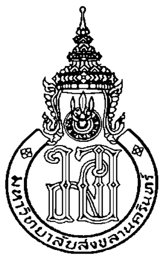

Effects of Mitragynine on Elimination of Permethrin in Rats

Kachamas Srichana

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

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Thesis Title Effects of Mitragynine on Elimination of Permethrin in Rats

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ชื่อวิทยานิพนธ์ ผลของมิตรากัยนีนต่อการกำจัดออกของเพอร์เมทรินในหนูขาว
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บทคัดย่อ

สารสกัดจากใบกระท่อมและยากันยุงมักถูกนำมาใช้เป็นส่วนผสมในสูตรเครื่องดื่มน้ำกระท่อม ใบกระท่อมประกอบด้วยมิตรากัยนีนซึ่งเป็นสารแอลคาลอยด์หลัก ในขณะที่ยากันยุงมีส่วนประกอบของไพรีทรอยด์สังเคราะห์เป็นหลัก สารประกอบทั้งสองจะเกิดปฏิกิริยาไฮโดรลัยซิสด้วยเอนไซม์คาร์บอกซิลเอสเทอเรสในร่างกาย งานวิจัยนี้มีจุดมุ่งหมายเพื่อศึกษาผลของมิตรากัยนีนต่อการกำจัดออกและประเมินความเป็นพิษต่อระบบประสาทของเพอร์เมทรินในหนูขาว

การทดลองประกอบด้วย 2 ระยะ ระยะที่ 1 สัตว์ทดลองได้รับเพอร์เมทรินทางปากขนาด 460 มิลลิกรัม/กิโลกรัม ระยะที่ 2 สัตว์ทดลองตัวเดียวกันได้รับมิตรากัยนีนทางปากขนาด 40 มิลลิกรัม/กิโลกรัม หรือสารสกัดแอลคาลอยด์ทางปากขนาด 100 มิลลิกรัม/กิโลกรัม ก่อนได้รับเพอร์เมทรินทางปากขนาด 460 มิลลิกรัม/กิโลกรัม เป็นเวลา 2 ชั่วโมง ใช้หนูขาวเพศผู้สายพันธุ์วิสตาร์ โดยแบ่งเป็น 4 กลุ่ม (กลุ่มละ 6 ตัว) ตามการได้รับยา กลุ่มที่ 1 ได้รับมิตรากัยนีนทางปากครั้งเดียว ขนาด 40 มิลลิกรัม/กิโลกรัม กลุ่มที่ 2 ได้รับสารสกัดแอลคาลอยด์ทางปากครั้งเดียว ขนาด 100 มิลลิกรัม/กิโลกรัม กลุ่มที่ 3 ได้รับมิตรากัยนีนทางปากขนาด 40 มิลลิกรัม/กิโลกรัม วันละครั้งเป็นเวลา 4 วัน กลุ่มที่ 4 ได้รับสารสกัดแอลคาลอยด์ทางปากขนาด 100 มิลลิกรัม/กิโลกรัม วันละครั้งเป็นเวลา 4 วัน วัดความเข้มข้นของเพอร์เมทรินและเมแทบอไลท์ (เฟน็อกซีเบนซิล อัลกอฮอล์) ในพลาสมาที่เวลา 8, 14, 18, และ 22 ชั่วโมง หลังจากได้รับเพอร์เมทริน โดยเทคนิคโครมาโทรกราฟีแบบของเหลวสมรรถนะสูง หาค่าอัตราส่วนเมแทบอลิคของเพอร์เมทริน เปอร์เซนต์การเปลี่ยนแปลงของอัตราส่วนเมแทบอลิคของเพอร์เมทริน ค่าคงที่ของอัตราการกำจัดออก ค่าครึ่งชีวิตของการกำจัดออก รวมทั้งเวลาเริ่มเกิด และระยะเวลาการเกิดการตอบสนองต่อเสียงโดยการสะดุ้ง และเปรียบเทียบค่าดังกล่าวในระยะที่ 1 และระยะที่ 2

ผลการทดลองแสดงว่า ค่าอัตราส่วนเมแทบอลิคของเพอร์เมทรินในระยะที่ 1 ของแต่ละกลุ่มของสัตว์ทดลอง เพิ่มขึ้นอย่างมีนัยสำคัญ (p<0.05) เมื่อเวลาผ่านไปหลังจากได้รับเพอร์เมทริน การได้รับมิตรากัยนีนครั้งเดียวและหลายครั้งทำให้ค่าอัตราส่วนเมแทบอลิคของเพอร์เมทรินที่เวลาต่างๆในระยะที่ 2 ลดลงอย่างมีนัยสำคัญ (p<0.05) โดยลดลงประมาณ 50-

80% ในช่วง 14-22 ชั่วโมง หลังจากได้รับเพอร์เมทริน ($p<0.05$) การได้รับสารสกัดแอลคาลอยด์ครั้งเดียวและหลายครั้งทำให้ค่าอัตราส่วนเมแทบอลิคของเพอร์เมทรินลดลงอย่างมีนัยสำคัญเช่นเดียวกัน โดยลดลงประมาณ 30-60% ในช่วง 14-22 ชั่วโมง หลังจากได้รับเพอร์เมทริน เปอร์เซ็นต์การลดลงของค่าอัตราส่วนเมแทบอลิคของเพอร์เมทรินในแต่ละกลุ่มของสัตว์ทดลองที่เวลา 8, 14, 18, และ 22 ชั่วโมง เพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) เปอร์เซ็นต์การลดลงของค่าอัตราส่วนเมแทบอลิคของเพอร์เมทรินหลังจากได้รับมิตรากัยนีนหรือสารสกัดแอลคาลอยด์แบบหลายครั้งไม่แตกต่างกันอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับค่าที่ได้หลังจากได้รับสารดังกล่าวแบบครั้งเดียว เปอร์เซ็นต์การลดลงของค่าอัตราส่วนเมแทบอลิคของเพอร์เมทรินหลังจากการได้รับสารมิตรากัยนีนแบบครั้งเดียวและหลายครั้งมีค่ามากกว่าอย่างมีนัยสำคัญเมื่อเทียบกับค่าที่ได้หลังจากการได้รับสารสกัดแอลคาลอยด์แบบครั้งเดียวและหลายครั้ง

การได้รับมิตรากัยนีนและสารสกัดแอลคาลอยด์แบบครั้งเดียวและหลายครั้งทำให้ลดค่าเฉลี่ยของค่าคงที่อัตราการกำจัดออกของเพอร์เมทริน จาก 0.059─0.062 ต่อชั่วโมง ในระยะที่ 1 เป็น 0.024─0.035 ต่อชั่วโมง ในระยะที่ 2 ($p<0.000$) การได้รับมิตรากัยนีนแบบครั้งเดียวและหลายครั้งและการได้รับสารสกัดแอลคาลอยด์แบบครั้งเดียวทำให้เพิ่มค่าครึ่งชีวิตการกำจัดออกของเพอร์เมทรินอย่างมีนัยสำคัญขึ้นเป็น 2 เท่า ($p<0.000$) (จาก 11.42 เป็น ถึง 21.77 ชั่วโมง) การเพิ่มขึ้นของค่าครึ่งชีวิตของการกำจัดออกของเพอร์เมทรินมีค่าสูงสุดหลังจากได้รับสารสกัดแอลคาลอยด์แบบหลายครั้ง (29.08 ชั่วโมง)

เวลาเริ่มเกิดการตอบสนองต่อเสียงโดยการสะดุ้งเพิ่มขึ้นหลังจากได้รับมิตรากัยนีนและสารสกัดแอลคาลอยด์แบบครั้งเดียว ($p<0.008$) เวลาเริ่มเกิดการตอบสนองต่อเสียงโดยการสะดุ้งหลังจากได้รับสารสกัดแอลคาลอยด์แบบครั้งเดียวนานกว่าเมื่อได้รับมิตรากัยนีนแบบครั้งเดียวอย่างมีนัยสำคัญ ($p<0.05$) ระยะเวลาการเกิดการตอบสนองต่อเสียงโดยการสะดุ้งนานขึ้นอย่างมีนัยสำคัญหลังจากได้รับมิตรากัยนีนและสารสกัดแอลคาลอย์แบบครั้งเดียว ($p<0.008$) ระยะเวลาการเกิดการตอบสนองต่อเสียงโดยการสะดุ้งหลังจากได้รับสารสกัดแอลคาลอยด์แบบครั้งเดียวนานกว่าอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับกลุ่มที่ได้รับสารมิตรากัยนีนแบบครั้งเดียว ($p<0.05$)

โดยสรุป การได้รับสารมิตรากัยนีน และสารสกัดแอลคาลอยด์ ทั้งแบบครั้งเดียวและหลายครั้ง ทำให้ลดค่าอัตราส่วนเมแทบอลิคของเพอร์เมทริน และทำให้การกำจัดออกของเพอร์เมทรินช้าลง ซึ่งเป็นไปได้ว่าอาจเกิดจากการขัดขวางการเกิดฮัยโดรลัยซิสของเพอร์เมทริน เหตุนี้ส่งผลให้เพิ่มความเป็นพิษของเพอร์เมทริน ผลการศึกษาชี้ให้เห็นว่า การได้รับสารอัลคาลอยด์จากกระท่อมและสารฆ่าแมลงไพรีทรอยด์อาจเปลี่ยนแปลงพฤติกรรมทางจลนศาสตร์และเพิ่มความเสี่ยงในการเกิดพิษจากสารไพรีทรอยด์ในร่างกายของสิ่งมีชีวิตได้

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ABSTRACT

Water extracts of kratom leaves and mosquito coils are usually mixed for kratom cocktail formulas. Kratom leaves contain a major alkaloid mitragynine (MG), while mosquito coils contain neurotoxic synthetic pyrethroid insecticides. Both compounds are subjected to a hydrolytic reaction catalyzed by carboxylesterase *in vivo*. This research is aimed to investigate the effects of MG on the elimination and assess the neurotoxicity of permethrin (PM) in rats.

The experiment consisted of two phases, phase I: single oral administration of PM (460 mg/kg) and phase II: pretreatment with MG (40 mg/kg) or AE (100 mg/kg) 2 h before receiving PM. Male wistar rats were divided into four groups (n=6 each); group 1, single oral dose of MG (40 mg/kg); group 2, single oral dose of AE (100 mg/kg); group 3, 4-day multiple oral doses of MG (40 mg/kg); group 4, 4-day multiple oral doses of AE (100 mg/kg). Plasma concentrations of PM and its metabolite, phenoxybenzyl alcohol (PBAlc), at 8, 14, 18, and 22 h after receiving PM were determined by the validated HPLC method. Permethrin metabolic ratio (PMR), percent change in PMR, elimination rate constant (k_{el}) and elimination half-life ($t_{1/2\ el}$) as well as the onset and duration of the acoustic startle response (ASR) of PM in phase I and phase II were determined and compared.

The results showed that phase I PMRs of each group of rats significantly increased with time after receiving PM. A single and multiple dose(s) of MG caused a significant decrease in phase II PMRs over time ($p< 0.05$) approximately 50-80% at 14-22 h after PM administration. A single and multiple dose(s) of AE also caused a significant reduction of PMRs approximately 30-60% at 14-22 h after PM dose. The percentages of decrease in PMRs of each group of rats at 8, 14, 18, and 22 h were significantly increased with time ($p< 0.05$). The percentage

of decrease in PMRs after multiple doses of MG or AE was not significantly different from that after a single dose. The percentage of decrease in PMRs after a single and multiple dose(s) of MG were significant greater than that produced by a single and multiple dose(s) of AE.

A single and multiple dose(s) of MG and AE significantly decreased the average k_{el} of PM, from 0.059–0.062 h^{-1} in phase I to 0.024–0.035 h^{-1} in phase II ($p<0.000$). A single and multiple dose(s) of MG and a single dose of AE significantly prolonged $t_{1/2\ el}$ of PM for 2-folds (from 11.42 to 21.77 h) ($p<0.000$). The increase in $t_{1/2\ el}$ of PM was the highest after pretreatment with multiple dose(s) of AE (29.08 h).

The onset of ASR of PM was significantly delayed by a single dose of MG and AE ($p<0.008$). The onset of ASR of PM after a single dose of AE was significantly delayed after a single dose of MG ($p<0.05$). The duration of ASR was significantly prolonged by a single dose of MG and AE ($p<0.008$). The duration of ASR in phase II in rats given a single dose of AE was significantly longer than those resulted from a single dose of MG ($p<0.05$).

In conclusion, a single and multiple dose(s) of MG and AE caused a decrease in PMRs and delayed elimination of PM probably by interfering with PM hydrolysis. That effect leaded to prolonged ASR a neurotoxicity of PM. The results suggest that concurrent ingestion of kratom alkaloids and pyrethroid insecticides could alter kinetic behavior and increase risk of toxicity of pyrethroids *in vivo*.

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Kachamas Srichana

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Figure **Page**

4.9 Permethrin metabolic ratios (PMRs) compared between phase I and phase II in rats receiving a single and multiple dose(s) of MG and AE; (A) MG single dose; (B) MG multiple dose; (C) AE single dose; (D) AE multiple dose. Significance differences were considered at $p < 0.05$ (Mean ± SEM). 76

* compared between phase I and phase II using Student paired *t*-test.

^{a} compared with phase I at 8 h, ^{b} compared with 14 h, and ^{c} compared with 18 h using ANOVA.

^{A} compared with phase II at 8 h, ^{B} compared with 14 h, and ^{C} compared with 18 h using ANOVA.

4.10 Percentage of the decrease in permethrin metabolic ratios (PMRs) after the oral administration of mitragynine (MG; 40 mg/kg) and alkaloid extract (AE; 100 mg/kg) in rats (n=6). Significance differences were considered at $p < 0.05$ (Mean ± SEM). 78

^{a} compared with at 8 h, ^{b} compared with 14 h, and ^{c} compared with 18 h using ANOVA

* compared with pretreatment MG single dose, $^{\#}$ compared with pretreatment MG multiple doses, $^{\alpha}$ compared with pretreatment AE single dose using ANOVA.

List of Abbreviations

ADH	Alcohol dehydrogenase
AE	Alkaloid extract
ALDH	Aldehyde dehydrogenase
ASR	Acoustic startle response
CES	Carboxylesterase
cis- PM	Cis-permethrin
Cl/F	Clearance
C_{max}	Maximum serum concentration
CNS	Central nervous system
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CS syndrome	Choreoathetosis/Salivation syndrome
DEV	Deviation
GJIC	Gap junctional intercellular communication
2G	9-O-demethyl MG glucuronide
3G	16-carboxy MG glucuronide
4G	9-O-demethyl-16-carboxy MG glucuronide
5G	17-O-demethyl-16,17-dihydro MG glucuronide
6G	9,17-O-bisdemethyl-16,17-dihydro MG glucuronide
H	Human
HPLC	High performance liquid chromatographic
i.e.	Id est
k_{ab}	Absorption rate constant
k_{el}	Elimination rate constant
kg	Kilogram
LD_{50}	Median lethal dose
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide

List of Abbreviations (cont.)

LSD	Lysergic acid diethylamide
MG	Mitragynine
mg	Milligram
mRNA	Messenger ribonucleic acid
min	Minute
mL	Milliliter
N	Number
ng	Nanogram
nm	Nanometer
p	*p*-values
PBAcid	Phenoxybenzyl acid
PBAlc	Phenoxybenzyl alcohol
PBAld	Phenoxybenzyl aldehyde
PGE_2	Prostaglandins E_2
PM	Permethrin
PMR	Permethrin metabolic ratio
p.o.	Oral administration
QC	Quality control
R	Rat
r	Correlation coefficient
RSD	Relative standard deviation
2S	9-O-demethyl MG sulfate
4S	9-O-demethyl-16-carboxy MG sulfate
6S	9,17-O-bisdemethyl-16,17-dihydro MG sulfate
SD	Standard deviation
sec	Second
SEM	Standard error of mean

List of Abbreviations (cont.)

S/N	Signal-to-noise
SPE	Solid-phase extraction
$t_{1/2\ el}$	Elimination half-life
T_{max}	Time to maximal serum concentration
trans-PM	Trans-permethrin
T syndrome	Tremor syndrome
UV	Ultraviolet
V_d/F	Volume of distribution
vs.	Versus
v/v	Volume by volume
μg	Microgram
μL	Microliter

CHAPTER 1

Introduction

1.1 Background and rationale

Mitragynine is a major alkaloid found in *Mitragyna speciosa* Korth. (Murple, 2005), so called 'kratom' in Thai, an indigenous plant of Thailand and other countries in South East Asia. Kratom is traditionally used by southern villagers by chewing their fresh leaves to treat common illness such as coughing, diarrhea, muscle, and hypertension. It is also used to relieve tiredness and increase sun heat tolerance during working in the farms. Most Kratom users are middle aged labor workers such as farmers, gardeners and fishermen (Junsirimongkol *et al.*, 2005). However, kratom leaves were abused by employing as a major ingredient in a particular narcotic preparation so called '4×100 Kratom cocktail'. It is also called 'OTOP' or boiled Kratom leaves.

As a narcotic, kratom is popularly prepared as a cocktail '4x100', which has been widespread since 2004 among four provinces of southern border of Thailand, i.e. Songkhla, Yala, Pattani and Narathiwas. The '4×100' is a mixture of boiled leaves extract, coke, cough syrup and mosquito coil usually containing pyrethroid insecticide such as permethrin. Permethrin which has two stereoisomers; cis- and trans-forms, is classified as a type I synthetic pyrethroid. It produces a neurotoxic 'T syndrome' including tremor, exaggerated startle response, hypersensitivity and skin paresthesia. Cis-permethrin is more toxic than trans-isomer. In humans and other mammals, permethrin, mainly trans-isomer, is rapidly inactivated by hydrolysis catalyzed by carboxylesterase in the liver to phenoxybenzyl alcohol (PBAlc) followed by oxidation and conjugation prior to excretion into urine and feces. Since mitragynine was also metabolized via hydrolysis of the methylester. Therefore, it would be possible that miyragynine may affect hydrolytic metabolism of permethrin and may increase the risk of neurotoxicity of permethrin in '4x100' kratom users.

Therefore the aim of this study was to investigate any effects of MG and alkaloid extract (AE) derived from kratom leaves on the elimination of PM by determining plasma metabolic ratio, elimination rate constant, and elimination half-life of PM in rats. The toxicity of PM expressed as the acoustic startle response (ASR) of PM was also determined.

CHAPTER 2

Review of Literature

2.1 Kratom

2.1.1 Botanical origin

Kratom (Figure 2.1) is a Thai local name of *Mitragyna speciosa* Korth. which is a plant indigenous to Southeast Asia, notably in Thailand and Malaysia peninsular. Other names include 'Kakuam', 'Ithang', and 'Thom' in the southern regions of Thailand, and 'Ketum' or 'Baik-Baik' in Malaysia (Suwanlert, 1975). The botanical classification of Kratom is as follows:

Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Gentianales
Family: Rubiaceae
Genus: *Mitragyna*
Species: *Mitragyna speciosa*

Kratom grows into a large shrub or small tree. The plants can grow to a normal height of 12-15 feet, but some specimens can reach a height of 50 feet. The stem is erect and branching. The leaves are evergreen and dark glossy green color, smooth, ovate-acuminate in shape and tapered ends. The size is over 18 cm long and 10 cm wide. There are two major types of *Mitragyna speciosa*, identifiable by the color of the veins found in the leaves. One type of Kratom plant has red veins in the leaves, while the other major type has green or white veins in the leaves. The red vein Kratom is more potent than white vein type. The deep yellow flowers grow in globular clusters attached to the leaf axils on long stalks, bearing up to 120 florets each (Shellard, 1974). The seeds are winged.

