 0.05$) ผลการทดลองสอดคล้องกับค่าเฉลี่ยของค่าครึ่งชีวิตของการกำจัดสารมิทรากัยนีนและค่าเฉลี่ยของเวลาที่สารมิทรากัยนีนที่เหลือในร่างกายของหนูที่ได้รับสารสกัดแอลคาลอยด์ร่วมกับแคฟเฟอีนน้อยกว่าอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับหนูที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (39.88±10.96 ชั่วโมง v.s. 184.03± 72.70 ชั่วโมงและ 45.92±13.84 ชั่วโมง v.s. 255.45±106.55 ชั่วโมง; $p < 0.05$) ปริมาตรการกระจายสารมิทรากัยนีนในหนูที่ได้รับสารสกัดแอลคาลอยด์ร่วมกับแคฟเฟอีนน้อยกว่าอย่างมีนัยสำคัญเมื่อเทียบกับหนูที่ได้รับสารสกัดแอลคาลอยด์ (61.96±10.30 ลิตรต่อกิโลกรัม v.s. 118.02±23.10 ลิตรต่อกิโลกรัม; $p < 0.05$) อัตราการชำระยาของสารมิทรากัยนีนในหนูที่ได้รับสารสกัดแอลคาลอยด์ร่วมกับแคฟเฟอีนสูงกว่าหนูที่ได้รับสารสกัดแอลคาลอยด์จากใบกระท่อมอย่างมีนัยสำคัญเมื่อเทียบกับหนูที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (1.51±0.34 ลิตรต่อชั่วโมง v.s. 0.75±0.25 ลิตรต่อชั่วโมง; $p < 0.05$) ส่วนโคเดอีนให้ผลที่แตกต่างจากแคฟเฟอีนโดยมีผลทำให้ค่าครึ่งชีวิตของการดูดซึมของสารมิทรากัยนีนในหนูที่ให้สารสกัดแอลคาลอยด์ลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับหนูกลุ่มที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (2.81±0.30 ชั่วโมง v.s. 4.58±0.66 ชั่วโมง; $p < 0.05$) เมื่อให้แคฟเฟอีนร่วมกับโคเดอีนพบว่าการดูดซึมของสารมิทรากัยนีนเพิ่มขึ้นโดยค่าคงที่ในการดูดซึมของมิทรากัยนีนเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับหนูกลุ่มที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (0.38±0.11 ต่อชั่วโมง v.s. 0.17±0.03 ต่อชั่วโมง; $p < 0.05$) และค่าครึ่งชีวิตของการดูดซึมของสารมิทรากัยนีนลดลงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับหนูกลุ่มที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว (2.62±0.60 ชั่วโมง v.s. 4.58±0.66 ชั่วโมง; $p < 0.05$)

การศึกษาผลของแคฟเฟอีนและโคเดอีนต่อการออกฤทธิ์ลดปวดของสารสกัดแอลคาลอยด์พบว่า หนูกลุ่มที่ได้รับสารสกัดแอลคาลอยด์ร่วมกับแคฟเฟอีนมีระยะเวลาที่สารสกัดแอลคาลอยด์เริ่มออกฤทธิ์ลดปวดมากกว่า 30 นาทีและฤทธิ์ลดปวดของสารสกัดแอลคาลอยด์ใบ

กระท่อมที่เวลา 120 นาทีลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ส่วนฤทธิ์ลดปวดของสารสกัดแอลคาลอยด์ในหนูที่ได้รับสารสกัดแอลคาลอยด์ร่วมกับโคเดอีน ลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) ที่เวลา 120 นาทีเมื่อเทียบกับหนูที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว แต่อย่างไรก็ตามพบว่าเมื่อให้สารสกัดแอลคาลอยด์ร่วมกับแคฟเฟอีนและโคเดอีน ทางปากเพียงครั้งเดียวในหนู พบว่าฤทธิ์ลดปวดที่เวลา 30 นาทีหลังให้สารทั้ง 3 ชนิดร่วมกันมากกว่าหนูที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียวอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) และฤทธิ์ลดปวดที่เวลา 90 และ 120 นาที ลดลงอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเทียบกับหนูที่ได้รับสารสกัดแอลคาลอยด์เพียงอย่างเดียว

ดังนั้นจากการทดลองพบว่าแคฟเฟอีนและโคเดอีนมีผลต่อเภสัชวิทยาของพืชกระท่อม โดยแคฟเฟอีนเพิ่มการกำจัดออกของสารมิทรากัยนีน ส่วนโคเดอีนเพิ่มการดูดซึมของสารมิทรากัยนีน เมื่อให้แคฟเฟอีนร่วมกับโคเดอีนสามารถเพิ่มการดูดซึมของสารมิทรากัยนีน โดยที่ผลในการกำจัดออกไม่เปลี่ยนแปลง สำหรับผลทางเภสัชพลศาสตร์พบว่าแคฟเฟอีนทำให้ระยะเวลาในการออกฤทธิ์ของสารสกัดแอลคาลอยด์นานขึ้นและระยะเวลาในการออกฤทธิ์ลดลง ส่วนโคเดอีนไม่มีผลต่อระยะเวลาในการเริ่มออกฤทธิ์ แต่ทำให้ระยะเวลาในการออกฤทธิ์ลดลงเช่นกัน เมื่อให้แคฟเฟอีนร่วมกับโคเดอีนมีผลเพิ่มฤทธิ์ลดปวดของสารสกัดแอลคาลอยด์ในช่วงแรกของการออกฤทธิ์และทำให้ระยะเวลาในการออกฤทธิ์ลดลง ดังนั้นการใช้แคฟเฟอีนและโคเดอีนร่วมกับสารสกัดแอลคาลอยด์จากใบกระท่อมจะมีผลทำให้เกิดการเปลี่ยนแปลงทางเภสัชจลนศาสตร์และเภสัชพลศาสตร์ของสารสกัดแอลคาลอยด์จากพืชกระท่อม

Thesis title	Effects of Caffeine and Codeine on Pharmacokinetics and Antinociceptive Activity of Alkaloid Extract from Leaves of Kratom (*Mitragyna speciosa* Korth.)
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Abstract

Leaves of kratom (*Mitragyna speciosa* Korth.), an indigenous plant in Southern Thailand, is commonly used by the villagers as a herbal medicine. Kratom is also abused by combining with other psychoactive substances such as caffeine from cola beverage and codeine from cough syrup and well known among drug abusers as "4×100 kratom cocktail". Since kratom which contains a major alkaloid, mitragynine, possesses several pharmacological activities and its interaction with caffeine and codeine has never been reported, therefore, the present study was aimed to investigate the effects of caffeine and codeine on pharmacokinetics and pharmacodynamics, focused on antinociceptive activity, of alkaloid extract of kratom leaves in rats.

In pharmacokinetic study, Wistar male rats were divided into 4 groups (n=6) and orally given a single oral dose of different regimens as follows: Group AE, alkaloid extract from kratom leaves (100 mg/kg); Group AE+CF, alkaloid extract from kratom leaves (100 mg/kg) and caffeine (25 mg/kg); Group AE+CD alkaloid extract from kratom leaves (100 mg/kg) and codeine (3 mg/kg) and Group AE+CF+CD, alkaloid extract from kratom leaves (100 mg/kg), caffeine (25 mg/kg) and codeine (3 mg/kg). Serial blood and urine samples were collected during 24 hours post dose. Concentrations of mitragynine in serum and urine were measured by a high performance liquid chromatography (HPLC) with Ultraviolet (UV) detection. For antinociceptive study, the effects of caffeine and codeine on antinociceptive activity of alkaloid extract from kratom leaves using hot plate test model were also examined in rat. The latency of nociceptive response was determined every 30 minute for 150 minute. Rats were divided into 6 groups (n=6). Each group was received a

single oral of 5 mL/kg distilled water (Group DW), 2 mL/kg propylene glycol (Group PG), 100 mg/kg alkaloid extract from kratom leaves (Group AE), 100 mg/kg alkaloid extract from kratom leaves and 25 mg/kg caffeine (Group AE+CF), 100 mg/kg alkaloid extract from kratom leaves and 3 mg/kg codeine (Group AE+CD) and co-administered of 100 mg/kg alkaloid extract from kratom leaves, 25 mg/kg caffeine and 3 mg/kg codeine (Group AE+CF+CD).

The pharmacokinetic parameters of mitragynine were analyzed based on the non-compartmental analysis. The results are shown that pharmacokinetic parameters of mitragynine after co-administration with caffeine and/or codeine was significantly, different from alkaloid extract treated alone. In combination with caffeine, C_{max} of mitragynine after orally administered AE and CF was significantly higher than AE treated alone (1320.00±185.26 ng/mL v.s. 835.00±123.52 ng/mL; $p < 0.05$). The k_{el} of rats treated with AE and CF was significantly higher than those given AE alone (0.0308±0.0108 h^{-1} v.s. 0.0067±0.0021 h^{-1}; $p < 0.05$). Its result was according with $t_{1/2el}$ and $MRT_{0\rightarrow\infty}$ of rats treated with AE+CF which was significantly less than that of AE treated alone (39.88±10.96 h v.s. 184.03± 72.70 h and 45.92±13.84 v.s. 255.45±106.55 h; $p < 0.05$). The V_d/F of mitragynine of group AE+CF was significantly less than that of group AE (61.96±10.30 L/kg v.s. 118.02±23.10 L/k g*; p* < 0.05). The Cl/F of mitragynine in rats treated with AE+CF was significantly higher than AE treated alone (1.51±0.34 L/h v.s. 0.75±0.25 L/h; $p < 0.05$). Codeine showed different effect, compared with those of caffeine, on pharmacokinetic of mitragynine. Absorption of mitragynine appeared to be accerelated in rats receiving AE+CD. The $t_{1/2\ ab}$ of group AE+CD was significantly less than that of AE treated alone (2.81±0.30 h v.s. 4.58±0.66 h; $p < 0.05$). Combination of CF and CD obviously increased absorption of mitragynine in rats given AE+CF+CD compared with those received AE alone (0.38±0.11 h^{-1} v.s. 0.17±0.03 h^{-1} for k_{ab} and 2.62±0.6 h vs 4.58±0.66 h for $t_{1/2\ ab}$; $p < 0.05$).

In this pharmacodynamic study, it was found that co-administration of CF or CD with AE had no effect on the antinociceptive response in rats when compared with AE alone. For the co-administer of 3 compounds, the results showed that in

AE+CF+CD treated rats significantly exhibited the increasing of latency period of antinociceptive effect only in the first 30 minutes after orally administered when compared with AE treated ($p < 0.05$).

In conclusion, the results suggested that caffeine and codeine have affect on pharmacology of kratom. Caffeine altered the pharmacokinetic of AE by increasing its elimination phase. Codeine increasd the absorption phase of the pharmacokinetic of alkaloid extract from kratom leaves, while codeine especially when combined with caffeine increased the absorption phase of the pharmacokinetic of alkaloid extract from kratom leaves but no effect on the elimination phase. In addition, caffeine and codeine had altered the pharmacodynamic activity of alkaloid extract from kratom leaves by shorting the onset and increasing the antinociceptive response of this alkaloid. Therefore, caffeine and codeine altered both pharmacokinetics and pharmacodynamic of alkaloid derived from leaves of kratom.

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Ornchuma Botpiboon

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List of Abbreviations

AE	Alkaloid extract from kratom leaves
AUC	Area under the concentration-time curve
AUMC	Area under the first-moment of the concentration-time curve
CD	Codeine
CF	Caffeine
Cl/F	Total body clearance
Cl_h	Hepatic clearance
Cl_r	Renal clearance
CNS	Central nervous system
C_{max}	Maximum serum concentration
CV	Coefficient of variation
DEV	Deviation
DW	Distilled water
GC	Gas chromatography
GC-FID	Gas chromatography–flame ionization detector
GC-MS	Gas chromatography–mass spectrometry
h	Hour
HLB	Hydrophlilic-lipophilic balanced
HPLC	High performance liquid chromatography
i.c.v.	Intracerebroventricular
i.e.	Id est
i.p.	Intraperitoneal
i.v.	Intravenous
k_{ab}	Absorption rate constant
k_{el}	Elimination rate constant
kg	Kilogram
L	Litre
LC	Liquid chromatography

List of Abbreviations (cont.)

LC-MS	Liquid chromatography-mass spectrometry
LC-ESI/MS	Liquid chromatography-electrospray ionization tandem mass spectrometry
LD_{50}	Median lethal dose
LLOQ	Lower limit of quantification
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
MRT	Mean residence time
mw.	Molecular weight
N	Number
ng	Nanogram
nm	Nanometer
p	*p*-value
PG	Propylene glycol
r	Correlation coefficient
S.D.	Standard deviation
S.E.	Standard error
sec	Second
SPE	Solid phase extraction
$t_{½\ ab}$	Half-life absorption
$t_{½\ el}$	Half-life elimination
TLC	Thin Layer Chromatography
T_{max}	Time to maximal serum concentration
t_R	Retention time
UV	Ultraviolet
V_d/F	Volume of distribution

List of Abbreviations (cont.)

v.s.	Versus
v/v	Volume by volume
μg	Microgram
μm	Micrometer

CHAPTER 1

Introduction

1.1 Background and rationale

In recent years, drug addiction is a major problem in many countries including Thailand. This problem is combined to the other problems associated with society, economy, and domestic and international politics. In Thailand, the tendency of drug distribution has been increasing continuously. Until the Thai government has announced a war to fight against drug of abuse during the year 2001-2002, the spreading of drugs of abuse is decreased. At present, the different varieties of drugs are epidemic in Thailand such as methamphetamine, cannabis, volatile substances, kratom, opium, heroine and club drugs (ice, ketamine, cocaine, and ecstacy). In southern Thailand, spreading of kratom is widely recognized. The endemic areas accounts for 79.1%, including Nakhonsrithammarat, Ranong, Chumphon, Songkhla, Suratthani, Pattani, Yala and Narathiwat (NCCD, 2008a).

Kratom is classified as category V in the Narcotic Act B.E. 2522 (1979). In fact, kratom has been used as Thai folk medicine. Its leaf is used to treat diarrhea, diabetes, cough and pain. In addition, the leaf is also used to substitute opium, increase work efficiency and tolerance to hard work under a scoring sun. Kratom can be used in different forms such as drinking, smoking and chewing. Kratom is used alone or mixed with the other ingredients such as coffee, sugar, salt and some herbs. Most of kratom users are adults working as labor (Junsirimongkol *et al.*, 2005). Furthermore the pattern of kratom use is now abused. Its leaves were used as a main ingredient in a combination called "4×100 kratom cocktail".

The "4×100 kratom cocktail" is firstly known in 2004 and is a mixture of boiled kratom leaves, caffeine in Coke®, codeine in cough syrup, and mosquito coil. The formula of 4×100 kratom cocktail is currently modified to many different formulas such as "3×100 kratom cocktail", "8×100 kratom cocktail" and "10×100 kratom cocktail"(ONCB, 2008). In principle, co-administration of any two drugs may

alter the drug response. Such circumstance is called “drug interaction”. The mechanism of a drug interaction may be pharmacokinetics or pharmacodynamics. Changed response of a drug resulting from an interaction may be expressed either as increased risk of toxicity or decreased drug efficacy or therapeutic failure. In case of kratom, which possesses many pharmacological actions, combined use with other pharmacologically active substances could cause drug interaction. Caffeine and codeine both are psychostimulant which are commonly used, especially caffeine is usually contained in beverages. Whether any or both of them affect (s) pharmacology of kratom is not known.

In the present study, the effects of either caffeine, or codeine, or their combination on the pharmacokinetics and the pharmacodynamics of kratom were investigated by using rats as an animal model. Following a single oral dose administration of crude alkaloid derived from kratom leaves, levels of mitragynine, a major alkaloid, in serum and urine were determined and used to examine pharmacokinetic behavior. In term of pharmacodyamics, alkaloid antinociceptive activity was investigated.

1.2 Literature review

1.2.1 Kratom

1.2.1.1 Botany of kratom

The botanical classification of *Mitragyna speciosa* is as follows:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Gentianales

Family: Rubiaceae

Genus: *Mitragyna*

Species: *Mitragyna speciosa*

Mitragyna speciosa (Figure 1.1) is the botanical name of a plant more commonly known as kratom. Kratom is a tree indigenous to Southeast Asia. It can be found growing wild in Thailand, especially in the central and southern regions, and

Malaysia peninsular. Kratom is also called “Kakuam”, “Ithang” and in southern regions of Thailand, “Thom”, while in Malaysia, it is commonly called “Ketum” or “Biak” (Kiang *et al.*, 2005; Suwanlert, 1975). It is first documented by Dutch colonial botanist Korthals. Kratom grows into a large shrub or small tree. In most cases, plants will grow to a height of 12-15 feet, but some specimens can reach a height of 50 feet. The stem is erect and branching. Flowers are yellow and grow in ball-shaped clusters. Leaves are evergreen and are a dark glossy green in color, smooth, ovate-acuminate in shape and opposite in growth pattern. Leaves can grow over 18 cm long and 10 cm wide. Kratom is evergreen rather than deciduous and leaves are constantly being shed and being replaced but there is some quasi-seasonal leaf shedding due to environmental conditions. During the dry season of the year leaf fall in more abundant and new growth is more plentiful during the rainy season. When grown outside their natural tropical habitat, leaf fall occurs with colder temperature, around 4 degrees Celsius. Kratom prefers wet, humusy soil in a protected position. Being a heavy feeder, it requires very rich, fertile soil. It is drought sensitive and if grown out of its native habitat, sensitive to frost. Propagation is by planting very fresh seed or cuttings (Murple, 2000).

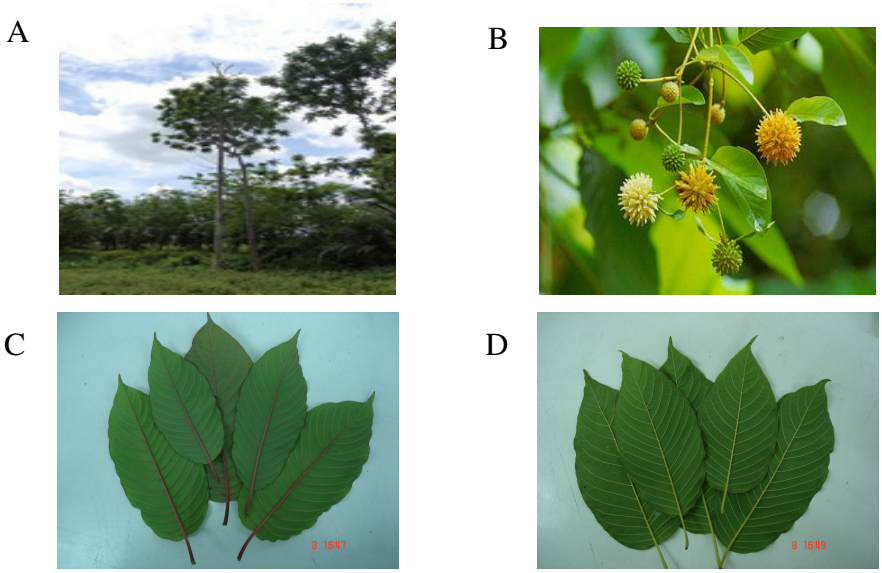


Figure 1.1 Tree (A), flower (B), red veins leaves (C) and white veins leaves (D) of kratom (sources of Figures: 1.1A and 1.1B from http://www.kratom.com/, Figures 1.1C and 1.1D photographed by Ornchuma)

1.2.1.2 Kratom situation in Thailand

Since the year 1897, kratom has been used and related with way of Thai life. In the past, kratom was widely used among opium users for its substitution. The Thai government realized the danger of using kratom which may cause addiction. Then in 1943, the government passed the Kratom Act B.E. 2486. This law stated that planting kratom is illegal and kratom trees have to be cut down. However, this law was not effective, since kratom is indigenous to the country (Withayanartpaisarn, 2007). Later, this Act was canceled and the Narcotics Act of B.E. 2522 (1979) was issued, of which kratom is classified as a category V narcotic substance. Category V consists of narcotics which are not included in category I to category IV such as marijuana and kratom plant. This means that it is illegal to buy, sell, import, or possess this plant. Exceptions are made with permission from Minister of Public Health in accordance with approval from Office of the Narcotics Control Board. Requirements and penalties were enacted in the Narcotics Act of B.E. 2522 (1979). Any person who produces, disposes, imports or exports narcotics of category V shall be liable to imprisonment for a term of two to fifteen years and to a fine from twenty thousands to

one hundred and fifty thousand Baht. Any person who possesses narcotics of category V shall be liable to imprisonment for a term not exceeding five years and to a fine not exceeding fifty thousand Baht (MOPH, 1996).

Despite, planting and using of kratom are slightly found, kratom use is traditional among Thai villagers. It has been use for treatment of illnesses, cooking, recreation and meeting, hard working, sex enhancement. Most kratom users are middle aged labor workers such as farmers, gardeners and fishermen (Junsirimongkol *et al.*, 2005). The continual usage of kratom was found during 1992-2000, which the statistics of kratom seized were increased from 506 cases to 1755 cases. Whereas during 2001-2002, kratom seizing was reduced because the government had announced policies to fight against drug abuse nationwide. However, during 2003-2008, kratom seizing increased continuously, especially in 2007 with the number of cases were up to 3,359 cases. Kratom weight of 41,015 kg was seized in southern area (NCCD, 2008b). The number of cases of kratom seized has increased because users and pattern of using kratom were changed. In the past, most of kratom users were middle-aged men. Fifteen percents of use was taking kratom with tea, chewing fresh and smoking. Eighty-five percents were taking with drinking water, energy-drink, coffee, salt and sugar. Nowadays, kratom leaves become a main ingredient in a particular drug abuse formula so called “4×100 kratom cocktail” . Such termination has been widespread since 2004 among 4 provinces of southern border of Thailand (i.e. Songkhla, Yala, Pattani and Narathiwat).

The “4×100 kratom cocktail” is a name of kratom drink. It is a mixture of boiled water of kratom leaves, Coca Cola beverage (“Coke”) which contains caffeine, cough syrup containing codeine, and mosquito coil containing pyrethroid insecticide. It is also called “OTOP” or boiled kratom leaves. This formula is currently modified to many other related ones aiming to meet out the needs of different groups of people, for example, “3×100” (3 ingredients in a mixture), “10×100” (many more ingredients in a mixture) and “One two call” (kratom powder mixed with yogurt).

According to the investigation of 119 samples of “4×100 kratom cocktail” in southern border provinces by the Office of the Narcotic Control Board in 2007, 5 main formulas of the cocktail were classified as follows.

Formula 1: A combination of boiled kratom leaves, Coke® and cough syrup

Formula 2: A combination of boiled kratom leaves, cough syrup and antihistamine

Formula 3: A combination of boiled kratom leaves and mosquito coil

Formula 4: A combination of boiled kratom leaves and many ingredients such as ketamine, actifed®

Formula 5: A combination of Coke®, cough syrup, antihistamine and antiemetic drugs etc. excluding boiled kratom leaves

Kratom cocktails contain several components each affecting the central nervous system (CNS). Kratom at a low dose produces a stimulating effect but while at a high dose it causes sedation. Caffeine is a CNS stimulant. The ingredients of cough syrup such as diphenhydramine and codeine produces a sedating effect (ONCB, 2008). Hence, taking 4×100 kratom cocktail produces a mix up effect such as euphoria, befuddle, brave, edgy, drowsy and faint. The typical behavior of taking 4×100 kratom cocktail is mixing a glass of ice with the cocktail. The 4×100 kratom cocktail is gaining popularity among muslim youngsters in several districts of Southern Thailand such as Yala, Pattani, Narathiwas and Songkhla (NCCD, 2008b).

1.2.1.3 Chemical properties of kratom

Mitragyna speciosa Korth. contains many phytochemicals, mainly indole alkaloids (Table 1.1). Other constituents include flavonoids, phenylpropanoids and tannins. Alkaloids isolated from kratom leaves vary in contents from location to location and from time to time (Shellard, 1974; Takayama, 2004).

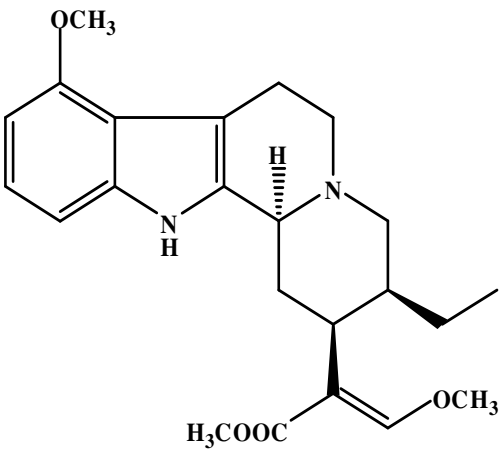
Mitragynine ($C_{23}H_{30}N_2O_4$, mw. 398.49, Figure 1.2) has been reported as the most abundant indole alkaloid in the leaves of *Mitragyna speciosa* (Ponglux *et al.*, 1994). It was first isolated in 1907 by Hooper and this process was repeated in 1921 by Field, who named the alkaloid. Its structure was first fully determined in 1964 by Zacharias and colleagues. In 1995, Takayama and coworkers at Chiba University were able to synthesize mitragynine. It is structurally related to both an alkaloid yohimbine and voacangine. It is more distantly related to tryptamine-based psychcdelic drugs such as psilocybin, ibogain or lysergic acid diethylamide (LSD) (Murple, 2005). Its weak base form is white, amorphous powder with melting point of

102–106 °C and boiling point of 230–240 °C. It is soluble in alcohol, chloroform and acetic acid (DMSC, 2008).

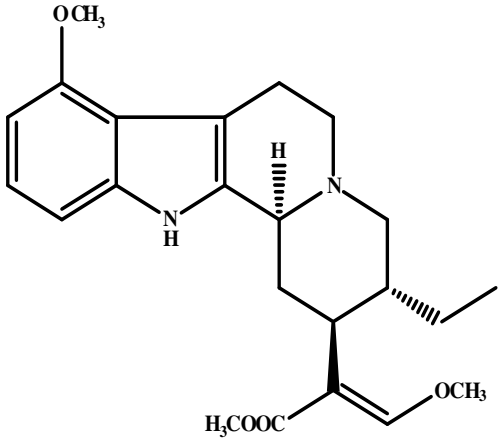
Table 1.1 Types and chemical constituents in different parts of kratom (Data are referenced from Assanangkornchai S. and Sirivonrse na Ayudhya A., 2005.)

Plant part	Types	Chemical compound	References
Leaves	Indole alkaloid	ajmalicine, akuammigine, angustine, corynantheidine, corynantheidaline, corynantheidalinic acid, corynoxeine, corynoxine, corynoxine B, hirsutine, hirsuteine, isocorynoxeine, isomitraphyline, isorhynchophylline, isocorynantheidine, javaphylline, mitraciliatine, mitragynine oxindole B, mitrajavine, mitraphylline, mitrasulgynine, mitragynaline, mitragynalinic acid, mitralactonal, paynantheine, mitragynine, pinoresinol, speciociliatine, speciogynine, 3-isoajmalicine, 3, 4, 5, 6-tetradehydromitragynine, 7α-hydroxyl-7H- mitragynine	Phillipson *et al*., 1973; Shellard *et al*., 1966 ; Shellard *et al*., 1978a ; Shellard *et al*., 1978b Takayama, 2004 Hinou and Harvala, 1988
	Flavones	apigenin, apigenin-7-O-rhamnoglucoside, cosmosiin	
	Flavonol	astragalin, hyperoside, kaempferol, quercetin, quercitrin, quercetin-3-galactoside-7-rhamnoside, quercitrin, rutin	Harvala and Hinou, 1988
	Phenylpropanoid	caffeic acid, chloronic acid	Hinou and Harvala, 1988 Houghton and Said, 1986 Takayama *et al*., 1998 Said *et al*., 1991
	Flavonoid	(-)-epicatechin	
	Lignin	(+)-pinoresinol	

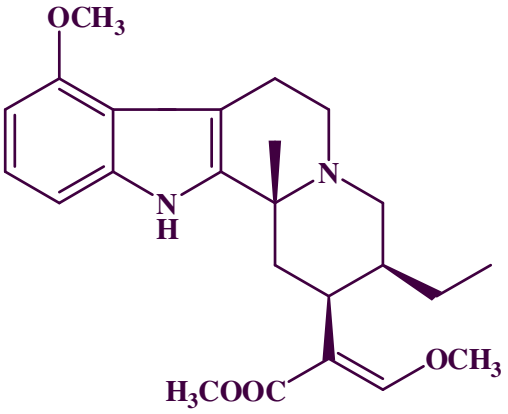
Plant part	Types	Chemical compound	References
	Triterpene	Ursolic acid	
Stem, trunk barks	Indole alkaloid	ciliaphylline, rhynchociline, ciliaphylline, isomitraphylline, isorhynchophylline, isospecionoxeine, javaphylline, mitraciliatine, mitragynine oxindole A, mitragynine oxindole B, mitraphylline, rhynchociline, rhynchophylline, speciogynine, speciociliatine, specionoxeine	Shellard *et al*., 1978a ; Shellard *et al*., 1978b
Root barks	Indole alkaloid	ciliaphylline, corynoxeine, isocorynoceine, isomitraphylline, isorhynchophylline, isospecionoxeine, mitraciliatine, mitraphylline, rhynchociline, rhynchophylline, speciociliatine, speciogynine, specionoxeine	Shellard *et al*., 1978b Houghton and Shellard, 1974



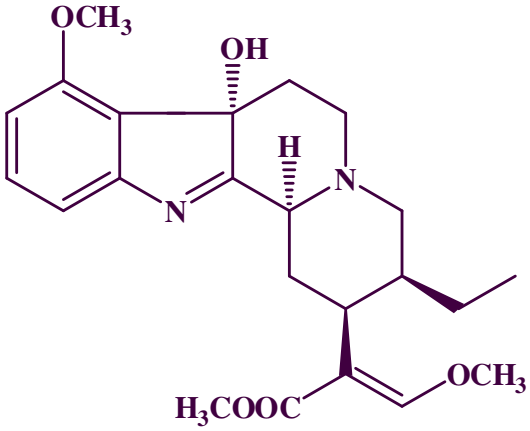
mitragynine



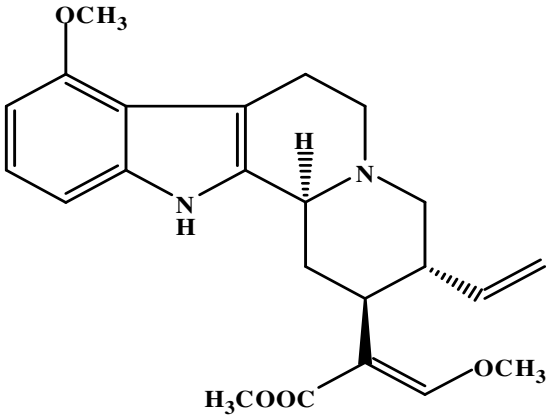
speciogynine



speciociliatine



7-hydroxymitragynine



paynantheine

Figure 1.2 Chemical structures of selected indole alkaloids in kratom leaves

1.2.1.4 Identification of kratom and determination of its constituents in kratom

Identification of *Mitragyna speciosa* Korth. involves botanical and chemical methods. Initially, the botanical procedure is needed so that the morphological features including macroscopical and microscopical characteristics (epidermis, palisade parenchyma, trichomes) will be identified (Kolkikumjorn, 1986). This method is however limited to fresh leaves only, not for dry- or powered leaves. Then, the chemical method is required since it is more specific to authenticate the plant and identify its constituents.