Kratom trees are evergreen rather than deciduous. Leaves are constantly being shed and replaced, but there is some quasi-seasonal leaf shedding due to environmental conditions. The leaves fall abundantly during the dry season of

the year 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 grows best in wet, humid, fertile soil, with medium to full sun exposure in areas protected from strong winds. It is drought sensitive and if grown out of its native habitat, sensitive to frost. Propagation is by planting very fresh seed or cuttings. The plant parts used for consumption are the leaf and smaller stems of the trees (Macko *et al.*, 1972).

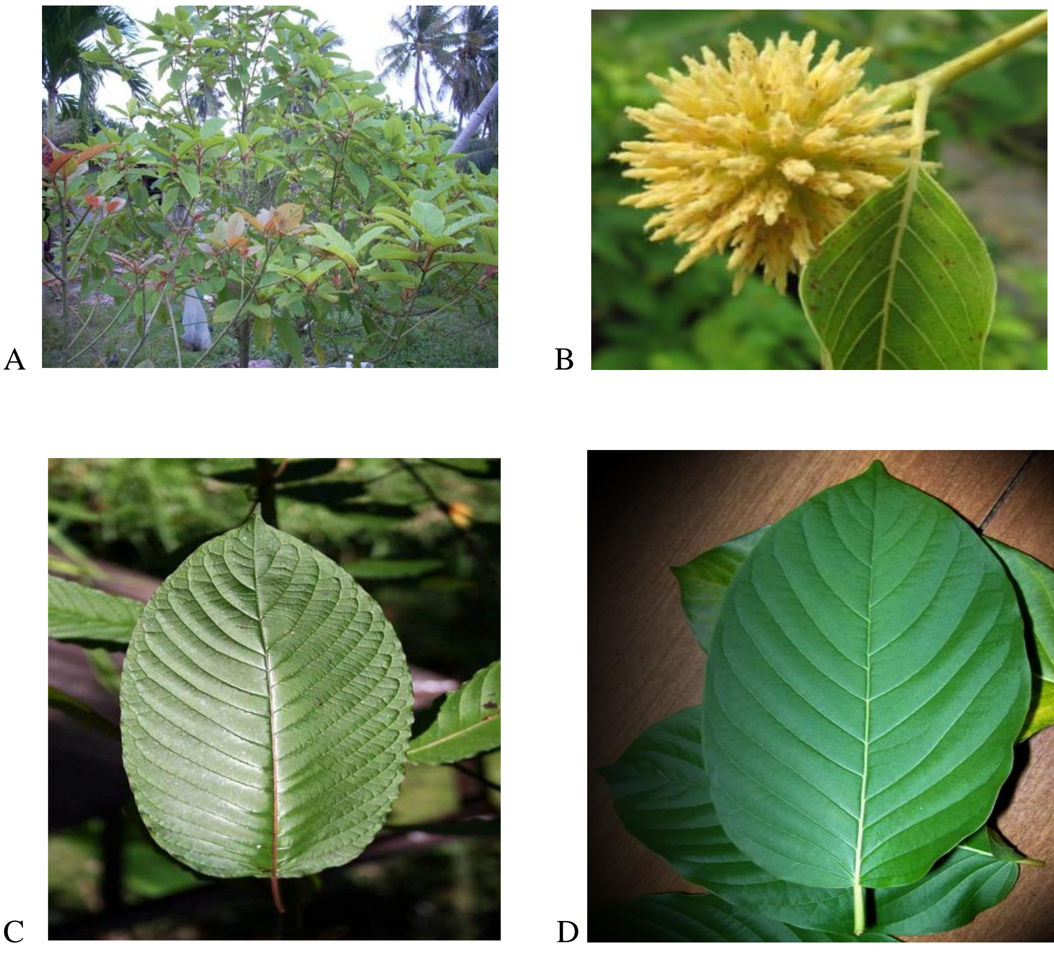

Figure 2.1 Tree (A), flower (B), red vein leaves (C) and white vein leaves (D) of kratom (sources of Figures: 1.1A and 1.1B from http://www.psychoactiveherbs.com, Figures 1.1C and 1.1D from http://www.kratom.com.

2.1.2 Traditional use

Kratom is traditionally used by people in Thailand, especially in the southern peninsula, and neighboring countries in Southeast Asia. In the nineteenth century, *Mitragyna speciosa* was reported to work as an opium substitute in the treatment of opium addiction in Malaysia and Thailand (Tanguay, 2011). Malay and Thai natives use Kratom by chewing raw leaves to enhance tolerance for hard work under the hot sun. Moreover, it has been used for cooking, recreaction, and sex enhancement. Most Kratom users are middle aged labor workers such as farmers, gardeners and fishermen (Junsirimongkol *et al.*, 2005). The leaves are also traditionally used to treat intestinal infections, muscle pain, coughing, and diarrhea (Suwanlert, 1975). Several studies suggested that *Mitragyna speciosa* preparations have analgesic, antipyretic, antidiarrheal, euphoric, anti-depressant, and anxiolytic effects. For analgesic effect have reported mitragynine less than morphine, but it does not cause significant dependence, making it potentially useful in treating minor to moderate pain. Generally, the amount of mitragynine is so high that can be practically extracted. 7-Hydrohydroxymitragynine has been shown to have much stronger analgesic effects than, but it is less abundant in the Kratom plant. They may work as immune booster, lower blood pressure, and have anti-viral, antidiabetes and appetite suppressing effects (Macko *et al.*, 1972).

In addition, many consumers use the plant as self-treatment in modulating opiate withdrawal, alcohol withdrawal, and chronic pain (Ward *et al.*, 2011). According to this study, the daily consumption of Kratom solution to alleviate opiate withdrawal symptoms is 3 × 250 mL, which contains approximately 68–75 mg mitragynine (Vicknasingam *et al.*, 2010). In fact, it is a cheaper alternative to the established opioid-replacement therapies and is obtainable without medical prescription.

2.1.3 Kratom abuse in Thailand

Since the year 1897, Kratom has been used and related with way of Thai life. Kratom was widely used among opium users for its substitution. The Thai government realized the danger of using Kratom, because it may cause addiction. Then in 1943, the government passed 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 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 that 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).

The continual usage of Kratom was found during 1922-2000, which the statistics of Kratom seized were increased from 506 cases to 1,755 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 (National Command Center for Drugs (NCCD), 2008b). The number of cases of Kratom seized has increased because users and pattern of using Kratom were changed.

Prevalence of Kratom use among secondary school students and Muslim teenagers in southern Thailand was between 2.3-4.9% in 2002–2004. The most recent National Household Survey of Substance Use found that Kratom was the

most prevalent substance used by the Thai population aged 12–65 year olds, it is 3.76% in the year 2007 (Assanangkornchai *et al.*, 2007).

Kratom leaves were used as a major 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 Narathiwas). The ‘4×100 Kratom cocktail’ is a mixture of boiled water of Kratom leaves, Coca Cola beverage (‘Coke’) which containing 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), ‘One two call’ (Kratom powder mixed with yogurt) and ‘10×100’ (many more ingredients in a mixture).

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 of 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 at a high dose it causes sedation. Caffeine is a CNS stimulant. The ingredients of cough syrup such as diphenhydramine and codeine produce a sedating effect (Office of the Narcotics Control Board (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 (National Command Center for Drugs (NCCD), 2008b).

Suwanlert (1975) investigated 30 Thai Kratom users, who were old and married men abusing Kratom over 5 years. The stimulant effect occurred 5-10 min after chewing the leaves and lasted 1-1.5 h. Users reported an increase in work output and tolerance of hot sunlight. They also reported that the drug 'helps to work harder' (76.6–87.5%), 'makes more active' (76.6–86.1%), 'increases sexual desire' (73.4–84.7%), and 'increase in appetite' (57.8–77.8%). These effects were self-perceived benefits. Interestingly, self-perceived response was higher among short-term than long term users. These findings differ from those in neighbouring countries where Kratom was used primarily to increase physical endurance.

2.1.4 Chemical properties of kratom leaves

Mitragyna speciosa Korth. contains over 25 alkaloids (Table 2.1), among which the alkaloid mitragynine is the majority (66.2% base on the crude base) together with its analogues, speciogynine (6.6%), speciociliatine (0.8%), and paynantheine (8.6%). In addition, a new alkaloid, 7α-hydroxy-7H-mitragynine was isolated as a minor constituent (2.0%). The structure of each compound is shown in Figure 2.2. Other constituents include flavonoids, phenylpropanoids and tannins. Alkaloids isolated from Kratom leaves vary in contents from location to location of planting and from time to time of collecting (Shellard, 1974; Takayama, 2004).

Table 2.1 Types and chemical constituents in different parts of Kratom (Assanangkornchai and Sirivonrse na Ayudhya, 2005)

Plant part	Type	Chemical compound	References
Leaves	Indole alkaloid	ajmalicine, akuammigine, angustine, corynsntheidine, 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, mitragynine, paynantheine, 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
	Flavone	apigenin, apigenin-7-O-rhamnoglucoside, cosmosiin	
	Flavonol	astragalin, hyperoside, kaempferol, quercetin, quercitrin, quercetin-3-galactoside-7-rhamnoside, quercitrin, rutin	Hinou and Harvala, 1988
	Phenylpropanoid	caffeic acid, chloronic acid	Hinou and Harvala, 1988 Houghton and Said 1986 Takayama et al., 1998
	Flavonoid	(-)-epicatechin	
	Lignin	(+)-pinoresinol	

Plant part	Type	Chemical compound	References
	Triterpene	ursolic acid	
Stem, Trunk barks	Indole alkaloid	ciliaphylline, rhynchociline, ciliaphylline, isomitraphylline, isorhynchophylline, isospecionoxeine, javaphylline, mitraciliatine, miytragynine 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 2.2 Chemical structures of selected indole alkaloids in Kratom leaves (Takayama, 2004)

2.2 Mitragynine

Mitragynine (MG; Figure 2.2) was first isolated from *Mitragyna speciosa* in 1907 by Hooper and Field, who named the alkaloid, and repeated this process in 1921. Its structure was first fully determined in 1964 by Zacharias and colleagues. In 1995, Takayama and coworkers at Chiba University were able to synthesize MG. It is structurally related to both an alkaloid yohimbine and voacangine. It is more distantly related to tryptamine-based psychedelic drugs such as psilocybin, ibogain or lysergic acid diethylamide (LSD) (Murple, 2005).

The synonym of MG is (E)-2-[(2S,3S)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahy-droindolo[3,2-h] quinolizin-2-yl]-3-methoxyprop-2-enoic acid methyl ester. Chemical formula is $C_{23}H_{30}N_2O_4$ with a molecular weight of 398.49 g/mol. Its weak base form is white, amorphous powder with melting point of 102-106 ° C and boiling point of 230-240 °C. MG is soluble in alcohol, chloroform and acetic acid (DMSC, 2008).

2.2.1 Pharmacology

Both pharmacokinetics and pharmacological actions of MG in rats and humans have been documented in several studies as followed.

1) Pharmacokinetics

1.1) Absorption

Many researchers studied the absorption of MG derived from kratom. In 2007, Janchawee and colleages studied the pharmacokinetics of MG in rats. It was found that MG was rapidly absorbed from GI tract after a single oral administration at the dose of 40 mg/kg body weight. The 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}. The half-lives of absorption ($t_{1/2\ ab}$) were 0.48 ± 0.36 h. In contrast to the study of de Moraes and colleagues (2009), the T_{max} at 1.26 ± 0.20 h and C_{max} of 0.42 μg/mL following an oral on ingestion of 20 mg/kg of MG were observed. The k_{ab} was 0.04±0.01 h^{-1}. In addition, the pharmacokinetics of MG in rats plasma after oral administration at the dose of 50 mg/kg body weight demonstrated

that MG showed an erratic and prolonged absorption. This dose led to a C_{max} of 0.70±0.21 μg/mL after T_{max} 4.5±3.6 h. The calculated absolute bioavailability for MG was 3.03±1.47 %. The low oral bioavailability may be caused by the poor aqueous solubility of MG (Parthasarathy *et al.*, 2010).

1.2) Distribution

MG had a high volume of distribution (V_d/F, 89.50 ± 30.30 L/kg). This may be due to its distribution to highly perfused and lipid-containing tissues, especially the brain, which is its site of action (Janchawee *et al.*, 2007). High V_d was also reported by Parthasarathy and colleages, i.e. 64±23 L/kg. In contrast, de Moraes and colleages (2009) reported the smaller V_d of 37.90±5.41 L/kg.

1.3) Metabolism

The phase I and II metabolism of MG in rats and humans urine were extensively studied using liquid chromatography-linear ion trap mass spectrometry. The metabolic pathways were postulated for rats and humans shown in Figure 2.3 (Philipp *et al.*, 2009). MG was metabolized in phase I by hydrolysis of methylester in position 16 (3,4). In addition, MG was O-demethylated at 9-methoxy group (2, 4, 6, 8) and at the 17-methoxy group (5-8). MG was also oxidized via the intermediate aldehydes, to carboxylic acid or reduced to alcohols and combinations of some steps (4, 6, 8). Finally, in rats four phase I metabolites were conjugated to glucuronides (2G, 3G, 4G, 6G) and one to sulfate conjugate (4S), but in human three metabolites to glucuronides (2G, 3G, 5G) and three to sulfates (2S,4S, 6S).

Keawklum and coworkers (2005) analyzed the urine samples collected from a person who had ingested kratom leaves. The three major alkaloids (mitragynine, paynantheine, speciogynine) and their metabolites can be detected. Two metabolites were identified as 9-Hydroxycorynantheidine desmethyl ester and 9-hydroxycorynantheidine. The 9-hydroxycorynantheidine desmethyl ester is a metabolite of speciogynine. These pharmacokinetic data were consistent with the study of Pingsuthiwong and coworkers (2009). Two major alkaloids (mitragynine and speciogynine) were detected in urine samples.

Figure 2.3 Postulated metabolic pathways of MG in rats and humans. Assumed intermediate metabolites in square brackets. (Philipp *et al.*, 2009)

Abbreviations; Rats (R); Humans (H); MG(1); 9-O-demethyl MG (9-O-DM-MG,2); 16-carboxy MG (16-COOH-MG, 3); 9-O-demethyl-16-carboxy MG(9-O-DM-16-COOH-MG, 4); 17-O-demethyl-16,17-dihydro MG (17-O-DM-DH-MG, 5); 9,17-O-bisdemethyl-16,17-dihydro MG (9,17-O-BDM-DH-MG, 6); 17carboxy-16,17-dihydro MG (17-COOH-DH-MG,7); 9-O-demethyl-17-carboxy-16,17-dihydro MG (9-O-DM-17-COOH-DH-MG, 8); 9-O-demethyl MG glucuronide (9-O-DM-G-MG, 2G); 9-O-demethyl MG sulfate (9-O-DM-S-MG, 2S);16-carboxy MG glucuronide (16-COOH-G-MG, 3G); 9-O-demethyl-16-carboxy MG glucuronide (9-O-DM-16-COOH-G-MG, 4G); 9-O-demethyl-16-carboxy MG sulfate (9-O-DM-16-COOH-S-MG, 4S); 17-O-demethyl-16,17-dihydro MG glucuronide (17-O-DM-DH-G-MG,5G); 9,17-O-bisdemethyl-16,17-dihydro MG glucuronide (9,17-O-BDM-DH-G-MG, 6G); and 9,17-O-bisdemethyl-16,17-dihydro MG sulfate (9,17-O-BDM-DH-S-MG, 6S).

1.4) Elimination

An animal study has shown that MG was slowly eliminated. The elimination rate constant (k_{el}) of 0.07 ± 0.01 h^{-1} and a clearance (Cl/F) of 1.60 ± 0.58 L/h were reported (Janchawee *et al.*, 2007). The elimination half-life was 9.43 ± 1.74 h. MG was gradually eliminated within 24 h from the plasma with a mean half-life of 6.6±1.3 h and a Cl/F of 7.0 ± 3.0 L/h/kg (Parthasarathy *et al.*, 2010). de Moraes and colleagues (2009) reported that the total Cl/F of MG obtained in this study was 6.35±0.43 L/h/kg, it is higher than that reported by Janchawee and colleagues. The elimination half-life of MG was 3.85±0.51 h, which was lower than that obtained from the study by Janchawee and coworkers.

2) Pharmacodynamics

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

2.1) Antinociceptive effects

Mutsumoto and coworkers (1996a) investigated an antinociceptive activity of MG in mice receiving MG by intraperitoneal and intracerebroventricular injections using pinch and hot plate test models. Its mechanism of action was evaluated by using naloxone, an opioid receptor antagonist. The results indicated that MG itself possesses antinociceptive activity by acting in the brain and the supraspinal opioid system. Thongpradichote and coworkers (1998) suggested that antinociceptive activity cause by MG dominantly mediated via μ and δ opioid receptor subtypes. In addition, the selectivity of MG for the supraspinal opioid receptor subtypes differs from that of morphine.

Mutsumoto et al., (1996b) investigated the roles of central monoaminergic system in the antinociceptive action of MG by means of the tail pinch and hot plate test in mice. It was found that both descending noradrenergic and serotonin systems involved in the antinociceptive activity of supraspinally administered MG on the mechanical noxious stimulation, while the descending

noradrenergic system predominantly contributed to the effect of supraspinal MG on the terminal noxious stimulation.

In addition, Idid *et al*., (1998) compared the antinociceptive activity of morphine and paracetamol with that of MG. Mice orally given MG (200 mg/kg) were subjected to acetic acid induced writhing, hot tail flick and cold tail flick tests. The results indicated that MG has analgesic property similar to morphine. Those effects were less pronounced than that of 5 mg/kg morphine but more evident than after 100 mg/kg paracetamol.

The opioid receptor agonistic action was assessed using the twitch contraction of the guinea pig ileum induced by electrical stimulation. Opioid agonist activity is measured as the inhibition of the twitch contraction, which is reversed by the opioid receptor antagonist naloxone. *Mitragyna speciosa* preparations, MG, and other isolated *Mitragyna speciosa* indole alkaloids as well as MG derivatives inhibited the electrically stimulated contraction. The antinociceptive effects of indole alkaloids could be blocked by naloxone, suggesting an opioid receptor mediated mechanism (Takayama *et al.*, 2002; Horie *et al.*, 2005).

Reanmongkol and coworkers (2007) studied the effect of the methanol extract from the *Mitragyna speciosa* Korth. leaves on the antinociceptive 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 day continued administration of the alkaloid extract. These results suggest that *Mitragyna speciosa* Korth. extract possesses analgesic activity which may be centrally mediated through μ receptor and no analgesic tolerance after continued administration.

In accordance with these findings was a study by Shaik Mossadeq and colleagues who showed that the methanolic extract of *Mitragyna speciosa* increased the latency of nociceptive responses in hot-plate test in mice. Findings in acetic-acid-induced writhing and the formalin test further proved that the methanolic extract has an antinociceptive activity as it significantly inhibits the writhing responses and pain sensation in both tests (Shaik Mossadeq *et al.*, 2009).