The chemical methods concern with the investigation of physical and chemical properties and may use specific instruments. The methods have been used for qualitative- and quantitative analysis. The chemical method used for qualitative analysis of dry macerated leaves or powered leaves is color-based test. For example, Duquinois'test, which was established by Kolkikumjorn in 1986, is an observation of a purple color in samples after adding Duquinois'reagent. The Duquenois reagent is prepared by adding 0.4 g of vanillin and 5 drops of acetaldehyde to 20 mL of 95% ethanol. The test is performed by placing of a target substance in a glass test tube, then 2 mL of the Duquenois reagent is added. After shaking, concentrated hydrochloric acid is added and the tube is again shaken. Two mL of chloroform is combined and the mixture is vortexed, then allows to settle and separate into two layers. A purple color in the upper layer (aqueous layer) indicates a positive result for kratom. Furthermore, Department of Medical Science, Ministry of Public Health has prepared the coloring kit test for boiled kratom leaves samples. The positive result of testing is blue solution. Color test is not practically to make a definite conclusion since it has poor specificity and false positive can occur. Therefore, other types of chemical methods are developed to determine kratom and substances extracted from kratom leaves such as mitragynine (Takayama, 2004).

Thin Layer Chromatography (TLC) is a type of simple chromatography that is used to separate mixtures of two or more compounds. The separation is accomplished by the distribution of the mixture between two phases: one is stationary and the other one is moving. The stationary phase is coated plate. The moving phase or mobile

phase is a suitable solvent containing in closed vessel (Sherma and Fried, 2003). The TLC system for kratom analysis was first reported by Kolkijkumjorn in 1986. The solvent system I was chloroform: acetone: diethylamine (5: 4: 1). The solvent system II was ethyl acetate: isopropanol: concentrated ammonia (100: 2: 1). The stationary phase was a thin layer of silica, which containing ultraviolet indicator. Spots were visualized by Dragendorff's spray, $FeCl_3$ solution spray, dried and then examined under UV light. The results showed that TLC could separate mitragynine by measuring of R_f (relative frontal mobility) values. In addition, Mongkoltran and Pariyavatee (2009) have developed a method for analysing "4×100" kratom cocktail (a boiled kratom leaves, diphenhydramine, caffeine and alprazolam). The solvent system I was a mixture of cyclohexane: toluene: diethylamine (75: 15: 10) and solvent system II was a combination of ethyl acetate: chloroform (2: 1). Spots were visualized by spray reagents ($FeCl_3$ solution, acidified iodoplatinate solution and Modified Ehrlich's reagent), dried and then examined under UV light at wavelength 254 and 366 nm. The results showed that TLC could separate mitragynine, diphenhydramine, caffeine and alprazolam by the measure of R_f (relative frontal mobility) values.

Gas chromatography is another chromatographic technique for determining mitragynine and other constituents in kratom leaves. GC can be used to separate volatile organic compounds. A gas chromatograph consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, and a detector. The organic compounds are separated based on different partition behaviors between the mobile phase and the stationary phase in the column. Many studies adopted the GC technique for kratom analysis. For example, Chan and coworkers (2005) determined mitragynine in various kratom preparations (dry or fresh leaves, powdered leaves, drinks and teas). Samples needed to be treated by liquid-liquid extraction before chromatographic analysis. Regarding to extraction procedure, macerated leaves and powered leaves were extracted with chloroform and methanol (1:4), while liquid samples such as drinks and tea were acidified and extracted with diethyl ether. Then the aqueous portion was alkalized and re-extracted with chloroform. The dry extract was reconstituted with methanol. Mitragynine was identified by comparing samples against a reference standard using a gas chromatography-flame ionization detector

(GC-FID) and gas chromatography-mass spectrometry (GC-MS). Kaewklum and coworkers (2005) used gas chromatography–mass spectrometry (GC-MS) detecting mitragynine and its metabolites in urine of kratom users following ingestion of kratom leaves. This method was able to detect mitragynine, speciogynine, paynantheine and three metabolites. Two of them were identified as mitragynine desmethyl and 9-hydroxycorynantheidine. By using GC-MS, Pingsuthiwong and coworkers (2009) also found mitragynine and speciogynine in urine. Both compounds can be used as markers of kratom consumption.

Liquid chromatography (LC) including high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS), liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS) is commonly used for mitragynine analysis. Liquid chromatography is an analytical chromatographic technique that involves passing of a mobile phase through a stationary phase.

Wasiman and coworkers (2002) reported a procedure for isolating and analyzing mitragynine and it metabolites using C_{18} SPE and HPLC. The extracts were separated on C_8 HPLC column with acetonitrile: water (90: 10) as a mobile phase. The analytes were monitored using ultraviolet (UV) detector at 230 nm. The extraction recovery was in a range of 61-98% and no interfering matrix was found.

In 2007, Janchawee and coworkers have developed the HPLC technique for determining mitragynine in serum. The separation system consisted of a C_{18} column heated to 35 $^{\circ}$C, a methanol-water (80: 20 v/v) mobile phase, a flow rate of 0.8 mL/min and detection in the ultraviolet at 225 nm. The calibration curve was linear from 0.1 to 10 μg/mL (r= 0.9995). Extraction of mitragynine from alkalinized serum using diethyl ether gave a high recovery (85-94%). The intra and inter day precisions were within 4.29-8.45% C.V. and the accuracy ranged from -9.54 to +0.67% DEV. The limit of detection and the lower limit of quantification were 0.03 and 0.1 μg/mL, respectively. The method was successfully applied to determine the pharmacokinetic characteristics of mitragynine levels in the serum of rats after it was administered orally.

Moraes *et al.,* (2009) developed and validated technique for quantify mitragynine in rat plasma by LC-MS. The method was successfully applied to pharmacokinetic studies of mitragynine after oral administration to rat. In the experiment, plasma samples were extracted with hexane-isoamyl alcohol (99: 1 v/v) and recoveries were about 95% and 90% for mitragynine and amitriptyline (internal standard), respectively. Mitragynine and internal standard were resolved on a Lichrospher® RP-18 column using 20 mmol/L ammonium acetate: acetonitrile: formic acid (70: 30: 0.5 v/v/v) at a flow rate of 1.2 mL/min as a mobile phase. Method validation showed precision and accuracy lower than 15 %. The quantification limit was 0.2 ng/mL and the linear range was 0.2-1000 ng/mL.

In 2009, Lu and coworkers reported the determination of mitragynine in human urine was determined by LC-ESI/MS. Mitragynine was extracted by methyl t-butyl ether (MTBE) and separated on a HILIC column. Ajmalicine was used as internal standard for method development. Quality control was performed at three concentration of mitragynine in urine. This method resulted in mean recoveries about 109% with average relative standard deviation < 22%. The regression linearity of mitragynine calibration ranged from 0.1 to 5.0 ng/mL was achieved with correlation coefficient greater than 0.995. A limit of detection was 0.01 ng/mL and high precision data within-day and between days analysis were obtained.

Kikura-Hanajiri and coworkers (2009) reported a method for simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids (speciogynine, speciociliatine, and paynantheine) which were present in the raw materials and commercial products of kratom in Japan by using LC-ESI/MS. The content of mitragynine in the products ranged from 1% to 6%, and that of 7-OH-mitragynine from 0.01% to 0.04%.

1.2.1.5 Pharmacology of kratom

Pharmacological information of kratom in animals (rats), both pharmacokinetics and pharmacological actions, has been documented. The animals were administrated kratom or its alkaloids and concentrations of the alkaloids or their actions were evaluated. Its pharmacology was reported as follows.

1) Pharmacokinetics of kratom

- Route of administration

Kratom is mainly administered via oral by chewing fresh leaves. It is also taken as ground fresh leaves or dried or reconstituted dried leaves. Some villagers use the leaves for cooking. During preparing fresh leaf, the vein is excluded and some salts are added to prevent constipation. Consumption of the leaf is usually followed by drinking something hot such as warm water or coffee. Leaves can also be prepared as a tea or a crude resin extract. This resin extract is made by preparing a water extract of the leaves, it is boiled until becomes viscous, and then shaping it into small balls which are rolled in a material such as flour, then stored until use. In addition, kratom leaves can be smoked (Marple, 2005).

- **Absorption**

The absorption of mitragynine from kratom was studied by many researchers. In 2007, Janchawee and coworkers studied the pharmacokinetics of mitragynine in rats. It was found that mitragynine was rapidly absorbed from GI tract after a single oral administration at the dose of 40 mg/kg body weight. Maximum serum concentration (C_{max}) of 0.63±0.18 μg/mL was achieved at 1.83±1.25 h (T_{max}) with an absorption rate constant (k_{ab}) of 1.43±0.90 h^{-1}. In contrast to the study of Moraes and coworkers (2009), the peak plasma concentration at 1.26±0.20 h and the maximum concentration of 0.42 μg/mL following an oral on ingestion of 20 mg/kg of mitragynine were observed. The absorption rate constant (k_{ab}) was 0.04±0.01 h^{-1}.

- Distribution

Mitragynine had a high volume of distribution (V_d/F, 89.50±30.30 L/kg) (Janchawee *et al.,* 2007). This may be due to its distribution to highly perfused and lipid-containing tissues, especially the brain, which is its site of action. Moraes and coworkers (2009) reported the smaller volume of distribution of 37.90±5.41 L/kg.

- **Metabolism**

Mitragynine was metabolized by hydrolysis of the methylester in position 16, *O*-demethylation of the 9-methoxy group and of the 17-methoxy group, followed, via the intermediate aldehydes, by oxidation to carboxylic acids or reduction to alcohols and combinations of some steps (Philipp *et al.*, 2009). Keawklum *et al.,* (2005) analyzed the urine from a person who had ingested the kratom leaves. The three major alkaloids (mitragynine, paynantheine, speciogynine) and the metabolites can be detected. The two metabolites were identified as 9-hydroxycorynantheidine desmethyl ester and 9-hydroxycorynantheidine. The 9-hydroxycorynantheidine desmethyl ester is a metabolite of paynantheine. The 9-hydroxycorynantheidine is a metabolite of speciogynine. These pharmacokinetic data were consistent with the study of Pingsuthiwong and coworkers (2007). Two major alkaloids (mitragynine and speciogynine) were detected in urine samples.

- Elimination

An animal study has shown that mitragynine was slowly eliminated. The elimination rate constant (k_{el}) of 0.07±0.01 h^{-1} and clearance (Cl/F) of 1.60±0.58 L/h were reported (Janchawee *et al.,* 2007). The corresponding elimination half-life was 9.43 h which was longer than that was recently reported in another study, i.e. 3.85 h (Moraes *et al.,* 2009).

2) Pharmacodymamics of kratom

Pharmacological effects of kratom have been reported as those of the major alkaloid, mitragynine, and other constituents of the leaves as follows.

- Mitragynine

Mutsumoto and coworkers (1996a) investigated an antinociceptive activity of mitragynine in mice receiving mitragynine by intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injections using pinch and hot plate test models. Its mechanism of action was evaluated by using naloxone, an opioid receptor antagonist. The result indicated that mitragynine itself possesses antinociceptive activity by acting in the brain and the supraspinal opioid system. Thongpradichote and coworkers

(1998) suggested that antinociceptive activity caused by mitragynine dominantly mediated via μ and δ opioid receptor subtypes. In addition, the selectivity of mitragynine for the supraspinal opioid receptor subtypes differs from that of morphine. Matusmoto *et al.,* (1996b) investigated the roles of central monoaminergic systems in the antinociceptive action of mitragynine by means of the tail pinch and hot plate test in mice. It was found that both descending noradrenergic and serotonergic systems involved in the antinociceptive activity of supraspinally administered mitragynine on the mechanical noxious stimulation, while the descending noradrenergic system predominantly contributed to the effect of supraspinal mitragynine on the thermal noxious stimulation.

In addition, Idid et *al.* (1998) compared the antinociceptive activity of morphine and paracetamol with that of mitragynine. Mice orally given mitragynine were subjected to acetic acid induced writhing, hot tail flick and cold tail flick. The results indicated that mitragynine has analgesic property similar to morphine. Reanmongkol and coworkers (2007) studied the effect of the methanol extract from the *Mitragyna speciosa* Korth. leaves on the nociceptive response using hot plate test in mice and tail flick test in rats. In addition, the analgesic tolerance was also investigated using hot plate test in mice by 14 days continued administration of the alkaloid extract. These results suggest that *M.speciosa* Korth. extract possesses analgesic activity which may be centrally mediated through μ receptor and no analgesic tolerance after continued administration.

Mitragynine has an effect on gastric acid secretion. Mitragynine inhibited 2-deoxy-*d*-glucose-stimulated gastric acid secretion through μ opioid receptors in anesthetized rats. It was suggested that mitragynine has a morphine-like effect on gastric acid secretion in the central nervous system (Tsuchiya *et al.,* 2002).

Matsumoto and coworkers (1997) investigated the effect of mitragynine on the 5-HT_{2A} receptor-mediated head-twitch response in mice. It was found that mitragynine suppressed 5-HT_{2A} receptor-mediated head-twitch by stimulated the postsynaptic α_2-adrenoceptor or blocked 5-HT_{2A} receptors. The 5-HT_{2A} receptor is known to participate in various psychiatric disorders such as depression, hallucination. Thus, this study suggested that mitragynine might have an effect to reduce psychiatric disorders.

- Mitragynine pseudoindoxyl

Mitragynine pseudoindoxyl was first isolated as a metabolite of mitragynine by microbial biotransformation. Its structure is different from other opioid agonists. It acts on opioid receptors leading to potent inhibition of eletrically stimulated contraction of ileum through the μ receptors and of mouse vas deferens through δ receptors (Yamamoto *et al.,* 1999).

In spite of its potent opioid effect, mitragynine pseudoindoxyl induced only a weak antinociceptive effect in mouse tail flick test in comparison with morphine (Takayama *et al.,* 2002; Matsumoto *et al.,* 2004).

- 7-Hydroxymitragynine

7-Hydroxymitragynine is a minor constituent of kratom leaves. In 2004, Matsumoto and colleagues studied antinociceptive effect of 7-hydroxymitragynine in mice. The results showed that 7-hydroxymitragynine induced dose-dependent antinociceptive effects in tail flick and hot plate tests after an oral administration in mice. Its effect was more potent than that of morphine. In 2006, Matsumoto and coworkers studied the mechanism of antinociception and the inhibitory effect on gastrointestinal transit of 7-hydroxymitragynine and compared its effects with these of morphine. The results indicated that both effects were mediated via μ opioid receptor. The result also showed that its constipation effect in animal was less potent than that of morphine.

1.2.1.6 Toxicity of kratom

- Acute effects

Acute effects of kratom is mainly on the central nervous system, of which is dose-dependent. At a low dose, the stimulating effect was predominant, which may cause alertness, energy and mild euphoria. A higher dose tends to be more tranquilizing causing an opiate-like dreamy reverie. Excessive dose can cause severe nausea. Sometimes kratom is used as an opium substitute and has been found to suppress symptoms of opiate withdrawal. Similarly to opium, it causes constipation. Some visual effects are open-eye and closed-eye effects. Wavering, shifting and strobing are also effects in visual field.

- Chronic effects

Health problems are unlikely unless one is consuming large quantities of kratom every day. In Thailand, some people use kratom every day. They become kratom dependent and may experience weight loss, dark pigmentation, especially around face region. They may have physical withdrawal symptoms if they quit abruptly. The withdrawal symptoms may include muscle aches, irritability, crying, runny nose, diarrhea and muscle jerking. Health problems are unlikely to occur in occasional kratom users. Like any drug or medicine, people's reactions vary and some people could possibly have an allergic or other unusual reaction to kratom, even if they used it responsibly. Kratom should not be combined with yohimbine, cocaine, amphetamine-like drugs or large dose of caffeine because of the possibility of over-stimulation or increases blood pressure. In addition, kratom should not concurrently use with large amounts of alcohol, benzodiazepines, opiates or other drugs that depress the nervous system. This is because of the possibility that such combination might cause over-sedation or even possible respiratory depression (Erowid, 2009).

1.2.2 Caffeine

Caffeine is the most widely used as a psychoactive substance in the world. It occurs naturally in coffee beans, cocoa beans, kola nuts and tea leaves. Caffeine is mainly consumed in drinks such as coffee, tea, soft drink and energy drink aiming for its stimulating effect (Riesselmann *et al.*, 1999). In addition, caffeine is an active ingredient in many headache medicines, both by prescription and sold over the counter, as well as in nonprescription aids and herbal prescription for alertness and dieting. Many abused illegal drugs or even some drugs sold legally may contain caffeine for added effect.

1.2.2.1 Chemistry of caffeine

Caffeine (1,3,7-trimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione; Figure 1.3) is a plant derived alkaloid. It was discovered by Friedrich Ferdinand Runge in 1819. Alternative names of caffeine are 1,3,7-trimethylxanthine, trimethylxanthine, theine and methyltheobromine. Chemical formula is $C_8H_{10}N_4O_2$ with a molecular weight of

194.19 g/mol. Purified caffeine is a white crystalline powder with a bitter taste and odorless. Caffeine is soluble in hot water, pyridine, ethyl acetate, partially soluble in cold water, acetone, alcohol and very slightly soluble in diethyl ether and petroleum ether. Caffeine behaves as a very free base, pH 6.9 in 1% aqueous solution and a polar compound with a pK_a around 14.2. Boiling point and melting point of caffeine are 178 $^{\circ}$C and 238 $^{\circ}$C, respectively (Science lab, 2008).

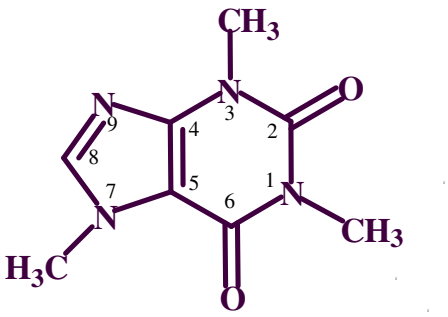


Figure 1.3 Chemical structure of caffeine

1.2.2.2 Pharmacology of caffeine

1) Pharmacokinetic of caffeine

- Routes of Administration

Oral administration is the most common route by which humans receive a beverage such as coffee, tea, soft drink and energy drink. Beyond beverages, caffeine is also consumed in a form of snack such as chocolate candy bars or as a component in medications (Mandel, 2002). Caffeine is a common street heroin constitute, inhalation of caffeine vapours is common in heroin users who take the drug by "chasing the dragon" carefully heating the drug on aluminium foil and inhaling the fumes through a straw (Huizer, 1987; Zandvliet *et al.*, 2005). Beside, in experimental of animals, they were obtained by intravenous or intraperitoneally injection (Wang and Lau, 1998).

- Absorption

In human, caffeine is rapidly absorbed from the gastrointestinal tract and reaching 99% in about 45 minutes after ingestion (Blanchard and Sawers, 1983). Caffeine absorption is also complete in animals (Nehlig, 1999). Beach and coworkers (1984) reported that after oral administration of caffeine at the doses of 200 and 400 mg, the maximum concentrations of caffeine in blood of 7.4 mg/mL for the 400 mg-dose and 3.4 mg/mL for the 200 mg-dose were achieved at 0.76±0.12 h. Stavchansky and coworkers (1988) reported the peak plasma concentration at 0.5-1 h following an ingestion of 100 mg of caffeine to health lactating women producing peak concentrations between 3.60 and 6.15 mg/mL. In summary, caffeine is rapidly transferred to breast milk. Liguori and coworkers (1997) reported peak caffeine absorption, time to peak absorption and subjective effect do not appear to be influenced by cola, or coffee vehicle. Perceived difference in the effects of coffee and cola may be due to the difference in dose, time of the day, added sweetener, environment setting or contingencies.

There is no difference in the elimination half-life of caffeine in young and elderly humans. The mean values for adult males and nonpregnant females were 3.4 h (range 2-5 h, n= 25) and 8.3 h (range 3-16 h, n= 57) for pregnant women (Knutti *et al.*, 1981).

- Distribution

Caffeine is extensively distributed throughout the body. Caffeine undergoes placental transfer. This is the reason for limiting dietary intake of caffeine during pregnancy (Kerrigan and Lindsey, 2005). The mean values of volume of distribution were 50 and 47 liters after 400 and 200 mg oral doses, respectively (Beach *et al.*, 1984). From the data reported by Lelo and coworkers (1986), the volume of distribution of caffeine and the other methylxanthines ranged from 0.63 to 0.72 L/kg. The unbound volume of distribution of caffeine was 1.06 L/kg. Nakazawa and coworkers (1985) reported the decreased volume of distribution of caffeine in rats given caffeine during pregnancy. The volume of distribution of caffeine in rats given

caffeine was 0.65 L/kg after intravenous administration and 0.80 L/kg after oral administration.

- Metabolism

In human, caffeine undergoes extensively hepatic metabolism to various methylxanthines, methylurates and uracil derivatives. The initial metabolism of caffeine leads to 1-, 3-, and 7- demethylations (yielding theobromine, paraxanthine and theophylline, respectively) and to 8-hydroxylation to 1, 3, 7-trimethyluric acid. Characteristic of caffeine biotransformation in rats is the formation of 6-amino-5-(N-formylmethylamino)-1, 3-dimethyluracil (1, 3, 7-DAU).

The three dimethylxanthines are further metabolized by demethylation to form 1-, 3-, or 7-methylxanthines or, alternatively, by 8-hydroxylation to generate the corresponding dimethyluric acids. Eventually, monomethylxanthines are 8-hydroxylated to the corresponding monomethyluric acids. The formation of paraxanthine is predominant in comparison to other demethylations (Schrader *et al.*, 1999).

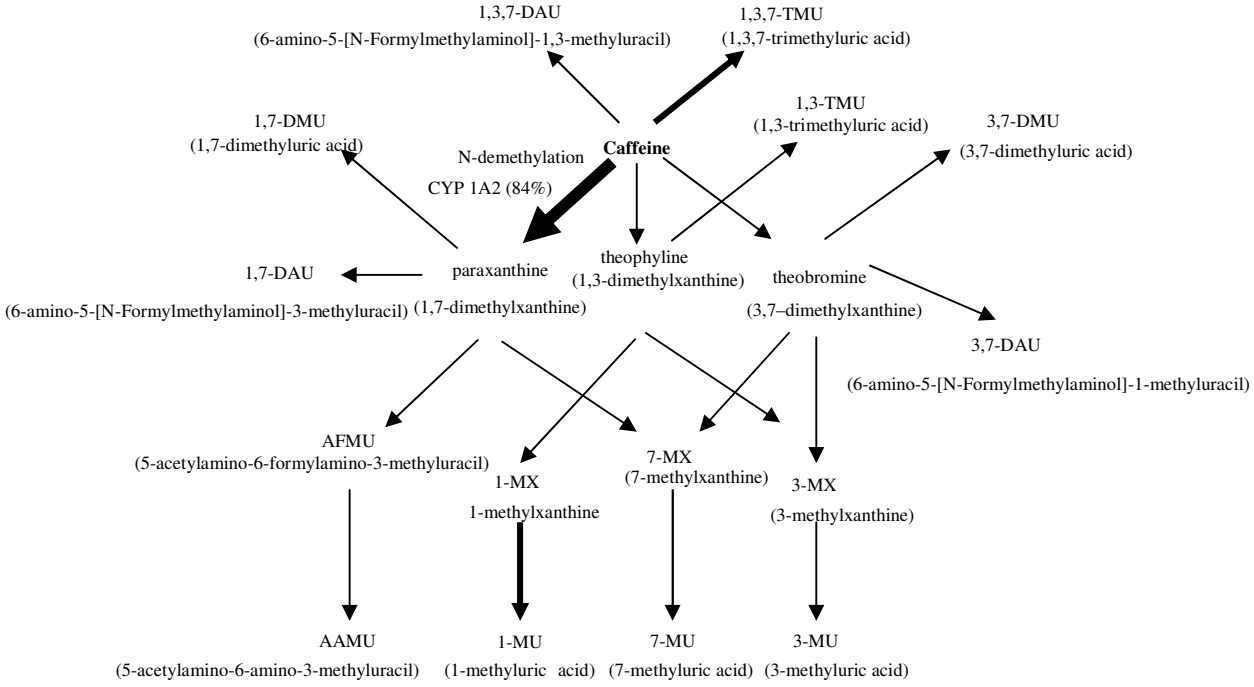


Figure 1.4 Metabolic pathway of caffeine in humans

- Elimination

During elimination, approximately 85% of a dose is excreted in the urine within 48 h, with approximately 1% as unchanged drug (Kerrigan and Lindsey, 2005). Newton and coworkers (1981) reported that total body clearance of caffeine was 0.98±0.38 mL/min/kg and unaffected by dose. From the data reported by Lelo and coworkers (1986), following oral administration of caffeine, the total plasma clearance was 2.07 mL/min/kg and the unbound plasma clearance was 3.11 mL/min/kg. The elimination half-life of caffeine is 4.1 h which was similar to that reported of Kuntti and coworkers (1981).

Rats receiving caffeine during premating and pregnant periods had a relatively high total body plasma clearance of caffeine (Kazuharn *et al.,* 1984).

2) Pharmacodynamics of caffeine

Caffeine has mild central nervous system stimulating effect. Caffeine acts by several mechanisms, including translocation of extracellular calcium, increase in cyclic adenosine monophophate (cAMP) and cyclic guanosine monophosphate (cGMP) caused by inhibition of phosphodiesterase, and blockade of adenosine receptors. Caffeine exerts a variety of pharmacological actions both centrally and peripherally at multiple sites as follows

- Central nervous system

The caffeine contained in 1-2 cups of coffee (100-200 mg) causes a decrease in fatigue and increased mental alertness as a result of stimulating the cortex and other areas of the brain. Consumption of 1.5 g of caffeine (12-15 cups of coffee) produces anxiety and tremors. The spinal cord is stimulated only by very high doses (2-5 g) of caffeine (Myeek *et al.*, 1997).

- Gastrointestinal system

Caffeine and all methylxanthines stimulate secretion of hydrochloric acid from the gastric mucosa. Individuals with peptic ulcers should avoid beverages containing methylxanthines.

- Cardiovascular system

Caffeine inhibits phosphodiesterase which activates cyclic AMP in directly resulting in an increased heart rate, an increase in cardiac output and blood pressure at rest (Sangdee and Jantraraksri, 1998).

- Diuretic action

Caffeine has a mild diuretic action that increases urinary output of sodium, chloride and potassium.

- Skeletal muscle

Its mechanisms of action, caffeine can increases skeletal muscle are to the intracellular calcium concentration, cause noradrenaline release, and sensitize dopamine receptor.

1.2.2.3 Toxicity of caffeine

- Acute effects

The median lethal dose (LD_{50}), given orally is 192 mg/kg body weight in rats. The LD_{50} of caffeine in humans is dependent on weight and individual sensitivity. Caffeine enters the body through the blood stream and goes to the central nervous system. Short-term effects of caffeine appear soon after a single dose and disappear within hour. An acute overdose of caffeine, usually occurs when it is given in excess of about 300 mg which is dependent on body weight and the level of caffeine tolerance, can result in a state of CNS over-stimulation called caffeine intoxication. At high dose, caffeine can cause considerable nausea vomiting dizziness headache and anxiety. Severe acute effects include peptic ulcer, delirium, coma, seizures and various types of arrhythmias (George, 2000).

- Chronic effects

Chronic or long-term effects of caffeine include chronic insomnia, persistent anxiety, depression and stomach ulcers. Caffeine use appears to be associated with irregular heartbeat and may raise cholesterol levels. But in large amounts and

especially over extended period of time, caffeine can lead to a condition known as caffeinism. Caffeinisim usually combines caffeine dependency with a wide range of unpleasant physical and mental conditions including nervousness, irritability, anxiety, tremulous, muscle twitching, insomnia, headache, respiratory alkalosis and heart palpitations. Furthermore because caffeine increases the production of stomach acid, extensive use over time can lead to peptic ulcer, erosive esophagitis and gastroesophageal reflux disease (George, 2000).

1.2.3 Codeine

Codeine is an opioid alkaloid which is used as antitussive, analgesic and antidiarrhea drug. Codeine can be found in many pharmaceutical products all around the world. It is present as tablet, capsule and syrup, etc. In most countries codeine is a controlled substance. In Thailand codeine is classified as narcotic Level 2 according to the Narcotics Act of B.E. 2522 (1979).

1.2.3.1 Chemistry of codeine

Codeine (Figure 1.5) is obtained either naturally from opium or by methylation of morphine. It was first isolated in 1830 in France by Jean-Pierre Robiquet. Codeine or (5α,6α)-7,8-didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol ($C_{18}H_{21}NO_3$; mw. 299.37 g/mol) is a phenanthrene-derivative opiate agonist. Alternative chemical names include methylmorphine, morphine monomethyl ether and morphine 3-methyl ether. Codeine occurs as colourless or white crystals or as a white, crystalline powder. It is slightly soluble in water and freely soluble in alcohol. Codeine is a weak basic, pH of more than 9 in 0.5% solution of codeine in water. Its pKa is 8.2. Boiling point and melting point of codeine are 250 $^{\circ}$C (at 22 mm/Hg) and 154-156 $^{\circ}$C, respectively. Codeine is commercially available as water soluble hydrochloride, sulfate or phosphate. Codeine phosphate ($C_{18}H_{21}NO_3 \cdot H_3PO_4$; mw. 397.40 g/mol) occurs as fine, white needle shaped crystals or as a white crystalline powder which is freely soluble in water and slightly soluble in alcohol. Codeine sulfate ((C_{18} $H_{21}NO_3)_2 \cdot H_2SO_4$; mw. 696.81 g/mol) occurs as white needle shaped crystal or as a white crystalline powder which is soluble in water and

very slightly soluble in alcohol. Owing to codeine phosphate is freely soluble in water then it is widely used as an ingredient in pharmaceutical products such as antitussive formulations. (Paolino, 1990)

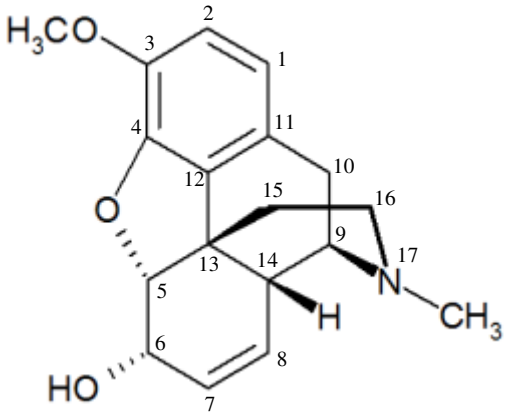


Figure 1.5 Chemical structures of codeine

1.2.3.2 Pharmacology of codeine

1) Pharmacokinetics of codeine

- Route of Administration

Codeine can be administered orally, subcutaneously, intramuscularly, intravenous and rectally. Oral administration is the most common route while the intravenous route may be used by drug abusers (Paolino, 1990).

- Absorption

Codeine is readily absorbed from the gastrointestinal tract and reaches its peak concentration in the plasma at about 1.1 h after oral administration. In human, codeine dosing of 60 mg produced peak plasma concentration of 138.8 ng/mL (Band *et al.*, 1994). Kim and coworkers (2002) reported after ingestion of codeine at the doses of 60 and 120 mg, the mean peak plasma concentrations were found to be 214.2 ± 27.6 and 474.3 ± 77.0 ng/mLat 1.2 and 1.3 h, respectively. In oral fluid, the mean peak concentrations of 1.7 and 1.6 h were observed, respectively.