Sabetghadam *et al*. (2010) compared the antinociceptive effects of various orally administered *Mitragyna speciosa* extracts with that of morphine in rats. Alkaloid (20 mg/kg), methanolic (200 mg/kg) as well as aqueous (100–400 mg/kg) *Mitragyna speciosa* extracts significantly prolonged the latency of nociceptive responses in both the hot plate- and the tail flick tests. These and the morphine effects could be blocked by pre-administration of the opioid antagonist naloxone, which suggests an opioid-receptor mediated effect for the *Mitragyna speciosa* extracts. The antinociceptive effect of 100 mg/kg (p.o.) *Mitragyna speciosa* alkaloid extract could be further potentiated by co-administration of caffeine (25 mg/kg, p.o.) and codeine (3 mg/kg, p.o.) in a hot plate test in rats (Botpiboon *et al.*, 2007).

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. 7-hydroxymitragynine is a 46-fold more analgesic than MG (Matsumoto *et al.*, 2004; Matsumoto *et al.*, 2005). The antinociceptive mechanism of 7-hydroxymitragynine was mediated via μ opioid receptor and more potent antinociceptive activity than morphine in the tail-flick and hot-plate tests when administered orally or subcutaneously (Matsumoto *et al.*, 2006).

2.2) Anti-inflammatory effects

An intraperitoneal administration of the methanol extract of *Mitragyna speciosa* at the dose 100 and 200 mg/kg inhibited the development of a carrageenan-induced paw edema with a maximum inhibition during first 3 h after carrageenan injection (Shaik Mossadeq *et al.*, 2009). The extract may provide anti-inflammatory effect by inhibiting the synthesis, release and action of number of hyperalgesic mediators. Therefore, it suppresses the early phase of the edema, which is the characteristic of acute inflammation. Arachidonic acid and metabolites might be responsible for the inhibitory activity of the extract at a dose of 200 mg/kg for a

period of 4 h, which at this stage produces edema dependent on neutrophil mobilization.

Daily administration of *Mitragyna speciosa* extract (200 mg/kg) inhibited the growth of granuloma tissue, fibroblasts as well as the multiplication of blood vessels producing a highly vascularized and reddened mass known as granulation tissue (Shaik Mossadeq *et al.*, 2009). The authors suggested that the inhibition of pro-inflammatory mediator release and vascular permeability in addition to enhanced immunity, stimulation of tissue repair and healing processes may have contributed to the anti-inflammatory activity of *Mitragyna speciosa*.

Raja and coworker (2005) studied the anti-inflammatory property of the alkaloid extract from the leaves of *Mitragyna speciosa.* It was evaluated on the basis of the inhibitory effect of the extract on carrageenan-induced hind paw edema in rats. The intraperitoneal administration at a concentration of 50 mg/kg produced almost 100% inhibition of the carrageenan-induced paw edema 1 hour after administration. These findings suggest that the alkaloid extracts of the leaves of *Mitragyna speciosa* have potent anti-inflammatory properties.

In addition, MG was shown to inhibit mRNA expression of COX-2 induced by LPS and protein expression as well as PGE_2 formation in a dose-dependent manner in RAW 264.7 macrophage cells. However, the effect of MG on COX-1 mRNA and protein expression is dependent on concentration. It did not affect at lower concentrations, but might inhibit them at higher concentrations (Utar *et al.*, 2011).

2.3) Gastrointestinal effects

Effect of MG on electrically stimulated contraction was studied in the guinea-pig ileum. MG inhibited the ileum contraction elicited by electrical stimulation. MG was 10 fold less potent than morphine (Watanabe *et al.*, 1997). The methanolic extract of *Mitragyna speciosa* reduced the defecation frequency and fecal weight in castor oil-induced diarrhea in rats. However, the methanolic extract of *Mitragyna speciosa* may affect mechanisms other than opioid-receptor mediated since naloxone pre-treatment showed no effect on the inhibition of the defecation frequency

and fecal weight. A single dose of the methanolic extract of the *Mitragyna speciosa* also resulted in a dose-dependent reduction of the intestinal transit and fluid (Chittrakarn *et al.*, 2008).

Tsuchiya and colleagues (2002) investigated the effect of MG on gastric acid secretion induced by 2-deoxy-D-glucose. MG inhibited 2-deoxy-D-glucose-stimulated gastric acid secretion through μ opioid receptors in anesthetized rats. It was suggested that MG has a morphine-like effect on gastric acid secretion in the central nervous system. This inhibition was reversed by naloxone indicating the involvement of opioid receptors. The effects of MG, particularly anorexia and weight loss, might be related to direct inhibition of neurons in the lateral hypothalamus.

2.4) Behavioral effects

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

2.5) Other effects

Acute oral administration at doses of 100, 500 and 1000 mg/kg of standardized methanolic extract of *Mitragyna speciosa* increased blood pressure in rats 1 h after administration (Harizal *et al.*, 2010).

Chittrakan et al. (2010) reported that methanolic extract of kratom leaves caused muscle relaxation in rats. Thereby, the extract had greater effect at the neuromuscular junction than on the skeletal muscle or the somatic nerve. The kratom extract and MG (2 mg/mL) blocked the nerve conduction, amplitude and duration of compound nerve action potential.

2.2.2 Toxicity

1) Acute effects

Acute effects of kratom is dose-dependent and mainly on the central nervous system. 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 effect in visual field. MG causes euphoria similar to that of cocaine (Grewal, 1932). In people who have been received mitragynine acetate of 50 mg, nausea and vomiting were reported (Jansen and Prast, 1988). Six subjects who had received mitragynine acetate 50-100 mg or kratom powder 650-1300 mg reported increase in sun heat tolerance during working and skin red, propably because of increase in blood flow to the skin . Eating high amounts of kratom cause numbing and nausea (Suwanlert, 1975).

2) Chronic effects

Health problems are unlikely unless one is consuming large quantities of kratom everyday. In Thailand, people who use kratom everyday 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).

2.3 Pyrethrins and synthetic pyrethroids

Plant insecticides are chemicals extracted or derived from naturally poisonous materials containing in plants one of them is pyrethrins (Fingure 2.4).

H_3C CH_3 CH_3 H_3C H CH_2 R O R_1 O O

Figure 2.4 Chemical structure of pyrethrins (Perger and Szadkowski, 1994)

Pyrethrins are derived from crude extract of the Chrysanthemum flowers (Pyrethrum). The plant extract, called pyrethrum contains six insecticidal active compounds (Table 2.2). The insecticidal properties of pyrethrins are derived from ketoalcoholic esters of chrysanthemic and pyrethroic acids. These acids are strongly lipophilic and rapidly penetrate many insects and paralyze their nervous system. It has been used to control pests for over a century and is generally considered quite safe. They have the ability to 'knock down' insects quickly, and low toxicity to mammals (including humans). Formulations containing pyrethrins usually also contain a chemical such as piperonyl butoxide, which is known as a 'synergist', because it enhances the effects of the pyrethrins. It prevents insects from recovering from the effects of the pyrethrins.

Table 2.2 Structures of the six natural pyrethrins (Soderlund *et al.*, 2002)

Compound	Chemical structure
Pyrethrin I	H_3C, CH_3, CH_3, O, O, CH_2, H_3C, CH_3, O
Pyrethrin II	H_3C, CH_3, CH_3, CH_3, O, H_3C, O, O, CH_2, O
Cinerin I	H_3C, CH_3, CH_3, O, CH_3, O, H_3C, CH_3, O
Cinerin II	H_3C, CH_3, CH_3, CH_3, O, O, H_3C, O, O, CH_3, O

Compound	Chemical structure
Jasmolin I	
Jasmolin II	

Synthetic pyrethroids (Table 2.3) are synthesized analogues of naturally occurring pyrethrins. Synthetic pyrethroids are sold as commercial pesticides used to control pest insects in agriculture, homes, communities, restaurants, hospitals, schools, and as a topical head lice treatment. Various formulations of these pesticides are often combined with other chemicals, known as synergists, to increase potency and persistence in the environment.

While chemically and toxicologically similar, pyrethrins are extremely sensitive to light, heat and moisture. In direct sunlight, its half-lives can be measured in hours. However, the synthetic pyrethroids were developed to capture the effective insecticidal activity of this botanical insecticide, with increased stability in light and air. The development of synthetic pyrethroids is the result of efforts to modify the structure of the natural pyrethrins in order to increase photostability while retaining the potent and rapid insecticidal activity and relatively low acute mammalian toxicity of pyrethrum (Pimsamarn, 1997).

Allethrin, one of the earliest of synthetic pyrethroids still in current use, represents one initial synthetic approach, the replacement of the pentadienyl side chain of pyrethrin I with a simpler, synthetically more accessible moiety having similar steric and electronic properties. Permethrin proved to be the first synthetic pyrethroid with sufficient photostability for agricultural use. There is now great progress in the development of synthetic pyrethriod including cypermethrin, deltamethrin and fenvalerate (Soderlund *et al.*, 2002)**.** Synthetic pyrethriod advantage is low toxicity to mammals and does not cause problems of residues in the environment.

Synthetic pyrethriods are divided into two classes based on their structures and toxic effects. Type I (Table 2.4) compounds do not contain a cyano group, but Type II (Table 2.5) compounds do so called 'α-cyanopyrethriods'. Type I compounds are generally considered to produce the 'tremor syndrome' (T syndrome) of intoxication such as fine tremor, hyperexcitability, exaggerated startle response and sympathetic activation and Type II compounds are considered to produce the 'choreoathetosis/salivation' (CS syndrome) including salivation, coarse tremor, choreoathetosis, hyperreflexia, sympathetic activation and convulsion (Table 2.6) (Soderlund *et al.*, 2002). The symptoms are dose-dependent, acute responses to overt pyrethriod toxicity. Both synthetic pyrethroid classes have a similar range of mammalian toxicity but, for commercial pesticides, type II pyrethroids such as deltamethrin and cypermethrin are generally more toxic than type I compounds such as permethrin (Ray and Forshaw, 2000).

Table 2.3 Synthetic pyrethroid insecticides and LD_{50} (Pimsamarn, 1997).

Name	Chemical name	$LD_{50\ rat}$ (mg/kg)	
		oral	skin
allethrin	(*RS*)-3-allyl-2-methyl-4-oxocyclopent-2-enyl (1*R*)-*cis, trans*chrysanthemate	685-1,100	>2,500
alpha-cypermethrin	Cyclopropane-carboxilic acid 3-(2,2-dichloroethenyl)-2,2-dimethylcyano(3-phenoxypheny) methyl ester, [1-alpha(s),3-alpha]-(±)	79	>2,000
s-bioallethrin	De-2-allyl-4-hydroxy-3-methyl-2-cyclopentane-1-one ester of d-trans chrysanthemummonocarboxylic acid	680-784	
bioresmethrin	5-benzyl-3-furylmethyl(1R,3R)-2,2-dimethyl-3-(2-methylprop-1-enyl)cyclopropanecarboxylate	7,070-8,000	>10,000
biphenthrin	(2-methyl[1,1´ biphenyl]-3-yl) methyl-3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethylcyclopropanecarboxylate	375	
cyfluthrin	Cyano(4-fluoro-3-phenoxybenzyl)methyl-3-(2,2dichloroethenyl)-2,2-dimethylcyclopropanecarboxylate	900	>5,000
cyhalothrin	(RS)-alpha-cyano-3-phenoxybenzyl(Z)-(1RS,3RS)-(2-chloro-3,3,3-trifluoropropenyl)-2,2-Z dimethylcyclopropanecarboxylate	144-243	
cypermethrin	(±)-alpha-cyano(3-phenoxybenzyl (±)cis,trans-3-(2,2-dichlorovinyl) (±)2,2 dimethylcyclopropanecarboxylate	251-4,123	>2,000 (rabbit)

Name	Chemical name	$LD_{50\ rat}$ (mg/kg)	
		oral	skin
deltamethrin	(S)-alpha-cyano-m-phenoxybenzyl(1R,3R)-3,(2,2-dichlorovinyl)-2, dimethylcyclopropanecarboxylate	128.5	>2,000 (rabbit)
fenpropathrin	(RS)-alpha-cyano-3- phenoxybenzyl 2,2,3,3-tetramethylcyclopropane carboxylate	71.8-72.4	>2,000 (rabbit)
fenvalerate	(RS)-alpha-cyano-3- phenoxybenzyl(RS)-2-(4-chlorophenyl)-3-methylbutyrate	>451	>5,000
fluvalinate	(RS)-alpha-cyano-3- phenoxybenzyl N-(2-chloro-alpha-alpha-alpha-trifluoro-p-tolyl-D-valinate)	261-282	>20,000
permethrin	3-phenoxybenzyl(1RS,3RS:1RS,3RS)-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate	430-4,000	>4,000
d-phenothrin	3-phenoxybenzyl(1RS,3RS:1RS,3SR)-2,2-dimethyl-3-(2-methylprop-1-enyl)=cyclopropanecarboxylate	>10,000	>10,000
tetramethrin	3,4,5,6-tetrahydrophthalimidomethyl(IRS)-cis-trans-chrysanthemate	>5,000	>5,000
tralomethrin	(1R,3S) 3[(1´,RS)(1´2´2´2´-tetrabromoethyl)]-2,2-dimethylcyclopropanecarboxylic acid(S)-alpha-cyano-3-phenoxybenzylester	1,070-1,250	>2,000 (rabbit)

Table 2.4 The chemicals structure of synthetic pyrethriods (type I) (Soderlund *et al.*, 2002)**.**

Compound	Chemical structure
Allethrin	
Bifenthrin	
Resmethrin	
Tefluthrin	

Compound	Chemical structure
Tetramethrin	
Permethrin	

Table 2.5 The chemicals structure of synthetic pyrethriods (type II) (Soderlund *et al.*, 2002)**.**

Compound	Chemical structure
Deltamethrin	
Cyhalothrin	

Compound	Chemical structure
Cypermethrin	
Fenpropathrin	
Cyfluthrin	
Tralomethrin	
Fenvalerate	

Table 2.6 Toxic symptoms of type I and type II pyrethroid in rats (Ray and Forshaw, 2000)

Type I poisoning	Type II poisoning
Severe fine tremor	Coarse tremor
Marked reflex hyperexcitability	Moderate reflex hyperexcitability
Sympathetic activation	Sympathetic activation
Paresthesia (dermal exposure)	Paresthesia (dermal exposure)
Clonic seizures	Tonic seizures
Hyperthermia	Choreoathetosis
Uncoordinated twitches	Increased extensor tone
Aggressive sparring	Profuse watery salivation
	Sinuous writhing
	Rolling gait
	Chewing nosing; pawing; burrowing

2.4 Permethrin

Permethrin (PM) is a type I synthetic pyrethroid. It produces 'tremor syndrome' including tremor, hyper-reflexia, convulsions and skin paresthesia in rats**.** It is proved to be the first synthetic pyrethroid with sufficient photostability and themostability for agricultural use (Soderlund *et al.*, 2002)**.** PM has two stereoisomers; cis- PM and trans-PM (Figure 2.5). Cis-PM is more toxic than trans-PM.

cis-PM trans-PM

Figure 2.5 Chemical structures of cis-PM and trans-PM (Garcia *et al.*, 2001)

PM (3-phenoxybenzyl(1RS,3RS;1RS,3RS)-3-(2,2-dichlorovinyl)-2,2 dimethylcyclopropanecarboxylate) was formulated by Michael Elliott and colleagues at Rothampstead Experimental Station (UK) in 1973. The commercial names of PM include ambush, atroban, biomist and coopex. Chemical formula is $C_{21}H_{20}Cl_2O_3$ with molecular weight of 391.3 g/mol. PM is a colorless crystal to a pale yellow viscous liquid. It is soluble in water at less than 1 ppm, and is miscible in most organic solvents except ethylene glycol. PM is soluble in acetone, ethanol, ether, and xylene. It behaves as a weak acid, pH 4.8-5.0 in 6% water (20 °C). Boiling point and melting point of PM are 198-200 °C (0.3 mmHg) and 34-35 °C, respectively (Laskowski, 2002; Holmstead and Soderlund, 1977).

2.4.1 Permethrin toxicology

1) Toxicokinetics

1.1) Absorption

In 1999, Anadon and coworkers studied the toxicokinetics of PM in male rats after single oral administration at the dose of PM 460 mg/kg body weight. It was found that PM was slowly and partially absorbed. The absorption half-life was calculated to be 0.91 h. Within 4 h the peak concentrations were attained. The value for T_{max} in plasma was 3.52 h. The maximum concentration (C_{max}) was 49.46 µg/mL. The bioavailability of PM after oral administration was 60.69% (Anadon *et al.*, 1991).

1.2) Distribution

PM rapidly distributes to in the nervous tissue. After oral administration of PM at the dose 460 mg/kg and intravenous administration of PM at the dose of 46 mg/kg, the distribution half-lives were 0.46 and 4.85 h, respectively. The apparent volumes of distribution during the elimination phase and the steady state after an oral dose were 1.70 and 1.03 L. The maximum amounts of PM in cerebellum, hippocampus, caudate putamen, frontal cortex, hypothalamus, and sciatic nerve were about 1.5, 2, 2, 2.7, 4.8, and 7.5 times higher than in plasma, respectively. The maximum PM concentrations in the central and peripheral nervous system were higher than plasma. This indicates an accumulation of PM by the nervous tissues.

Nervous tissue accumulation of PM was also reflected by the area under the concentration curve ratios of tissue/plasma (1.16, 3.71, 1.57, 4.27, 3.48, and 8.77, respectively) (Anadon *et al.*, 1991).

1.3) Metabolism

Nakamura and coworkers (2007) studied *in vitro* metabolism of PM and its hydrolysis products in rats. Metabolic partway was proposed as shown in Figure 2.6. Cis- and trans-PM were mainly hydrolyzed by liver and small intestine microsomes of rats. Trans-isomer is hydrolysed more effectively than the cis-isomer. Hydrolysis of trans-PM is catalyzed by carboxylesterase (CES), ES-3 and ES-10 isoforms; while that of cis-PM was mediated by ES-3 isoform. (Ross M.K *et al.*, 2006; Ueyama *et al.*, 2010). The hydrolytic product was 3-phenoxybenzyl alcohol (PBAlc) which was further oxidized to 3-phenoxybenzyl aldehyde (PBAld) by CYP2C9. Finally, PBAld was oxidized to 3-phenoxybenzyl acid (PBAcid) by CYP1A2, CYP2B1, CYP2C6, CYP2D1, and CYP3A1. Oxidation catalyzed by CYPs required NADPH. Oxidation reactions to the PBAld and PBAcid were also occurred by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in liver cytosolic fraction contribute to reaction.

ES-10, ES-3

ES-3

3-phenoxybenzyl alcohol (PBAlc)

CYP2C9

Alcohol dehydrogenase

3-phenoxybenzyl aldehyde (PBAld)

CYP1A2, 2B1, 2C6, 2D1, 3A1

Aldehyde dehydrogenase

3-phenoxybenzyl acid (PBAcid)

Figure 2.6 Postulate metabolic pathways of cis- and trans-PM by rat liver preparations. (Nakamura *et al.*, 2007)

1.4) Elimination

In male rats receiving PM by a single oral dose of 460 mg/kg and an intravenous administration of 46 mg/kg, the elimination half-life of PM in plasma was 8.67 and 12.37 h, respectively. PM levels in plasma declined slowly. The total plasma clearance of PM was 0.058 L/h irrespective of the dosing route. The elimination half-life of PM was greater for the hippocampus, medulla oblongata, frontal cortex, and sciatic nerve, i.e. 23.10, 22.36, 13.86, and 16.27 h, respectively (Anadon *et al.*, 1991).