- Distribution

Codeine is rapidly distributed from the intravascular (i.v.) spaces to the tissues with preferential uptake by the liver, spleen and kidney. The volume of distribution is 5.1 ± 1.7 L/kg following i.v. bolus (3 mg/kg) to male Sprague-Dawley rats (Shah and Mason, 1990).

- Metabolism

Codeine metabolism, which mainly occurs in liver, is complex and includes 3 metabolic pathways (Figure 1.6). Codeine (70%) is mainly metabolized to codeine-6-glucuronide by glucuronidation catalyzed by UDPGT (uridine-diphophate-glucuronosyl-tranferases). Codeine (10%) is also N-demethylated by CYP 3A4 to norcodeine which is further glucuronidated to morphine-3-glucuronide. At least 5-10% of codeine is O-demethylated by either CYP 2D6 in human or CYP 2D1 in rat to morphine which there is converted to morphine-3-glucuronide and morphine-6-glucuronide (Vree and Wissen, 1992; Popa *et al.*, 2003).

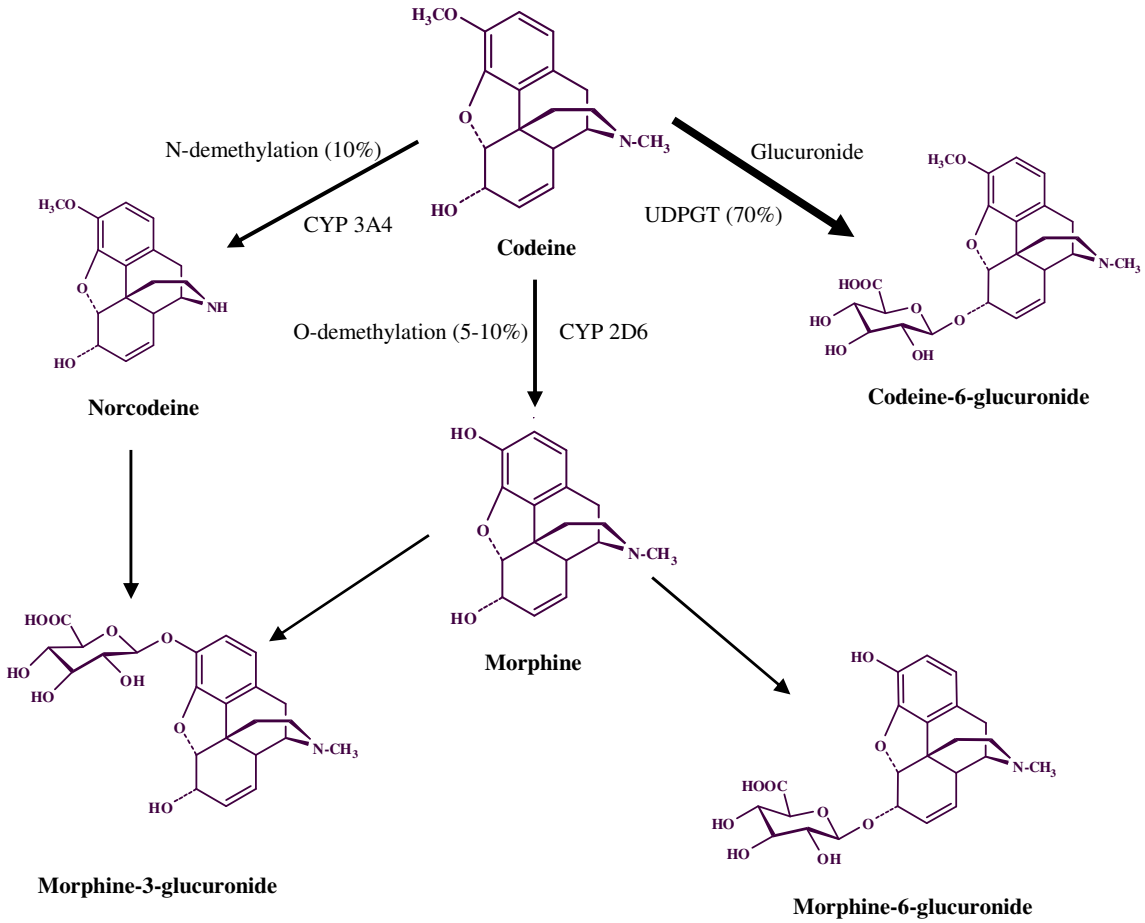


Figure 1.6 Metabolic pathway of codeine

- Excretion

Codeine is primarily excreted via the kidney within 24 h. After a single dose administration of 30 mg to human, 86.1±11.4% of the dose was recovered in urine which 59.8±10.3% was codeine-6-glucuronide, 7.1±1.1% was total morphine, 6.9±2.1% was total norcodeine and 11.8±3.9% was unchanged codeine. The renal clearance of codeine was 183±59 mL/min (Chen *et al.*, 1991; Lotsch *et al.*, 2009). Following an intravascular codeine administration to Sprague-Dawley rats, the total body clearance was 6.2±1.5 L/kg/h (Shah and Mason, 1990). A small amount of codeine can be found in breast milk and bile (Paolino, 1990).

2) Pharmacodynamics of codeine

Codeine produces the major effect on the central nervous system by binding with stereospecific receptors. Codeine has only 20% or less of the analgesic potency than morphine. Metabolites of codeine (morphine and codeine-6-glucuronide) have the effect on the analgesia (Srinivasan *et al.*, 1997). Codeine and binding of codeine and its metabolites to mu receptors are supportive of an analgesic effect of codeine itself. The antitussive property of codeine is a result of acting centrally on the medullary cough center. For the action of codeine-like drug on the gastrointestinal system is a decrease in motility. The mechanism of action of this effect is probably a combination of both local effects on the intestine as well as on CNS center regulating intestinal motility. The effects of codeine vary depending on the dose, frequency and duration of use.

1.2.3.3 Toxicity of codeine

- Acute effects

The desired effects of codeine are used in the treatment of mild to moderate pain, suppressing coughs and treatment for diarrhea. There are benefits of therapeutic use. The mental effects of codeine are euphoria, well being and calmness. Codeine may produce a mild stimulant effect, and a feeling of elation. In addition, codeine produced a felling of dysphasia, a general feeling of discomfort, restlessness and drowsy, with no noticeable positive or negative effect on mood. The most common physiological effects produced by medicinal quantities of codeine are nausea, vomiting, constipation and itching of the skin (Homsi *et al.*, 2001). These symptoms would be considered relatively harmful effects and typically disappear after taking codeine for several days or by reducing the dosage. The high dose codeine is concerned because of its respiratory depression resulting from the effect on the area of the brain that controls breathing. A large enough single dose of codeine can stop breathing completely, resulting in death. Combining codeine with another central nervous system depressant, such as alcohol or sedatives is especially risky.

- Chronic effects

Chronic effects are very few adverse health effects as far as organs or tissues damage are concerned from long term use of codeine. As an individual continues to use codeine (including therapeutic benefit) the body can develop tolerance to the drug. A high dose risk of codeine addiction also present for a long term use which can include either years of continual use or a repeated cycle of use and abstinence. The latter can be especially difficult with codeine given the potentially serious withdrawal symptoms associated with stopping the drug suddenly. Long term use also can lead to physical dependence, a process in which the body adapts to the presence of codeine and withdrawal symptoms occur if its use is reduced or eliminated abruptly. Symptoms of withdrawal can include restlessness, muscle and bone pain, insomnia, diarrhea, runny nose, chills with goose bump, and involuntary leg movements (Purdue Pharma, 2008).

1.2.4 Drug interaction

A drug interaction is a situation in which a substance affects the activity of another drug when they are administered together i.e. the effects are increased or decreased, or they produce a new effect that neither produces on its own. Typically, interaction between drugs comes to mind (drug-drug interaction). Drug interactions occur not only with other medications but also with herbal preparations, dietary supplements and foods. The various mechanisms of drug-drug, drug-herb and drug-food interactions with emphasis placed on the interactions most likely to cause harm. As pharmaceutical technology continues to expand at a phenomenal rate, so does the incidence of drug interaction. A regimen of 2 or more drugs, during admission to a critical care unit, and increasing age are risk factors for experiencing a drug interaction. These interactions can range in severity from theoretical to clinically significant, including prolonged morbidity and even death (Manzi and Shannon, 2005). Drug interactions may be the result of various processes. Most interactions are either pharmacokinetic, or pharmacodynamic. Some drug interactions are due to a combination of there two mechanisms.

These processes may include

1.2.4.1 Pharmacokinetic drug interaction

Pharmacokinetic drug-drug interaction is caused by abnormally increased or decreased drug concentrations in the body as a result of an alteration in the pharmacokinetics such as the absorption, distribution, metabolism, and excretion of a drug (Hisaka *et al.*, 2010).

1) Alteration in absorption

Drug interactions can occur where one drug changes the absorption characteristics of another drug. The binding of one drug to another, changes in gastric pH and changes in gastrointestinal motility can cause these drug interactions (Delafuente, 2003). For example, Kuwayama and coworkers (2007) have shown that the area under the plasma concentration-time curve of 3,4-methylenedioxymethamphetamine (MDMA) significantly increased by co-administration with caffeine in comparison to MDMA alone and that caffeine affects the absorption of MDMA in a different way from the transport system.

Codeine, another example, may delay the absorption of a number of drugs. Codeine will slow gastrointestinal motility, while drug such as metoclopramide will increase motility. Codeine may antagonize the effects of metoclopramide on gastrointestinal motility (Paolino, 1990).

2) Alteration in distribution

The most common mechanism of drug-drug interaction affecting drug distribution is alteration in protein binding. This type of interaction occurs when there is competitive inhibition for protein binding sites. This allows for the unbound fraction of the drugs to be increased and it is the free fraction that is responsible for pharmacological activity. The most clinically significant interactions involve drug that are highly protein bound and have a narrow therapeutic index (Delafuente, 2003).

3) Alteration in metabolism

The most clinically important types of pharmacokinetic drug-drug interactions are those altering a drug's metabolism. The cytochrome P450 enzymes are the major drug metabolizing enzymes. There are unique isoenzymes found primarily in the liver

and are responsible for the metabolism of many drugs and toxins. These enzymes may inactivate a drug by producing a metabolite or alternatively activate the drug. Drug interactions should be more predictable on the basic of the knowledge of which compounds induce and inhibit specific P450 enzymes.

- Cytochrome P450 inhibition

Competitive binding at the enzyme's binding site between two drugs is often responsible for inhibition of a drug's metabolism. The onset of CYP450 inhibition depends on the inhibiting drug's half-life. For drug with short half-lives, enzyme inhibition occurs quickly and clinically significant interactions can be apparent with 1 or 2 days. Inhibition of CYP450 is also dose-dependent. Higher dose of an inhibitory drug will cause greater amounts competitive inhibition than lower doses (Shapiro and Shear, 2001).

An example of this type of drug-drug interaction is codeine and glutethimide. Popa and coworkers (2003) investigated codeine-glutethimide pharmacokinetic interaction in rats. The results revealed that glutethimide was responsible for a significant increase of morphine plasma levels and for a significant decrease of M-3-G plasma levels, explained by the inhibition of morphine glucuronidation. In conclusion, glutethimide potentiates and prolongs the analgesic effect of codeine by a pharmacokinetic mechanism.

- Cytochrome P450 induction

The onset for enzyme induction is usually longer than that of enzyme inhibition. Enzyme induction is dependent on synthesis of new CYP450 isoenzymes and is dependent on the half-life of the inducing drug. Like inhibition of CYP450 enzymes, shorter half-life drugs will have a shorter onset of induction (Shapiro and Shear, 2001).

4) Altered renal elimination

Excretion and elimination of drugs occur primarily via the kidneys. Biliary secretion, plasma esterase and other minor pathways are important routes, albeit less

common than renal elimination. Many drugs and metabolites are excreted in the urine via renal tubular secretion. Two drugs can compete for the same active secretion sites in the tubule allowing for decreased elimination and potentially toxic serum concentration. Alteration in urine pH can also affect drug elimination. Alkalinization of the urine will decrease elimination of drugs that are weak bases and decrease in urine pH will increase their elimination. Acidification of the urine will decrease renal elimination drugs that are weak acids. These more common and potentially more significant are drug-drug interactions that affect renal function. Glomerular filtration rates decline with advanced aging. To compensate for this physiologic change a compensatory production of vasodilatory renal prostaglandins occurs (Delafuente, 2003).

1.2.4.2 Pharmacodynamic drug interaction

Pharmacodynamic interactions involve one drug changing the clinical effects of another drug. These types of interactions are probably more common than pharmacokinetic interactions. Pharmacodynamic interactions occur when two or more drugs used that have additive or synergistic pharmacological activities or have antagonistic pharmacological activities. Pharmacodynamic interactions are more difficult to detect and study than pharmacokinetic interactions.

Some examples of pharmacodynamic drug interaction are documented. Caffeine produced additive effect when combined with analgesic drugs such as paracetamol and aspirin (Gayawali *et al.,* 1991).

The administration of caffeine plus MDMA would induce an increase in dopamine activity. An increase in dopamine release in the mesolimbic system may precipitate purposive movement increasing motivation and alertness, reducing appetite and inducing insomnia, acute effects that are often seen when ingesting ecstasy pills (Camarasa *et al.*, 2006) In addition, co-administration of caffeine with MDMA induced a profound tachycardia response compared to rats treated with either alone (McNamara *et al.*, 2007)

Coadministration of codeine and diclofenac resulted in synergistic effect of antinociception (Jimnez-Andrade *et al.,* 2003). The highest synergism observed after

systemic administration suggested that the interaction is occurring at several anatomical site. Codeine given concurrently with an opioid may result in increased constipation, paralytic ileus, as well as an increased risk of respiratory depression. When given together with antihypertensive drugs, codeine may potentiate hypotension and increase the risk of orthostatic hypotension (Paolino, 1990).

1.2.5 Pain

According to The International Association for the Study of Pain, "pain" is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in term of such as damage. Pain is a body defense mechanism and is a warning of a problem, particularly when it is acute and may become chronic where it outlasts any potential for healing and becomes modified centrally (Gould, 2002).

1.2.5.1 Types of pain

Pain can be divided into 2 types, i.e. acute and chronic pain. Both experiences alter the comfort level of the patient and cause different pain reaction behaviors.

1) Acute pain

Acute pain is characterized by its transitory nature, its sudden onset and limited duration for the time of on going physiologic injury. Acute pain is commonly associated with surgery, diagnostic procedures, burns and trauma (Bullock and Henze, 1999).

2) Chronic pain

Chronic pain is persist beyond the normal healing time as pain signals are repeatedly being generated making neural pathways hypersensitive to pain signals and resistant to antinociceptive input. Chronic pain is often prolonged beyond 6 months and less defined in its source and course (Bullock and Henze, 1999; McCurdy and Scully, 2005).

1.2.5.2 Pain mechanism

1) Peripheral sensitization

After occurring of an injury which may be caused by mechanical, chemical or thermal stimuli on nociceptors (nociceptors is a specialized group of sensory receptors which stimulate from tissue-damaging chemical, thermal or mechanical agents). The biological molecules such as substance P, histamine, bradykinin are produced and released. Then, the pain impulses travel to the dorsal horn of the spinal cord through two types of nerve fiber: A-delta (Aδ) fiber are small myelinated conducting afferent neurons, 1-6 μm in diameters which conduct rate of 12-30 m/s so these fibers are rapid in transfer of information from the periphery to terminate in lamina I and V of the spinal cord. These fibers are high-threshold receptors which responses to mechanical stimulation such as firm pinch. Some of the fibers also respond to noxious heat (> 45°C). Aδ fiber activation results in sharp pain. The C fiber do not have myelin sheath, 0.4-1.2 μm in diameters which conduct rates of 0.5-2 m/s slower conducting. These receptors are sensitive to chemical and thermal stimulation and are frequency referred to as polymodal nociceptor. They terminate in larmina I and II of the spinal cord (substantia gelatinosa) and mediate dull, diffuse, aching or burning pain sometime called visceral pain. Aδ and C fibers release excitatory amino acids (glutamate) in dorsal horn, C fiber also releases neuropeptides, substance P (Stephen, 1998). This state is called peripheral sensitization.

2) Central sensitization

The repeated afferent impulse to the spinal cord as a result of the sensitizing biological molecules at the site of tissue damage, cause the dorsal horn neurons within the spinal cord to become hyperexcitable. When A-δ fibers produce the acute sensation of sharp and bright pain, their neurotransmitter in the dorsal horn is glutamate acting on alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors. The C-fibers can respond to a broad range of painful stimuli including mechanical, thermal or metabolic factors. The pain produced is slow, burning and long lasting. The neurotransmitter in the dorsal horn is glutamate along with certain peptide such as substance P. The receptors for glutamate are not only

AMPA, but also N-methyl-D-aspartate (NMDA). When AMPA receptor was activated and open following prolonged depolarization, continue stimulation of c fibers eventually causes greater excitation in the postsynaptic neurons in the dorsal horn as the NMDA receptors start added to the response. Recall that there only open with prolonged depolarization, such as it would occur with prolong pain. The resulting influx of Ca^{2+} triggers other long lasting cellular changes so signal transduction coming to sensory projection field in the cortex (postcentral gyrus). This part of the cortex, together with the thalamus is responsible for the conscious perception of pain and particularly localizing and registering the intensity of the pain. The ascending reticular activating system has an influence on evaluation. The limbic system is responsible for the emotional reaction trigged by pain while autonomic reactions are controlled by the hypothalamus. This state of hyperexcitability is called central sensitization.

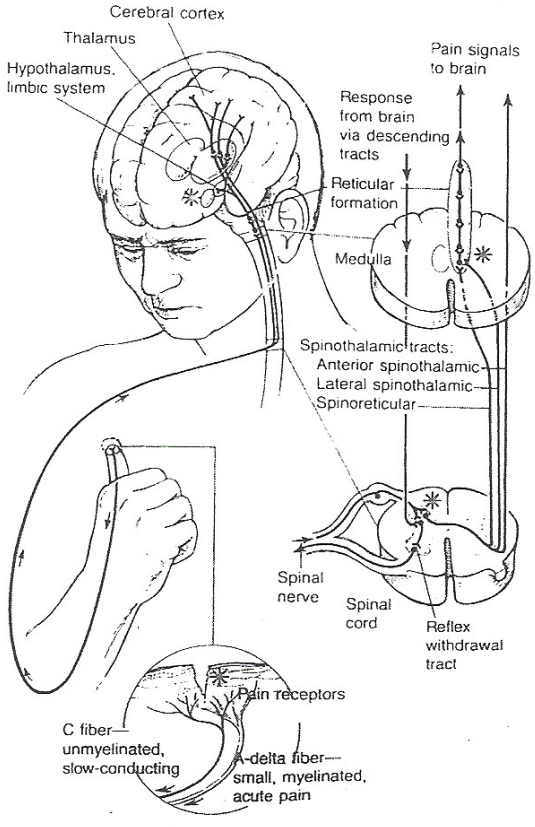


Figure 1.7 Pathway of pain work

1.3 Objectives

1. To study the effects of caffeine, codeine and the combination of caffeine and codeine on pharmacokinetics of alkaloid extract from leaves of kratom (*Mitragyna speciosa* Korth.)

2. To study the effects of caffeine, codeine and the combination of caffeine and codeine on antinociceptive activity of alkaloid extract from leaves of kratom (*Mitragyna speciosa* Korth.)

CHAPTER 2

Methodology

2.1 Chemicals materials and instruments

2.1.1 Chemicals

Chemicals employed in this study are listed in Table 2.1.

Table 2.1 A list of chemicals used in the study

Chemical	Grade	MW/FW/g/mol	Supplier
Acetic acid (Lot no.B44806)	A.C.S. Reagent	60.05	Mallinckrodt Baker Inc., NJ, U.S.A.
Ammonium hydroxide, 28-30% (Lot no.B31052)	A.C.S. Reagent	35.05	Mallinckrodt Baker Inc., NJ, U.S.A.
Anhydrous sodium sulfate (Lot no.A514897 422)	-	142.04	VWR International Ltd, Poole, U.K.
Caffeine (Lot no.391 DC-6074-86)	purity 99%	194.19	Alltech Applied Science Labs, State College, PA, U.S.A. (It was obtained from the Scientific Crime Detection Division 9, Office of the Police Forensic Science, Thailand)

Chemical	Grade	MW/FW/g/mol	Supplier
Chloroform (Lot no.K37228641 716)	GR	119.38	VWR International Ltd, Poole, U.K.
Codeine (Lot no.L1087 DC-5053-30)	purity 99%	299.36	Alltech Applied Science Labs, State College, PA, U.S.A. (It was Obtained from the Scientific Crime Detection Division 9, Office of the Police Forensic Science, Thailand)
Disodium phosphate	-	141.96	Mallinckrodt Baker Inc., NJ, U.S.A.
Ether (Lot no.H15 B16)	A.C.S. Reagent	74.12	Mallinckrodt Baker Inc., NJ, U.S.A.
Methanol (Lot no.C50B50)	AR	32.04	Mallinckrodt Baker Inc., NJ, U.S.A.
Methanol (Lot no.C06E65)	HPLC	32.04	Mallinckrodt Baker Inc., NJ, U.S.A.
Petroleum ether (Lot no.C51B08)	A.C.S. Reagent	86.16	Mallinckrodt Baker Inc., NJ, U.S.A.
Sodium hydroxide	-	40.00	Mallinckrodt Baker Inc., NJ, U.S.A

2.1.2 Materials and instruments

A list of materials and instruments employed in the study are shown in Table 2.2

Table 2.2 List of materials and instruments used in the study

	Item	Source
Material	Oasis® HLB cartridge (1 mL/30 mg)	Waters Corporation, Massachusetts, U.S.A.
	Sentry™ guard column (20×4.6 mm i.d., 5 μm)	Waters Corporation, Massachusetts, U.S.A.
	Sunfire™ C_{18} column (250×4.6 mm i.d., 5 μm)	Waters Corporation, Massachusetts, U.S.A.
Instrument	Agilent 1200 Series HPLC	Agilent, DE, U.S.A.
	Analytical balances model AB 204-S	METTLER TOLEDO, Greifensee, Switzerland
	Automatic pipette 100 μL	Eppendorf, Hamburg, Germany
	Automatic pipettes 200 and 1000 μL	SOCOREX, Isba S.A., Switzerland
	CentriVap Concentrator	LABCONCO, MO, U.S.
	Diode array detector	Agilent, DE, U.S.A.

	Item	Source
Instrument	Memmert Oven D 06064 Modell 800	Memmert GmbH + Co. KG, Schwabach, Germany
	pH Meter model SevenEasy	METTLER TOLEDO, Greifensee , Switzerland
	Refrigerated centrifuges	SORVALL RC-3B Plus, California, U.S.A.
	RE 120 Rotavapor and B 169 Vacuum system	BUCHI, Flawil, Switzerland
	Ultrasonic bath model JAC 2010	Kodo, Hwaseong, South Korea
	Vortex Genie-2	Sciencetific Industries, New York, USA

2.2 Source and identification of kratom

Kratom leaves were supplied from the Scientific Crime Detection Division 9, Office of the Police Forensic Science, Thailand. They were primarily identified by both botanical and chemical methods. For botanical method, the leaves were examined for both macroscopical and microscopical characters. For chemical test, a rapid Duquenois test was used. The Duquenois reagent was prepared by adding 0.4 g of vanillin and 5 drops of acetaldehyde to 20 mL of 95% ethanol. The test was performed by placing of a target substance in a glass test tube, then 2 mL of the Duquenois reagent was added. After shaking, concentrated hydrochloric acid (2 mL) was added and the tube was again shaken. Two mL of chloroform were added and the tube was vortexed, then allowed to settle and separate into two layers. Purple color in

the upper layer (aqueous layer) developed indicating a positive result for authentic kratom (Kolkijkumjorn, 1986).

2.3 Alkaloid extraction procedure

Extraction of crude alkaloid from kratom leaves was carried out as previously described by Houghton *et al.* (1991) and Ponglux *et al.* (1994) with some modifications as follows.

Fresh leaves were dried overnight at 45-50 °C by using Memmert Oven. The leaves of kratom were powdered and macerated with methanol for three times. Fluids were filtrated by using vacuum system. The methanol filtrates were combined and evaporated by using rotavapor under vacuum condition. The crude methanol extract was dissolved in 10% acetic acid solution, well shaken and left to stand overnight. The acidic filtrate was washed with petroleum ether, then brought to pH 9 with 28-30% ammonia hydroxide solution and extracted with chloroform. The chloroform extract was washed with distilled water, dried over anhydrous sodium sulfate and evaporated to yield a dry crude alkaloid extract (with an approximately 0.25% yield based on the fresh leaves weight).

2.4 Isolation of mitragynine

Kratom leaves were collected from natural sources in Songkhla and Satun provinces, Thailand, during 2004-2005. Authentication of the plant material was carried out at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Extraction and isolation of mitragynine were also carried out as previously described by Houghton *et al.* (1991) and Ponglux *et al.* (1994) with some modifications as follows.

The leaves were dried at 45-50 °C, powdered and macerated with methanol (repeated three times). The methanol filtrates were combined and evaporated under reduced pressure. The crude methanol extract was dissolved in 10% acetic acid solution, well shaken and left to stand overnight. The acidic filtrate was washed with petroleum ether, then brought to pH 9 with 25% ammonia solution and extracted with chloroform. The chloroform extract was washed with distilled water, dried over

anhydrous sodium sulfate and evaporated to yield a dry crude alkaloid extract (with an approximately 0.25% yield based on the fresh leaf weight). An aliquot (2.5 g) of alkaloid extract was subjected to silica gel column chromatography, elute with 5% methanol in chloroform to obtain a major alkaloid (1.27 g), which appeared as a single spot on TLC analysis (four solvent systems) and was found to be a pure compound upon spectroscopic analysis by MS (Thermofinnigan MAT 95 XL mass spectrometer: EIMS with direct insert probe), and ^{1}H-NMR and ^{13}C-NMR (Varian Unity Inova 500 NMR spectrometer) spectra. When the obtained spectral data were compared with the published assignments (Shellard *et al.,* 1978 and Houghton *et al.*, 1991), it was identified as mitragynine. The extraction procedure of mitragynine is shown in Figure 2.1.

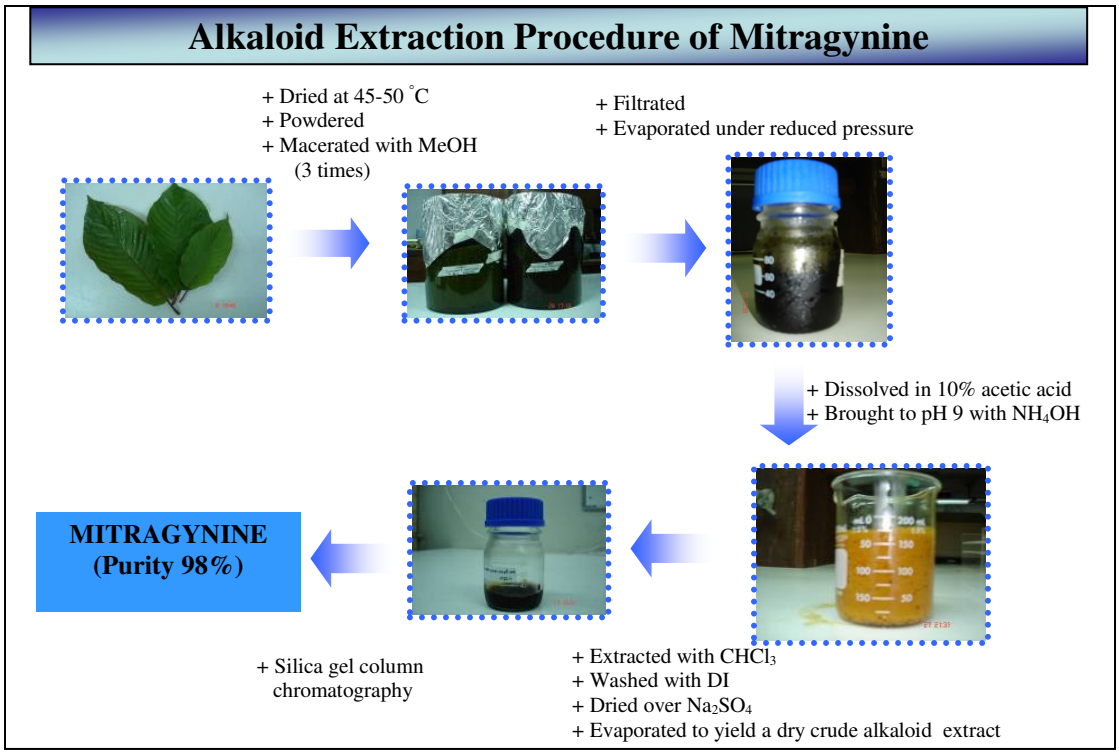


Figure 2.1 Alkaloid extraction procedure of mitragynine

2.5 Experimental animals

Male Wistar rats weighing between 220 and 260 g obtained from the Southern Laboratory Animal Facility, Prince of Songkla University were used. The animals were housed under control conditions with an ambient temperature of 23±2°C and a 12 h/light/dark cycle. There were freely accessed to food and water *ad libitum*. The experimental designs were approved by the Ethics Committee for Experimental Animals (no. Ref 16/50), Prince of Songkla University.

2.6 Experiment protocols

2.6.1 Experiment 1: Effect of caffeine and codeine on pharmacokinetics of alkaloid extract from kratom leaves

2.6.1.1 Selection dose of compound administration

1) Dose of alkaloid extract

Rats were divided into three groups for an optimum dose of alkaloid extract in the pharmacokinetic study. Each group contained 3 animals and orally given a single oral dose of treatment as follows: propylene glycol 2 mL/kg, alkaloid extract 100 and 150 mg/kg. After administration, rats were observed clinical effects and antinociceptive response. The schematic diagram for selection dose of alkaloid extract is shown in Figure 2.2.

2) Dose of caffeine

Twenty one rats were divided into seven groups (n=3). There were received a single dose of treatment as follows: distilled water 5 mL/kg, caffeine 10, 25, 40, 50, 75 and 100 mg/kg. Rats were examined latency of nociceptive response using hot plate analgesia meter. The schematic diagram for selection dose of caffeine is shown in Figure 2.2.

3) Dose of codeine

Fifteen rats were divided into five groups (n=3). There were received a single oral dose of treatment as follows: distilled water 5 mL/kg, dose of 1, 3, 5 and 10

mg/kg of codeine. There were also preliminary studied on the nociceptive response using hot plate test in rats after administration. The schematic diagram for selection dose of codeine is shown in Figure 2.2.

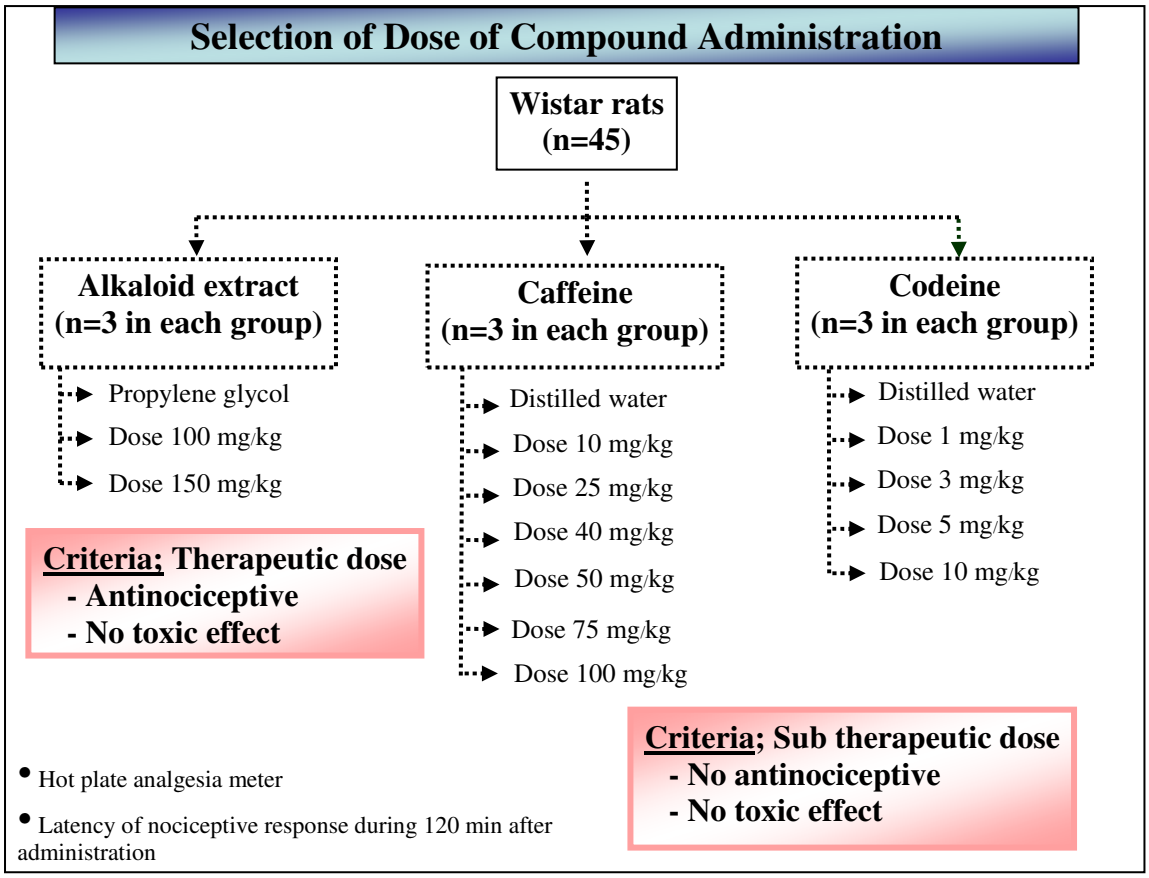


Figure 2.2 The schematic diagram for selection dose of alkaloid extract, caffeine and codeine.

2.6.1.2 Animal treatment

There were two groups of experimental animals. Each group contained 24 animals. One group was used for collecting serum sample. The other group was for collecting urine sample. Both groups were further divided into 4 groups (n=6) and received treatments as follows.

Group AE: The animals obtained 100 mg/kg alkaloid extract from kratom leaves (AE).

Group AE+CF: The animals simultaneously obtained 100 mg/kg alkaloid extract from kratom leaves (AE) and 25 mg/kg of caffeine (CF).

Group AE+CD: The animals simultaneously obtained 100 mg/kg alkaloid extract from kratom leaves (AE) and 3 mg/kg of codeine (CD).

Group AE+CF+CD: The animals simultaneously obtained 100 mg/kg alkaloid extract from kratom leaves (AE), 25 mg/kg of caffeine (CF) and 3 mg/kg of codeine (CD).

Prior to the drug administration, animals were fasted overnight but freely accessed to water. Then, animals were orally administrated a single dose of each compound. The formulation for the oral administration was prepared by dissolving alkaloid extract from kratom with a minimum volume of 100% propylene glycol (2 mL) while caffeine and codeine dissolving with distilled water (5 mL).

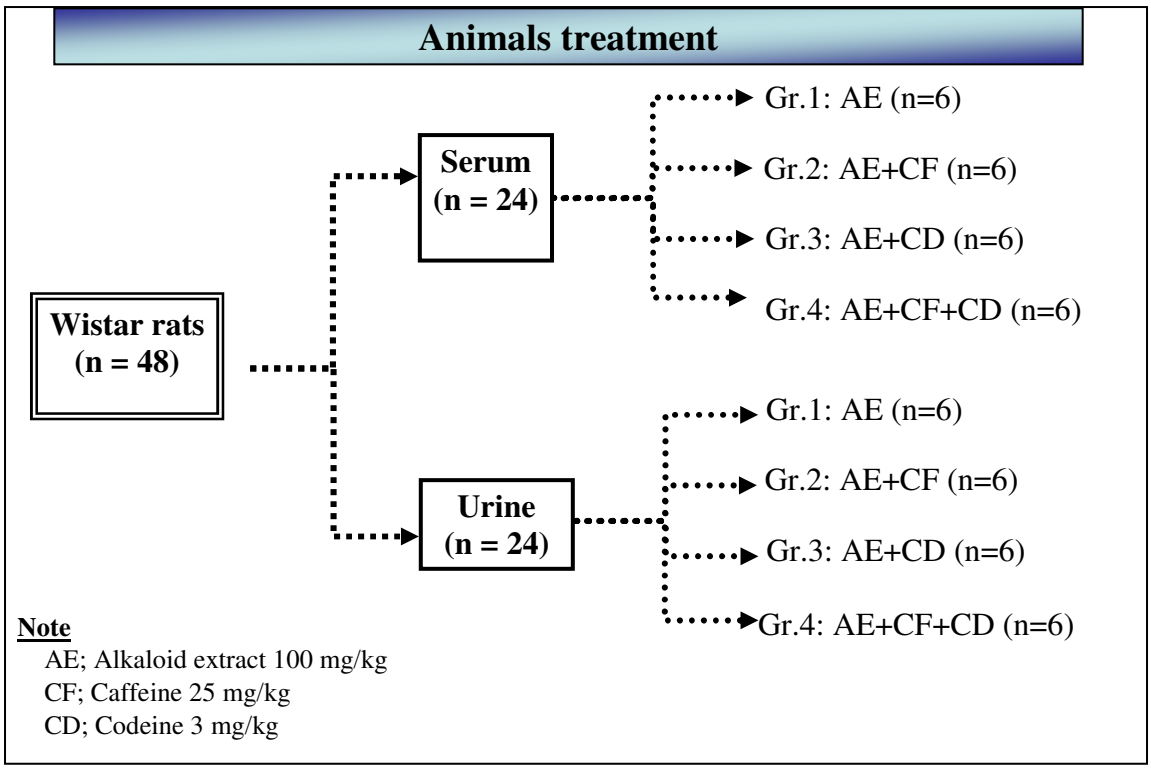


Figure 2.3 The schematic diagram of animals treatment for experiment 1

2.6.1.3 Serum sample collection

Blood samples (500 μL) were collected by puncture of the retro-orbital sinus under ether anesthesia into microcentrifuge tube at 0, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after drug administration. Serum samples were separated after centrifugation at 2000×*g* for 15 min at 4°C in refrigerated centrifuge and kept frozen at -70°C in an ultra-low temperature freezer until analysis. The schematic diagram for serum samples collection is shown in Figure 2.4.

2.6.1.4 Urine sample collection

In order to collect urine, the rats were housed individually in metabolic cage. Urine samples were collected during 8 h before drug administration and at 8, 16 and 24 h after drug administration. Urine samples were centrifuged at 2000×*g* for 15 min at 4°C. Supernatant was separated and kept frozen at -70°C until analysis. The schematic diagram for urine samples collection is shown in Figure 2.4.

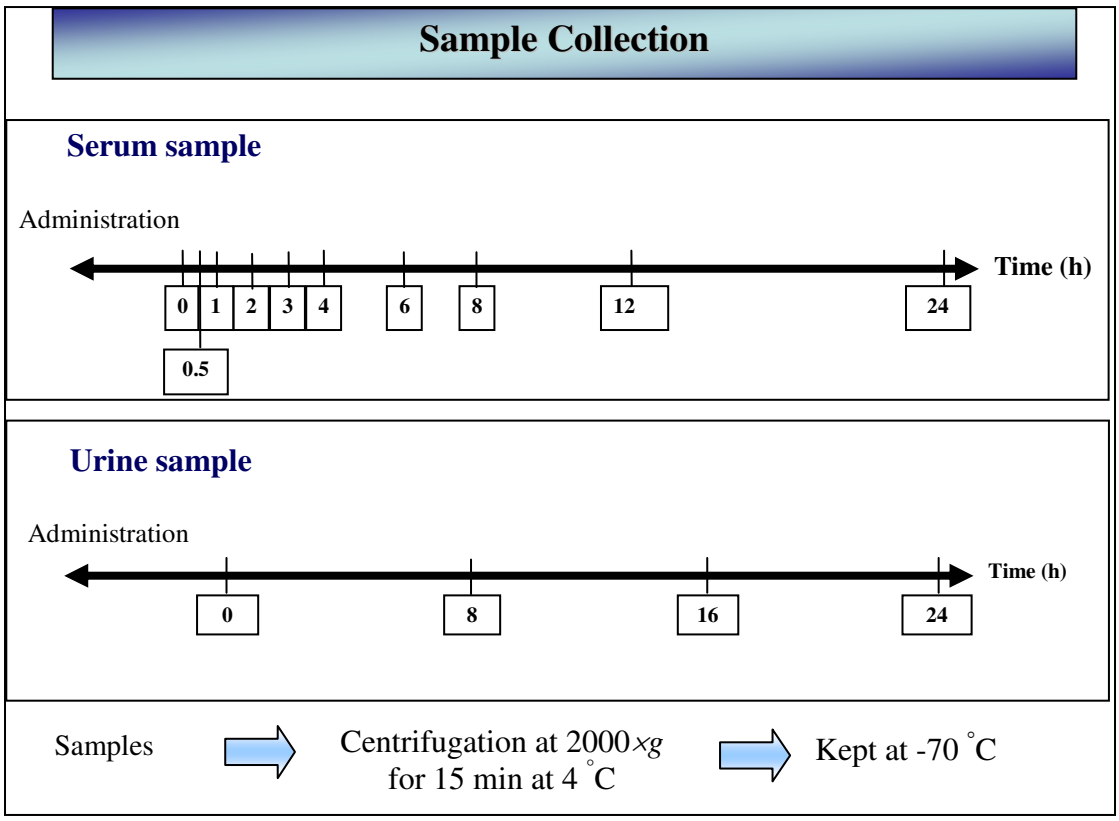


Figure 2.4 Serum and urine sample collection

2.6.1.5 Chromatographic conditions

An Agilent 1200 Series HPLC (Agilent, DE, U.S.A.) system was used, coupled with a diode array detector (Agilent, DE, U.S.A.). Data were collected and processed using the Agilent Chemstation Software System (CA, U.S.A). Concentrations of mitragynine in serum and urine were determined using method established by Janchawee *et al.* (2007). The HPLC column was reverse-phase Sunfire™ C_{18} column (250×4.6 mm i.d., 5 µm particle size) connected to Sentry™ guard column (20×4.6 mm i.d., 5 µm particle size) from Water (Milford, MA, U.S.A.). The temperature of the column oven was set at 35±1°C while that of the autosampler was at ambient temperature (25±1°C). The freshly prepared mobile phase was methanol-water mixture (80:20, v/v), which was filtered separately before mixing through a 0.22 µm nylon membrane filter and degassed ultrasonically for 20 min before use. The flow rate was 0.8 mL/min. A sample volume of 20 µL was injected and mitragynine was detected at wavelength of 225 nm.

2.6.1.6 Preparation of standard solutions

1) Stock standard solution

Stock solution (1 mg/mL) of standard mitragynine was prepared by dissolving a standard compound (1000 μg) with pure methanol (1 mL) in a 1.5 mL-microcentrifuge tube and stored at -20 °C.

2) Working standard solutions

Working standard solutions were prepared by diluting the stock solution with pure methanol to a concentration range of 0.5-100 μg/mL for standard mitragynine. Working standard solutions were stored at -20 °C until analysis.

3) Calibration standard solutions

Calibration standard solutions were prepared by adding appropriate working standard mitragynine solutions to blank rat serum (final volume 100 μL) and blank rat urine (final volume 1 mL) so that final concentrations of mitragynine were 0.1, 0.5, 1, 5, 10 μg/mL

2.6.1.7 Method validation for analysis of mitragynine in serum and urine

Prior to mitragynine analysis, validation characteristics, namely linearity, recovery, intra-day and inter-day precision, accuracy, lower limit of quantification were performed in accordance with ICH guideline recommendations (Swartz and Krull, 1997) and Guaidance for Industry Bioanalytical Method Validation of Food and Drug Administration in U.S. (FDA, 2001). Drug-free rat serum and urine were used throughout the validation.

1) Linearity and range

Linearity was determined by preparing standard serum and urine sample with different concentrations of mitragynine (0.1, 0.5, 1, 5, 10 μg/mL: 3 and 5 replicates of each concentration, respectively). The calibration curve is the relationship between instrument response and known concentration of the analysis. The calibration curve was constructed by plotting peak areas (Y) of mitragynine versus its concentrations

(X). Regression analysis was performed to obtain the calibration equation and correlation coefficient *(r)*.

2) Precision

Intra-day and inter-day precisions were determined by adding different concentrations of standard mitragynine (0.1, 0.5, 5, 10 μg/mL: 3 and 5 replicates of each concentration) into serum and urine, respectively. For serum samples, the analyses were performed within the same day and for 3 consecutive days, respectively. For urine samples, the analyses were performed within the same day and for 5 consecutive days, respectively. Precision was expressed as the coefficient of variation (C.V.) and calculated as follows.

$$\text{Coefficient of variation (\% C.V.)} = \frac{\text{Standard deviation (S.D.)}}{\text{Mean}} \times 100$$

The precision determined at each concentration level should not exceed 15% of the C.V., except for the LLOQ, where it should not exceed 20% of the C.V.

3) Accuracy

Accuracy was determined by adding the standard mitragynine into serum and urine at concentrations of 0.1, 0.5, 5, 10 μg/mL (3 replicates of each concentration for serum samples and 5 replicates of each concentration for urine sample). Accuracy was expressed as %deviation (DEV) which was calculated as follow.

$$\text{Deviation (\% DEV)} = \frac{(\text{measured concentration} - \text{nominal concentration})}{\text{nominal concentration}} \times 100$$

An acceptable accuracy was less than 15% of the nominal concentration value except at LLOQ, where it should not deviate by more than 20%.

4) Recovery

The recovery of mitragynine by extraction from samples (serum and urine) was determined at concentrations of 0.1, 0.5, 5, 10 μg/mL (3 replicates of each

concentration for serum samples and 5 replicates of each concentration for urine sample) by comparing responses obtained after extracting mitragynine from samples (serum and urine) with those obtained after the direct injection of standard mitragynine prepared in methanol. The percent recovery was calculated as follows.

$$\text{Recovery (\%)} = \frac{\text{responses after extraction}}{\text{responses after direct injection}} \times 100$$

5) Lower limit of quantification (LLOQ)

The lower limit of quantification were determined by adding different concentrations of standard mitragynine (0.1, 0.5, 1, 5, 10 μg/mL: 3 and 5 replicates of each concentration) into samples (serum and urine). Analyte peak (response) should be identifiable, discrete and reproducible with a precision of 20% and accuracy of 20%.

2.6.1.8 Sample preparation for determination of mitragynine

1) Serum samples

Mitragynine in serum spiked with mitragynine and real serum samples were extracted by liquid-liquid phase procedure following the technique previously described by Janchawee and coworkers (2007).

A serum sample (100 μL), containing known amounts of mitragynine, was placed in a 12×75 mm glass tube. The sample was treated by adding 50 μL of 0.5 M aqueous Na_2HPO_4, which was pre-adjusted to pH 11 with 25% aqueous NaOH and then mixed thoroughly using vortex for 30 sec. One mL of diethyl ether was added into the mixture and then vortexed for 1 min before centrifugation at 2000×*g* at 25°C for 15 min. The upper organic phase was transferred to a clean glass tube and evaporated to dryness by using CentriVap Concentrator at 40 °C. The residue was reconstituted with 100 μL of methanol for HPLC analysis. The schematic diagram for serum sample preparation is shown in Figure 2.5.

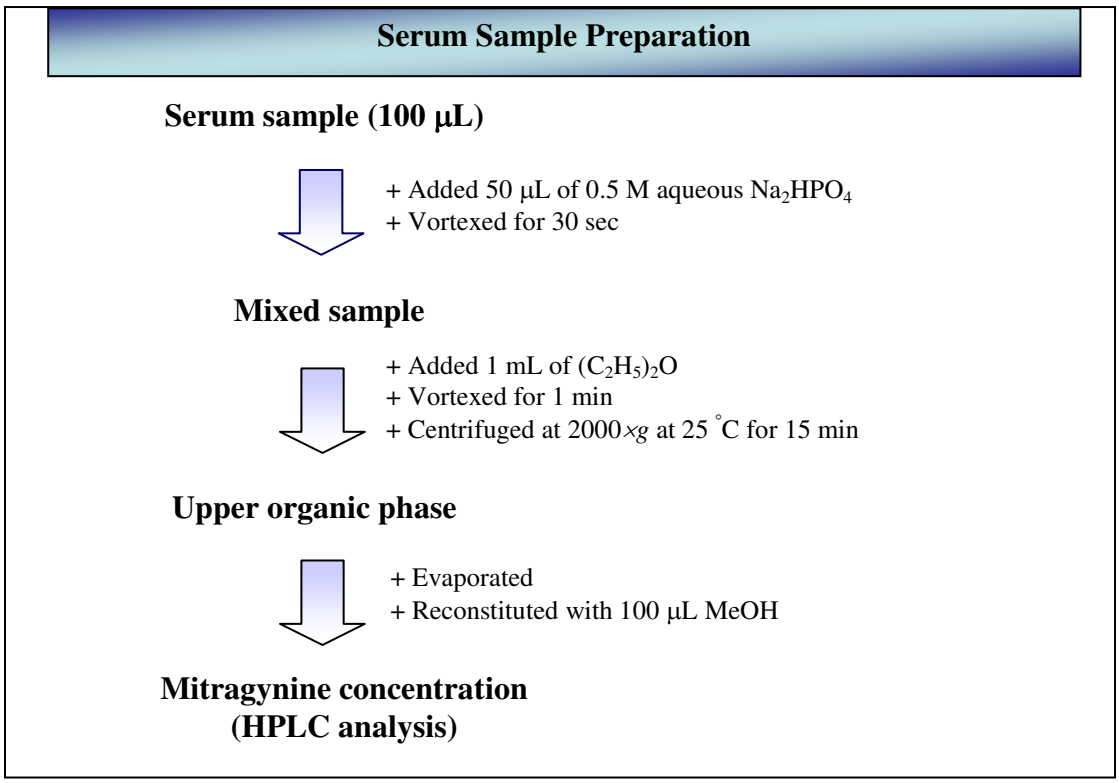


Figure 2.5 serum sample preparation for determination of mitragynine

2) Urine samples

2.1) Wash-elute study

Since method of extraction of mitragynine from urine has not been reported yet. Several methods for sample preparation were tried such as liquid-liquid extraction using organic solvents with different polarities. That resulted in low recovery of extraction and a lot of interferences, comparing with SPE technique. Therefore, the conditions affecting the efficiency of urine preparation using SPE include the solvents for washing and eluting sample. Three replicates of samples were done and each sample was injected three times. The optimum condition was considered based on the highest peak area response.

Wash-elute study is determines the percentage of methanol mixture needed in washing and eluting steps. The wash-elute study was carried out as described in Oasis® HLB method Development Guide (Cheng *et al.*, 1998). Ten SPE cartridges were pre-conditioned with 1 mL of methanol and equilibrated with 1 mL of deionized water. One mL of blank urine containing 10 µg/mL was loaded and the cartridges

were washed with different concentrations, i.e. 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% (v/v) of methanol-water mixtures containing 2% ammonium hydroxide. For the another set of ten SPE cartridges, the analyses was eluted from each column with methanol-water mixture, containing 2% acetic acid, of varying methanol concentration (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol). All fluids obtained from washing and eluting step were collected separately and evaporated to dryness by using CentriVap Concentrator at 55 °C. The residue was reconstituted with 1 mL of methanol for HPLC analysis. The solvent mixture making the analyses retained the most in the cartridge was selected for washing step while the one making the compound eluted the most from the cartridges was selected for eluting step. Figure 2.6 is shown the diagram for wash-elute study in urine.

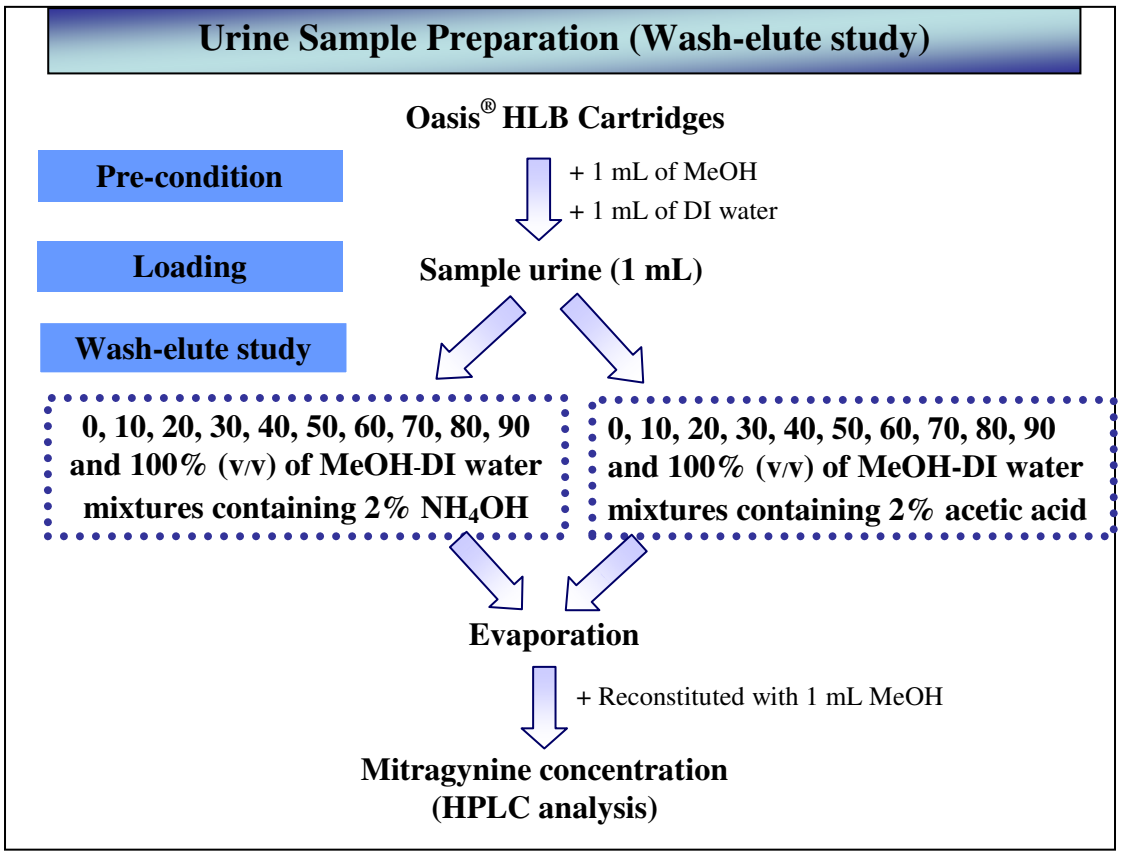


Figure 2.6 urine sample preparation for wash-elute study

2.2) SPE procedure

Urine samples were prepared by SPE procedure using the optimized condition obtained from wash-elute study. The cartridges was previously conditioned with 1 mL of methanol and equilibrated with 1 mL of DI water. The samples were loaded onto individual SPE column and washed twice with 1 mL of 5% methanol containing 2% ammonium hydroxide followed by 1 mL of 70% methanol containing 2% ammonium hydroxide. Then the analyte was eluted with 1 mL of 100% methanol containing 2% acetic acid. The eluate was collected in a clean glass tube and evaporated to dryness by using CentriVap Concentrator at 55 °C. This residue was reconstituted with 1 mL of methanol for HPLC analysis. The schematic diagram of urine sample preparation for determination mitragynine in urine by using SPE is shown in Figure 2.7.

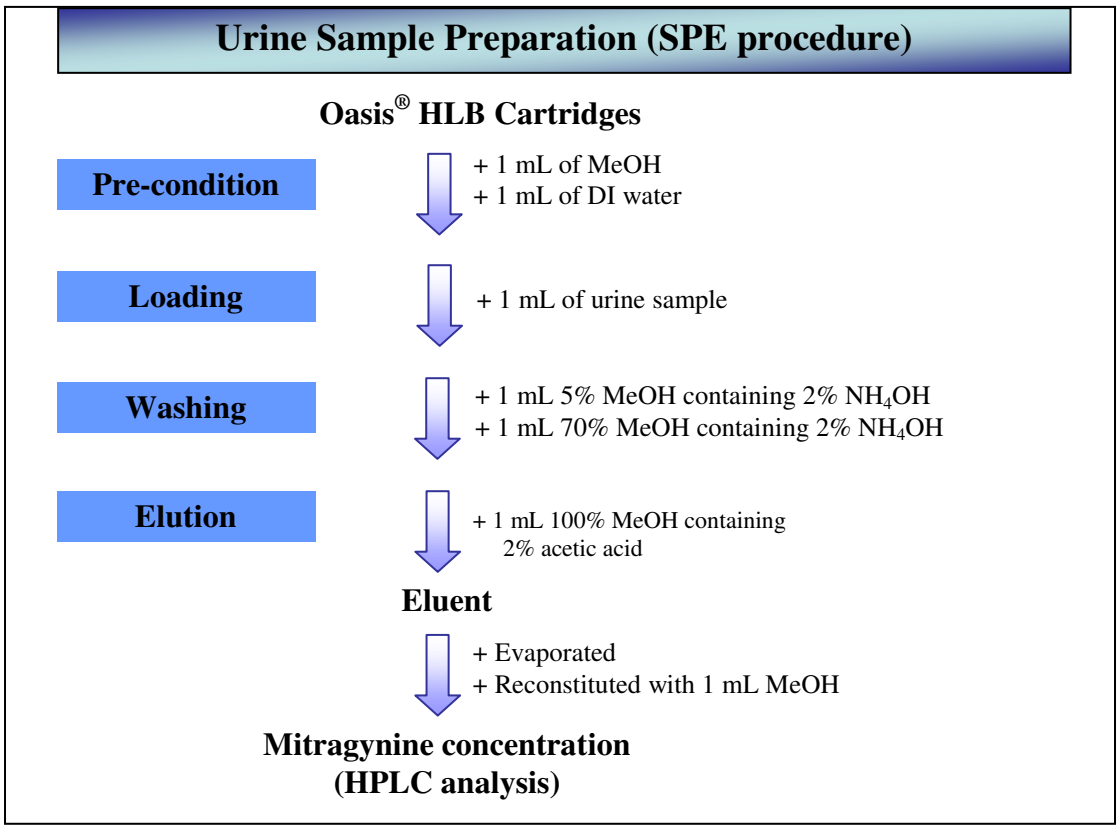


Figure 2.7 Urine sample preparation for determination mitragynine

2.6.1.9 Pharmacokinetic parameters analysis

The concentrations of mitragynine in each individual rat and the mean concentrations were plotted against time. Pharmacokinetic parameters of mitragynine were analysed based on the non-compartmental method (Gibaldi and Perrier, 1982) and calculated using Microsoft® Excel 2003 (Microsoft Corporation, U.S.A.). The maximum serum concentration (C_{max}) and the time to maximum concentration (T_{max}) were directly read from the concentration-time curve of each individual rat. The area under the concentration time curve from the time of dosing to the last quantifiable time point ($AUC_{0\rightarrow t}$) was calculated by using the linear trapezoidal rule. AUC_{last} is the area under the curve from the time of dosing extrapolated to infinity was calculated from the following formulas:

$$AUC_{last} = \frac{C_{last}}{k_{el}}$$

C_{last} represents the last concentration corresponding to T_{last}, which is time of last measurable concentration. Then, the area under the concentration time curve from the time of dosing to infinity ($AUC_{0\rightarrow\infty}$) was calculated from the following formulas:

$$AUC_{0\rightarrow\infty} = AUC_{0\rightarrow t} + AUC_{last}$$

The elimination rate constant (k_el) associated with the terminal (log linear) portion of the curve was estimated via linear regression of time- log concentration calculated as follow.

$$k_{el} = \frac{-(\text{slope of the terminal phase of log concentration-time curve})}{2.303}$$

The terminal half-life ($t_{½\,ke}$) was calculated as follows.

$$t_{½\,el} = \frac{0.693}{k_{el}}$$

The absorption rate constant (k_{ab}) was obtained by using the method of residuals as follows.

1) Plot curves of log concentration versus time

2) Back extrapolate the log linear portion of the decline phase. Let C'_1, C'_2,… denotes the serum concentration along this extrapolated line.

3) Subtract the observed serum concentration C_1, C_2,… from the corresponding

extrapolate value at each time point.

4) Plot the residuals (C'_1-C_1, C'_2-C_2,…) against time on the same logarithmic graph.

The absorption rate constant was obtained from the slope of the straight line fitted

to residuals calculated as follows.

$$k_{ab} = \frac{-(\text{slope of the residuals phase of log concentration-time curve})}{2.303}$$

The absorption half-life ($t_{½\ ab}$) was calculated as follows.

$$t_{½\ ab} = \frac{0.693}{k_{ab}}$$

The total body clearance (Cl/F) was calculated as follows.

$$Cl/F = \frac{\text{Dose}}{AUC_{0\rightarrow\infty}}$$

Renal clearance ($Cl_{r)}$ was calculated as follows.

$$Cl_r = \frac{\Sigma(\text{Concentration of mitragynine} \times \text{Volume of urine})}{AUC_{0\rightarrow\infty}}$$

Hepatic clearance (Cl_h) was calculated as follows.

Clh = Total body clearance(Cl/F) - Renal clearance ($Cl_{r)}$

The volume of distribution (V_d/F) based on the terminal phase, which was calculated as follows.

$$V_d/F = \frac{\text{Dose}}{k_{el} \cdot AUC_{0\rightarrow\infty}}$$

$AUMC_{0\rightarrow t}$ represents the area under the first-moment curve. It is calculated according to the trapezoidal rule. $AUMC_{last}$ is the area under the first moment curve from the time of dosing to the last measurable concentration. The area under the first moment curve extrapolated to infinity was calculated as follows.

$$AUMC_{last} = \frac{T_{last} \cdot C_{last}}{k_{el}} + \frac{C_{last}}{k_{el}^2}$$

Then, the area under first moment curve from the time of dosing to infinity ($AUMC_{0\rightarrow\infty}$) was calculated from the following formulas:

$$AUMC_{0\rightarrow\infty} = AUMC_{0\rightarrow t} + AUMC_{last}$$

The mean residence time extrapolated to infinity ($MRT_{0\rightarrow\infty}$) was calculated as follows.

$$MRT_{0\rightarrow\infty} = \frac{AUMC_{0\rightarrow\infty}}{AUC_{0\rightarrow\infty}}$$

All pharmacokinetic parameter were expressed as mean± S.E.