2) Mechanism of action

PM has an effect on central and peripheral nervous system of mammals and insects. Regarding to the basis of nerve stimulation, an action potential is generated by nerve membrane depolarization. Sodium channels open allowing to an influx of sodium ions into the nerve axon. The action potential is inactivated when the sodium ion influx decreases. Repolarization occurs when potassium channels open at the peak of the action potential and that allows movement of potassium out of the nerve cell. This returns the membrane potential to the resting state (Figure 2.7; left column).

When PM affects the nerve, the opening of sodium channels is prolonged and that leads to prolonged influx of sodium ions (Figure 2.7; right column). This phenomenon results to repetitive firing of action potentials (repetitive discharge) (Narahashi, 1996). Synaptic transmission is also disrupted. Cis-PM is over 10-fold more toxic on sodium channels than trans-isomer. The overall effect is to produce hyperexcitability in neurons and to increase their firing rate (Ray, 2001).

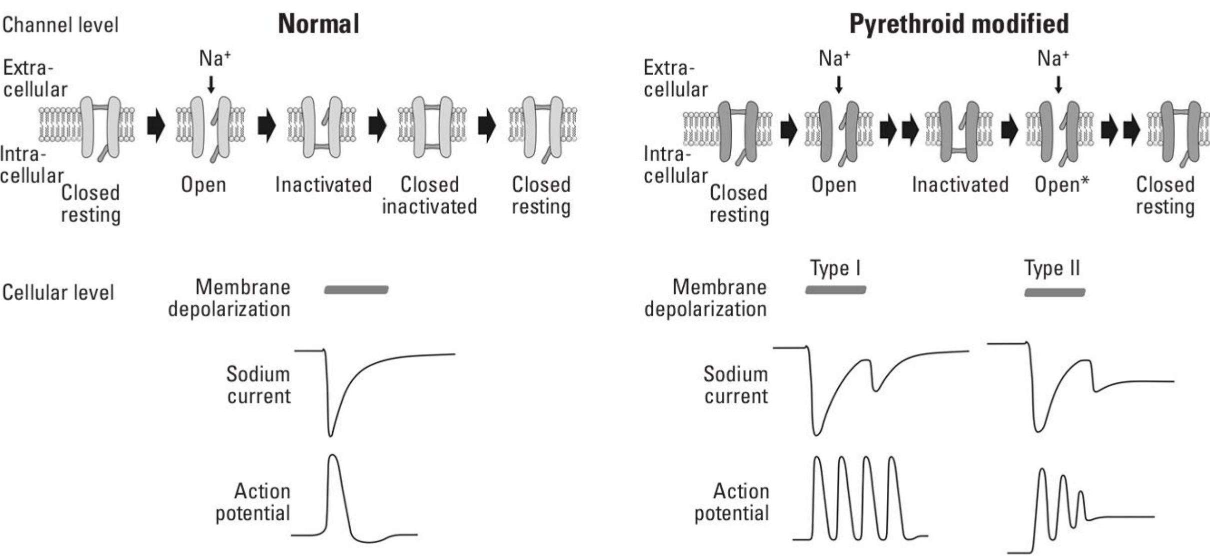

Figure 2.7 Mechanism of action of pyrethriod in neuron (Shafer *et al.*, 2005).

3) Toxic effects

3.1) Effect on nervous system

In mammals, high doses of PM produce neurotoxic symptoms include tremors, incoordination, hyperactivity, paralysis, and an increase in body temperature. These symptoms can persist up to three days. Other behavioral effects have been observed at lower doses. For example, sublethal exposure of mice to the PM-containing insecticide ‘Ambush’ increased activities like chewing. Sublethal exposure of rats to PM increased aggressive behavior, agitation, and resistance to being captured (Caroline, 1998).

3.2) Irritation

PM irritates eyes causing tearing, swelling, and blurred vision. It also irritates skin leading to red-ness, swelling, and possibly blistering of the skin (Caroline, 1998).

3.3) Effect on the immune system

Experiments with laboratory animals indicate toxicity of PM on the immune system. A multiple oral dose of PM reduces the ability of immune system cells called T-lymphocytes to recognize and respond to foreign proteins. Doses of PM equivalent to 1/100 of the LD50 inhibited T-lymphocytes over 40 percent. PM ingestion also reduced the activity of a second type of immune system cell, natural killer cells, by about 40 percent (Blaylock *et al.*, 1995). In tests using mouse cell cultures, PM had similar effects on the immune system, inhibition of two kinds of lymphocytes (Stelzer and Gordon, 1984). In addition, a study that applied varying doses of PM to shaved regions of mice revealed that dermal absorption caused the reduction of antibody production. The study concluded that low-level topical PM exposure might produce systemic immunotoxicity.

3.4) Effect on reproductive system

PM affects both male and female reproductive systems. It binds to receptors for androgen (a male sex hormone) in skin cells from human males (Eil and Nisula, 1990). PM also binds to a different receptor such as peripheral benzodiazepine receptor that stimulates production of the male sex hormone testosterone (Ramadan *et al.*, 1988). In addition, in a long-term feeding study of mice, PM was shown to cause reduced testes weights. In females, PM exposure has caused embryo loss in pregnant rabbits and in pregnant rats (Caroline, 1998).

3.5) Mutagenicity

PM was mutagenic (damaging to genetic material) in three tests with human cell cultures, one with hamster cells, and one with fruit fly larvae. In cultures of human lymphocytes (white blood cells), PM exposure caused an increase in chromosome aberrations, chromosome fragments, and DNA lesions. In the cultures of hamster ovary cells, PM exposure caused chromosome aberrations. Exposure to Ambush (a PM-containing insecticide) during larval development increased sex-linked lethal mutations in fruit flies (Caroline, 1998).

3.6) Carcinogenicity

According to the U.S. Environmental Protection Agency (EPA), PM is a possible human carcinogen (chemical that causes cancer). EPA reports that PM increased the frequency of lung tumors in female mice, and increased the frequency of liver tumors in male and female mice. The World Health Organization (WHO) reports that PM increased the frequency of lung tumors in females in two out of the three mouse studies it reviewed. Lung tumors increased with increasing PM exposure in the third study, but the increase was not statistically significant. In a study on the effects of PM on breast cancer cells, researchers found that PM increases the expression of a gene that is involved with proliferation of cells in the mammary gland. PM has also been linked to prostate cancer; in a study of farmers and professional pesticide applicators, PM was shown to increase the risk of prostate cancer in men with a family history of prostate cancer (Caroline, 1998).

Two molecular mechanisms could explain PM's carcinogenicity. First, PM reduces the activity of an enzyme involved in the breakdown of the amino acid tryptophan. This can lead to the buildup of carcinogenic tryptophan breakdown products (el-Toukhy *et al.*, 1989). Second, PM inhibits what is called "gap junctional intercellular communication" (GJIC), chemical communication between cells. GJIC plays an important role in the growth of cells, and some cancer promoting chemicals inhibit GJIC (Tateno *et al.*, 1993).

3.7) Chronic effects

In 17 medium and long-term studies performed by the EPA that exposed rats, mice, and dogs to PM, all the studies noted effects on the liver even at the lowest levels of PM. Other chronic effects include enlarged adrenal glands (in a rabbit feeding study) and increased kidney weights (in a rat feeding study). PM may also be linked to pediatric brain tumors. The findings of a 1997 study indicate that chemicals used in flea/tick products may increase risk of pediatric brain tumors. PM is one of the most commonly used chemicals in flea and tick products (Caroline, 1998).

4) Signs of toxicity

4.1) Animals

Dermal exposures to cats and dogs may cause temporary paresthesia and neurological signs as evidenced by paw flicking or ear, tail or skin twitching, or rolling on the ground. Cats exposed dermally to some PM products may experience hyperexcitability, depression, ataxia, vomiting, anorexia, tremors, or convulsions. Symptoms can begin within a few minutes or up to three days after the exposure. A report of 11 cats intentionally treated with products containing 45-65% PM described adverse effects including muscle tremors, seizures, incoordination and agitation after exposure. In addition, the symptoms of PM include tremors, agitation, aggressive behavior, convulsion, and paresthesia in rat (Toynton *et al.*, 2009).

4.2) Humans

In humans, PM has low toxicity. Dermal exposure to PM produced irritation, itching, or paresthesia (a tingly, prickly sensation) at the site of contact. These symptoms rarely last more than 24 hours. Ocular exposures may result in pain, redness, or a burning sensation. Ingestion of PM may cause sore throat, abdominal pain, nausea, and vomiting. Inhalation of PM may include headache, nasal and respiratory irritation, difficulty breathing, dizziness, nausea or vomiting. Due to low vapor pressure of PM, inhalation exposures are more likely to result from aerosols, spray droplets, and dust, than from actual vapors (Toynton *et al.*, 2009).

2.4.2 Treatment of PM toxicity

There is no specific antidote for treating PM toxicity. Typically, the treatment is symptomatic as follows.

1) Use diazepam or barbiturates to suppress seizures.
2) Gastric lavage and give activated charcoal to reduce the absorption of substances that may remain in the digestive tract.
3) To relieve allergic symptoms, antihistamines and corticosteroids may be necessary.

2.5 Analytical methods of permethrin

Several analytical methods have been used for identification and quantification of PM and its metabolites in plasma and urine samples. Most of them used the high performance liquid chromatographic (HPLC) techniques as listed in Table 2.7.

Anadon and coworkers (1991) studied toxicokinetics of PM (46 and 460 mg/kg) in rats. The plasma and homogenized tissue samples were extracted in three 8-mL portions of *n*-pentane. The combined pentane extracts were blown down to dryness under N_2 at 30 °C, and the residue was taken up in 0.5 mL methanol for HPLC analysis. Samples were separated by C_{18} column. The mobile phase was 5 mL acetic acid, 25 mL chloroform, and 470 mL of distilled deionized water (42%) in methanol (pH 2.4). The flow rate was 1.1mL/min and the detection was performed with the ultraviolet absorption at 254 nm.

In plasma, standard curves were linear (0.1-75 µg/mL) for PM and its metabolites. The intra- and inter-day coefficients of variation were less than 4%. The limit of detection was 0.1 µg/mL. In tissues, standard curves were linear for PM (0.2-75 µg/mL) and metabolites (0.2-50 µg/mL). In plasma and tissues, excepting liver, overall recoveries were >94%. In liver, recoveries were >80%. The intra- and inter-day coefficients of variation were <10%. The detection limits were 0.2 µg/mL.

Abu-Qare and Abu-Donia (2000) used HPLC and solid-phase extraction (SPE) to determine PM, PBAlc, and PBAcid, and other substances (pyridostignmine bromide, N-methyl-3-hydroxypyridinium bromide, N,N-diethyl-m-toluamide, m-toluamide and m-toluic acid) in rat plasma and urine. The SPE was performed by using C_{18} Sep-Pak® cartridges. The reversed-phase C_{18} column and gradient elution were used. The mobile phase was water (pH 3.20 adjusted with 1N acetic acid): acetonitrile, the gradient started at 1% acetonitrile, increased to 75% acetonitrile at 6 min, and then increased to 99% acetonitrile by 11 min. Then the system returned to 1% acetonitrile at 15 min where it was kept under this condition for 2 min to re-equilibrate. Flow rate ranging between 0.5 and 1.7 mL/min in a period of 17 min. The eluents were monitored by UV detection at 230 nm.

The calibration curve was linear over the range of 100-5000 ng/mL. The limits of detection were 20-50 ng/mL, while limits of quantitation were 100 ng/mL for PM and metabolites. The recovery was over 63% for plasma and urine.

In 2001a, Abu-Qare and Abu-Donia reported a procedure for analyzing PM, PBAlc, and PBAcid, and other substances (malathion, malaoxon, N,N-diethyl-m-toluamide, m-toluamide and m-toluic acid) in rat plasma and urine using reverse-phase HPLC and C_{18}-SPE. The extracts were separated on C_{18} column with water (adjusted to pH 3.50 using 1 N acetic acid): acetonitrile as a mobile phase. The flow rate programmed at 0.5 mL/min from 0-9 min, increased to 2 mL/min by 10 min, then returned to 0.5 mL/min at13 min. The gradient started at 45% acetonitrile until 9 min, increased to 90% acetonitrile by 10 min. Then the system returned to 45% acetonitrile at 13 min where it was kept under this condition for 2 min to re-equilibrate. The analytes were monitored using UV detector at a wavelength of 210 nm.

This method results in retention times of 10.2, 10.7 and 12.3 min for PBAlc, PBAcid and PM, respectively. The calibration curve was linear (100-1000 ng/mL). The limits of detection were 20-50 ng/mL, while limits of quantitation were 50–150 ng/mL for PM and metabolites. The recovery of extraction was over 80% for plasma and urine. (Abu-Qare and Abou-Donia, 2001a).

Another method using C_{18} Sep-Pak® cartridges and C_{18} reverse phase HPLC was developed for analysis of PM, PBAlc, PBAcid and other substances (chlorpyrifos, chlorpyrifos-oxon, and 3,5,6-trichloro-2-pyridinol) in rat plasma and urine (Abu-Qare and Abou-Donia, 2001b). The compounds were separated using a gradient program of acetonitrile in water (adjusted to pH 3.0 using 1 N acetic acid) at a flow rate ranging between 1 and 1.5 mL/min. The gradient started at 1% acetonitrile, increased to 55% acetonitrile at 6 min, and then increased to 80% acetonitrile at 11 min. Then the system returned to 1% acetonitrile at 15 min where it was kept under this condition for 2 min to re-equilibrate and absorbance detection at 210 nm for PM and metabolites.

Method showed concentration was linear over a range of 200-2000 ng/mL. The retention times were 14.5, 10.6 and 11.2 min for PM, PBAlc and PBAcid, respectively. The limit of detection ranged 20-50 ng/mL, while limits of quantitation

were 150 ng/mL for PM and metabolites. The recovery was over 80% for plasma and urine.

Garcia and coworker (2001) reported the determination of PM in raw material and pharmaceutical formulations using isocratic HPLC method. The sample was extracted by Liquid-Liquid extraction with n-pentane, and separated by C_{18} column at 35°C. Mobile phase consisted of methanol – water (78:22, v:v) at a flow rate of 1 mL/min. The UV detection was performed at 272 nm.

The method resulted to the correlation coefficients over 0.999. The relative standard deviations (RSD) ranged from 0.51 to 1.95% and average recoveries were over 98%.

Chunkhew and coworker (2010) studied effects of temperature, time and acidity on stability of PM in blood samples by using HPLC and C_{18} SPE to determine PM and PBAlc. The samples in plasma were extracted by using the SPE method modified from that of Junting and Chuichang (1991). The sample was acidified with 1 N acetic acid. VertiPak ^{TM} C_{18}- Tubes (200 mg/3mL) cartridges were preconditioned prior to loading of sample. The cartridges were washed with water, and eluted with chloroform. The eluate was evaporated to dryness at room temperature. The residue was reconstituted with acetonitrile. The reversed phase HPLC method with some modifications from that of Abu-Qare and Abu-Donia (2001a) was used. The mobile phase was deionized water and acetonitrile. The gradient elution was used by starting with 70% acetonitrile for 4 min, increased to 100% acetonitrile form 5 to 20 min under and decreased to 70% acetonitrile form 21 to 23 min. Flow rate was 1.4 mL/min and column temperature was set at 25°C. PM was detected at a wavelength of 210 nm.

Calibration curves were linear with the correlation coefficient of >0.9990. The method was precise and accurate for analyzing PM and PBAlc. The lower limits of quantification (LLOQ) were 0.2 μg/mL for trans-PM and 0.7 μg/mL for PBAlc. The recovery were over 77% for trans-PM and PBAlc.

Table 2.7 The HPLC method for determine PM and its metabolites in biological samples

References	Column	Mobile phase	Isocratic/gradient program	Flow rate (mL/min)	UV detector (nm)
Anadon *et al.*, 1991	C_{18}	Acetic acid : chloroform : water (5:25:470 mL) in methanol (pH2.4)	Isocratic	1.1	254
Abu-Qare and Abou-Donia, 2000	C_{18}	Acetonitrile : Water (pH 3.2)	Time %Acetonitrile %Water 0-5 1 99 6-10 75 25 11-15 99 1 15-17 1 99	0.5-1.7	230
Abu-Qare and Abou-Donia, 2001a	C_{18}	Acetonitrile : Water (pH 3.5)	Time %Acetonitrile %Water 0-9 45 55 10-12 90 10 13-15 45 55	0.5-2.0	210

References	Column	Mobile phase	Isocratic/gradient program	Flow rate (mL/min)	UV detector (nm)
Abu-Qare and Abou-Donia, 2001b	C_{18}	Acetonitrile : Water (pH 3.0)	Time / %Acetonitrile / %Water 0-5 / 1 / 99 6-10 / 55 / 45 11-14 / 80 / 20 15-17 / 1 / 99	1.0-1.5	210
Garcia *et al.*, 2001)	C_{18}	Methanol : Water (78:22, v:v)	Isocratic	1.0	272
Chunkhew *et al.*, 2010	C_{18}	Acetonitrile : Water	Time / %Acetonitrile / %Water 0-4 / 70 / 30 5-20 / 100 / 0 20-23 / 70 / 30	1.4	210

2.6 Acoustic startle response

Startle response is a motor reaction to a certain class of stimuli of different modalities. Behaviorally, the startle response consists of rapid contraction of head, neck, trunk and legs muscles (Szabo, 1965) in addition to the arrest of ongoing activity. Auditory, visual and several types of tactile stimuli are successfully used for eliciting startle. In laboratory practice, the most widely used stimuli is an intense auditory signal eliciting so called acoustic startle response (ASR; Figure 1.8)

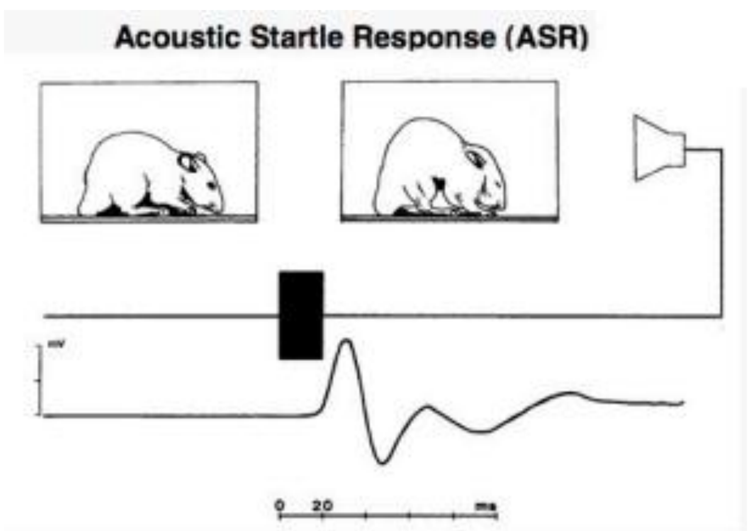

Figure 1.8 The acoustic startle response (ASR) in rat (Yeomans *et al.*, 2002)

Sensitivity of the ASR to a variety of experimental treatments make it an important research tool in studies of brain mechanisms of learning, memory, emotions and movement control. Although many studies are devoted to different aspect of ASR, the properties of a reliable acoustic stimulus to elicit ASR received relatively little attention. Fleshler (1965) was the first to show that pulses as short as 6 ms can elicit ASR, and further elongation of the stimulus has no effect on the magnitude of the response. In addition, in humans, changes in electrical activity in neck muscles can occur within 9 ms after the onset of an auditory stimulus and within 14 ms in jaw muscles. In rats, startle occurs within 5 ms in the neck and 8 ms in the hind leg.