Therefore, the pharmacokinetic parameters analyses are summarized as follows;

T_{max} = Time to maximum concentration (h)

C_{max} = Maximum concentration (ng/mL)

k_{ab} = Absorption rate constant (h^{-1})

k_{el} = Elimination rate constant (h^{-1})

$t_{1/2\ ab}$ = Absorption half-life (h)

$t_{1/2\ el}$ = Elimination half-life (h)

Cl/F = Total body clearance (L/h)

Cl_r = Renal clearance (L/h)

Cl_h = Hepatic clearance (L/h)

V_d/F = Volume of distribution (L/kg)

$AUC_{0\rightarrow t}$ = Area under the curve 0⟶t (ng·h/mL)

$AUMC_{0\rightarrow\infty}$ = Area under the first moment curve t⟶∞ (ng·h^2 /mL)

$MRT_{0\rightarrow\infty}$ = Mean residence time (h)

2.6.2 Experiment 2: Effect of caffeine and codeine on antinociceptive activity of alkaloid extract from kratom leaves

2.6.2.1 Animals treatment

Thirty-six rats were randomly divided into 6 groups (n=6) for studied antinociceptive activity. There animals were received treatments as follows and the schematic diagram of their treatment is shown in Figure 2.8.

Group DW: The animals obtained 5 mL/kg distilled water (DW).

Group PG: The animals obtained 2 mL/kg propylene glycol (PG).

Group AE: The animals obtained 100 mg/kg alkaloid extract from kratom leaves (AE).

Group AE+CF: The animals simultaneously obtained 100 mg/kg alkaloid extract from kratom leaves (AE) and 25 mg/kg of caffeine (CF).

Group AE+CD: The animals simultaneously obtained 100 mg/kg alkaloid extract from kratom leaves (AE) and 3 mg/kg of codeine (CD).

Group AE+CF+CD: The animals simultaneously obtained 100 mg/kg alkaloid extract from kratom leaves (AE), 25 mg/kg of caffeine (CF) and 3 mg/kg of codeine (CD).

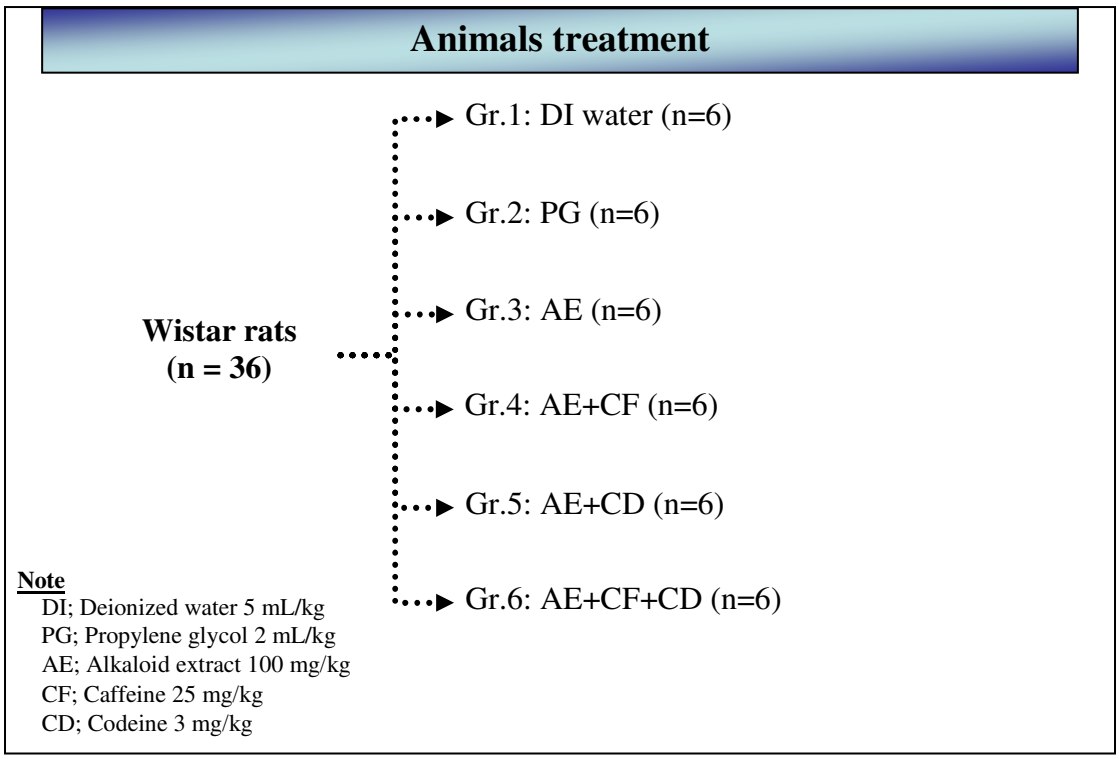


Figure 2.8 The schematic diagram of animals treatment for experiment 2

2.6.2.2 Antinociceptive activity test

Hot plate Analgesia Meter was used to study antinociceptive activity. Hot plate test was carried out according to the method described by Woolfe and MacDonald (1994). Rats were placed on hot plate maintained at 55±1°C. Latency of nociceptive response such as licking, flicking of hind limb or jumping was measured. The response was measured at thirty minutes after oral administration of alkaloid extract from kratom, co-administration of caffeine or codeine with alkaloid extract alkaloid extract from kratom and the co-administer of 3 compounds. The nociceptive response was measured every 30 min over a 120 min period. The cut-off time was 45 sec. Only the rats that showed nociceptive responses within 15 sec were used for the experiments. The schematic diagram for antinociceptive activity test is shown in Figure 2.9.

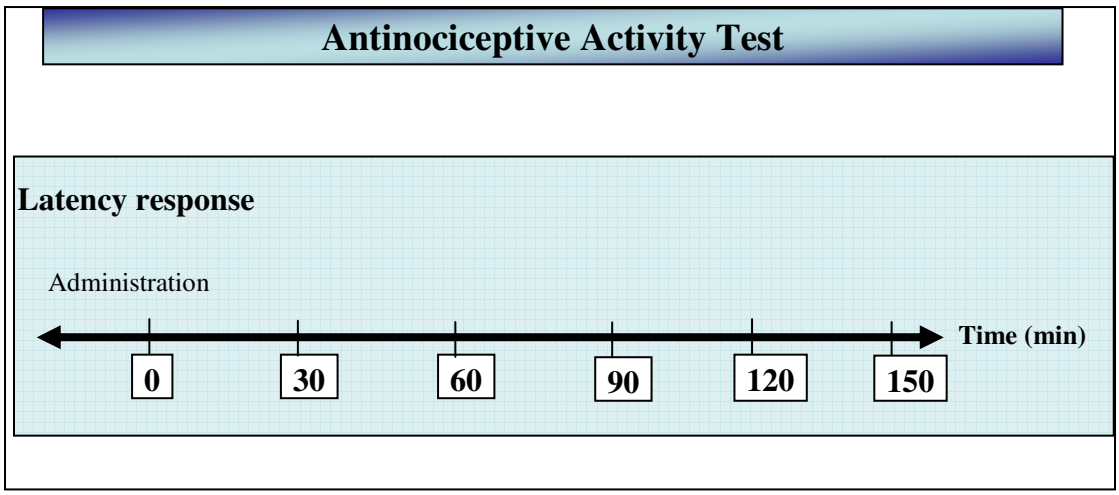


Figure 2.9 Antinociceptive activity test

2.7 Statistical analysis

The results of pharmacokinetic parameter were calculated using Microsoft® Excel 2003 (Microsoft Corporation, U.S.A.) and shown as the mean value ± S.E.. The results of antinociceptive activity were presented as the mean value ± S.E.. All data were compared by the application of analysis of variance (ANOVA) followed by LSD test. A difference was considered significant at $p<0.05$. The software used was the SPSS version 11.50 statistical software program.

CHAPTER 3

Results and discussions

3.1 Experiment 1: Effect of caffeine and codeine on pharmacokinetics of alkaloid extract from kratom leaves

3.1.1 Chromatographic conditions

The HPLC conditions for separation and quantitation of mitragynine in serum and urine were described in Chapter 2 (Methodology). The condition used for serum analysis followed the previous work (Janchawee *et al.*, 2007). The authors described that separation of mitragynine was dependant on the condition of the reversed-phase liquid chromatography method. Mitragynine is freely soluble in alcohol like methanol but insoluble in water, the composition of methanol and water in the mobile phase determines its selectivity and sensitivity of separation. At a high percentage of methanol in the mobile phase (>80%, v/v), mitragynine eluted quickly with a high detection response, but the peak overlapped with interfering peaks. In contrast, at a low percentage of methanol (<80%, v/v), i.e. the higher the composition of water in the system, the less soluble was mitragynine. Due to its hydrophobicity, mitragynine was retained in the column longer and although well separated from the polar interferences it eluted with a poor detection response and resulted in a broad peak. The final choice of solvent was an 80:20 v/v methanol : water mixture. The column temperature was set constantly at 35 $^{\circ}$C because separation of mitragynine was also influenced by the temperature and this resulted in better resolution. The relatively long column was preferred because it produced better resolution.

3.1.2 Sample preparation for determination of mitragynine

1) Serum sample

Janchawee and coworkers (2007) reported the method for serum preparation consisting of sample alkalinization followed by liquid-liquid extraction using organic solvents with different polarities. Extraction with diethyl ether gave the best recovery

of mitragynine from serum and resulted in fewer interfering peaks during HPLC separation compared with other organic solvents. Therefore, the extraction of serum samples with diethyl ether was chosen because it was a simple and most effective method. In this study, the method used to prepare serum sample, resulting in good separation and symmetric.

2) Urine sample

Several methods for sample preparation were trialed. Those included sample alkalinization with sodium tetraborate and sodium hydroxide at various of pH, and followed by liquid-liquid extraction using organic solvents with different polarities such as ethyl acetate, diethyl ether and chloroform. Extraction resulted in low recovery and interfering peaks. In addition, sample preparation required high volume of urine.

Then solid phase extraction (Oasis® HLB cartridges) was considered. There are different approaches to selectively isolate the analytes of interest from a complex sample solution. One is the pH and the other is the concentration of organic modifier. Mitragynine is free base but its pKa is not reported. Oasis® HLB is a macroporous copolymer consisting of two monomers, the lipophilic m-divinylbenzene and the hydrophilic N-vinylpyrrolidone. These two monomers construct a hydrophilic-lipophilic balance. They are capable of extracting acidic, basic and neutral compounds whether polar or nonpolar (Cheng *et al.,* 1998; Blahova and Brandteterova, 2004). The concentration of organic modifier or the conditions affecting the efficiency of urine preparation using SPE were studied.

- Wash-elute study

The objective of wash-elute study is to determine the percentage of methanol mixture required in the washing and eluting steps of SPE procedure. When the cartridge was passed with a methanol-water mixture with 2% ammonium hydroxide, no analyte was eluted until the methanol concentration reaches 80% (Figure 3.1). The highest peak response was produced at 100% methanol. The results

suggested that a methanol-water mixture, 0-80% v/v, could be used as washing solvent.

When the cartridge was passed with a methanol-water mixture mixed with 2% acetic acid, no analyte was eluted until the methanol concentration was beyond 40% (Figure 3.2). According to this finding, 100% methanol containing 2% acetic acid was chosen as the eluting solvent.

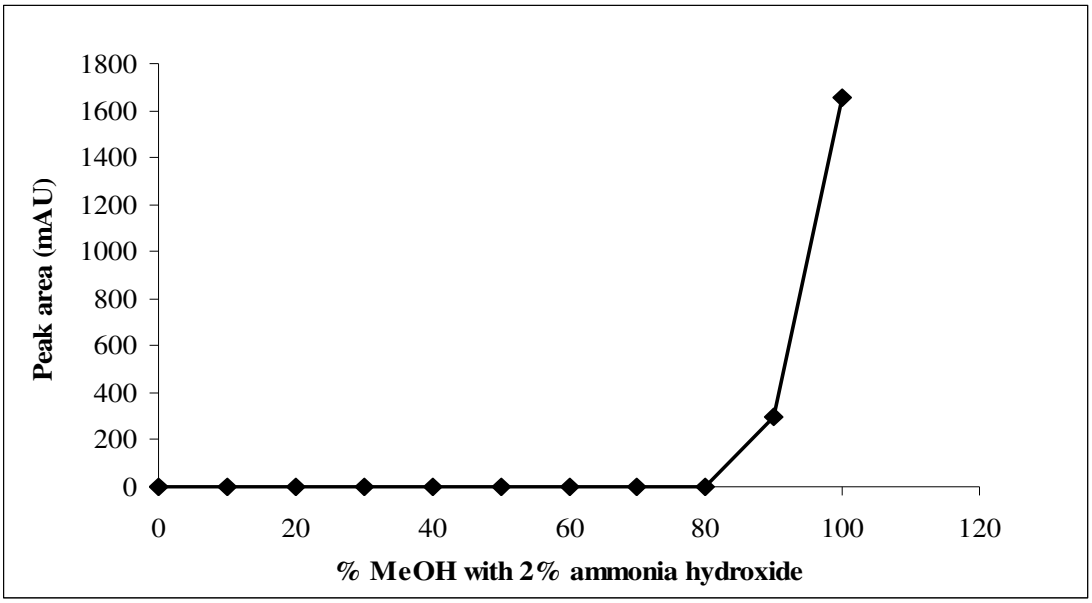


Figure 3.1 Peak area responses of mitragynine after loading spiked urine sample into the cartridge and eluting with varying percentage of a methanol-water mixture containing 2% ammonium hydroxide

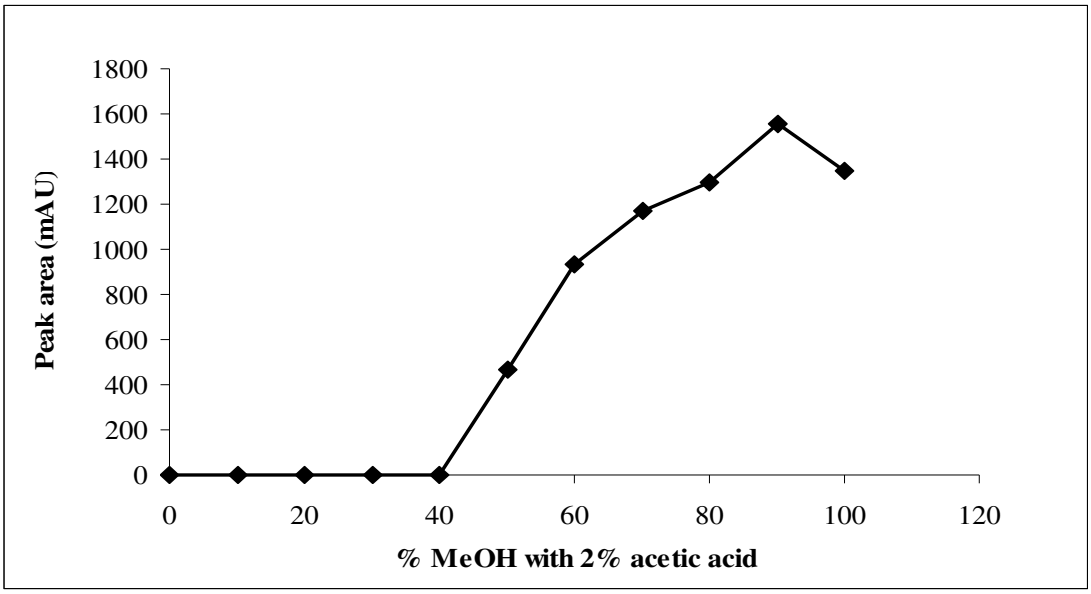


Figure 3.2 Peak area responses of mitragynine after loading spiked urine into the cartridge and eluting with varying percentage of a methanol-water mixture containing 2% acetic acid

- Comparison of SPE procedure with one- and two washing step(s)

It is interesting to compare the results of separating mitragynine in urine using different SPE methods: one washing step (either 5% methanol containing 2% ammonium hydroxide, or 70% methanol containing 2% ammonium hydroxide) and two washing steps (5% methanol containing 2% ammonium hydroxide followed by 70% methanol containing 2% ammonium hydroxide). After washing with only 1 mL of 5% methanol-water mixture containing 2% ammonium hydroxide prior to elution with 1 mL of 100% methanol-water mixture containing 2% acetic acid, the chromatogram showed some interfering peaks with high intensity (Figure 3.3A). When the cartridge was washed once with 1 mL of 70% methanol-water mixture containing 2% ammonium hydroxide prior to elution with 1 mL of 100% methanol-water mixture containing 2% acetic acid, chromatogram showed lesser interferences and better peak shape (Figure 3.3B). With two washing steps, 1 mL of 5% methanol-water mixture containing 2% ammonium hydroxide followed by 1 mL of 70% methanol-water mixture containing 2% ammonium hydroxide prior to usual elution more effective elimination of urine interferences was observed (Figure 3.3C). Hence,

two-wash procedure was chosen because it could effectively remove neutral and polar acidic interferences in urine leading to higher recovery and a cleaner background.

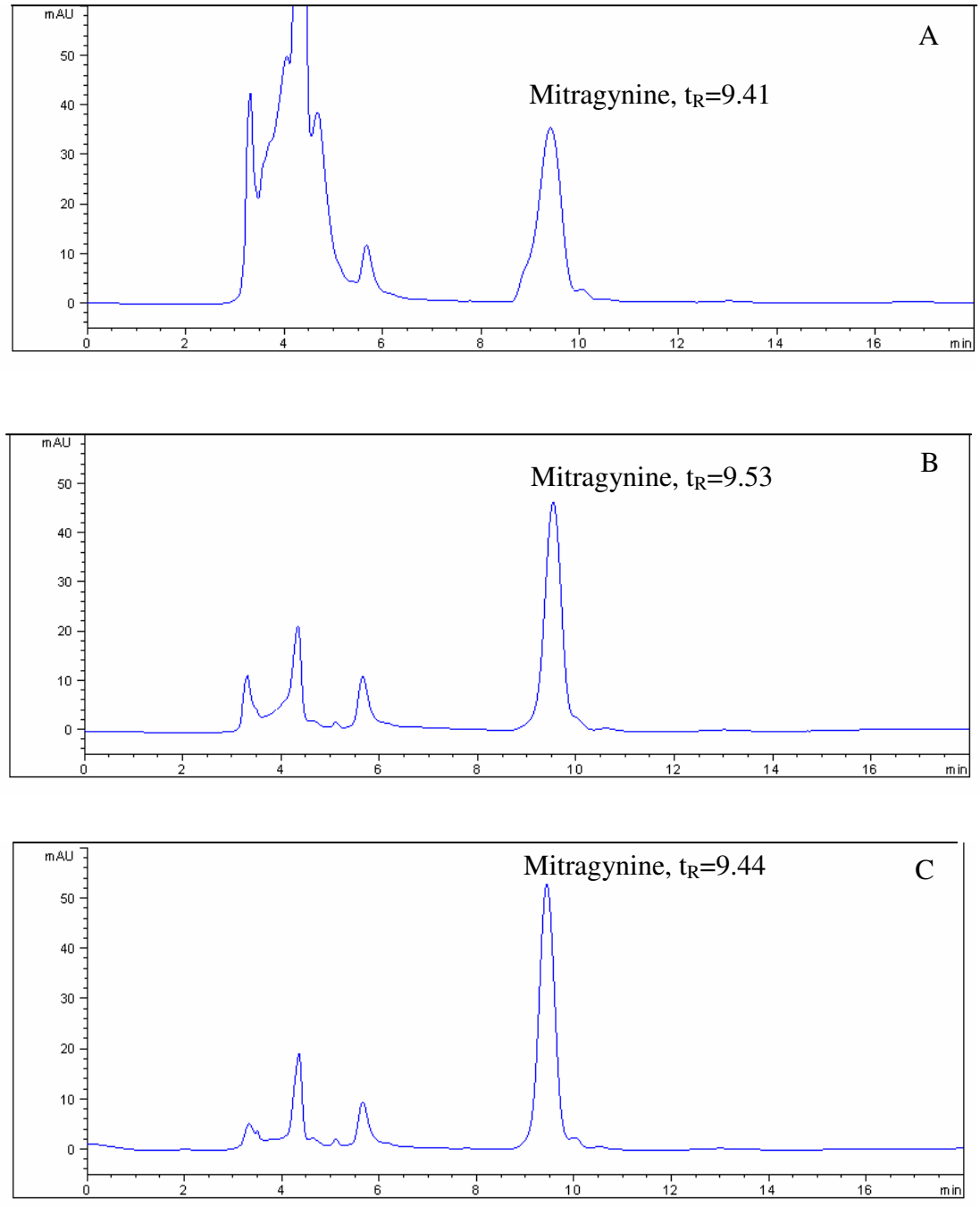


Figure 3.3 Representative chromatograms of mitragynine (10 µg/mL) spiked in rat urine undergoing SPE method; (A) one wash using 5% methanol-water mixture containing 2% ammonium hydroxide; (B) one wash using 70% methanol-water mixture containing 2% ammonium hydroxide; (C) two washes using 5% methanol-water mixture containing 2% ammonium hydroxide followed by 70% methanol-water mixture containing 2% ammonium hydroxide.

3.1.3 Method validation for analysis of mitragynine in serum and urine

1) Linearity and range

Linearity regression analysis of the calibration curve of samples (serum and urine) containing mitragynine at different concentrations ranging of 0.1-10 μg/mL (3 replicates of each concentration for serum samples and 5 replicates of each concentration for urine samples) showed good correlation (*r*= 0.9995 and 0.9991), as shown in Figures 3.4 and 3.5 respectively. The linear equation for mitragynine in serum was y = (93.17±1.58) x-(2.67±3.20). That for mitragynine in urine was y = (108.04±8.30) x-(5.96±13.95), where x is the mitragynine concentration and y is the peak area.

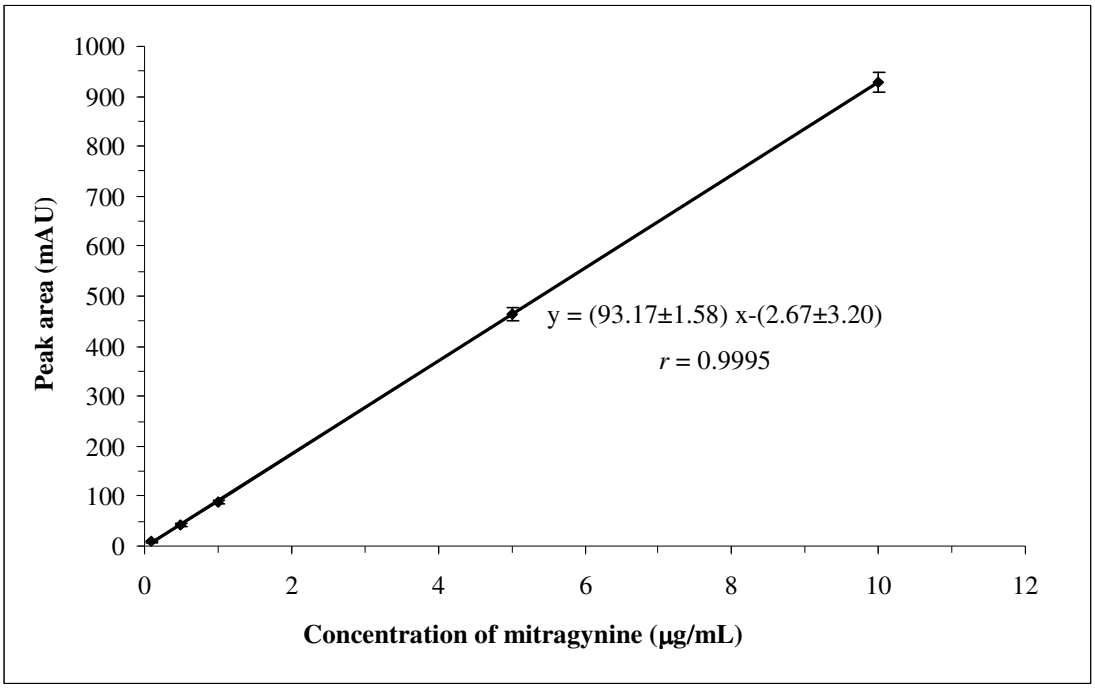


Figure 3.4 Linearity curve (mean±S.D.) of different concentrations of mitragynine spiked in serum (n=3); correlation coefficient *(r)* = 0.9995

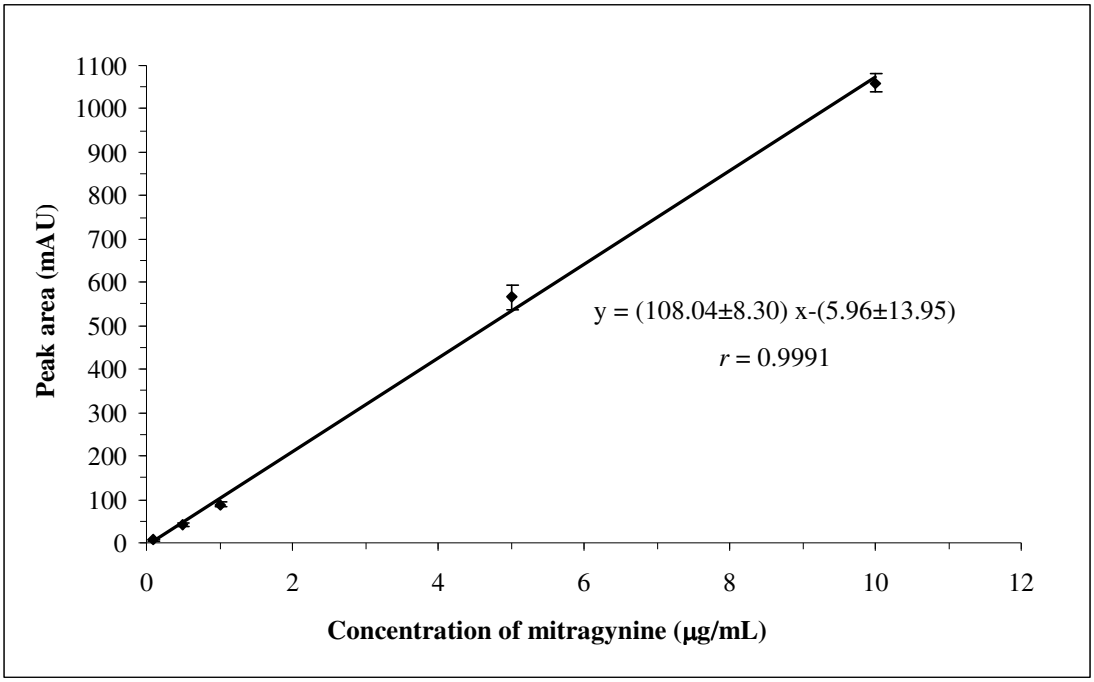


Figure 3.5 Linearity curve (mean±S.D.) of different concentrations of mitragynine spiked in urine (n=5); correlation coefficient *(r)* = 0.9991

2) Precision

Method reproducibility was determined by measuring repeatability of intra-day and inter-day injection by using 3 different concentrations of quality control samples of mitragynine (3 replicates of each concentration). Precisions of serum analysis expressed as coefficient of variation were within 2.11-9.04% and 2.82-9.94%, respectively (Table 3.1). For urine, intra-day and inter- day precisions determined from three different mitragynine concentrations (5 replicates of each concentration) were within ranges of 1.21-4.87% and 1.09-5.87%, respectively (Table 3.2). The precision are within the level of acceptance.

Table 3.1 Intra-day and Inter-day precisions of the method for determining mitragynine in serum

Concentration of mitragynine (μg/mL)	Intra-day		Inter-day	
	Mean peak area ± S.D. (n=3)	C.V. (%)	Mean peak area ± S.D. (n=3)	C.V. (%)
0.1	8.28±0.75	9.04	8.14±0.81	9.94
0.5	42.82±2.99	6.98	39.10±2.74	6.94
5	464.29±12.68	2.73	465.13±15.04	3.24
10	928.63±19.56	2.11	912.47±25.76	2.82

Table 3.2 Intra-day and Inter-day precisions of the method for determining mitragynine in urine

Concentration of mitragynine (μg/mL)	Intra-day		Inter-day	
	Mean peak area ± S.D. (n=5)	C.V. (%)	Mean peak area ± S.D. (n=5)	C.V. (%)
0.1	6.99±0.34	4.87	6.82±0.40	5.87
0.5	43.79±1.31	2.99	43.14±1.38	3.17
5	570.78±7.28	1.28	568.09±6.21	1.09
10	1129.26±13.67	1.21	1121.57±13.93	1.24

3) Accuracy

The accuracy of an analytical method is defined as the degree to which the determined value of analyte in a sample corresponds to the true values (APVMA, 2004). In this study, the method for determination of mitragynine at concentrations of 0.5, 5 and 10 μg/mL in serum (3 replicates of each concentration and urine (5

replicates of each concentration) was accurate. Percentage of accuracy for serum and urine ranged from (-3.50)-(11.31)%DEV (Table 3.3) and (-11.14)-19.48%DEV, respectively (Table 3.4). The accuracy was within ±15%, except ±20% for the concentration at LLOQ.

Table 3.3 Accuracy of the method for determining mitragynine in serum

Concentration of mitragynine (μg/ml)	Measured concentration Mean ±S.D. (n=3)	DEV (%)
0.1	0.11±0.04	11.31
0.5	0.48±0.05	-3.50
5	5.01±0.16	0.22
10	10.00±0.08	-0.05

Table 3.4 Accuracy of the method for determining mitragynine in urine

Concentration of mitragynine (μg/ml)	Measured concentration Mean ±S.D. (n=5)	DEV (%)
0.1	0.12±0.02	19.48
0.5	0.44±0.03	-11.14
5	5.08±0.08	1.56
10	9.94±0.05	-0.56

4) Recovery

The recovery of an analyte in an assay is the comparison of the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limit of variability (FDA, 2001).

Extraction of mitragynine at different concentrations of 0.1, 0.5, 5, 10 μg/ mL in serum (3 replicates of each concentration) and urine samples (5 replicates of each concentration) was complete. The percentage of recovery ranged from 92.16% to 99.43% and from 92.74% to 100.26% for serum (Table 3.5) and urine samples (Table 3.6), respectively.

Table 3.5 Recovery of extraction of mitragynine from serum

Concentration of mitragynine (μg/ml)	Mean peak area ± S.D. (n=3)		Recovery (%)
	Direct injection	After extraction	
0.1	9.02±0.52	8.28±0.86	92.16
0.5	44.70±3.90	42.82±2.99	96.65
5	467.50±11.90	464.29±12.68	99.39
10	934.03±6.80	928.63±19.56	99.43

Table 3.6 Recovery of extraction of mitragynine from urine

Concentration of mitragynine (μg/ml)	Mean peak area ± S.D. (n=5)		Recovery (%)
	Direct injection	After extraction	
0.1	7.55±0.44	6.99±0.34	92.74
0.5	45.91±2.35	43.79±1.31	95.50
5	569.36±6.50	570.76±7.28	100.26
10	1130.98±14.68	1129.26±13.67	100.00

These data indicated that practically no matrix effect was observed for mitragynine.

5) Lower limit of quantification

The lower limit of quantification was detemined by using three calibration curves of standard mitragynine spiked in serum samples and five calibration curves of standard mitragynine spiked in urine samples at concentrations of 0.1, 0.5, 1, 5, 10 μg/mL. The result shown that LLOQ of all analytes were 0.1 μg/mL.

3.1.4 Chromatographic analysis

Chromatograms of separation of mitragynine spiked into rat serum samples are shown in Figure 3.6. Peaks of mitragynine were well separated from other serum components. The retention time of mitragynine was 9.58 min. The elution time for each sample was approximately 30 min.

For urine analysis, we were able to obtain good peak shapes with a simple mobile phase (methanol:water , 80: 20 v/v). Peak of mitragynine was well separated from other urine components with a retention time of 9.38 min. The elution time for each sample was approximately 16 min. Chromatograms of separation of mitragynine spiked into rat urine are presented in Figure 3.7.