In a systematic study, it was found that the range of 80-125 dB for each stimulus intensity, the magnitude of response increased with increasing the duration of stimulus. The acoustic pulse lasting 4 ms were already adequate to elicit a near maximum response. The time constant of the neural system subserving ASR was around 3 ms, which is shorter than the time constant of the middle ear reflex (<10 ms).

In numerous studies, it was demonstrated that a crucial factor for the elicitation of ASR is a short rise time of the stimulus. Manipulation with stimulus rise time was found to cause pronounced changes in ASR amplitude (Blumenthal, 1988; Fleshler, 1965; Ison, 1978). Chabot and Taylor (1992) showed that in 65% of rats, startle occurred in response to 80 dB tone pulse with a short rise time. For a greater rise time even, a very high sound stimulus did not elicit startle.

2.7 Objectives

The objectives of this work are to study any effect of MG and AE on elimination of PM in rat plasma was investigated which studies the following subtopics:

2.7.1 Investigate the effect of a single dose of MG and AE

2.7.2 Investigate the effect of a multiple doses of MG and AE

CHAPTER 3

Methodology

3.1 Chemicals, materials, and instruments

3.1.1 Chemicals

Reference standard permethrin ($C_{21}H_{20}Cl_2O_3$; purity 98%; mixture of cis- and trans- isomers; PESTANAL®) and phenoxybenzyl alcohol ($C_{13}H_{12}O_2$; purity 98%) were purchased from Sigma-Aldrich (P.O., Germany) and Aldrich (USA), respectively. Methanol and acetonitrile (HPLC grade), and perchloric acid ($HClO_4$; AR grade) were purchased from Millinckrodt Baker Inc. (NJ, USA). Chloroform ($CHCl_3$; AR grade) was obtained from VWR International (Briare, France). Acetic acid (CH_3COOH; AR grade) was purchased from Merck (Frankfurter, Germany). Heparin (NUPARIN®) was purchased from Troikaa (Troikaa, India).

3.1.2 Materials

The VertiPak ™ C_{18} - Tubes (200 mg/3mL) and VertiPure™ nylon syringe filters (0.22 µm, 4mm, 100/PK) were purchased from Vertical Chromatography Co. Ltd. (Nonthaburi, Thailand). Fortis ™ C_{18} column (150 x 4.6 mm., 5 µm particle size) was obtained from Fortis ™ Technologies Ltd. (Cheshire West and Chester, UK). Sunfire ™ C_{18} guard column (20 x 4.6 mm., 5 µm particle size) was obtained from Waters Corporation (Massachusetts, U.S.A).

3.1.3 Instruments

The Waters 2695 Separation Module and a Water 5487 Dual λ Absorbance detector were obtained from Waters Corporation (Milford, MA, U.S.A.). Vortex-2 genie was purchased from Scientific Industries Inc. (New York, USA). Ultrasonic cleaner was purchased from TELSONIC AG (Bronschhofen, Switzerland). Automatic pipette 10, 100, and 1000 µL were purchased from Eppendorf AG (Hamburg, Germany), which 200 and 20 µL were purchased from Gilson Inc.

(Villiers-le-Bel, France). INC 553-BSRR Rodent Restrainers (8×2 in, 125-250g) was purchased from PLAS-LABS Inc. (Lansing, MI, USA). Thermometer was purchased from SOCOREX Isba S.A. (Switzerland). Ultrapure deionized water was produced from a MilliQ water purification system (Millipore, USA). Cubis® was obtained from Sartorius AG (Goettingen, Germany). Allegra® 64R Centrifuge was purchased from Beckman Coulter, Inc. (Sao Paulo, Brasil).

3.2 Preparation of alkaloid extract (AE) and isolation of mitragynine

The leaves of *M. speciosa* were collected from natural sources in Songkhla and Satun provinces, Thailand. 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, where the herbarium voucher specimens (no. PCOG/MS001-002) have been deposited. Extraction and isolation of the alkaloid were carried out as previously described by Houghton et al. (1991) and Ponglux et al. (1994) with some modifications.

3.2.1 Alkaloid extract (AE)

Fresh leaves were dried overnight at 45-50 º 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 filtrated was combined and evaporated under reduced pressure. The crude methanolic 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. Then, petroleum ether solution was extracted with chloroform. The chloroform extract was washed with distilled water, dried over anhydrous sodium sulfate and evaporated to yield a dried crude alkaloid extract (with an approximately 0.25% yield based on the fresh leaves weight). This extract was stored in a bottle and kept in a refrigerator at below -20 °C.

3.2.2 Isolation of mitragynine

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, eluted 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; Houghton *et al.*, 1991), it was identified as mitragynine.

3.3 Animals

The experimental protocol of using animals was approved by the Animal Ethic Committee, Prince of Songkla University (Ref. 13/55). Male Wistar rats weighing 200-220 g were obtained from the Southern Laboratory Animals Facility, Prince of Songkla University, Songkhla, Thailand. The animals were housed under controlled environment (temperature of 23±2 ºC, 12 h dark/light cycles) with food and water *ad libitum*. Before treatment, the animals were fasted overnight with free access to water.

3.4 Experimental design

The animals were divided into four groups (six animals each) based on drug pretreatments; group 1, an oral dose of 40 mg/kg MG; group 2, an oral dose of 100 mg/kg AE; group 3, 4-day multiple oral dose of 40 mg/kg MG; group 4, 4-day multiple oral dose of 100 mg/kg AE. MG and AE were prepared by dissolving in 20% tween 20.

Each group was subjected to two phases of the experiment (Figure 3.1). In phase I, the animals were given a single oral dose of 460 mg/kg of PM which were dissolved in corn oil. In phase II, the same animals were pretreated with MG or AE at 2 h before receiving the same dose of PM as in phase I. There was a five-day period between phase I and phase II to allow PM to be completely washed out. Blood samples were collected from the animals by tail clip bleed at 0, 8, 14, 18 and 22 h after PM administration in phase I and phase II of each group.

3.4.1 Blood sample preparation

A half milliliter of whole blood sample obtained from tail clip was collected in an ice-chilled microcentrifuge tube containing 50 µL of heparin (60 unit/mL) and 2.5 µL of perchloric acid (perchloric acid 1 µL: blood 200 µL). After thoroughly mixing, the mixture was centrifuged at 1600×g at 4°C for 15 min. Plasma was separated and kept at -20°C until analysis (Chunkhew, 2010).

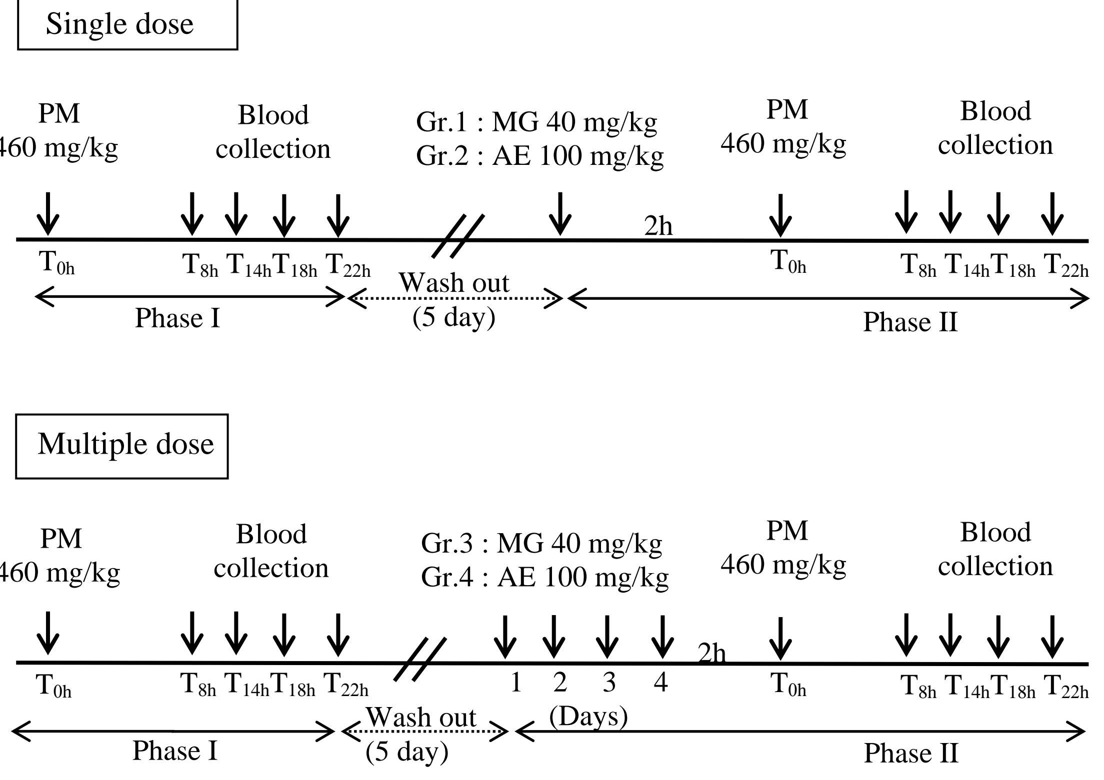

Figure 3.1 Experimental design

3.4.2 Acoustic startle response (ASR)

Acoustic startle response (ASR) testing of PM was used to test the symptom of toxicity of PM. The rats were tested in small plastic cages. The cages were placed on platforms that recorded the vertical reaction force of the animal's startle response.

In this studies, the experiments applied acoustic startle test using oscilloscope by fix frequency 4,000 Hz. At the start of each trial, the animals are given 5-10 min with a constant background white noise in acclimating to the testing apparatus. Acoustic stimulus is administered for 30-40 ms should be displayed every 30 min. The magnitude of the animals startle response is measured and recorded (Valsamis and Schmid, 2011).

3.5 Analysis of PM and PBAlc

3.4.1 Preparation of standard solution

1) Stock standard solution

An individual stock solution (10 mg/mL) of PM and PBAlc was prepared by dissolving with acetonitrile and stored at -20°C within 2 months.

2) Working standard solution

Working standard solutions of PM and PBAlc were prepared as a mixture by diluting the stock solution with acetonitrile to different concentrations of 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 µg/mL.

3) Calibration standard solution

Calibration standard solution were prepared by adding working standard mixture of PM and PBAlc at the concentration 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 51.2, 102.4, and 204.8 µg/mL to plasma blank samples (final volume 200 µL).

3.5.2 Chromatographic instruments and condition

Plasma concentrations of PM and PBAlc were analyzed by using the HPLC system consisting of a Waters 2695 Separation Module and a Waters 5487 Dual λ Absorbance detector (Milford, MA, U.S.A.). Data were collected and processed using the Empower ™ Software System.

Concentrations of PM and PBAlc in plasma were determined using the reversed phase HPLC method described by Abu-Qare and Abu-Donia (2001a) with some modifications. The analytical column was Fortis ™ C_{18} column (150 × 4.6 mm i.d., 5 µm particle size) connecting to a Sunfire ™ C_{18} guard column (20×4.6 mm i.d., 5 µm particle size). The mobile phase was water and acetonitrile, freshly prepared by filtering separately through a 0.22 µm nylon membrane filter and degassing using ultrasonic bath for 20 min before use. Elution was performed using the following gradient program started at 65% acetonitrile for 5 min, increased to 100% acetonitrile from 5 to 15 min under curve 6 and then decreased to 65% acetonitrile from 15 to 20 min under curve 7; with a post run of 7 min in order to equilibrate the column between injections. Flow rate was 1.4 mL/min and column temperature was set at 25±2°C. An injection volume was 20 µL. PM and PBAlc were detected at a wavelength of 230 nm.

3.5.3 Plasma sample extraction

PM and PBAlc in plasma were extracted by using solid phase extraction (SPE) technique by Junting and Chuichang (1991) with some modifications (Figure 3.2). The sample (200 µL) was acidified with 60 µL of 1 N acetic acid and mixed thoroughly by vortex for 30 s. The VertiPak ™ C_{18}- Tubes (200 mg/3mL) cartridges were preconditioned with 3 mL of the following solvent; chloroform, methanol, methanol-water (50%, v/v), and water, respectively, prior to loading of sample. The cartridges were washed with 3 mL of methanol-water (10%, v/v), and eluted by 3 mL of chloroform. The eluate was evaporated to dryness with stream of nitrogen at room temperature. The residue was reconstituted with 200 µL of acetonitrile and a 20 µL aliquot was injected into the HPLC system for analysis.

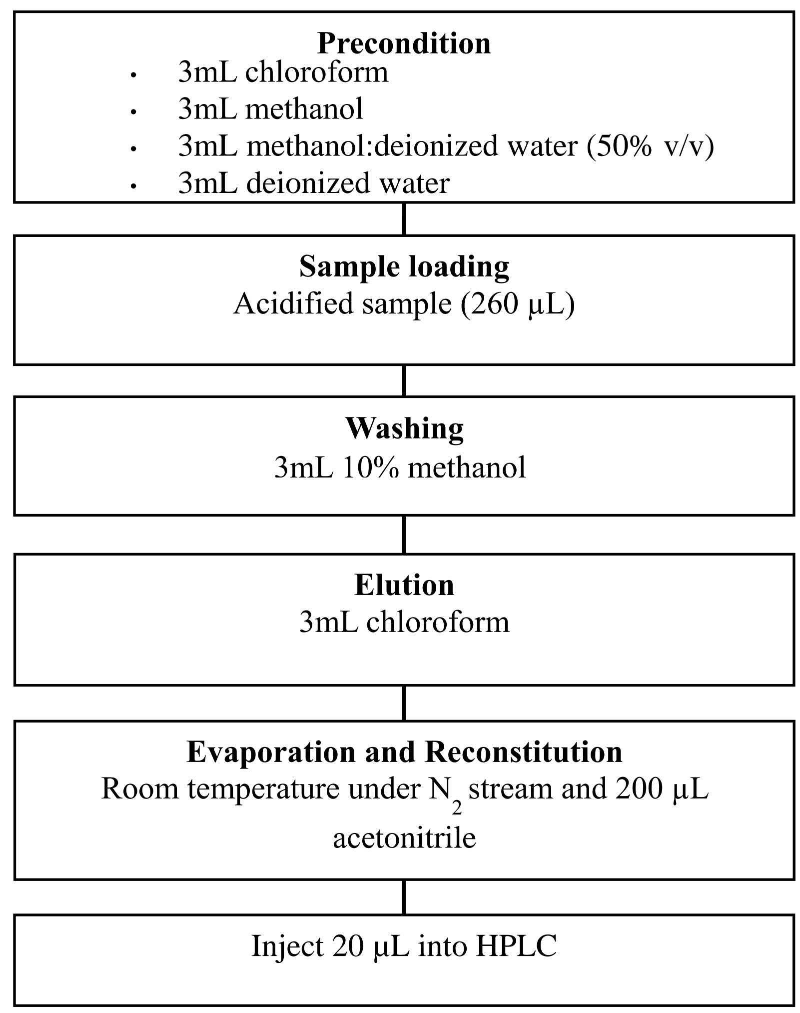

Figure 3.2 Plasma sample preparation for determination of PM and PBAlc.

3.5.4 Method validation

The method of analysis was validated in accordance with the Guidance for Industry Bioanalytical Method Validation of Food and Drug Administration in U.S. (FDA, 2001) and ICH Harmonized Tripartite Guideline Topic Q2B (ICH Steering Committee, 1996). Parameters included linearity, intra-day and inter-day precision and accuracy, recovery and lower limit of quantification (LLOQ).

1) Linearity

Linearity was determined by preparing standard plasma samples with different concentrations of PM (0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 µg/mL; four replicates of each concentration) and PBAlc (0.1, 0.8, 6.4, 51.2, 102.4, and 204.8 µg/mL; four replicates of each concentration). The calibration curve was constructed by plotting peak area of the analyte (Y) versus its concentrations (X). Regression analysis for each calibration curve was performed to obtain the calibration equation and correlation coefficient (r).

2) Precision

Precision was evaluated by using four quality control (QC) samples of PM (0.1, 0.2, 0.8, and 3.2 µg/mL) and PBAlc (0.1, 0.8, 51.2, and 204.8 µg/mL) which were prepared by adding different concentrations of standard solutions into plasma blank samples. Intra-day precision was determined by assaying five samples of each concentration during the same day under the same experimental condition while the inter-day precision was determined by daily assay of the samples for five consecutive days. The precision was expressed as the relative standard deviation (RSD) and calculated as follows.

$$RSD\ (\%) = \frac{\text{standard deviation (SD)}}{\text{mean(x)}} \times 100$$

The level of acceptance for precision is within 15% RSD except at the LLOQ where 20% RSD is accepted.

3) Accuracy

The same concentration of four QC samples was used for determining the accuracy. Intra-day accuracy was determined by assaying five samples of each concentration on the same day under the same experimental condition while the inter-day accuracy was determined by assaying five consecutive days. The accuracy was expressed as the deviation (DEV), which was calculated as follow.

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

It is acceptable when the DEV is within ±15% except at the LLOQ where ±20% was accepted.

4) Recovery

Recovery of plasma extraction of PM and PBAlc was determined at 0.1, 0.2, 0.8, and 3.2 µg/mL for PM; and at 0.1, 0.8, 51.2, and 204.8 µg/mL for PBAlc; five replicates of each concentration. The extraction recovery were evaluated by comparing responses obtained after extraction from plasma samples with those obtained after the direct injection of standard PM and PBAlc. The percentage recovery was calculated using the following expression.

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

5) Lower limit of quantification (LLOQ)

Lower limit of quantification were determined as the lowest concentration on the calibration curve that could be determined with a signal-to-noise ratio (S/N) of 5.

3.6 Data analysis

Permethrin metabolic ratio (PMR) in phases I and II were calculated as the ratio of the plasma concentration of PBAlc and that of trans-PM expressed as formula:

$$PMR = \frac{[PBAlc]}{[trans\text{-}PM]}$$

Percent change was calculated using the expression:

$$\%\ change\ in\ PMR = \frac{(PMR\ in\ phase\ II - PMR\ in\ phase\ I) \times 100}{PMR\ in\ phase\ I}$$

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.

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

Terminal elimination half-life from the time of fourteen to twenty-two ($t_{1/2\ el}$) was calculated by:

$$t_{1/2el} = \frac{0.693}{k_{el}}$$

All values are expressed as mean±standard error of mean (SEM). The data were analyzed using SPSS software version 16.0. Statistical was compared different group using a one-way analysis of variance (ANOVA) followed by post hoc-LDS test. The statistical analysis was compared between group using student and paired *t*-test. A difference was considered significant at *p* values less than 0.05 ($p<0.05$).

CHAPTER 4

Results

4.1 Chromatographic profile of permethrin and phenoxybenzyl alcohol

Chromatographic profile of separation of a standard mixture of 3.2 μg/mL of PM and PBAlc spiked into rat plasma samples and real samples are shown in Figure 4.1 (B & C). PM, both trans- and cis- isomers, and PBA were well separated from plasma interferences. Separation of trans- and cis-forms by using the present method provided higher resolution compared with that reported by Junting and Chuichang (1991). The retention times of PBAlc, trans-PM and cis-PM were 3.07, 16.07 and 16.43 min, respectively.

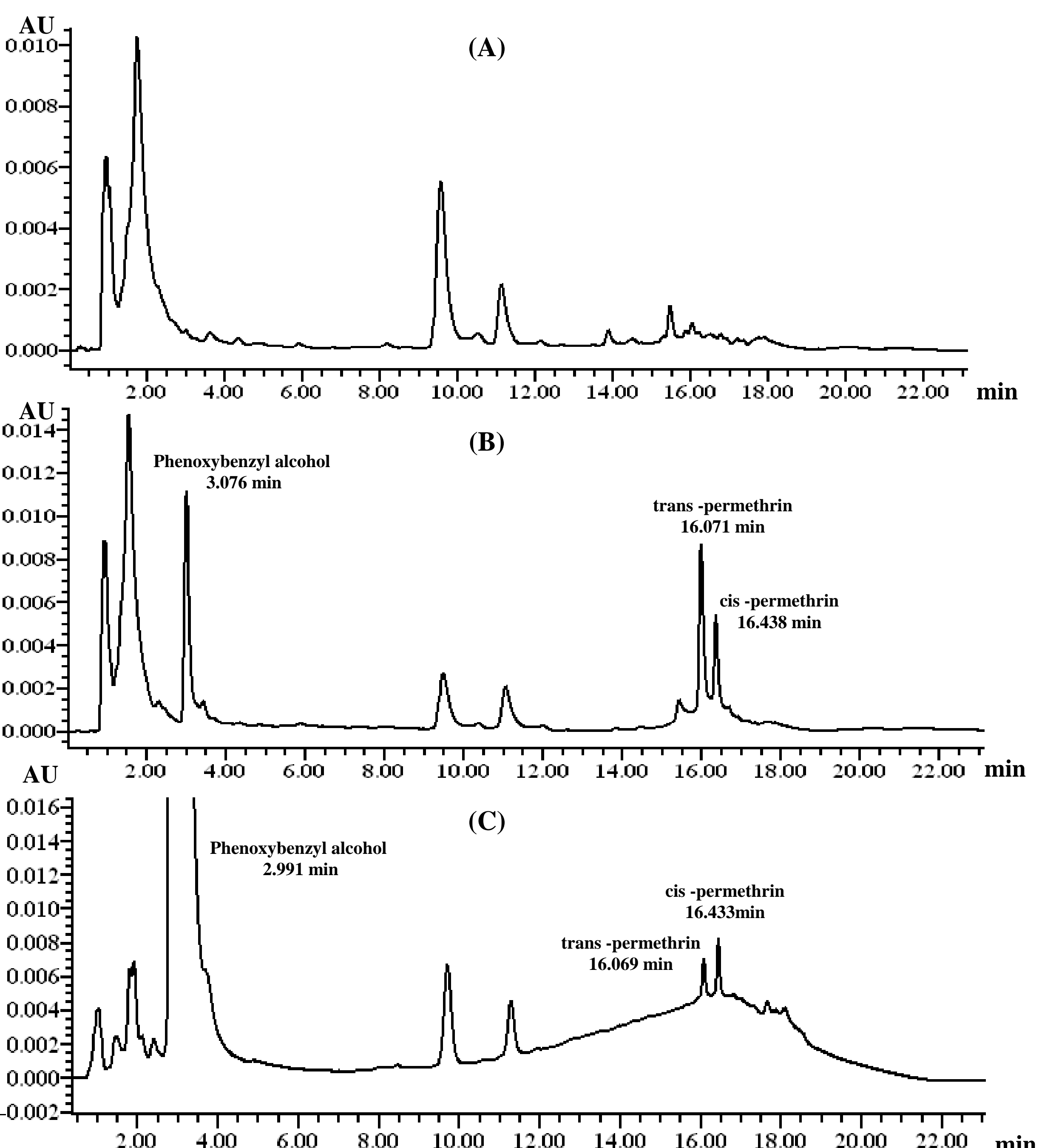

Figure 4.1 Representative chromatograms for separation of trans-permethrin, cis-permethrin and phenoxybenzyl alcohol; (A) blank plasma; (B) standards mixture of permethrin and phenoxybenzyl alcohol (3.2 µg/mL) spiked in rat blank plasma and (C) trans-permethrin (0.43 µg/mL), cis-permethrin (1.13 µg/mL) and phenoxybenzyl alcohol (98.42 µg/mL) detected from plasma of rat receiving permethrin (460 µg/mL, p.o.) at 8 h.

4.2 Method validation for analysis of permethrin and phenoxybenzyl alcohol in plasma

1) Linearity

For the analysis of plasma samples containing PM and PBAlc, regression analysis result showed that the calibration curves were linear over the concentration ranges of 0.1-3.2 µg/mL for PM and 0.1-208.4 µg/mL for PBAlc (four replicates of each concentration), as shown in Figure 4.2 and 4.3. Correlation coefficient (r) of each analytes was good value. The linear equations were y = (22491±374.08)x – (239.08±107.93) (r = 0.9999) for trans-PM and y = (34378±400.79)x + (806.31±419.26) (r = 1) for PBAlc.

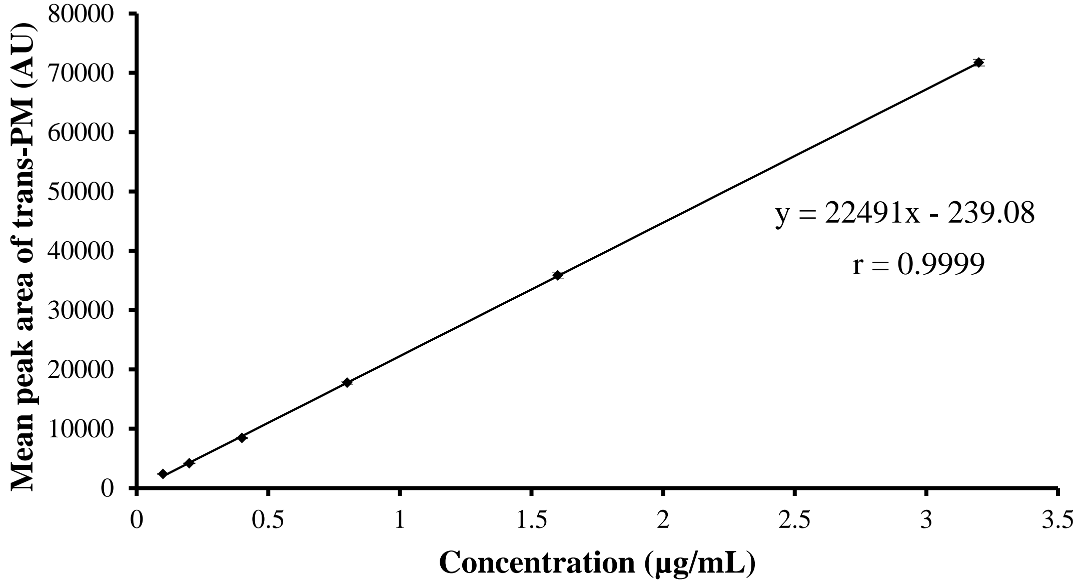

Figure 4.2 Linearity plots of mean peak area ±SEM against different concentrations of trans-PM spiked in rat plasma blank samples (n=4); correlation coefficient (r) = 0.9999

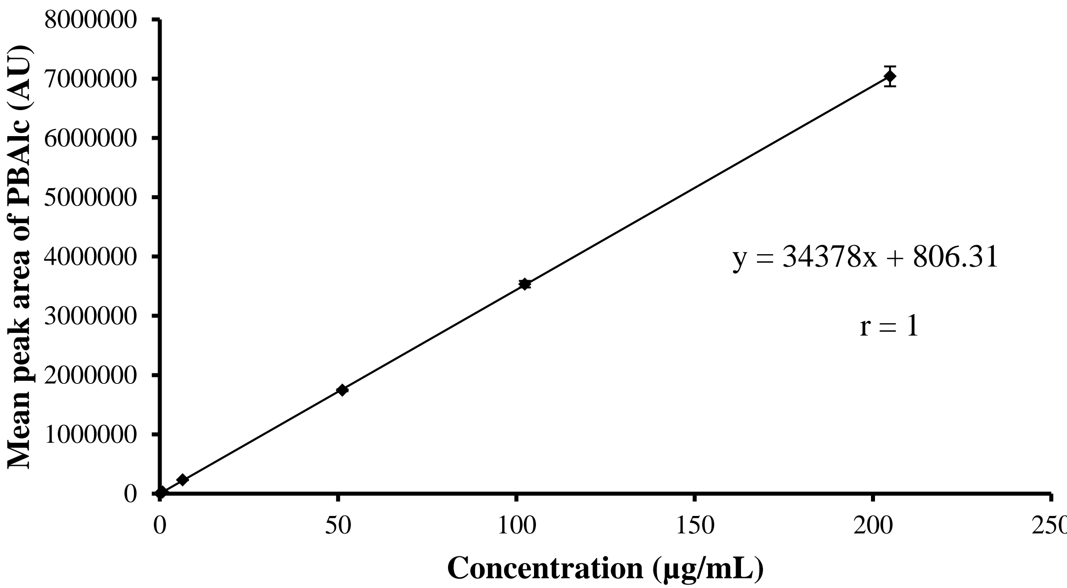

Figure 4.3 Linearity plots of mean peak area ±SEM against different concentrations of PBAlc spiked in rat plasma blank samples (n=4); correlation coefficient (r) = 1

2) Precision

Intra- and inter-day precisions were evaluated using four QC samples prepared by dissolving PM (0.1, 0.2, 0.8, and 3.2 µg/mL) and PBAlc (0.1, 0.8, 51.2 and 204.8 µg/mL) in rat plasma blank. Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same experimental condition. Inter-day precision was determined by analyzing of samples for five consecutive days. Intra- and inter-day precisions for analysis expressed with relative standard deviation (%RSD) were within 2.24-7.63% for trans-PM (Table 4.1) and 1.18-3.66% for PBAlc (Table 4.2). Both intra- and inter-day precisions for all analytes were found to be within the level of acceptance (± 15%RSD and ± 20%RSD for the concentration at LLOQ) (FDA, 2001). The results indicated that the method of analysis was precise.

Table 4.1 Intra-day and Inter-day precisions of the method for determining trans-PM in rat plasma (n=5)

Concentration of trans-PM (μg/mL)	Intra-day		Inter-day	
	Mean peak area ± SEM	%RSD	Mean peak area ± SEM	%RSD
0.1	2.25×10^{3}±76.90	7.63	2.08×10^{3}±62.90	6.75
0.2	4.34×10^{3}±98.04	5.05	4.34×10^{3}±128.61	6.63
0.8	1.67×10^{4}±195.06	2.62	1.63×10^{4}±227.65	3.12
3.2	6.99×10^{4}±701.28	2.24	6.85×10^{4}±1256.48	4.10

Table 4.2 Intra-day and Inter-day precisions of the method for determining PBAlc in rat plasma (n=5)

Concentration of PBAlc (μg/mL)	Intra-day		Inter-day	
	Mean peak area ± SEM	%RSD	Mean peak area ± SEM	%RSD
0.1	3.80×10^{3}±39.28	2.31	3.83×10^{3}±47.36	2.76
0.8	2.65×10^{4}±433.64	3.66	2.55×10^{4}±303.75	2.66
51.2	1.75×10^{6}±9220.56	1.18	1.74×10^{6}±15753.35	2.02
204.8	7.17×10^{6}±55450.14	1.73	7.03×10^{6}±108655.19	3.46

3) Accuracy

The accuracy of analysis method was determined using four quality controls (QC) samples by adding different concentrations of PM (0.1, 0.2, 0.8, and 3.2 μg/mL) and PBAlc (0.1, 0.8, 51.2, and 204.8 μg/mL) into rat plasma blank. Intra- and inter-day accuracies were expressed as the deviation (%DEV) values which ranged from (-) 7.85 to (+) 10.92% for trans-PM (Table 4.3) and (-) 12.89 to (+) 1.79% for PBAlc (Table 4.4). The accuracy was within ±15%, except ±20% for concentration at LLOQ (FDA, 2001).

Table 4.3 Intra- and inter-day accuracies of the method for determining trans-PM in rat plasma (n=5)

Concentration of tran-PM (µg/mL)	Intra-day		Inter-day	
	Measured concentration ± SEM	%DEV	Measured concentration ± SEM	%DEV
0.1	0.11±0.01	10.92	0.10±0.01	3.44
0.2	0.20±0.01	1.86	0.20±0.01	1.91
0.8	0.75±0.01	-5.95	0.74±0.01	-7.85
3.2	3.21±0.03	-2.37	3.06±0.06	-4.44

Table 4.4 Intra- and inter-day accuracies of the method for determining PBAlc in rat plasma (n=5)

Concentration of PBAlc (µg/mL)	Intra-day		Inter-day	
	Measured concentration ± SEM	%DEV	Measured concentration ± SEM	%DEV
0.1	0.09±0.01	-12.89	0.09±0.01	-11.91
0.8	0.75±0.01	-6.78	0.72±0.01	-10.03
51.2	50.86±0.27	-0.66	50.58±0.46	-1.20
204.8	208.46±1.61	1.79	204.38±3.16	-0.21

4) Recovery

The recovery of extraction was determined by comparing of the response obtained from extracting the analyte addedto the plasma samples with those obtained after the direct injection of pure authentic standard. Results of the recovery extraction of trans-PM from rat plasma is shown in Table 4.5 and that of PBAlc is shown in Table 4.6. The mean percentages of recovery ranged from 81.07 to 82.34% for trans-PM and 91.53 to 95.07% for PBAlc. The percentage of recovery in this study was similar to that reported by Sarinthip and colleagues (2010) for trans-PM, but the percentage of recovery of PBAlc is higher. The results showed that the method of extraction was highly effective.

Table 4.5 Extraction recovery for determination of trans-PM in rat plasma (n= 5)

Concentration of trans-PM (μg/mL)	Mean peak area (AU)		%Recovery ± SEM
	Direct injection	After extraction	
0.1	2.74×10^{3}	2.25×10^{3}	82.34±2.66
0.2	5.35×10^{3}	4.34×10^{3}	81.07±1.96
0.8	2.07×10^{4}	1.67×10^{4}	80.43±0.96
3.2	8.52×10^{4}	6.99×10^{4}	82.12±0.84

Table 4.6 Extraction recovery for determination of PBAlc in rat plasma (n=5)

Concentration of PBAlc (μg/mL)	Mean peak area (AU)		%Recovery ± SEM
	Direct injection	After extraction	
0.1	4.00×10^{3}	3.80×10^{3}	95.07±1.10
0.8	2.89×10^{4}	2.65×10^{4}	91.72±1.54
51.2	1.91×10^{6}	1.75×10^{6}	91.53±0.48
204.8	7.55×10^{6}	7.17×10^{6}	94.93±0.73

5) Lower limit of quantification (LLOQ)

The LLOQ was determined as the lowest plasma concentration resulting to a signal-to-noise ratio (S/N) of five. The LLOQs of trans-PM and PBAlc in rat plasma samples is shown in Table 4.7 and Table 4.8 respectively. The LLOQs were found to be 0.1 μg/mL for both trans-PM (S/N=5.99. *n* =5) and PBAlc (S/N=5.49, *n* =5). The value was lower than those shown by Sarinthip and colleagues (2010) who reported the LLOQs of 0.2 μg/mL for trans-PM and 0.7 μg/mL for PBAlc. This study showed high sensitivity of the present method for determining trans-PM and PBAlc in rat plasma samples.

Table 4.7 Lower limit of quantification (LLOQ) for determination of trans-PM in rat plasma

Concentration (μg/mL)	(n)	Peak area (AU)		S/N
		trans-PM	Noise	
0.1	1	2078	309	6.72
	2	2172	387	5.61
	3	1964	343	5.73
	4	2272	358	6.35
	5	1937	344	5.63
mean		2084.60	348.20	5.99

Table 4.8 Lower limit of quantification (LLOQ) for determination of PBAlc in rat plasma

Concentration (μg/mL)	(n)	Peak area (AU)		S/N
		PBAlc	Noise	
0.1	1	3595	663	5.42
	2	3854	668	5.77
	3	3551	679	5.23
	4	3679	672	5.47
	5	3786	684	5.54
mean		3693.00	673.20	5.49

4.3 Plasma concentration-time profile

Plasma concentration-time profiles expressed on arithmetic and logarithmic scales during 8-22 h of trans-PM and PBAlc in rats receiving a single dose and multiple doses of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.) pretreatment were shown in Figure 4.4 and Figure 4.5, respectively.

PBAlc and trans-PM concentrations were detectable in plasma from 8 to 22 h post dose. The concentrations of trans-PM and PBAlc in plasma were gradually decreased over time. After 22 h, the levels of trans-PM in plasma of rats given a single dose and multiple doses pretreatment of MG were below its LLOQ.