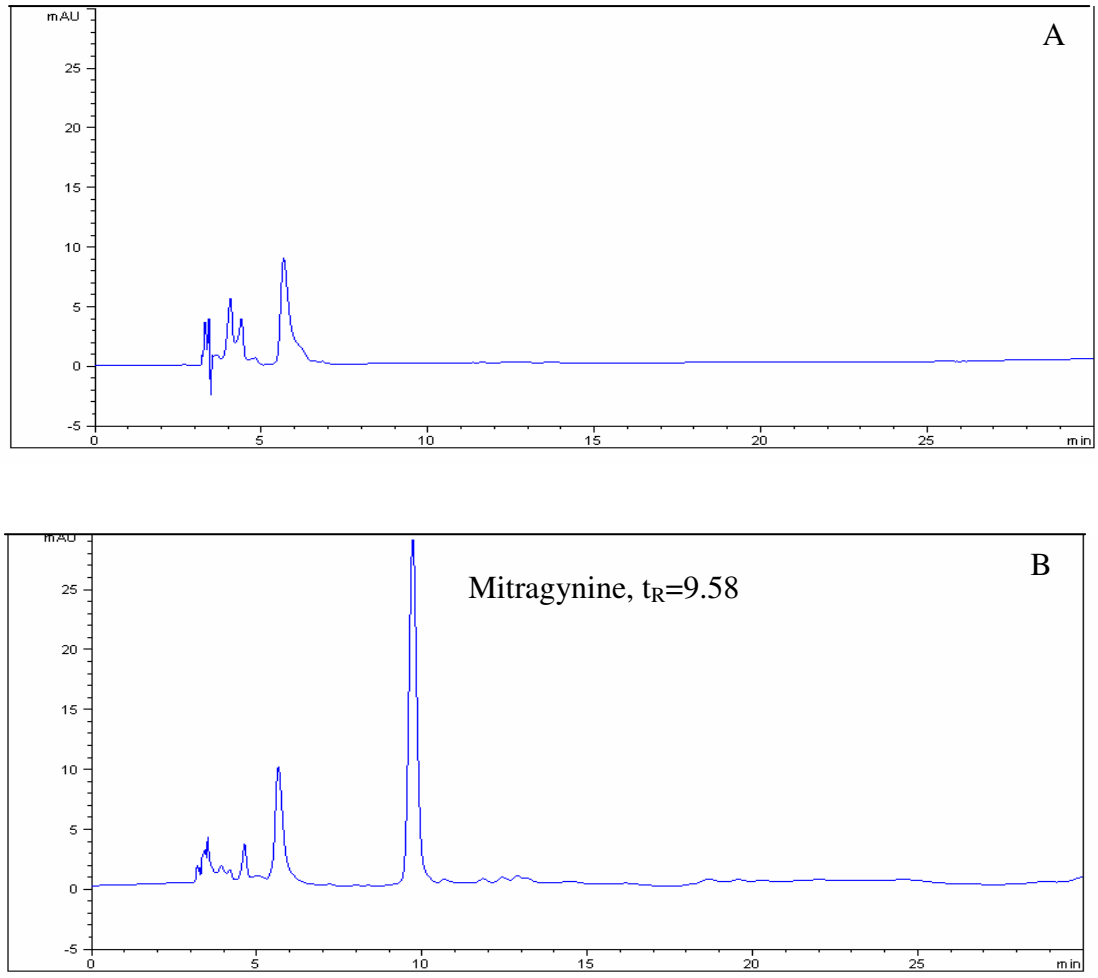


Figure 3.6 Representative chromatograms for mitragynine in rat serum; (A) blank serum; (B) blank serum spiked with standard mitragynine (5 µg/mL)

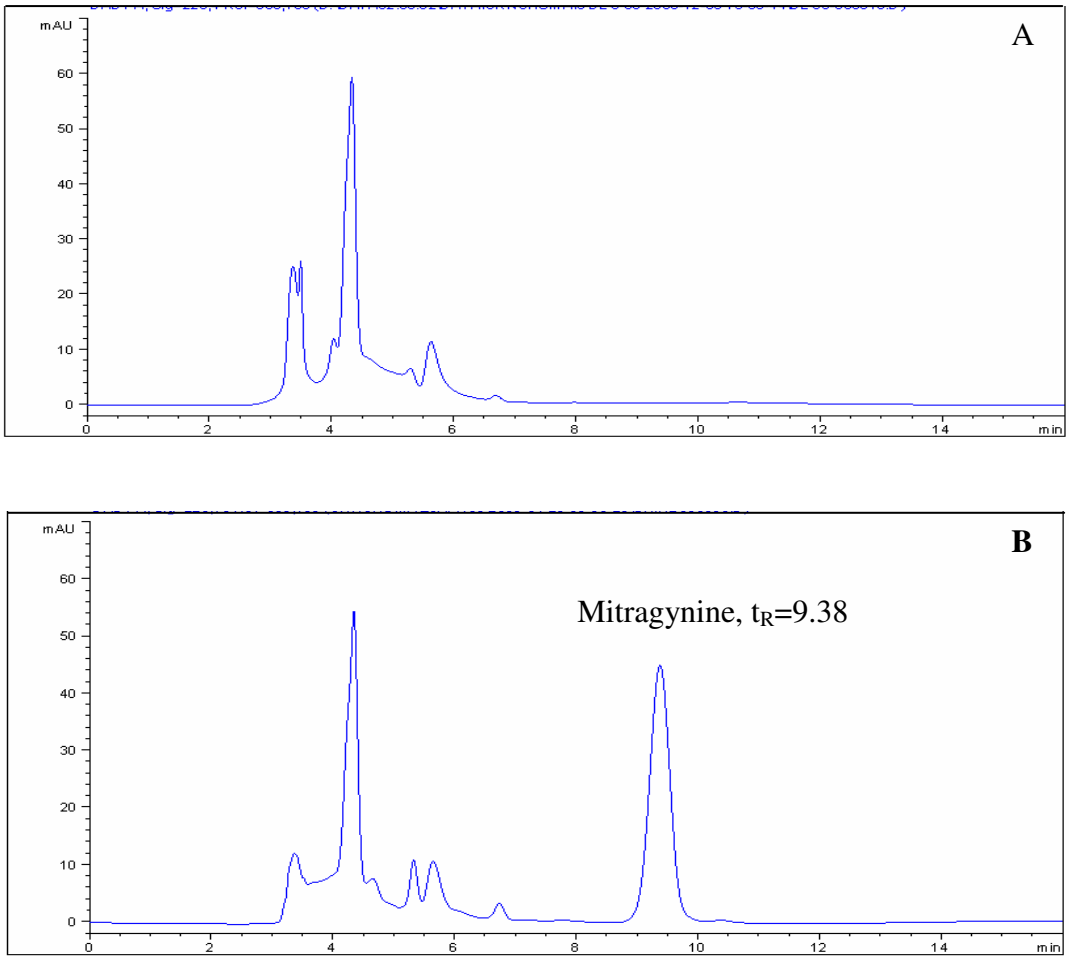


Figure 3.7 Representative chromatograms for mitragynine in rat urine; (A) blank urine; (B) blank urine spiked with standard mitragynine (10 μg/mL)

3.1.5 Pharmacokinetics of alkaloid extract from kratom leaves in rats

3.1.5.1 Selection of dose of compound administration

1) Dose of alkaloid extract

An optimum dose of alkaloid extract for the pharmacokinetic study should be lower than LD_{50}. The LD_{50} of alkaloid extract after an oral administration in mice was 173.20 mg/kg (Reanmongkol *et al.*, 2007). Prior to pharmacokinetic investigation, a preliminary study to find an optimum dose of alkaloid extract was examined. Rats (n=3) were given a single dose of either 100 mg/kg, or 150 mg/kg and clinical effects were observed. At the dose of 150 mg/kg of alkaloid extract, some unwanted effects

such as drowsy and tremor were excluded. These symptoms were not seen in rats receiving the dose of 100 mg/ kg. Therefore, that dose was chosen.

2) Dose of caffeine

Since caffeine itself produces antinociception, so effect of various dosages of caffeine (10, 25, 40, 50, 75 and 100 mg/kg) on the nociceptive response using hot plate analgesia meter were preliminary studied in rats (n=3). Latency of nociceptive response during 120 min after caffeine administration is presented in Table 3.7.

Table 3.7 The effect of caffeine on latency of nociceptive response stimulated by heat in rats

Compound	Dose (mL, mg/kg)	Latency of nociceptive response (sec)			
		30 min	**60 min**	**90 min**	**120 min**
Distilled water	5	7.60±0.10	7.60±0.37	7.45±0.40	7.47±0.35
Caffeine	10	7.10±0.46	7.27±0.18	8.50±0.38	7.80±0.20
	25	7.37±0.33	7.30±0.32	8.20±0.42	8.73±0.42
	40	7.67±0.68	8.73±0.34[a,b,c]	8.07±0.47	8.77±0.30
	50	10.00±0.32[a,b,c,d]	8.07±0.32	8.57±0.35[a]	9.63±1.03[a]
	75	11.00±0.23[a,b,c,d]	9.20±0.75[a,b,c]	8.37±0.46	8.67±0.68
	100	10.47±0.16[a,b,c,d]	9.93±0.99[a,b,c,e]	10.37±0.22[a,b,c,d,e,f]	8.90±1.04

Values are presented as mean ± S.E. (n =3)

a $p < 0.05$, significantly different compared to the distilled water (one way ANOVA)

b $p < 0.05$, significantly different compared to the dose of 10 mg/kg (one way ANOVA)

c $p < 0.05$, significantly different compared to the dose of 25 mg/kg (one way ANOVA)

d $p < 0.05$, significantly different compared to the dose of 40 mg/kg (one way ANOVA)

e $p < 0.05$, significantly different compared to the dose of 50 mg/kg (one way ANOVA)

f $p < 0.05$, significantly different compared to the dose of 75 mg/kg (one way ANOVA)

Throughout the 120 min duration, caffeine at the doses of 50, 75 and 100 mg/kg exhibited significant antinociception at 30, 60 and 90 min when compared with the distilled water group ($p< 0.05$). Lower doses of caffeine (10 and 25 mg/kg) did not significantly alter the latent period when compared with the distilled water group ($p< 0.05$). At 60 min, the latency of nociceptive response of dose of 40 mg/kg was significantly increased antinociceptive response when compared with rats receiving distilled water, 10 and 25 mg/kg of caffeine. Therefore, the dose of 25 mg/kg of caffeine was chosen for the pharmacokinetic study.

3) Dose of codeine

Due to its antinociceptive action, effect of different doses of 1, 3, 5 and 10 mg/kg of codeine were also preliminary studied on the nociceptive response using hot plate test in rats (n=3). Results are presented in Table 3.8. It has been shown that administration of 5 and 10 mg/kg of caffeine exhibited significant antinociception at 60 min when compared with the distilled water group ($p<0.05$). The doses of 1 and 3 mg/kg of codeine did not produce such activity during all time of measurement. Hence, codeine at the dose of 3 mg/kg was selected.

Table 3.8 The effect of codeine on latency of nociceptive response stimulated by heat in rats

Compound	**Dose (mL, mg/kg)**	**Latency of nociceptive response (sec)**			
		30 min	**60 min**	**90 min**	**120 min**
Distilled water	5	7.80±0.15	8.10±0.79	7.40±0.12	7.60±0.60
Codeine	1	8.37±1.08	7.77±0.47	7.47±0.74	7.50±0.06
	3	9.13±0.62	7.40±0.25	7.87±0.52	7.37±0.07
	5	8.10±0.46	10.13±0.29[a,b,c]	7.30±0.23	8.47±0.38
	10	9.37±0.07	10.53±0.07[a,b,c]	7.73±0.03	7.40±0.50

Values are presented as mean ± S.E. (n =3)

a $p < 0.05$, significantly different compared to the distilled water (one way ANOVA)

b $p < 0.05$, significantly different compared to the dose of 1 mg/kg (one way ANOVA)

c $p < 0.05$, significantly different compared to the dose of 3 mg/kg (one way ANOVA)

3.1.5.2 Chromatographic analysis

Chromatograms of separation of mitragynine spiked in rat samples (serum and urine) and found in real samples are shown in Figure 3.8.

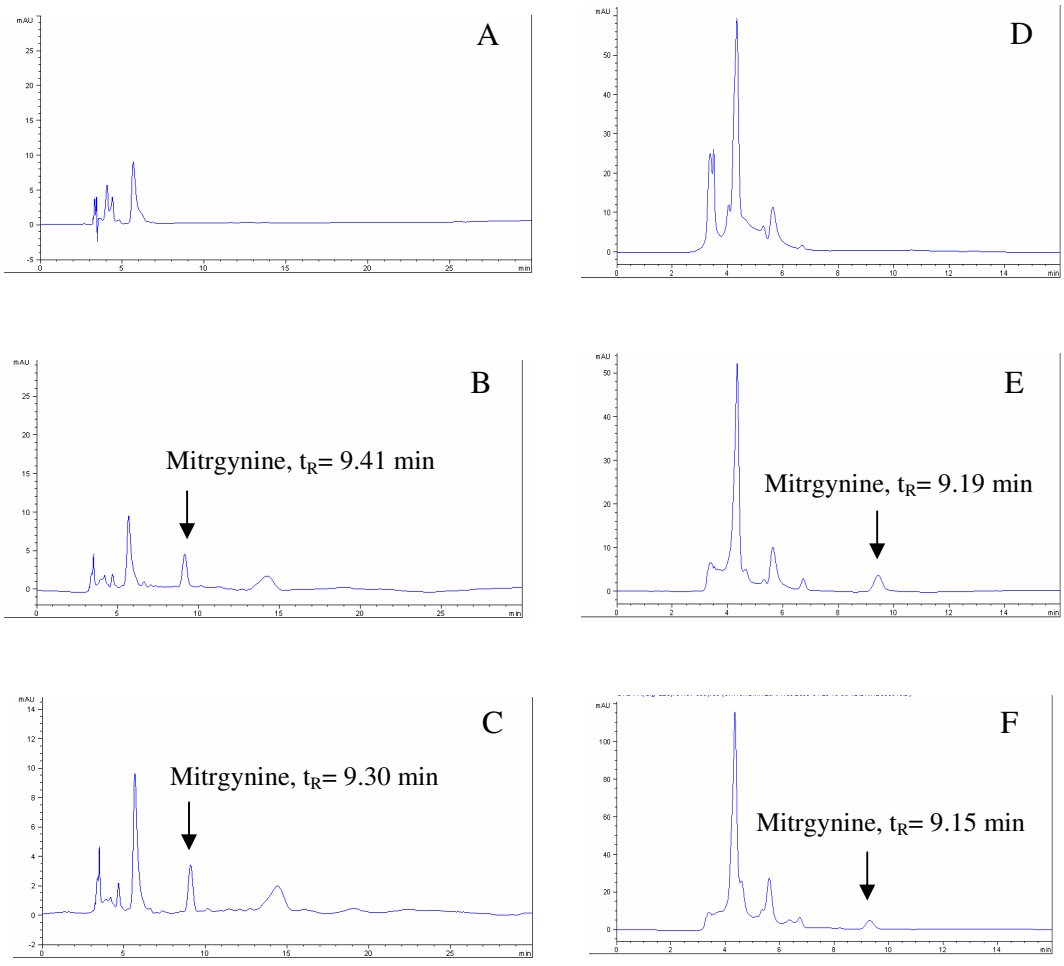


Figure 3.8 Representative chromatograms of mitragynine; (A) rat serum blank, (B) rat serum spiked with mitragynine (1 µg/mL), (C) serum sample collected from one rat at 6 hour after administration of alkaloid extract (100 mg/kg); measured concentration of 0.59 µg/mL, (D) rat urine blank, (E) rat urine spiked with mitragynine (1 µg/mL), (F) urine sample collected from one rat during 8-16 hours after administration of alkaloid extract (100 mg/kg); measured concentration of 0.91 µg/mL

3.1.5.3 Serum concentration-time profile

Serum concentration-time profiles of mitragynine in rats receiving a single oral dose of 100 mg/kg of alkaloid extract (AE), 100 mg/kg of alkaloid extract and 25 mg/kg of caffeine (AE+CF), 100 mg/kg of alkaloid extract and 3 mg/kg of codeine (AE+CD), and 100 mg/kg of alkaloid extract, 25 mg/kg of caffeine and 3 mg/kg of codeine (AE+CF+CD) were shown in Figure 3.9 and in Figure 3.10 which was expressed the concentration of mitragynine as logarithm scale. Mitragynine concentrations were detectable in serum sample from 0 up to 24 hour post-dose. After 24 hour, the level of mitragynine in serum was below the LLOQ. Levels of mitragynine in serum of some rats in AE+CF groups, i.e. n_1, n_4 and n_5, could not be detected at 24 hours.

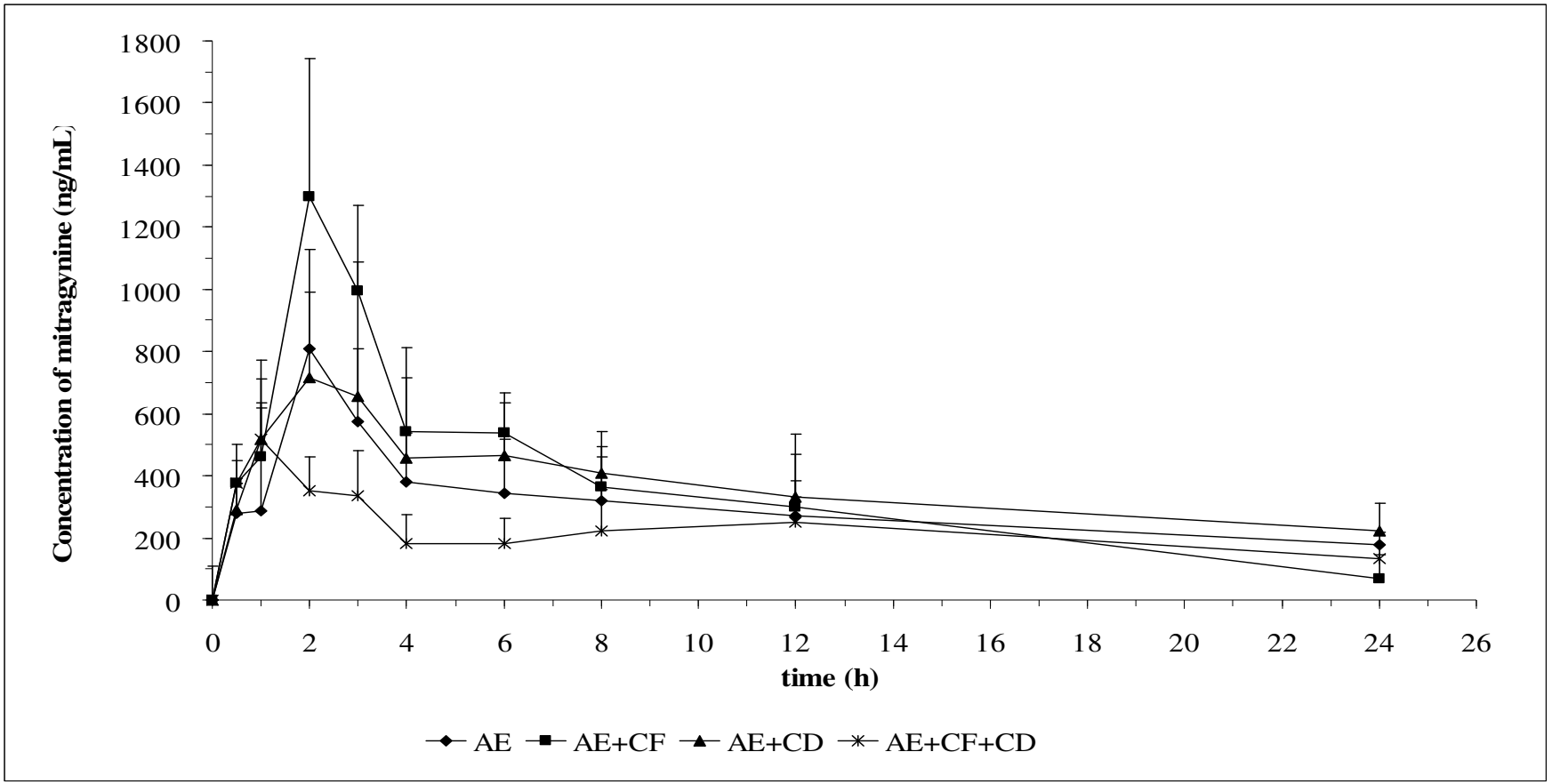


Figure 3.9 Serum mitragynine concentration-time profiles of mitragynine (mean±S.E.) in rats receiving a single oral dose of following compounds; 100 mg/kg of alkaloid extract (AE), 100 mg/kg of alkaloid extract and caffeine 25 mg/kg (AE+CF), 100 mg/kg of alkaloid extract and codeine 3 mg/kg (AE+CD), 100 mg/kg of alkaloid extract, caffeine 25 mg/kg and codeine 3 mg/kg (AE+CF+CD)

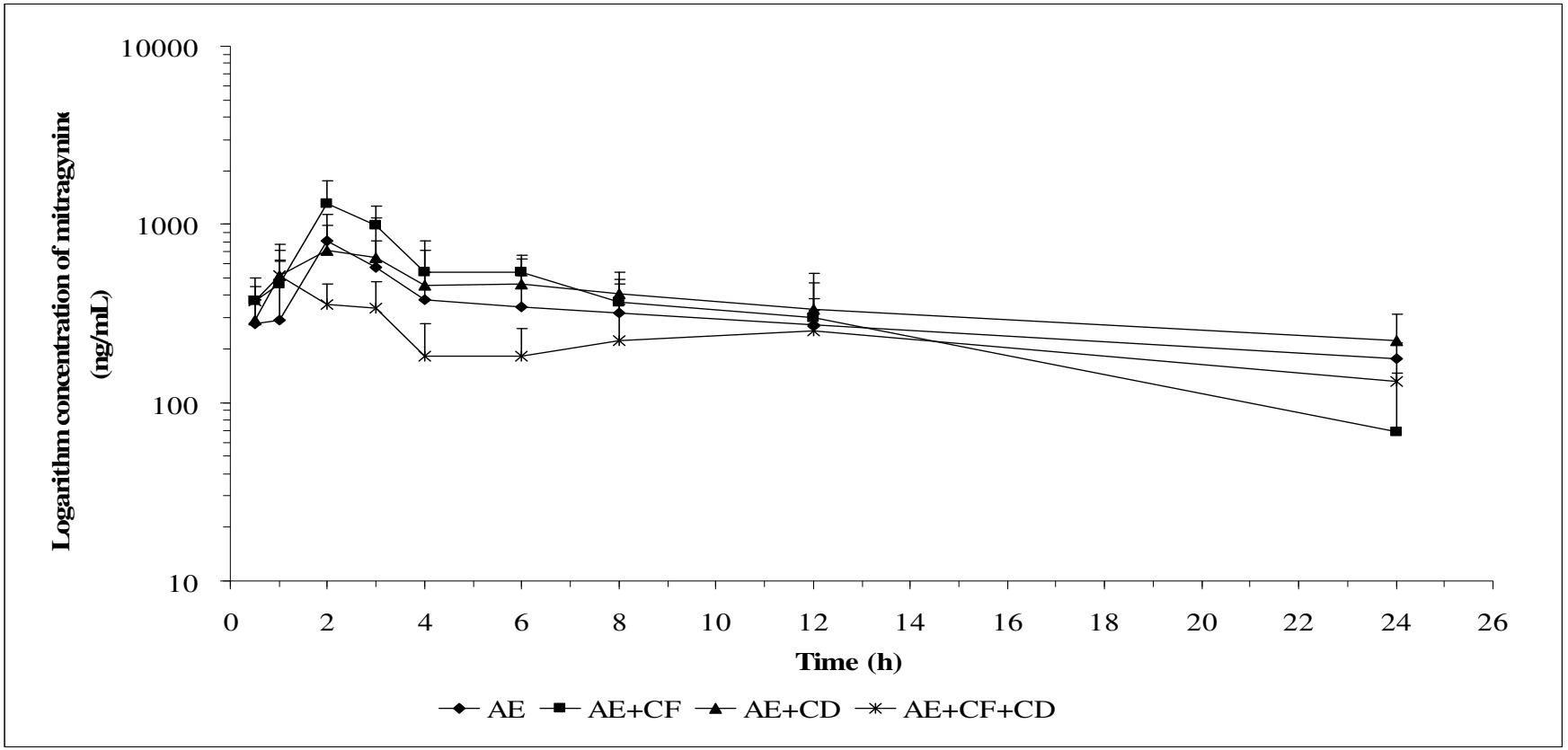


Figure 3.10 Semi-logarithm plots of mean serum concentrations of mitragynine and standard error bars in rats receiving a single oral dose of following compounds; 100 mg/kg of alkaloid extract (AE), 100 mg/kg of alkaloid extract and caffeine 25 mg/kg (AE+CF), 100 mg/kg of alkaloid extract and codeine 3 mg/kg (AE+CD), 100 mg/kg of alkaloid extract, caffeine 25 mg/kg and codeine 3 mg/kg (AE+CF+CD

3.1.5.4 Pharmacokinetic parameter analyses

Pharmacokinetic parameters of mitragynine derived from the non-compartmental method are presented in Table 3.9 and raw data are presented in Appendix-7.

Table 3.9 Pharmacokinetic parameters derived from a non-compartmental analysis of mitragynine in rats receiving alkaloid extract, alone or combined with caffeine and/or codeine

Parameters	Units	Compounds			
		AE	AE+CF	AE+CD	AE+CF+CD
C_{max}	ng/mL	835.00±123.52	1320.00±185.26*	820.00±120.19	621.97±58.97
T_{max}	h	1.83±0.17	1.51±0.34	1.83±0.17	1.67±0.33
k_{ab}	h^{-1}	0.17±0.03	0.18±0.02	0.26±0.02	0.38±0.11*
$t_{1/2\ ab}$	h	4.58±0.66	4.15±0.50	2.81±0.30*	2.62±0.60*
k_{el}	h^{-1}	0.007±0.002	0.031±0.011*	0.008±0.00	0.005±0.000
$t_{1/2\ el}$	h	184.03± 72.70	39.88±10.96*	102.39±16.60	168.91±25.22
V_d/F	L /kg	118.02±23.10	61.96±10.30*	90.18±14.34	112.57±22.19
Cl/F	L/h	0.75±0.25	1.51±0.34*	0.76±0.26	0.48±0.007
Cl_h	L/h	0.7483±0.2543	1.5132±0.3447	0.7556±0.2584	0.4766±0.0654
Cl_r	L/h	0.0004±0.0000	0.0011±0.0003	0.0004±0.0001	0.0006±0.0004
$AUC_{0\rightarrow\infty}$	ng h/mL	57090.33±21442.90	21085.82±5667.82	43068.93±8283.78	55293.46±10372.06
$AUMC_{0\rightarrow\infty}$	ng h^2/mL	$(25\times10^{6)}\pm(19\times10^{6})$	$(1\times10^{6)}\pm(0.6\times10^{6})$	$(6\times10^{6)}\pm(2\times10^{6})$	$(13\times10^{6)}\pm(4\times10^{6})$
$MRT_{0\rightarrow\infty}$	h	255.45±106.55	45.92±13.84*	138.26±24.20	236.50±35.82

Values are presented as mean ± S.E. (n =6)

* $p < 0.05$, significantly different compared to the alkaloid extract of kratom leaves (one way ANOVA)

In this study, serum concentration-time profiles for each group of animals showed different models. Hence, non-compartment model was chosen. In the study of Janchawee and coworkers (2007), pharmacokinetics of mitragynine was also described based on non-compartment model.

Pharmacokinetic parameters of mitragynine analyzed using non-compartment model are shown in Table 3.9. In rats receiving a single oral dose of 100 mg of AE per kg body weight (n=6), the mean±S.E. values of maximum serum concentration (C_{max}) of 835.00±123.52 ng/mL was achieved at 1.83±0.17 h (T_{max}) with an absorption rate constant (k_{ab}) of 0.17±0.03 h^{-1}. Mitragynine had a volume of distribution (V_d/F) 118.02±23.10 L/kg. It was eliminated with an elimination rate constant (k_{el}) of 0.007±0.002 h^{-1} and clearance (Cl/F) of 0.75±0.25 L/h. The hepatic clearance and renal clearance were 0.75±0.25 L/h and 0.0004±0.0000 L/h, respectively. The half-life of absorption ($t_{1/2\ ab}$) and elimination ($t_{1/2\ kel}$) were 4.58±0.66 h and 184.03± 72.70 h, respectively. The mean residence time ($MRT_{0\rightarrow\infty}$) was 255.45±106.55 h.

Caffeine appeared to affect pharmacokinetics of mitragynine. In rats receiving AE and CF, k_{el} was significantly higher than those given AE alone (0.031±0.011 h^{-1} v.s. 0.007±0.002 h^{-1}; $p < 0.05$). In addition, $t_{1/2\ el}$ and $MRT_{0\rightarrow\infty}$ in this group were significantly lower than those given AE alone (39.88±10.96 h v.s. 184.03± 72.70 h and 45.92±13.84 h v.s. 255.45±106.55 h, respectively; $p < 0.05$). These finding suggest that caffeine enhanced eliminating process of mitragynine. Volume of distribution (V_d/F) of mitragynine were significantly decreased by caffeine (61.96±10.30 L /kg v.s. 118.02±23.10 L /kg; $p < 0.05$). The increase in elimination and the decrease in distribution from blood to tissue caused a significant increase in total clearance (1.51±0.34 L/h v.s. 0.75±0.25 L/h; $p < 0.05$). Both hepatic (Cl_h) and renal clearances (Cl_r) tended to be increased but those were not significantly different (1.5132±0.3447 L/h v.s. 0.7483±0.2543 L/h and 0.0011±0.0003 L/h v.s. 0.0004±0.0000 L/h, respectively; $p < 0.05$). Effect of caffeine in increasing elimination of mitragynine may be due to its diuretic effect. Caffeine may increase

renal blood flow and glomerular filtration rate. Hence, urinary excretion of drugs can be increased.

Codeine showed different effects, compared with those of caffeine, on pharmacokinetics of mitragynine. Absorption of mitragynine appeared to be accerelated in rats receiving AE+CD. This was shown by a significant decrease in $t_{1/2\ ab}$, i.e. 2.81±0.30 h v.s. 4.58±0.66 h; $p < 0.05$, despite of a non significant increase in k_{ab}, i.e. 0.26±0.02 h^{-1} v.s. 0.17±0.03 h^{-1}; $p < 0.05$. Other parameters related to absorption process such as C_{max}, T_{max} and $AUC_{0\rightarrow\infty}$ were also unchanged.

Combination of CF and CD obviously increased absorption of mitragynine in rats given AE, CF and CD, compared with those received AE alone (0.38±0.11 h^{-1} v.s. 0.17±0.03 h^{-1} for k_{ab} and 2.62±0.60 h v.s. 4.58±0.66 h for $t_{1/2\ ab}$; $p < 0.05$). However effects of caffeine to increase elimination and decrease distribution as observed in rats administered AE+CF were not seen.

3.1.5.5 Percentage amount excreted of mitragynine in urine

After administration of each compound, profiles of urinary concentrations of mitragynine during 24 h were presented in Figure 3.11. After 24 hour, the level of mitragynine in urine was below the LLOQ. Urinary concentrations of mitragynine could not be detected in some rats of each interval. Between 0-8 h, urinary concentration of mitragynine could not be detected in some rats of group AE+CD (n_4) and group AE+CF+CD (n_1, n_2, n_5). Between 9-16 h, its concentration of mitragynine could not be detected in some rats of group AE+CD (n_2). Urinary concentrations of mitragynine could not be detected between 17 and 24 h in some rats of groups AE (n_1, n_5), AE+CF (n_3, n_4, n_6), AE+CD (n_1, n_2, n_5, n_6) and AE+CF+CD (n_1, n_3).