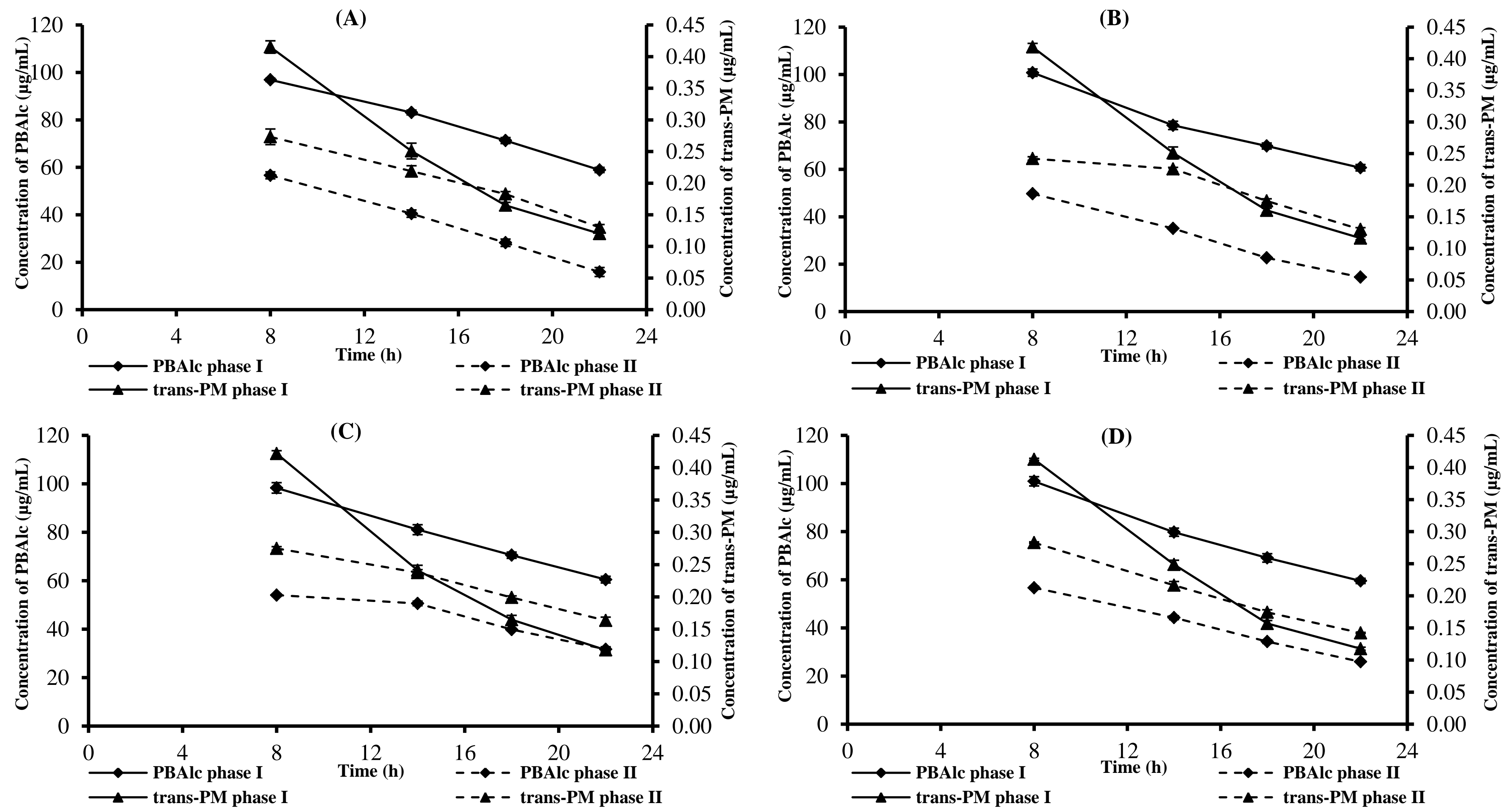

Figure 4.4 Plasma concentration-time profiles of trans-PM and PBAlc (mean ± SEM) during 8-22 h in rats receiving a single- and multiple dose(s) of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.); (A) MG single dose; (B) MG multiple dose; (C) AE single dose; (D) AE multiple doses

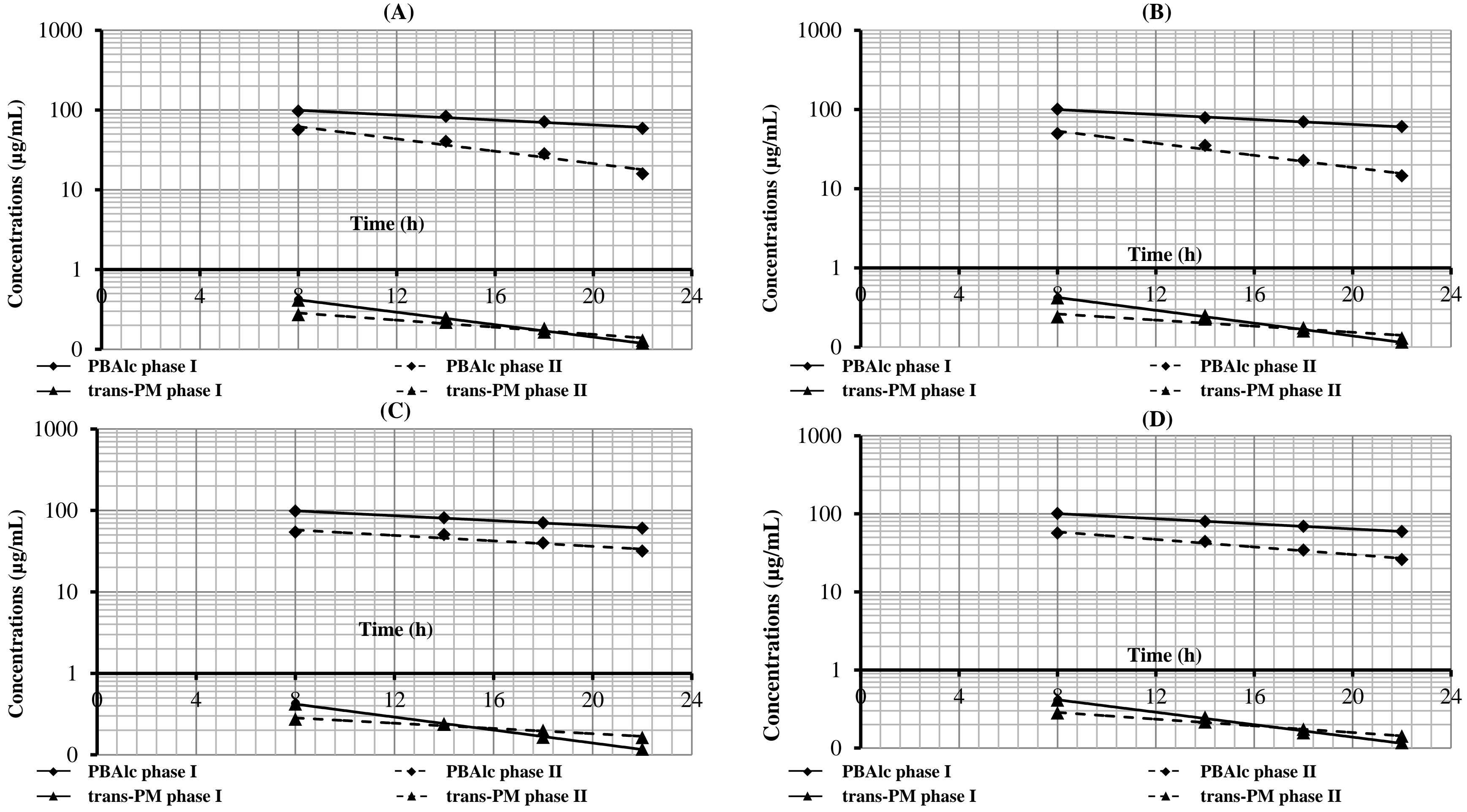

Figure 4.5 Semilog plasma concentration-time profiles of trans-PM and PBAlc (mean ± SEM) during 8-22 h in rats receiving a single- and multiple dose(s) of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.); (A) MG single dose; (B) MG multiple dose; (C) AE single dose; (D) AE multiple doses

4.4 Concentrations of PBAlc and trans-PM in rats before (phase I) and after (phase II) pretreatment with a single dose and multiple doses of MG and AE

Figure 4.6 shows concentrations of PBAlc in phase II compared with those in phase I of rats receiving a single dose and multiple doses of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.). Concentrations of PBAlc of each group of rats were significantly decreased over time.

Figure 4.7 shows concentrations of trans-PM in phase II compared with those in phase I of rats receiving a single dose and multiple doses of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.).

Results show that pretreatment of each group were significantly increased concentrations of trans-PM at 18-22 h in phase II compared with those in phase I ($p < 0.05$) after PM administration.

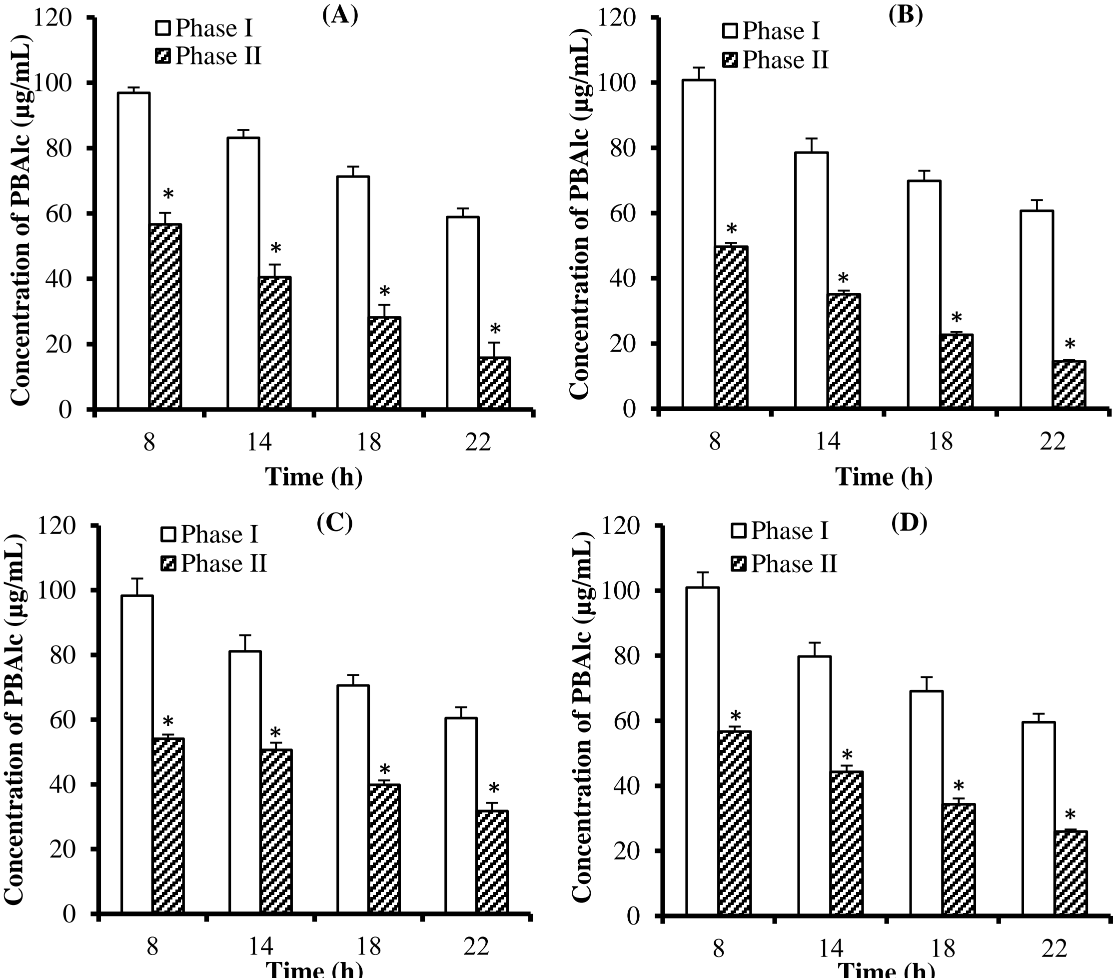

Figure 4.6 Concentrations of PBAlc compared between phase I and phase II in rats receiving a single and multiple dose(s) of MG and AE; (A) MG single dose; (B) MG multiple dose; (C) AE single dose; (D) AE multiple dose. Significance differences were considered at $p < 0.05$ (mean ± SEM).

* compared between phase I and phase II using Paired *t*-test.

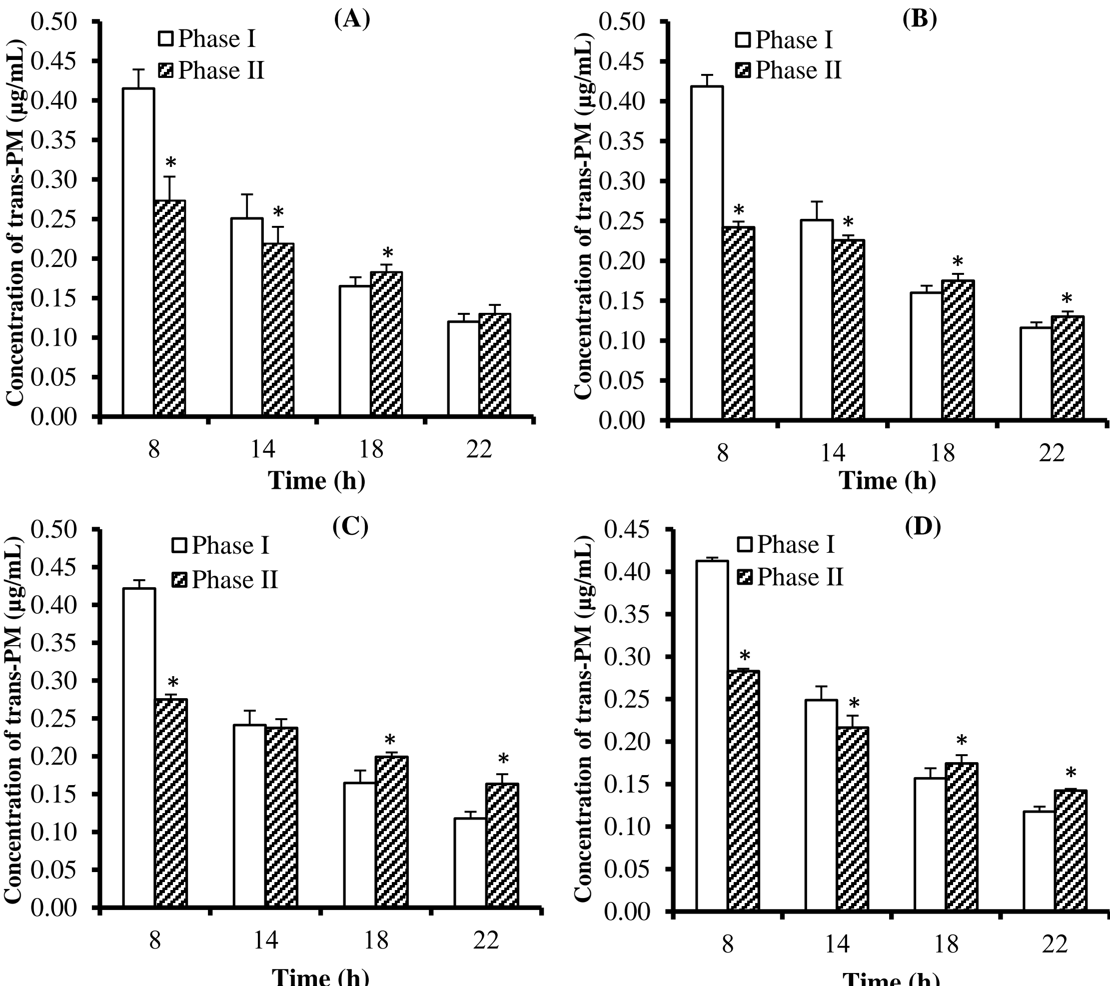

Figure 4.7 Concentrations of trans-PM compared between phase I and phase II in rats receiving a single and multiple dose(s) of MG and AE; (A) MG single dose; (B) MG multiple dose; (C) AE single dose; (D) AE multiple dose. Significance differences were considered at $p < 0.05$ (mean ± SEM).

* compared between phase I and phase II using Paired *t*-test.

4.5 Permethrin metabolic ratio (PMR) in rats before (phase I) and after (phase II) pretreatment with a single dose and multiple doses of MG and AE

Figure 4.8A shows phase I PMRs in different groups of rats receiving PM alone prior to pretreatment with MG or AE. At 8, 14, 18, and 22 h after receiving PM, PMRs of each group of rats significantly increased with time. No significant difference of phase I PMRs was observed among 4 groups of rats at any given time.

Figure 4.8B presents phase II PMRs in the same group of rats as in phase I. PMRs in rats pretreatment with single and multiple dose(s) of MG significantly decreased with time. In contrast, PMRs in rats given single and multiple dose(s) of AE did not significantly change with time.

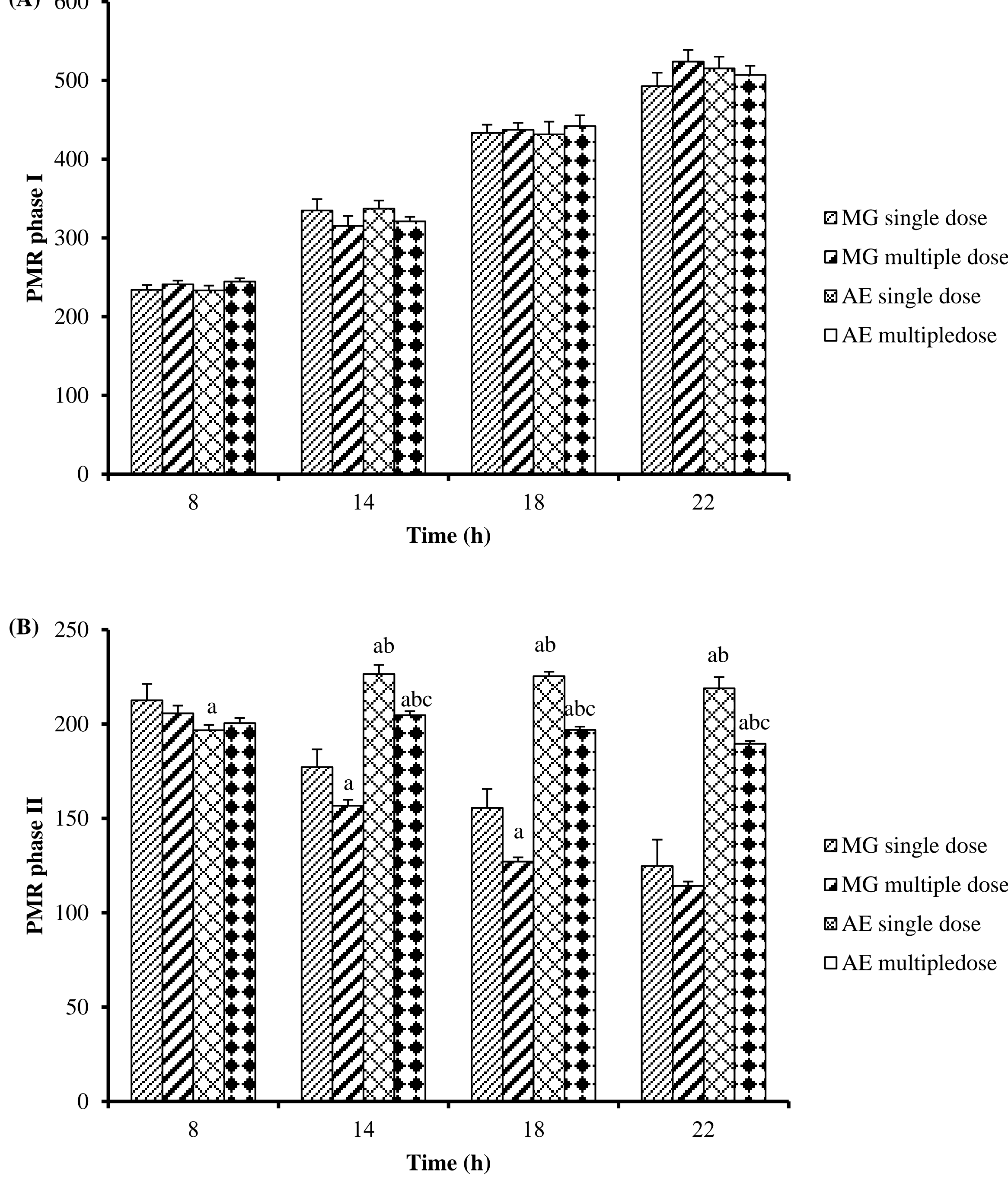

Figure 4.8 Permethrin metabolic ratio (PMRs) in each groups of rats assigned for pretreatment with a single or multiple dose(s) of MG or AE; (A) Phase I; (B) Phase II. Significance differences were considered at $p < 0.05$ (mean ± SEM).

[a] compared with pretreatment MG single dose, [b] compared with pretreatment MG multiple dose, and [c] compared with pretreatment AE single dose using ANOVA by post hoc LSD.

PMRs in phase II (MG,AE+PM) compared with those in phase I (PM alone) of rats receiving a single dose and multiple doses of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.) were shown in Figure 4.9.

Results show that pretreatment with a single dose of MG caused a significant decreased in PMRs (phase II vs. phase I) at 8, 14, 18, and 22 h after PM administration (Figure 4.9A). Similar results were observed in rats pretreated with multiple doses of MG (Figure 4.9B). Likewise, pretreatment with either a single or multiple doses of AE significantly decreased PMRs (Figure 4.9C and D).