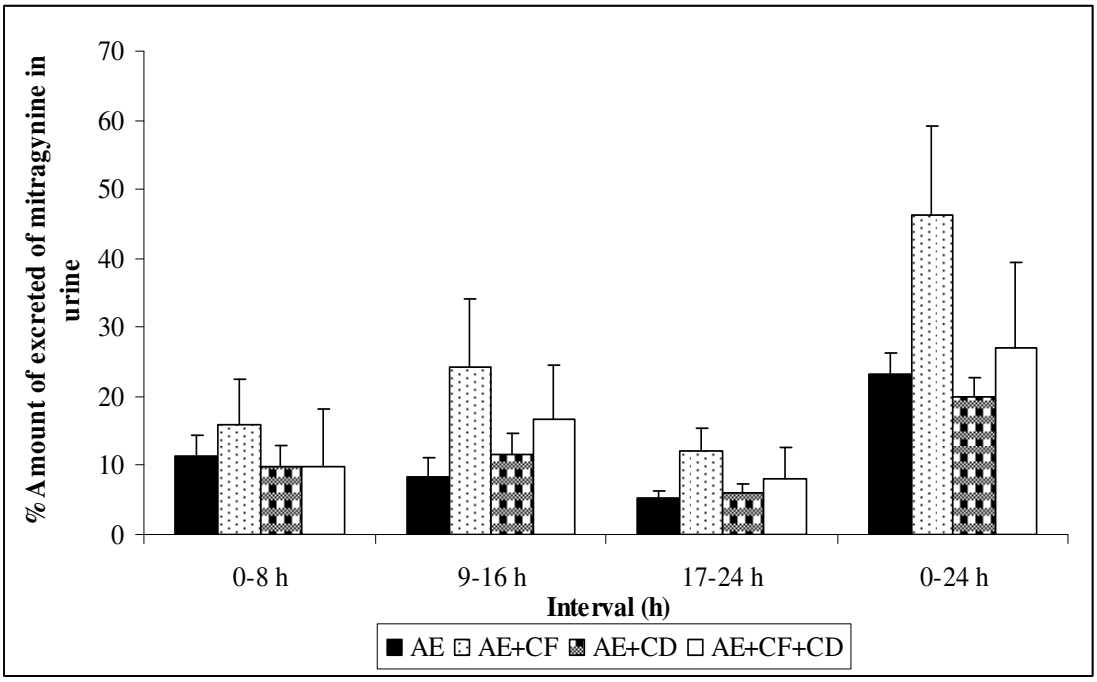


Figure 3.11 Percentage amount excreted of mitragynine (mean±S.E.) in urine of rats given a single oral dose of test compounds; 100 mg/kg of alkaloid extract (AE, n=6), 100 mg/kg of alkaloid extract and caffeine 25 mg/kg (AE+CF, n=6), 100 mg/kg of alkaloid extract and codeine 3 mg/kg (AE+CD, n=6), 100 mg/kg of alkaloid extract, caffeine 25 mg/kg and codeine 3 mg/kg (AE+CF+CD, n=6)

After administration of AE, The percentage amounts excreted of mitragynine in urine were gradually decreasing (Figure 3.11). For interval (0-8 h, 9-16 h and 17-24 h), the %amount excreted of mitragynine in urine of group AE was ranging from 5.08-22.64%, 2.10-19.44%, 3.11-7.13%, respectively and was not significantly different from each interval. Post dose 24 h, the % amount excreted of mitragynine in urine of Group AE was ranging from 9.70-24.49%. Each the interval (0-8 h, 9-16 h, 17-24 h), the %amount excreted of mitragynine in urine of group AE+CF were ranging from 3.38-38.43%, 2.69-64.89% and 6.45-21.59%, respectively. The %amount excreted of this group was highest in 9-16 h post dose but did not significantly different from other intervals. The %amount excreted of mitragynine of group AE+CD in interval 0-8h, 9-16h and 17-24 h were ranging from 6.14-21.71%,

4.89-19.58%, 3.99-8.14%, respectively. In the group of three combination (AE+CF+CD), the %amount excreted of mitragynine of interval 0-8 h, 9-16 h, 17-24 h were 1.73-26.44%, 3.41-52.47%, 2.73-21.61%, respectively. When overall %amount excreted of mitragynine in urine during 0-24 h was considered, group AE+CF was highly excreted mitragynine in urine when compaired to other treatments but did not significantly different (see raw data in Appendix-8). These results were shown that caffeine increased elimination of mitragynine in urine and correlated with pharmacokinetic parameter in which k_{el} of rat receiving AE and CF was significantly higher than AE treated alone.

3.2 Experiment 2 : Effect of caffeine and codeine on antinociceptive activity of alkaloid extract from kratom leaves

The present work examined antinociceptive activity by using hot plate test which is commonly used to evaluate the analgesic effect of a drug in rodents. The measured parameter was a latent period that the animals responded to heat stimulus. The animals only showed nociceptive response to hot plate test such as jumping or licking within 15 seconds were included. Values of latency of nociceptive response stimulated by heat in rats receiving different compounds at 30, 60, 90, 120 and 150 min after administration are presented in Table 3.10 and raw data are present in Appendix-9.

Table 3.10 The effect of caffeine and codeine on latency of nociceptive response of alkaloid extract from leaves of kratom.

Compound	Latency of nociceptive response (sec)				
	30 min	**60 min**	**90 min**	**120 min**	**150 min**
DW	8.73 ± 0.43	8.63 ± 0.49	9.07 ± 0.33	8.57 ± 0.35	8.55 ± 0.33
PG	8.25 ± 0.51	8.48± 0.58	8.97 ± 0.51	8.48 ± 0.46	9.37 ± 0.45
AE	10.47 ± 0.39*	11.70 ± 0.53*	12.10 ± 0.34*	11.45 ± 0.61*	9.00 ± 0.71
AE+CF	9.80 ± 0.53	11.32 ± 1.23*	11.18 ± 0.67*	9.30 ± 0.85**	8.28 ± 0.52
AE+CD	11.43 ± 1.01*	11.55 ± 0.77*	11.73 ± 0.81*	9.43 ± 0.62**	8.32 ± 0.55
AE+CF+CD	12.48 ± 1.02*, **	12.05 ± 0.74*	10.40 ± 0.69**	9.03 ± 0.75**	9.07 ± 0.84

Values are presented as mean ± S.E.M. (n =6)

* $p < 0.05$, significantly different compared to the control vehicle group, propylene glycol (one way ANOVA)

** $p < 0.05$, significantly different compared to the alkaloid extract from leaves of kratom (one way ANOVA)

Both DW and PG did not have antinociceptive activity throughout 150 min duration of observation. AE at the dose of 100 mg/kg exhibited antinociception at 30 min and the effect remained significant until 120 min following an oral administration, compared with the vehicle control group (PG). Combination of CF at the dose of 25 mg/kg with AE revealed antinociception of AE only at 60 and 90 min, compared with the PG treated group. The latency of nociceptive response in this group (AE+CF) during 60 and 90 min were not significantly different from those in AE treated group. It is apparent that onset of action was delayed and duration of action was shortened. This may be due to an increased elimination rate of mitragynine by caffeine.

After administration of AE and CD, the antinociceptive activity of AE was significantly increased at 30, 60, and 90 min, compared with PG treated group. This effect disappeared at 120 min. The latency of nociceptive response at 30 min in rats given AE+CD was slightly increased compared with those received AE alone. Co-administration of AE, CF and CD resulted in antinociceptive activity of AE only at 30 and 60 min when compared with PG treated group. The latency period in rats receiving AE, CF and CD at 30 min was significantly longer than those given AE alone. This finding shows that the onset of action seemed to be shorten and the action was more efficacious, especially at 30 min after administration. This may be due to an enhanced absorption rate of AE by codeine.

CHAPTER 4

Conclusion

The present study has shown that either caffeine, or codeine and their combination altered both pharmacokinetics and pharmacodynamics of alkaloid extract derived from leaves of kratom. In terms of pharmacokinetics, caffeine, when co-administered with an alkaloid extract, significantly increased the elimination and decreased the distribution of mitragynine, a major alkaloid of kratom leaves. Codeine influenced differently from caffeine did. Although codeine alone tended to slightly increase the absorption rate of mitragynine, but combined codeine and caffeine caused a significant increase in its absorption. Additionally, pharmacological action of the alkaloid extract also changed by these substances. Caffeine and codeine at sub-analgesic doses either alone, or combination changed the antinociception of the alkaloid extract. Caffeine alone decreased the efficacy and duration of action of the alkaloid extract, while codeine especially combined with caffeine significantly increased the efficacy and may be the onset. These effects can be explained by changed pharmacokinetic behaviors of the alkaloid extract.

These results indicates significant pharmacokinetic and pharmacodynamic interactions between the alkaloid extract from leaves of kratom and caffeine or codeine, either alone or their combination. Caffeine is a safe substance commonly contained in beverages while codeine is frequently used as an antitussive drug. However, these two compounds usually abused by mix Coke® and cough syrup with the boiled water extract of kratom leaves so called "4×100 kratom cocktail". The present works support the increased effects of kratom consumed by drug abusers. Those effects are desired by those individuals but actually are considered to be unwanted effects by the pharmacologists or toxicologists. Since kratom possesses several pharmacological actions, concomitant use of kratom, as an herbal medicine, and caffeine or codeine by non-abusers should be careful. Moreover, something may be undiscovered because the present study clarified only the effects of either caffeine,

or codeine, and their combination on pharmacology of kratom but the interaction between caffeine and codeine is not yet revealed.

In addition, as mentioned earlier, alkaloid extract derived from kratom leaves are compose of many types of alkaloid, including 7-hydroxymitragynine, that has antinociceptive effect also. Hence, the effect of caffeine or codeine and their combination on pharmacology of other types of alkaloid should be clarified further.

In view of the forensic scientists, screening of kratom abusers is of importance because kratom is classified as category V drug in Narcotic Act B.E. 2522 (1979) and consumption of kratom is illegal. There have been a number of methods successfully shown to detect substances which are constituents in kratom leaves, for example, mitragynine, in serum or urine of animals and humans. Those included samples collected after administration of mitragynine or kratom leaves. From the findings that elimination of mitragynine can be enhanced by even a small amount of caffeine co-administered, a sensitive method is needed to identify abusers consuming 4×100 kratom cocktail. Additionally, biological samples especially urine which was successfully developed in this study should be obtained as early as possible before the interesting analyte will be cleared from the body.

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Appendix-1

Method validation for analysis of mitragynine in serum

Table A1 Raw data for determination of linearity and lower limit of quantification of the method for analyzing mitragynine in serum

Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)				Measured concentration (μg/mL)			
			Individual	Mean	S.D.	% C.V.	Individual	Mean	S.D.	%DEV
0.1	1	9.429	8.65	8.28	0.86	10.39	0.16	0.12	0.04	14.24
	2	9.730	7.30				0.09			
	3	9.607	8.90				0.09			
0.5	1	9.701	43.68	42.82	2.99	6.98	0.53	0.49	0.06	-2.97
	2	9.115	45.29				0.51			
	3	9.719	39.50				0.42			
1	1	9.692	89.00	89.22	3.79	4.24	1.01	0.99	0.03	-1.70
	2	9.012	85.55				0.95			
	3	9.715	93.11				1.00			
5	1	9.897	451.00	464.29	12.68	2.73	4.82	5.01	0.16	0.21
	2	9.669	465.63				5.09			
	3	9.715	476.25				5.12			
10	1	9.710	950.00	928.63	19.56	2.11	10.09	10.00	0.08	-0.08
	2	9.641	911.63				9.96			
	3	9.661	924.25				9.93			

Table A2 Raw data for determination of intra-day precision of the method for analyzing mitragynine in serum

Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V
			Individual	Mean	S.D.	
0.1	1	9.929	8.50	8.28	0.75	9.04
	2	9.730	7.45			
	3	9.607	8.90			
0.5	1	9.701	43.68	42.82	2.99	6.98
	2	9.115	45.29			
	3	9.719	39.50			
5	1	9.897	451.00	464.29	12.68	2.73
	2	9.669	465.63			
	3	9.715	476.25			
10	1	9.710	950.00	928.63	19.56	2.11
	2	9.641	911.63			
	3	9.661	924.25			

Table A3 Raw data for determination of inter-day of the method for analyzing mitragynine in serum

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
1	0.1	1	9.353	9.18	8.18	0.89	10.87
		2	9.347	7.88			
		3	9.382	7.48			
	0.5	1	9.375	40.88	40.75	4.19	10.28
		2	9.415	44.88			
		3	9.394	36.50			
	5	1	9.165	461.13	463.96	15.82	3.41
		2	9.348	449.75			
		3	9.336	481.00			
	10	1	9.424	915.63	915.04	24.38	2.66
		2	9.378	890.38			
		3	9.278	939.13			

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
2	0.1	1	9.402	8.35	8.26	0.86	10.40
		2	9.566	9.08			
		3	9.498	7.36			
	0.5	1	9.400	38.38	40.29	2.01	4.98
		2	9.051	42.38			
		3	9.534	40.13			
	5	1	9.416	459.88	454.71	14.82	3.26
		2	9.490	466.25			
		3	9.394	438.00			
	10	1	9.070	925.50	900.75	26.67	2.96
		2	9.589	904.25			
		3	9.409	872.50			

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
3	0.1	1	9.579	8.65	7.99	0.68	8.54
		2	9.601	7.29			
		3	9.496	8.03			
	0.5	1	9.572	35.39	36.25	2.02	5.57
		2	9.644	38.56			
		3	9.543	34.81			
	5	1	9.676	492.13	476.71	14.49	3.04
		2	9.646	463.38			
		3	9.655	474.63			
	10	1	9.751	930.00	921.63	26.21	2.84
		2	9.789	892.25			
		3	9.777	942.63			

Table A4 Raw data for determination of accuracy (intra-day) of the method for analyzing mitragynine in serum

Nominal concentration (μg/mL)	N	t_R (min)	Peak area (mAU)	Measured concentration (μg/mL)			%DEV
				Individual	Mean	S.D.	
0.1	1	9.929	8.50	0.16	0.11	0.04	11.31
	2	9.730	7.45	0.07			
	3	9.607	8.90	0.10			
0.5	1	9.701	43.68	0.53	0.48	0.05	-3.50
	2	9.115	45.29	0.49			
	3	9.719	39.50	0.43			
5	1	9.897	451.00	4.82	5.01	0.16	0.22
	2	9.669	465.63	5.08			
	3	9.715	476.25	5.13			
10	1	9.710	950.00	10.09	10.00	0.08	-0.05
	2	9.641	911.63	9.96			
	3	9.661	924.25	9.94			

Table A5 Raw data for determination of recovery of the method for analyzing mitragynine in serum

Concentration of mitragynine (μg/mL)	N	Direct injection		After extraction		Recovery		
		t_R (min)	Peak area (mAU)	t_R (min)	Peak area (mAU)	Individual	Mean	S.D.
0.1	1	9.626	9.50	9.429	8.65	91.05	92.16	12.53
	2	9.589	9.10	9.730	7.30	80.22		
	3	9.624	8.46	9.607	8.90	105.20		
0.5	1	9.578	44.00	9.701	43.68	99.26	96.65	14.75
	2	9.616	41.20	9.115	45.29	109.92		
	3	9.623	48.90	9.719	39.50	80.78		
5	1	9.689	480.10	9.897	451.00	93.94	99.39	4.98
	2	9.656	463.20	9.669	465.63	100.52		
	3	9.676	459.20	9.715	476.25	103.71		
10	1	9.689	927.20	9.710	950.00	102.46	99.43	2.64
	2	9.593	934.10	9.641	911.63	97.59		
	3	9.669	940.80	9.661	924.25	98.24		

Appendix-2

Method validation for analysis of mitragynine in urine

Table A6 Raw data for determination of linearity and lower limit of quantification of the method for analyzing mitragynine in urine

Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)				Measured concentration (μg/mL)			
			Individual	Mean	S.D.	% C.V.	Individual	Mean	S.D.	%DEV
0.1	1	9.331	6.41	6.75	1.24	18.39	0.14	0.12	0.05	17.93
	2	9.314	6.31				0.20			
	3	9.341	7.30				0.09			
	4	9.315	5.20				0.09			
	5	9.338	8.52				0.07			
0.5	1	9.466	39.20	42.69	3.40	7.97	0.55	0.45	0.08	-10.87
	2	9.366	40.83				0.49			
	3	9.351	43.82				0.37			
	4	9.458	41.64				0.37			
	5	9.000	48.00				0.44			
1	1	9.252	84.60	87.69	5.90	6.72	0.94	0.86	0.06	-13.67
	2	9.539	86.00				0.88			
	3	9.064	90.30				0.84			
	4	9.141	81.11				0.77			
	5	9.011	96.44				0.89			

Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)				Measured concentration (μg/mL)			
			Individual	Mean	S.D.	% C.V.	Individual	Mean	S.D.	%DEV
5	1	9.499	516.62	566.65	28.41	5.01	4.66	5.33	0.48	6.56
	2	9.480	570.92				5.04			
	3	9.226	579.61				5.72			
	4	9.392	583.90				5.84			
	5	9.496	582.20				5.37			
10	1	9.567	1155.50	1059.95	29.69	2.80	10.17	9.85	0.23	-1.48
	2	9.480	1146.72				9.99			
	3	9.167	974.20				9.66			
	4	9.082	958.32				9.61			
	5	9.242	1065.03				9.83			

Table A7 Raw data for determination of intra-day precision of the method for analyzing mitragynine in urine

Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
			Individual	Mean	S.D.	
0.1	1	9.466	7.36	6.99	0.34	4.87
	2	9.323	7.09			
	3	9.349	6.50			
	4	9.267	7.20			
	5	9.611	6.81			
0.5	1	9.420	43.04	43.79	1.31	2.99
	2	9.363	42.20			
	3	9.281	44.10			
	4	9.365	43.90			
	5	9.445	45.70			
5	1	9.254	565.33	570.78	7.28	1.28
	2	9.378	580.30			
	3	9.419	574.06			
	4	9.332	572.30			
	5	9.369	561.92			
10	1	9.415	1143.64	1129.26	13.67	1.21
	2	9.303	1109.70			
	3	9.370	1134.40			
	4	9.408	1121.15			
	5	9.396	1137.40			

Table A8 Raw data for determination of inter-day precision of the method for analyzing mitragynine in urine

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
1	0.1	1	9.350	5.91	6.69	0.57	8.58
		2	9.298	6.43			
		3	9.333	6.60			
		4	9.200	7.20			
		5	9.296	7.30			
	0.5	1	9.200	39.89	41.13	1.07	2.60
		2	9.256	42.60			
		3	9.304	41.53			
		4	9.288	40.31			
		5	9.376	41.30			
	5	1	9.359	553.25	560.31	5.23	0.93
		2	9.352	567.70			
		3	9.264	559.10			
		4	9.360	562.02			
		5	9.398	559.50			
	10	1	9.239	1115.23	1101.63	9.37	0.85
		2	9.304	1100.00			
		3	9.358	1103.00			
		4	9.359	1088.92			
		5	9.386	1101.00			

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
2	0.1	1	9.366	7.18	6.92	0.35	5.07
	0.1	2	9.423	7.30			
	0.1	3	9.494	6.90			
	0.1	4	9.462	6.82			
	0.1	5	9.311	6.40			
	0.5	1	9.455	46.00	43.71	1.32	3.02
	0.5	2	9.430	42.83			
	0.5	3	9.484	43.12			
	0.5	4	9.350	42.92			
	0.5	5	9.345	43.70			
	5	1	9.384	582.37	570.40	7.35	1.29
	5	2	9.395	569.30			
	5	3	9.419	564.00			
	5	4	9.412	571.36			
	5	5	9.424	564.95			
	10	1	9.480	1137.65	1128.07	14.10	1.25
	10	2	9.403	1109.70			
	10	3	9.470	1145.44			
	10	4	9.407	1120.10			
	10	5	9.448	1127.48			

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
3	0.1	1	9.362	7.09	6.99	0.26	3.69
		2	9.490	6.57			
		3	9.369	7.20			
		4	9.315	7.17			
		5	9.336	6.93			
	0.5	1	9.445	42.66	42.90	1.14	2.66
		2	9.443	43.50			
		3	9.392	44.56			
		4	9.367	41.80			
		5	9.289	42.00			
	5	1	9.312	566.70	568.33	6.63	1.17
		2	9.220	562.46			
		3	9.328	579.60			
		4	9.330	564.90			
		5	9.444	567.98			
	10	1	9.306	1107.95	1121.43	10.28	0.92
		2	9.325	1132.71			
		3	9.401	1115.40			
		4	9.410	1130.20			
		5	9.300	1120.90			

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
4	0.1	1	9.359	7.03	6.58	0.36	5.48
	0.1	2	9.463	6.72			
	0.1	3	9.284	6.04			
	0.1	4	9.296	6.50			
	0.1	5	9.277	6.61			
	0.5	1	9.295	41.90	42.99	1.16	2.70
	0.5	2	9.319	43.33			
	0.5	3	9.259	42.00			
	0.5	4	9.225	44.75			
	0.5	5	9.106	42.97			
	5	1	9.262	576.51	571.69	5.55	0.97
	5	2	9.271	570.40			
	5	3	9.310	563.40			
	5	4	9.273	577.10			
	5	5	9.266	571.06			
	10	1	9.312	1112.96	1126.56	19.80	1.76
	10	2	9.420	1138.90			
	10	3	9.205	1151.30			
	10	4	9.303	1101.71			
	10	5	9.308	1127.92			

Day	Concentration of mitragynine (μg/mL)	N	t_R (min)	Peak area (mAU)			% C.V.
				Individual	Mean	S.D.	
5	0.1	1	9.164	7.68	6.94	0.45	6.52
		2	9.176	6.84			
		3	9.223	6.51			
		4	9.017	7.00			
		5	9.000	6.67			
	0.5	1	9.063	44.69	44.97	2.20	4.89
		2	9.108	47.90			
		3	9.185	46.20			
		4	9.023	43.91			
		5	9.004	42.14			
	5	1	9.094	579.20	569.73	6.28	1.10
		2	9.092	571.40			
		3	9.044	563.00			
		4	9.100	565.27			
		5	9.102	569.80			
	10	1	9.100	1112.18	1130.17	16.09	1.42
		2	9.071	1150.97			
		3	9.023	1139.30			
		4	9.114	1116.30			
		5	9.067	1132.10			

Table A9 Raw data determination for accuracy (intra-day) of the method for analyzing mitragynine in urine

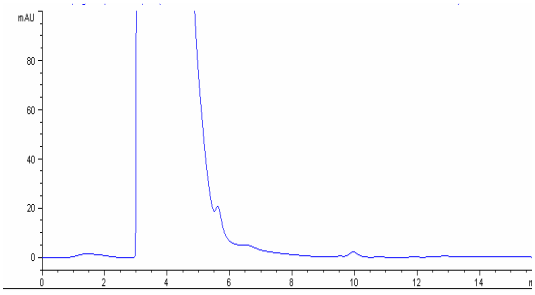
Nominal concentration (μg/mL)	N	t_R (min)	Peak area (mAU)	Measured concentration (μg/mL)			%DEV
				Individual	Mean	S.D.	
0.1	1	9.466	7.36	0.14	0.12	0.02	19.48
	2	9.323	7.09	0.09			
	3	9.349	6.50	0.12			
	4	9.267	7.20	0.11			
	5	9.611	6.81	0.13			
0.5	1	9.420	43.04	0.46	0.44	0.03	-11.14
	2	9.363	42.20	0.41			
	3	9.281	44.10	0.44			
	4	9.365	43.90	0.43			
	5	9.445	45.70	0.47			
5	1	9.254	565.33	5.04	5.08	0.08	1.56
	2	9.378	580.30	5.20			
	3	9.419	574.06	5.07			
	4	9.332	572.30	5.10			
	5	9.369	561.92	4.98			
10	1	9.415	1143.64	9.88	9.94	0.05	-0.56
	2	9.303	1109.70	9.91			
	3	9.370	1134.40	9.97			
	4	9.408	1121.15	9.95			
	5	9.396	1137.40	10.01			

Table A10 Raw data for determination of recovery of the method for analyzing mitragynine in urine

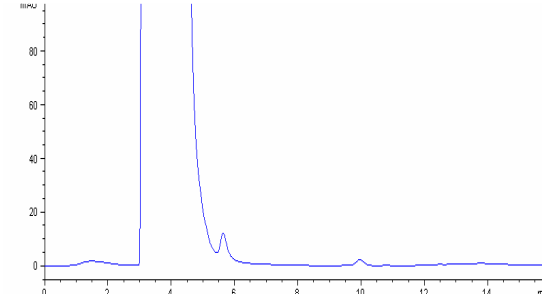
Concentration of mitragynine (μg/mL)	N	Direct injection		After extraction		Recovery		
		t_R (min)	Peak area (mAU)	t_R (min)	Peak area (mAU)	Individual	Mean	S.D.
0.1	1	9.372	7.74	9.466	7.36	95.09	92.74	4.10
	2	9.234	7.50	9.323	7.09	94.53		
	3	9.367	7.34	9.349	6.50	88.56		
	4	9.393	8.16	9.267	7.20	88.24		
	5	9.444	7.00	9.611	6.81	97.29		
0.5	1	9.386	45.51	9.420	43.04	94.57	95.50	3.78
	2	9.222	44.50	9.363	42.20	94.83		
	3	9.312	43.20	9.281	44.10	102.08		
	4	9.487	47.10	9.365	43.90	93.21		
	5	9.387	49.24	9.445	45.70	92.81		
5	1	9.341	575.50	9.254	565.33	98.23	100.26	1.64
	2	9.297	564.52	9.378	580.30	102.80		
	3	9.339	575.20	9.419	574.06	99.80		
	4	9.277	570.75	9.332	572.30	100.27		
	5	9.353	560.91	9.369	561.92	100.18		
10	1	9.488	1100.60	9.415	1143.64	103.91	100.00	4.78
	2	9.318	1149.22	9.303	1109.70	96.56		
	3	9.258	1095.10	9.370	1134.40	103.59		
	4	9.224	1201.30	9.408	1121.15	93.33		
	5	9.282	1108.70	9.396	1137.40	102.59		

Appendix-3

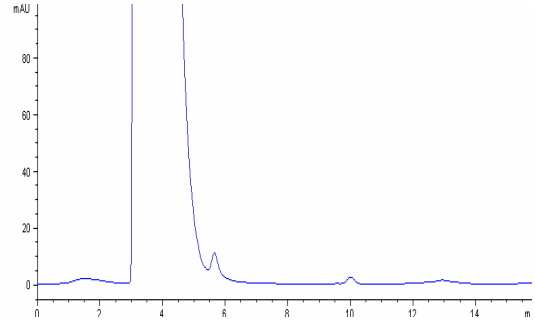
Chromatographic analysis of wash-elute study



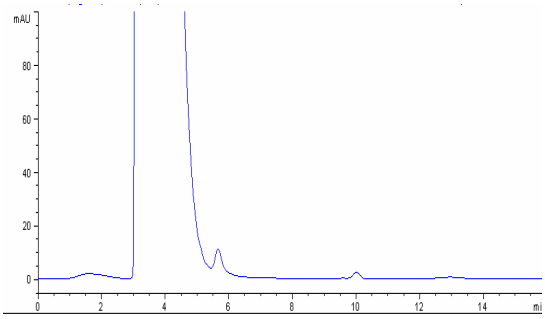
0% MeOH with 2% ammonium hydroxide



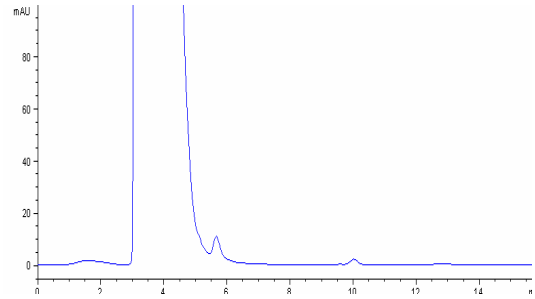
10% MeOH with 2% ammonium hydroxide



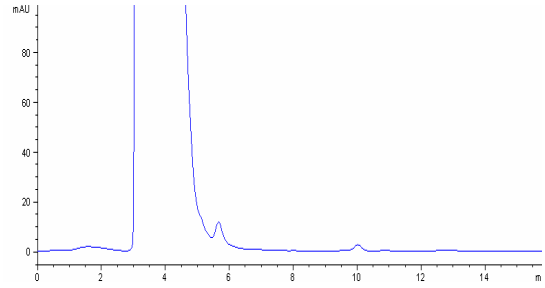
20% MeOH with 2% ammonium hydroxide



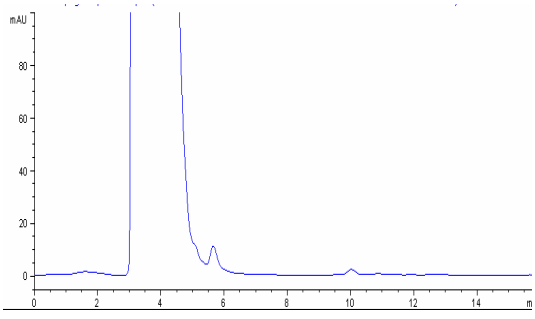
30% MeOH with 2% ammonium hydroxide



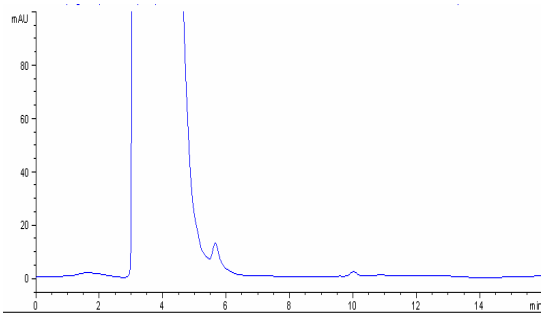
40% MeOH with 2% ammonium hydroxide



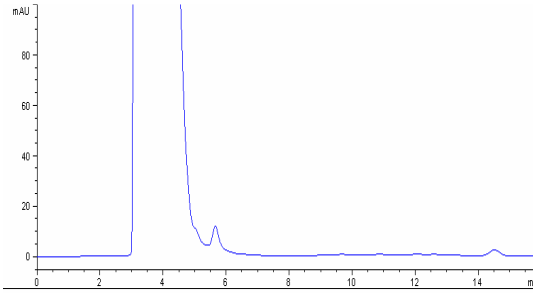
50% MeOH with 2% ammonium hydroxide



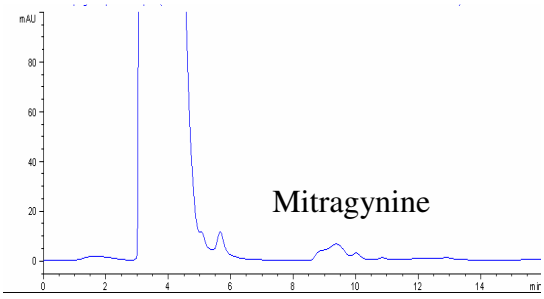
60% MeOH with 2% ammonium hydroxide



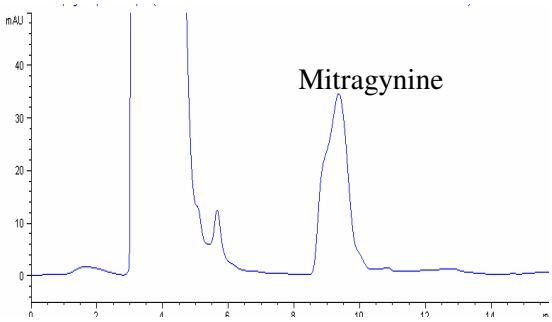
70% MeOH with 2% ammonium hydroxide



80% MeOH with 2% ammonium hydroxide

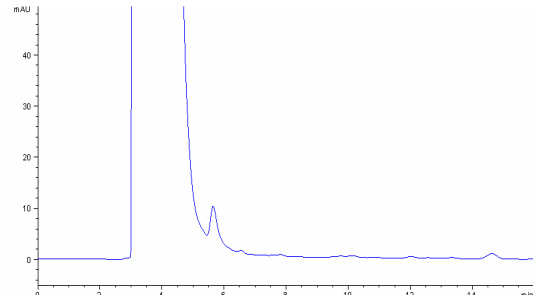


90% MeOH with 2% ammonium hydroxide

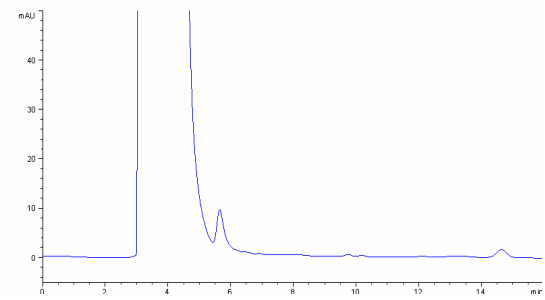


100% MeOH with 2% ammonium hydroxide

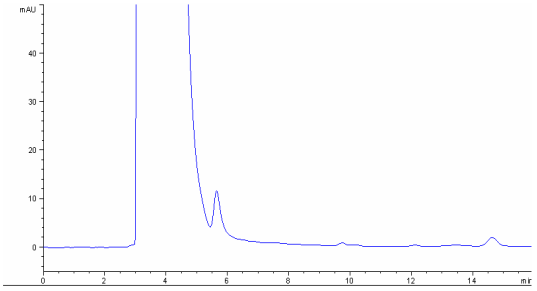
Figure A1 Chromatogram of mitragynine (10 μg/mL) in spiked rat urine passing through Oasis® HLB cartridge and eluted with varying percentage of a methanol-water mixture containing 2% ammonium hydroxide



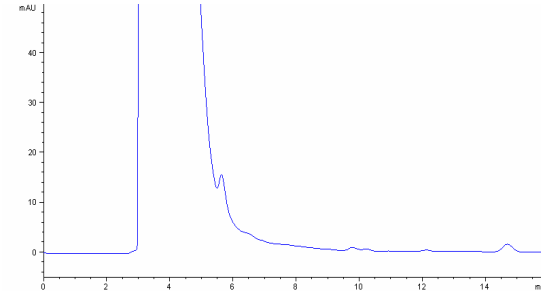
0% MeOH with 2% acetic acid



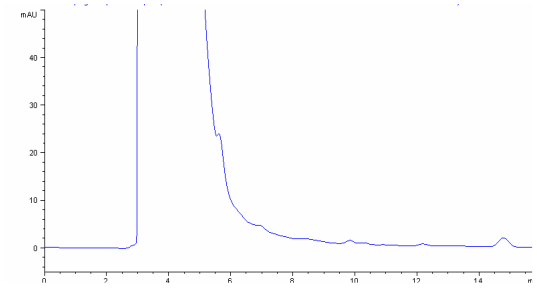
10% MeOH with 2% acetic acid



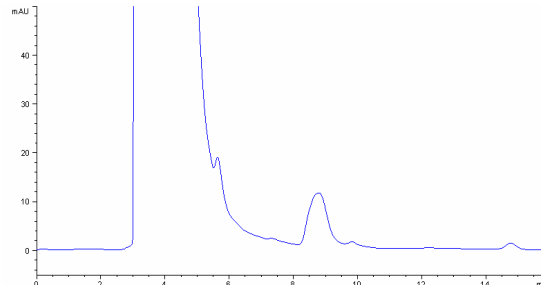
20% MeOH with 2% acetic acid



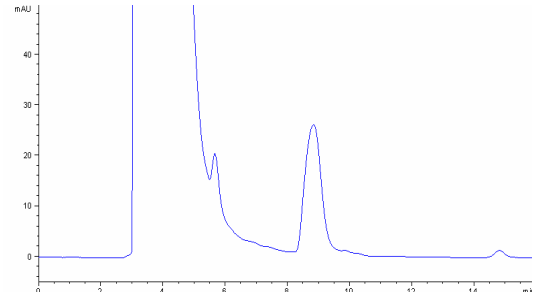
30% MeOH with 2% acetic acid



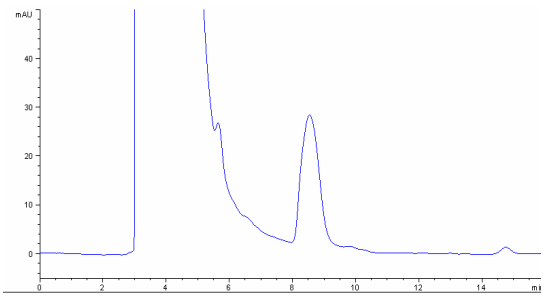
40% MeOH with 2% acetic acid



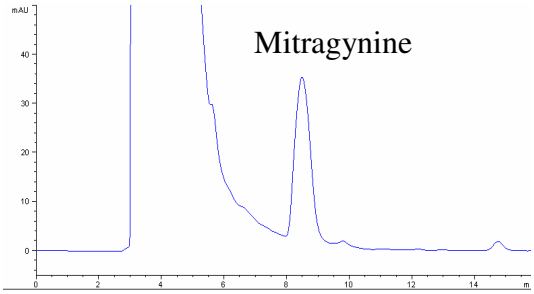
50% MeOH with 2% acetic acid



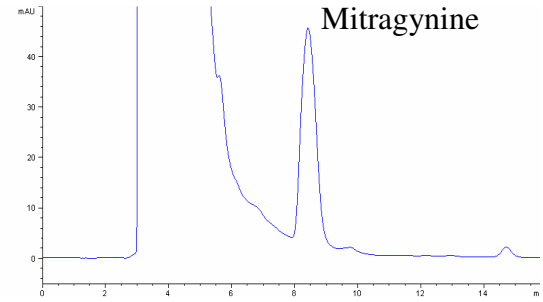
60% MeOH with 2% acetic acid



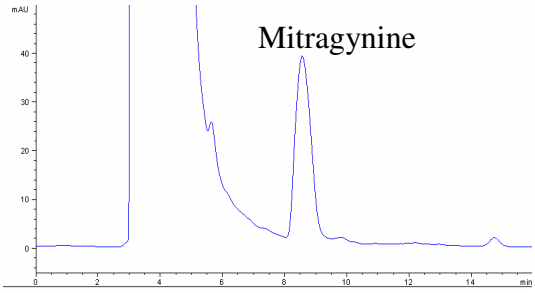
70% MeOH with 2% acetic acid



80% MeOH with 2% acetic acid



90% MeOH with 2% acetic acid



100% MeOH with 2% acetic acid

Figure A2 Chromatogram of mitragynine (10 μg/mL) in spiked rat urine passing through Oasis® HLB cartridge and eluted with varying percentage of a methanol-water mixture containing 2% acetic acid

Appendix-4

Weight of male Wistar rats

Table A11 Weight of male Wistar rats for collection serum sample

Group	Weight (g)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
AE	234	235	230	233	241	237	235.00	1.53
AE+CF	241	232	234	231	230	234	233.67	1.61
AE+CD	238	235	225	233	228	227	231.00	2.08
AE+CF+CD	229	231	227	232	230	235	230.67	1.12

Table A12 Weight of Wistar rats for collection urine sample

Group	Weight (g)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
AE	229	229	240	241	236	243	236.33	2.50
AE+CF	242	235	240	245	233	235	238.33	1.93
AE+CD	230	238	237	230	232	243	235.00	2.13
AE+CF+CD	235	235	237	236	239	229	235.17	1.38

Appendix-5

Serum concentrations of mitragynine in rats

Table A13 Serum concentrations of mitragynine at 0-24 h in rats administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves (Group AE)

Time (h)	Serum concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
0	0	0	0	0	0	0	0	0
0.5	420	180	300	330	120	320	278.33	44.61
1	250	410	290	400	180	200	288.33	40.13
2	1010	260	1130	1130	580	750	810.00	141.90
3	640	220	810	1060	350	370	575.00	130.84
4	600	240	690	440	180	120	378.33	95.81
6	430	230	590	410	180	220	343.33	65.21
8	220	230	610	420	130	300	318.33	70.41
12	200	210	500	340	90	290	271.67	57.48
24	160	200	170	260	80	190	176.67	24.04

Table A14 Serum concentrations of mitrgynine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 25 mg/kg of caffeine (Group AE+CF)

Time (h)	Serum concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
0	0	0	0	0	0	0	0	0
0.5	370	190	260	450	490	490	375.00	51.50
1	430	470	260	340	570	700	461.67	64.58
2	970	1420	790	1650	1940	1030	1300.00	181.55
3	870	1540	790	960	850	960	995.00	112.29
4	150	960	370	580	540	650	541.67	111.24
6	470	650	390	550	540	630	538.33	39.88
8	390	380	410	130	520	360	365.00	52.34
12	230	150	580	120	360	360	300.00	69.63
24	nd	130	180	nd	nd	100	68.33	32.30

Note; nd, not detectable

Table A15 Serum concentrations of mitragynine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 3 mg/kg of codeine (Group AE+CD)

Time (h)	Serum concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
0	0	0	0	0	0	0	0	0
0.5	340	350	300	120	300	330	290.00	35.03
1	470	250	830	490	440	630	518.33	79.78
2	220	620	940	980	770	770	716.67	112.71
3	160	270	1310	970	520	700	655.00	177.21
4	150	390	770	360	470	700	473.33	93.78
6	160	490	770	550	360	460	465.00	82.67
8	230	480	390	600	290	450	406.67	54.70
12	90	350	200	590	210	550	331.67	82.74
24	110	250	180	380	210	210	223.33	36.67

Table A16 Serum concentrations of mitragynine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves, 25 mg/kg of caffeine and 3 mg/kg of codeine (Group AE+CF+CD)

Time (h)	Serum concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
0	0	0	0	0	0	0	0	0
0.5	260	410	330	430	360	460	375.00	29.98
1	790	660	240	310	780	320	516.67	103.71
2	300	230	450	500	380	260	353.33	44.10
3	220	220	420	410	200	550	336.67	58.81
4	130	160	130	160	150	370	183.33	37.75
6	120	170	120	120	260	300	181.67	32.50
8	150	360	130	170	420	110	223.33	53.90
12	130	380	120	160	430	290	251.67	54.87
24	120	260	nd	100	150	160	131.67	34.69

Note; nd, not detectable

Appendix-6

Urine concentrations of mitragynine in rats

Table A17 Urine concentrations of mitragynine at 0-24 h in rats administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves (Group AE)

Interval	Urine concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	0	0	0	0	0	0	0.00	0.00
2 (0-8 h)	610	450	1360	320	1200	490	738.33	176.66
3 (9-16 h)	240	270	960	910	1090	270	623.33	164.34
4 (17-24 h)	nd	250	450	230	nd	270	200.00	70.91

Note; nd, not detectable

Table A18 Volume of urine at 0-24 h in rats administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves (Group AE)

Interval	Volume of urine (mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	4.30	4.80	5.00	5.80	2.00	7.10	4.83	0.69
2 (0-8 h)	8.50	9.00	1.90	4.50	1.00	3.00	4.65	1.38
3 (9-16 h)	2.00	2.00	2.90	2.70	1.00	17.50	4.68	2.58
4 (17-24 h)	3.50	6.00	3.80	4.80	1.50	2.80	3.73	0.64

Table A19 Urine concentrations of mitragynine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 25 mg/kg of caffeine (Group AE+CF)

Interval	Urine concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	0	0	0	0	0	0	0	0
2 (0-8 h)	600	250	310	360	540	110	362	74.64
3 (9-16 h)	110	1220	1630	150	410	370	648	255.84
4 (17-24 h)	150	430	nd	nd	280	nd	143	73.62

Note; nd, not detectable

Table A20 Volume of urine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 25 mg/kg of caffeine (Group AE+CF)

Interval	Volume of urine (mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	15.00	2.50	22.30	15.50	15.00	14.20	14.08	2.62
2 (0-8 h)	15.00	7.00	6.30	2.30	14.90	8.30	8.97	2.06
3 (9-16 h)	7.60	12.50	5.50	4.40	12.80	9.50	8.72	1.44
4 (17-24 h)	10.40	11.80	5.60	9.20	6.80	14.50	9.72	1.33

Table A21 Urine concentrations of mitragynine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 3 mg/kg of codeine (Group AE+CD)

Interval	Urine concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	0	0	0	0	0	0	0	0
2 (0-8 h)	1420	340	200	nd	570	240	461.67	206.25
3 (9-16 h)	290	nd	340	880	210	390	351.67	119.51
4 (17-24 h)	nd	nd	220	260	nd	nd	80.00	50.87

Note; nd, not detectable

Table A22 Volume of urine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 3 mg/kg of codeine (Group AE+CD)

Interval	Volume of urine (mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	14.50	14.60	14.40	15.00	15.00	15.00	14.75	0.11
2 (0-8 h)	1.50	15.20	5.50	3.00	2.50	8.10	5.97	2.09
3 (9-16 h)	13.00	14.00	3.50	3.30	5.40	12.20	8.57	2.05
4 (17-24 h)	6.80	14.10	4.30	7.20	5.90	8.50	7.80	1.38

Table A23 Urine concentrations of mitragynine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves, 25 mg/kg of caffeine and 3 mg/kg of codeine (Group AE+CF+CD)

Interval	Urine concentration of mitragynine (ng/mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	0	0	0	0	0	0	0	0
2 (0-8 h)	nd	nd	350	780	nd	180	218.33	126.16
3 (9-16 h)	1160	620	690	430	680	890	745.00	102.55
4 (17-24 h)	nd	350	nd	340	500	320	251.67	83.78

Note; nd, not detectable

Table A24 Volume of urine at 0-24 h in rats co-administrated a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves, 25 mg/kg of caffeine and 3 mg/kg of codeine (Group AE+CF+CD)

Interval	Volume of urine (mL)							
	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.E.
1 (baseline)	13.50	13.70	11.00	14.10	16.50	15.70	14.08	0.78
2 (0-8 h)	4.20	8.00	1.20	8.00	3.00	2.20	4.43	1.20
3 (9-16 h)	1.30	1.80	2.20	14.50	1.20	13.50	5.75	2.62
4 (17-24 h)	3.00	2.80	2.00	15.00	1.30	2.50	4.43	2.13

Appendix-7

Pharmacokinetic parameters of mitagynine

Table A25 Pharmacokinetic parameters derived from a non-compartmental analysis of mitragynine in rats receiving a single oral dose of 100 mg alkaloid extract from kratom leaves per kg of body weight (Group AE)

Parameters	**Units**	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.E.**
C_{max}	ng/mL	1010.00	410.00	1130.00	1130.00	580.00	750.00	835.00	123.52
T_{max}	h	2.00	1.00	2.00	2.00	2.00	2.00	1.83	0.17
kab	1/h	0.13	0.22	0.14	0.31	0.13	0.11	0.17	0.03
$t_{1/2\ ab}$	h	5.46	3.11	4.81	2.24	5.29	6.60	4.58	0.66
kel	1/h	0.00	0.00	0.02	0.01	0.01	0.00	0.01	0.00
$t_{1//2\ el}$	h	187.30	533.08	43.31	113.61	82.50	144.38	184.03	72.70
Vd/F	L /kg	125.81	113.61	65.35	73.04	222.95	107.35	118.02	23.10
Cl/F	L/h	0.47	0.15	1.05	0.45	1.87	0.52	0.75	0.25
Clh	L/h	0.47	0.15	1.05	0.45	1.87	0.51	0.75	0.25
Clr	L/h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$AUC_{0\rightarrow\infty}$	ng h/mL	50270.74	159113.65	21997.50	52297.95	12868.81	45993.33	57090.33	21442.90
$AUMC_{0\rightarrow\infty}$	ng h^2/mL	12785713.38	122095715.46	1022385.00	8103862.31	1391253.28	9265127.78	25777342.87	19358892.12
$MRT_{0\rightarrow\infty}$	h	254.34	767.35	46.48	154.96	108.11	201.45	255.45	106.55

Table A26 Pharmacokinetic parameters derived from a non-compartmental analysis of mitragynine in rats receiving a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 25 mg/kg of caffeine (Group AE+CF)

Parameters	**Units**	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.E.**
C_{max}	ng/mL	970.00	1540.00	790.00	1650.00	1940.00	1030.00	1320.00	185.26
T_{max}	h	2.00	3.00	2.00	2.00	2.00	2.00	2.17	0.17
kab	1/h	0.16	0.23	0.12	0.13	0.22	0.22	0.18	0.02
$t_{1/2\ ab}$	h	4.33	3.00	5.68	5.54	3.18	3.19	4.15	0.50
kel	1/h	0.02	0.05	0.01	0.07	0.01	0.01	0.03	0.01
$t_{1//2\ el}$	h	28.88	12.83	69.30	9.49	69.30	49.50	39.88	10.96
Vd/F	L /kg	62.35	36.80	83.01	39.29	50.23	100.10	61.96	10.30
Cl/F	L/h	1.50	1.99	0.83	2.87	0.50	1.40	1.51	0.34
Clh	L/h	1.49	1.99	0.83	2.87	0.50	1.40	1.51	0.34
Clr	L/h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$AUC_{0\rightarrow\infty}$	ng h/mL	16105.83	11674.91	28190.00	8053.84	45792.50	16697.86	21085.82	5667.82
$AUMC_{0\rightarrow\infty}$	ng h^2/mL	557250.56	166019.40	2334550.00	73511.82	4095740.00	756280.15	1330558.65	646099.92
$MRT_{0\rightarrow\infty}$	h	34.60	14.22	82.81	9.13	89.44	45.29	45.92	13.84

Table A27 Pharmacokinetic parameters derived from a non-compartmental analysis of mitragynine in rats receiving a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves and 3 mg/kg of codeine (group AE+CD)

Parameters	**Units**	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.E.**
C_{max}	ng/mL	470.00	620.00	1310.00	980.00	770.00	770.00	820.00	120.19
T_{max}	h	1.00	2.00	2.00	2.00	2.00	2.00	1.83	0.17
kab	1/h	0.30	0.20	0.28	0.29	0.17	0.32	0.26	0.02
$t_{1/2\ ab}$	h	2.33	3.47	2.50	2.37	4.01	2.17	2.81	0.30
kel	1/h	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.00
$t_{1//2\ el}$	h	53.31	113.61	76.15	99.00	173.25	99.00	102.39	16.60
Vd/F	L /kg	154.77	77.76	84.25	49.60	95.79	78.93	90.18	14.34
Cl/F	L/h	2.01	0.47	0.77	0.35	0.38	0.55	0.76	0.26
Clh	L/h	2.01	0.47	0.77	0.35	0.38	0.55	0.76	0.26
Clr	L/h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$AUC_{0\rightarrow\infty}$	ng h/mL	11829.04	49541.11	29347.72	67103.21	59505.00	41087.50	43068.93	8283.78
$AUMC_{0\rightarrow\infty}$	ng h^2/mL	887542.00	7793895.58	2723223.30	9197746.68	14453005.00	5113469.29	6694813.64	1997570.49
$MRT_{0\rightarrow\infty}$	h	75.03	157.32	92.79	137.07	242.89	124.45	138.26	24.20

Table A28 Pharmacokinetic parameters derived from a non-compartmental analysis of mitragynine in rats receiving a single oral dose of 100 mg/kg of alkaloid extract from kratom leaves, 25 mg/kg of caffeine and 3 mg/kg of codeine (Group AE+CF+CD)

Parameters	**Units**	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.E.**
C_{max}	ng/mL	790.00	660.00	450.00	500.00	780.00	550.00	621.67	58.97
T_{max}	h	1.00	1.00	2.00	2.00	1.00	3.00	1.67	0.33
kab	1/h	0.26	0.76	0.26	0.18	0.15	0.66	0.38	0.11
$t_{1/2\ ab}$	h	2.63	0.91	2.66	3.85	4.62	1.05	2.62	0.60
kel	1/h	0.00	0.00	0.00	0.00	0.01	0.00	0.01	0.00
$t_{1//2\ el}$	h	216.56	173.25	198.00	144.38	57.75	223.55	168.91	25.22
Vd/F	L /kg	172.91	79.75	173.95	128.68	44.04	76.12	112.57	22.19
Cl/F	L/h	0.55	0.32	0.61	0.62	0.53	0.24	0.48	0.07
Clh	L/h	0.55	0.32	0.61	0.62	0.53	0.24	0.48	0.07
Clr	L/h	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
$AUC_{0\rightarrow\infty}$	ng h/mL	41387.50	72410.00	37285.71	37560.83	43523.33	99593.39	55293.46	10372.06
$AUMC_{0\rightarrow\infty}$	ng h^2/mL	12656247.50	17897277.50	10226629.44	7383254.44	3495126.11	31361344.71	13836646.62	4028305.25
$MRT_{0\rightarrow\infty}$	h	305.80	247.17	274.28	196.57	80.30	314.89	236.50	35.82

Appendix-8

Amount and %amount excreted of mitragynine in urine

Table A29 Amount and %Amount of excreted of mitragynine in urine of rats given a single oral dose of 100 mg/kg of alkaloid extract (Group AE)

Interval / n	0-8 h		9-16 h		17-24 h		0-24 h	
	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount
n_1	5185	22.64	480	2.10	nd	nd	5665	24.74
n_2	4050	17.69	540	2.36	1500	6.55	6090	26.59
n_3	2584	10.77	2784	11.60	1710	7.13	7078	29.49
n_4	1440	5.98	2457	10.20	1104	4.58	5001	20.75
n_5	1200	5.08	1090	4.62	nd	nd	2290	9.70
n_6	1470	6.05	4725	19.44	756	3.11	6951	28.60
Mean	2654.83	11.37	2012.67	8.39	1267.50	5.34	5512.50	23.31
S.E.	667.20	2.97	671.41	2.75	211.78	0.92	719.47	3.00

Note; nd, not detectable

Table A30 Amount and %Amount of excreted of mitragynine in urine of rats given a single oral dose 100 mg/kg of alkaloid extract from kratom leaves and 25 mg/kg of caffeine (Group AE+CF)

Interval / n	0-8 h		9-16 h		17-24 h		0-24 h	
	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount
n_1	9300	38.43	836	3.45	1560	6.45	11696	48.33
n_2	1750	7.45	15250	64.89	5074	21.59	22074	93.93
n_3	1953	8.14	8965	37.35	nd	nd	10918	45.49
n_4	828	3.38	660	2.69	nd	nd	1488	6.07
n_5	8046	34.53	5248	22.52	1904	8.17	15198	65.23
n_6	913	3.89	3515	14.96	nd	nd	4428	18.84
Mean	3798.33	15.97	5745.67	24.31	2846.00	12.07	10967.00	46.32
S.E.	1560.87	6.55	2279.99	9.68	4929.56	3.39	3025.01	12.92

Note; nd, not detectable

Table A31 Amount and %Amount of excreted of mitragynine in urine of rats given a single oral dose 100 mg/kg of alkaloid extract from kratom leaves and 3 mg/kg of codeine (Group AE+CD)

Interval / n	0-8 h		9-16 h		17-24 h		0-24 h	
	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount
n_1	2130	9.26	3770	16.39	nd	nd	5900	25.65
n_2	5168	21.71	nd	nd	nd	nd	5168	21.71
n_3	1100	4.64	1190	5.02	946	3.99	3236	13.65
n_4	nd	nd	2904	12.63	1872	8.14	4776	20.77
n_5	1425	6.14	1134	4.89	nd	nd	2559	11.03
n_6	1944	8.00	4758	19.58	nd	nd	6702	27.58
Mean	2353.40	9.95	2751.20	11.70	1409.00	6.07	4723.50	20.07
S.E.	727.09	3.04	712.10	2.97	463.07	1.20	642.95	2.67

Note; nd, not detectable

Table A32 Amount and %Amount of excreted mitragynine in urine of rats given a single oral dose 100 mg/kg of alkaloid extract from kratom leaves, 25 mg/kg of caffeine and 3 mg/kg of codeine (Group AE+CF+CD)

Interval / n	0-8 h		9-16 h		17-24 h		0-24 h	
	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount	Amount (ng)	%Amount
n_1	nd	nd	1508	6.42	nd	nd	1508	6.42
n_2	nd	nd	1116	4.75	980	4.17	2096	8.92
n_3	420	1.77	1518	6.41	nd	nd	1938	8.17
n_4	6240	26.44	6235	26.42	5100	21.61	17575	74.47
n_5	nd	nd	816	3.41	650	2.72	1466	6.13
n_6	396	1.73	12015	52.47	800	3.49	13211	57.69
Mean	2352.00	9.98	3868.00	16.65	1882.50	8.00	6299.00	26.97
S.E.	1944.07	8.23	1825.71	7.97	1074.62	4.55	2932.70	12.57

Note; nd, not detectable

Appendix-9

Latency of nociceptive response

Table A33 Latency of nociceptive response in rats administered a single oral dose of distilled water

Group	Compound	n	Weight (g)	Latency of nociceptive response (sec)				
				30	**60**	**90**	**120**	**150**
DW	Distilled water	1	236	6.9	9.3	8.8	9.1	8.6
	(5 mL/kg)	2	242	8.3	8.5	10.2	8.6	9.8
		3	240	9.9	7.9	7.9	9.0	8.3
		4	229	9.2	10.1	9.6	8.6	8.8
		5	231	9.3	9.3	8.6	6.9	7.3
		6	231	8.8	6.7	9.3	9.2	8.5
		Mean ± S.E.	234.83 ± 2.18	8.73 ± 0.43	8.63 ± 0.49	9.07 ± 0.33	8.57 ± 0.35	8.55 ± 0.33

Table A34 Latency of nociceptive response in rats administered a single oral dose of propylene glycol

Group	Compound	n	Weight (g)	Latency of nociceptive response (sec)				
				30	**60**	**90**	**120**	**150**
PG	Propylene glycol (2 mL/kg)	1	243	8.7	9.4	9.1	8.3	9.8
		2	238	6.9	7.6	7.8	9.2	7.9
		3	237	8.2	7.8	9.0	8.9	10.4
		4	235	10.2	8.1	9.5	9.0	8.2
		5	233	8.6	7.1	7.9	9.2	10.5
		6	231	6.9	10.9	10.5	6.3	9.4
		Mean ± S.E.	236.17 ± 1.72	8.25 ± 0.51	8.48 ± 0.58	8.97 ± 0.41	8.48 ± 0.46	9.37 ± 0.45

Table A35 Latency of nociceptive response in rats administered a single oral dose of alkaloid extract from kratom leaves

Group	Compound	n	Weight (g)	Latency of nociceptive response (sec)				
				30	**60**	**90**	**120**	**150**
AE	Alkaloid extract	1	244	9.1	10.7	12.6	9.6	8.3
	(100 mg/kg)	2	242	10.3	10.1	11.8	13.4	11.4
		3	237	9.8	11.8	12.1	10.3	7.6
		4	238	11.5	11.5	12.2	12.8	8.3
		5	233	11.5	13.8	10.7	10.7	7.4
		6	228	10.6	12.3	13.2	11.9	11.0
		Mean ± S.E.	237.00 ± 2.39	10.47 ± 0.39	11.70 ± 0.53	12.10 ± 0.34	11.45 ± 0.61	9.00 ± 0.71

Table A36 Latency of nociceptive response in rats co-administered a single oral dose of alkaloid extract from kratom leaves and caffeine

Group	Compounds	n	Weight (g)	Latency of nociceptive response (sec)				
				30	**60**	**90**	**120**	**150**
AE+CF	Alkaloid extract	1	237	8.9	8.9	11.2	10.3	6.8
	(100 mg/kg) +	2	243	11.2	12.6	12.1	11.0	9.2
	Caffeine (25 mg/kg)	3	232	9.9	14.2	11.2	7.6	7.7
		4	237	10.9	15.1	9.0	8.1	7.2
		5	227	10.2	8.8	13.7	6.8	10.1
		6	229	7.7	8.3	9.9	12.0	8.7
		Mean ± S.E.	234.17 ± 2.43	9.80 ± 0.53	11.32 ± 1.23	11.18 ± 0.67	9.30 ± 0.85	8.28 ± 0.52

Table A37 Latency of nociceptive response in rats co-administered a single oral dose of alkaloid extract from kratom leaves and codeine

Group	Compounds	n	Weight (g)	Latency of nociceptive response (sec)				
				30	**60**	**90**	**120**	**150**
AE+CD	Alkaloid extract	1	240	11.8	14.3	13.8	9.7	7.3
	(100 mg/kg) +	2	236	9.8	12.3	8.2	7.8	6.5
	Codeine (3 mg/kg)	3	234	10.7	11.2	13.0	11.9	9.6
		4	231	16.2	12.5	12.7	10.3	9.6
		5	224	10.6	9.1	11.3	8.3	7.6
		6	224	9.5	9.9	11.4	8.6	9.3
		Mean ± S.E.	231.50 ± 2.66	11.43 ± 1.01	11.55 ± 0.77	11.73 ± 0.81	9.43 ± 0.62	8.32 ± 0.55

Table A38 Latency of nociceptive response in rats co-administered a single oral dose of alkaloid extract from kratom leaves, caffeine and codeine

Group	Compounds	n	Weight (g)	Latency of nociceptive response (sec)				
				30	60	90	120	150
AE+CF+CD	Alkaloid extract	1	246	14.4	11.2	11.2	9.6	7.4
	(100 mg/kg) +	2	231	14.3	14.7	13.0	9.0	9.7
	Caffeine (25 mg/kg)	3	235	10.4	13.7	11.2	12.3	10.5
	+Codeine (3 mg/kg)	4	230	13.8	11.8	9.2	8.0	12.0
		5	225	13.6	9.8	9.4	7.0	6.5
		6	225	8.4	11.1	8.4	8.3	8.3
		Mean ± S.E.	232.00 ± 3.20	12.48 ± 1.02	12.05 ± 0.74	10.40 ± 0.69	9.03 ± 0.75	9.07 ± 0.84

Appendix -10

ที่ ศธ 0521.11/ ๖๘๗

สำนักวิจัยและพัฒนา
มหาวิทยาลัยสงขลานครินทร์
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Ref.16/50

หนังสือรับรอง

โครงการวิจัย เรื่อง **ผลของแคฟเฟอีนและโคเดอีนต่อเภสัชจลนศาสตร์ และการออกฤทธิ์ลดปวดของสารแอลคาลอยด์จากใบกระท่อม**

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ได้ผ่านการพิจารณาและเห็นชอบจาก คณะกรรมการจรรยาบรรณการใช้สัตว์ทดลอง มหาวิทยาลัยสงขลานครินทร์

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List of Publications and Proceedings

Poster presentation

Botpiboon, O., Prutipanlai, S., Janchawee, B. and Thainchaiwattana, S. 2009. Effect of Caffeine and Codeine on Antinociceptive Activity of Alkaloid Extract from Leaves of Kratom *(Mitragyna speciosa* Korth.*)*. Proceeding of the 35th Congress on Science and Technology of Thailand (STT 35), Chonburi, Thailand, 15-17 October 2009.