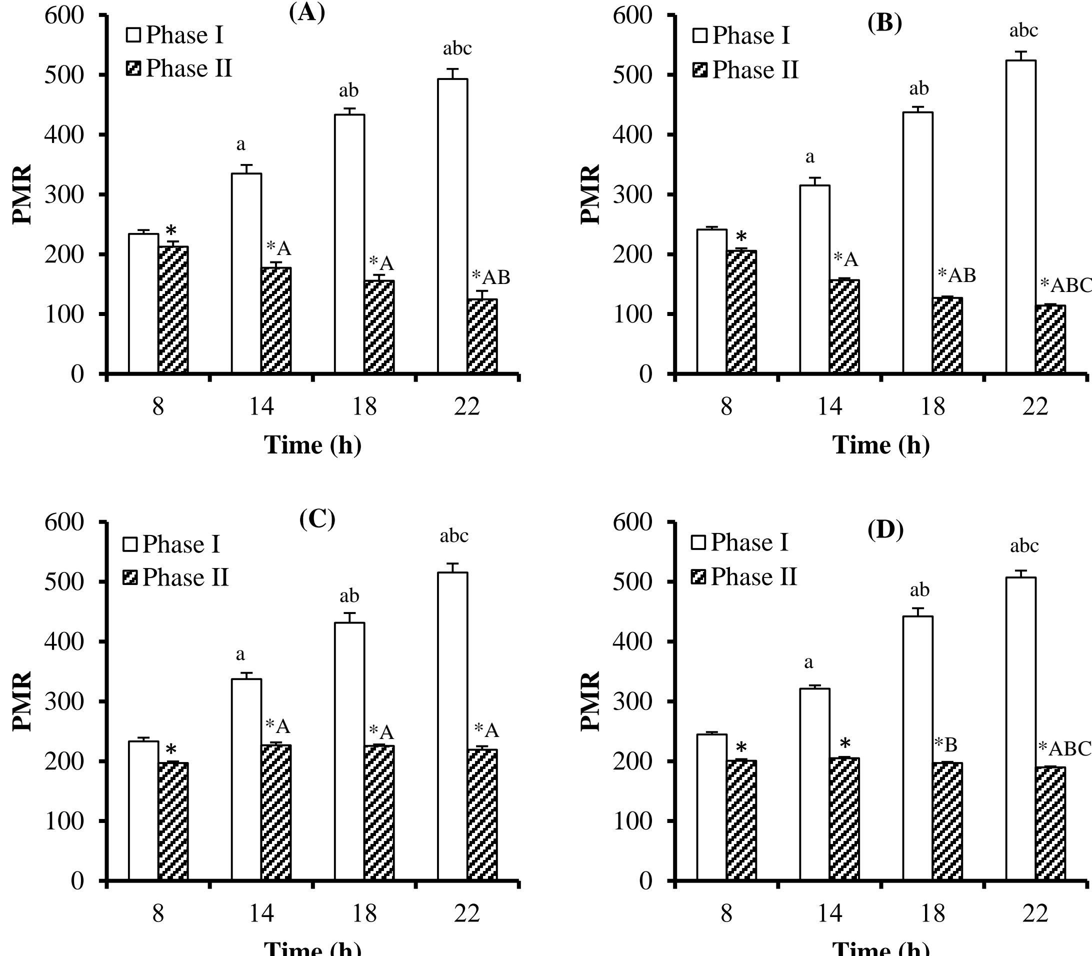

Figure 4.9 Permethrin metabolic ratios (PMRs) compared between phase I and phase II in rats receiving a single and multiple dose(s) of MG and AE; (A) MG single dose; (B) MG multiple dose; (C) AE single dose; (D) AE multiple doses. Significance differences were considered at $p < 0.05$ (mean ± SEM).

[*] compared between phase I and phase II using Student paired *t*-test.

[a] compared with phase I at 8 h, [b] compared with 14 h, and [c] compared with 18 h using ANOVA by post hoc LSD.

[A] compared with phase II at 8 h, [B] compared with 14 h, and [C] compared with 18 h using ANOVA by post hoc LSD.

4.6 The percentage of decrease in metabolic ratios of PM in rats receiving a single- and multiple dose(s) pretreatment with MG and AE

Figure 4.8 shows the percentages of decrease in PMRs of rats receiving pretreatment with a single and multiple dose(s) of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.) at 8, 14, 18, and 22 h, respectively.

At 8, 14, 18, and 22 h after administration PM, the percent reduction of PMRs of each group of rats were significantly increased with time.

At 8 h shows, the percent reduction of PMRs in rats receiving multiple dose of AE was significantly different with single dose of MG. In addition, the percent reduction of PMRs in rats pretreated with single and multiple dose(s) of MG did not significantly different at 14, 18, and 22 h. Similar results were observed in rats pretreated with single and multiple dose(s) of AE. At 14, 18, and 22 h in rats pretreated with single dose of AE, the percent reduction of PMRs was significantly decreased with single dose of MG. Similar results were observed in rats given with multiple doses of AE.

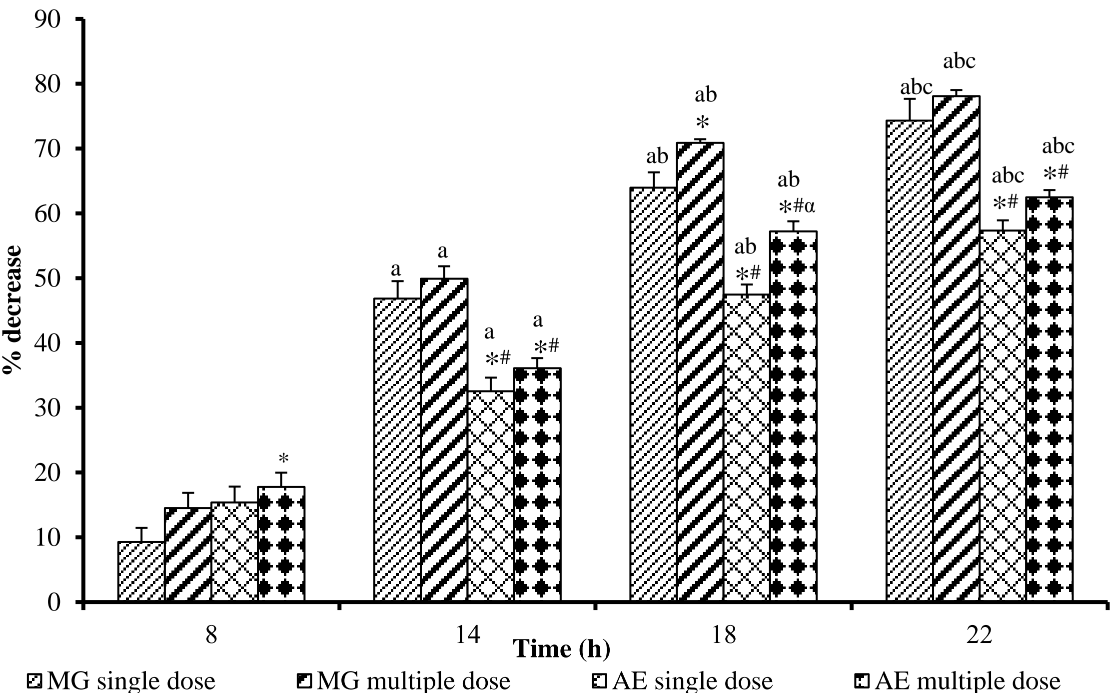

Figure 4.10 Percentage of the decrease in permethrin metabolic ratios (PMRs) after the oral administration of mitragynine (MG; 40 mg/kg) and alkaloid extract (AE; 100 mg/kg) in rats (n=6). Significance differences were considered at $p < 0.05$ (mean ± SEM).

[a] compared with at 8 h, [b] compared with 14 h, and [c] compared with 18 h using ANOVA

* compared with pretreatment MG single dose, [#] compared with pretreatment MG multiple doses, [α] compared with pretreatment AE single dose using ANOVA by post hoc LSD.

4.7 Effects of MG and AE on the elimination rate constant of PM

The elimination rate constants (k_{el}) of PM in phase I and phase II for rats receiving a single and multiple dose(s) pretreatment with MG and AE were shown in Table 4.9. The values of k_{el} of PM in phase I ranged from 0.059±0.001 to 0.062±0.005 h^{-1} while those in phase II ranged from 0.024±0.003 to 0.035±0.004 h^{-1}. The values of k_{el} of PM in phase II were significantly reduced compared with those in phase I (p<0.01).

The k_{el} of PM in phase I was not significantly different for each groups of pretreatment. In phase II, k_{el} of PM in phase II was significantly lower after AE multiple doses pretreatment (p<0.01).

Table 4.9 The average elimination rate constant (k_{el}) of PM in phase I and phase II

Group	k_{el} in phase I (h^{-1})	k_{el} in phase II (h^{-1})
MG single dose	0.061 ± 0.001	0.035 ± 0.002 *
MG multiple dose	0.062 ± 0.001	0.033 ± 0.002 *
AE single dose	0.060 ± 0.001	0.031 ± 0.001 *
AE multiple dose	0.059 ± 0.001	0.024 ± 0.001 * a b c

Values are presented as mean ± SEM (n=6)

*$p < 0.01$, compared between phase 1 and phase 2 using Paired t-test.

[a] Compared with pretreatment MG single dose, [b] compared with pretreatment MG multiple dose, and [c] compared with pretreatment AE single dose using ANOVA by post hoc LSD, $p < 0.01$.

4.8 Effects of MG and AE on the terminal half-life of PM

Table 4.10 shows the terminal half-lives ($t_{1/2\ el}$) of PM in phase I and phase II for rats receiving a single- and multiple dose(s) pretreatment with MG and AE.

After pretreatment with a single dose of MG, the average value of the elimination half-life was significantly increased i.e. 11.46 vs. 19.98 h. Pretreatment with multiple doses of MG also significantly increased $t_{1/2\ el}$ of PM, i.e. 11.19 vs. 22.65 h. Both single- and multiple pretreatment(s) with AE significantly increased $t_{1/2\ el}$ of PM i.e. 11.61 vs. 22.69 h and 11.71 vs. 29.08 h, respectively. The significant increase in the $t_{1/2\ el}$ of PM was the highest after pretreatment with multiple dose(s) of AE, i.e. 29.08 h.

Table 4.10 The average terminal elimination half-life from the time of fourteen to twenty-two ($t_{1/2\ el}$) of PM in phase I and phase II

Group	$t_{1/2\ el}$ in phase I (h)	$t_{1/2\ el}$ in phase II (h)
MG single dose	11.46 ± 0.13	19.98 ± 1.17*
MG multiple dose	11.19 ± 0.12	22.65 ± 0.85*
AE single dose	11.61 ± 0.22	22.69 ± 0.68*
AE multiple dose	11.71 ± 0.26	29.08 ± 1.47$^{*\ a\ b\ c}$

Values are presented as mean ± SEM (n=6)

*p < 0.001, compared between phase 1 and phase 2 using Paired *t*-test.

[a] compared with pretreatment MG single dose, [b] compared with pretreatment MG multiple dose, and [c] compared with pretreatment AE single dose using ANOVA by post hoc LSD, $p < 0.001$.

4.9 Effects of MG and AE on the acoustic startle response (ASR) of PM

The onset and duration of action of PM on ASR in phase I and phase II in rats receiving a single dose of MG and AE were shown in Table 4.11.

The onset of PM on ASR in phase II was significantly delayed compared with that in phase I. The significant delay was also observed when the rats were pretreated with AE single dose or MG single dose (p<0.01). In addition, pretreatment of AE single dose caused a significant difference in the onset of PM on ARS compared with that after MG single dose pretreatment (p<0.05).

The duration of action of PM on ASR in phase II was significantly prolonged compared with that in phase I after a single dose pretreatment with MG or AE (p<0.01). In addition, the duration of action of PM on ASR in phase II was significantly different after a single dose of AE when compared with that after a single dose of MG pretreatment (p<0.05).

Table 4.11 The onset and duration of action of acoustic startle response (ASR) of PM in phase I and phase II

Group	Onset ASR (h)		Duration of action ASR (h)	
	Phase I	Phase II	Phase I	Phase II
Control	ND.	ND.	ND.	ND.
MG single dose	6.70 ± 0.12	9.40 ± 0.29*	10.90 ± 0.19	12.40 ± 0.33*
AE single dose	6.90 ± 0.19	11.10 ± 0.29*#	10.90 ± 0.29	13.30 ± 0.25*#

Values are presented as mean ± SEM (n=5)

ND; Not detectable

*$p < 0.01$, compared between phase I and phase II using Paired *t*-test.

# $p < 0.05$, compared between MG single dose pretreatment using Student *t*-test.

CHAPTER 5

Discussion and Conclusion

HPLC method validation confirmed that an HPLC procedure was suitable for PM determination. PM and PBAlc were well separated with no interference in blank plasma. Separation of trans- and cis-PM using the present method provided higher resolution compared with that reported by Junting and Chuichang (1991). The retention times of trans-PM and cis-PM were 16.07 and 16.43 min, respectively, were shorter than that reported by Ramesh and Ravi (2004) which were 44.79 and 44.93 min, respectively.

The results indicated that the response of the detector showed a good linearity for quantification of trans-PM and PBAlc within the studied concentration ranges. The correlation coefficient (r) obtained from the regression line demonstrated the excellent relationship between peak area and concentrations of PM and PBAlc. The inter-day and intra-day precision and accuracy were found to be within the level of acceptance (±15 %RSD and ±20 %DEV for the concentration at LLOQ) indicating that the method of analysis was precise and accurate. The values of percentage of recovery obtained in this study were 81-82% and 92-95% for trans-PM and PBAlc, respectively. This was similar to that reported by Sarinthip and colleagues(2010) and Abu-Qare and Abou-Donia (2000) for trans-PM, but the percentage of recovery of PBAlc is higher. The results showed that the method is effective. The LLOQ values for trans-PM and PBAlc was 0.1µg/mL. This could be due to the difference in the wavelength of UV detection. This factor caused the decrease noise of all analytes. In this study, the values of LLOQ were lower than shown by Sarinthip and colleagues (2010) who reported the LLOQs of 0.2 µg/mL for trans-PM and 0.7 µg/mL for PBA, and less than 0.15 µg/mL for all analytes in rat urine and plasma as reported by Abu-Qare and Abou-Donia (Abu-Qare and Abou-Donia, 2001). This study showed high sensitivity of the present method for determining trans-PM and PBA in rat plasma samples.

Plasma concentration during 8-22 h of PBAlc and trans-PM in rats receiving a single and multiple dose(s) pretreatment of MG and AE gradually decreased over time. The result indicated that PM was metabolized in the rats probably by esterase enzyme before being excreted in the urine and feces (Soderlund *et al.*, 2002).

The concentration of trans-PM in rats receiving a single and multiple dose(s) pretreatment of MG and AE were increased when compared with those receiving PM alone. This result confirms that the MG and AE was interfere the metabolism of PM to PBAlc. However, during 8-14 h found that the level of trans-PM in rats receiving a single and multiple dose(s) pretreatment of MG and AE were decreased when compared with those receiving PM alone. Kratom extract (50-100 mg/kg) was shown to decreased gastrointestinal motility (Chittrakarn *et al.,* 2008). The results indicated that a single and multiple dose(s) pretreatment of MG and AE decreased absorption of PM.

In the present study, trans-PM and PBAlc were simultaneously determined in rat plasma. PMRs of each group of pretreatment in phase I (PM alone) was not different at the same time. The results suggested that among 4 group of pretreatment rats given PM alone had the same rate of metabolism. The effects that the animals were maintained under the same controlled condition i.e. weight range of 200-230 g, time of blood collection, and time of administration. Phase I PMRs at 8, 14, 18, and 22 h among different groups of rats receiving PM alone were increased with time because the levels of metabolite in the blood increased over time simultaneously substrate gradually decreased. These results indicate that metabolism of PM has been gradually increased over the studied range of time, resulting in increased PMRs.

In contrast to phase I baseline, phase II PMRs in rats receiving pretreatment with single or multiple dose(s) of MG (40 mg/kg, p.o.) before PM administration were significantly decreased with time. This result was probably attributable to an interference of MG to the hydrolysis of PM to PBAlc. It is likely that MG, which is also metabolized via hydrolysis at the ester bond (Philipp *et al.*, 2009), compete with PM for the same catalytic esterase. CESs are categorized as phase-I drug-metabolizing enzymes that can hydrolyze a variety of ester-containing

drugs and prodrugs, such as angiotensin-converting enzyme inhibitors, anti-tumor drugs, and narcotics (Hosokawa, M., 2008). Therefore, MG decreased of PM metabolism in this study. In contrast, phase II PMRs in rats given single and multiple dose(s) of AE (100 mg/kg, p.o.) did not significantly change with time. This may be due to AE contains a many alkaloids (Takayama, 2004). Alkaloids compound have ester group, they cause metabolized via hydrolysis by enzyme esterase. Therefore, substrate increased the activity of these enzymes in hydrolysis was rapid, which may cause enzyme saturation.

In addition, the pretreatment with a single and multiple dose(s) of MG caused a significant decreased in PMRs (phase II vs. phase I) at 8, 14, 18, and 22 h after PM administration. Similar results were observed in rats pretreated with a single or multiple doses of AE that significantly decreased PMRs. The result indicated that a single dose and multiple doses of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.) can also interfere the metabolism of PM to PBA, by competitively inhibiting with PM hydrolysis by esterase enzyme (Nakamura *et al.*, 2007).

The percentages of decrease in PMRs of rats receiving pretreatment with a single and multiple dose(s) of MG (40 mg/kg, p.o.) and AE (100 mg/kg, p.o.) at 8, 14, 18, and 22 h after administration of PM were significantly increased with time. The results that may offer a partial explanation the decreased PMRs induced by MG or AE.

In addition, the percentages of decrease in PMRs for rats receiving pretreatment a single dose and multiple doses of MG tended to significantly increase more than a single dose and multiple doses of AE. This results may be due to PMRs of MG pretreatement tended to decreased more than AE pretreatment.

The elimination rate constants of PM in phase I (PM alone) ranged from 0.059±0.001 to 0.062±0.005 h^{-1}while those in phase II (MG, AE+PM) ranged from 0.024±0.003 to 0.035±0.004 h^{-1}. The values of elimination rate constants of PM in phase II were significantly reduced compared with those in phase I. The reduction of elimination rate constants in phase II was probably resulted from the disturbance of MG and AE on the metabolism of PM to PBAlc. In addition, the elimination rate constants of PM in phase I (PM alone) of all groups of treatment were similar,

reflecting that the animals included in the study were well controlled. The result demonstrated that pretreatment with multiple doses of AE caused the lowest elimination rate constants of PM. This may be due to the accumulation of alkaloids in the body after multiple doses administration.

The terminal half-lives ($t_{1/2\ el}$) of PM in phase I for rats receiving a single- and multiple dose(s) pretreatment with MG and AE ranged from 11.19±2.98 to 11.71 ± 0.26h. That was similar to that reported earlier by Anadon and colleagues(1991), who had shown that $t_{1/2\ el}$ of PM in rats receiving a single oral dose of 460 mg/kg PM was 12.37 h. The average values of the elimination half-life in phase II for rats receiving a single- and multiple dose(s) pretreatment with MG and AE ranged from19.98 ± 1.17 to 29.08 ± 1.47 h.

After pretreatment with a single- and multiple dose(s) pretreatment of MG and AE, the average values of the elimination half-life were significantly increased, compared with those in phase I. The results indicated that MG and AE prolonged the elimination half-life of PM. This effect was likely to be due to a slow rate of elimination of PM. The significant increase in the $t_{1/2\ el}$ of PM was the highest after pretreatment with multiple dose(s) of AE. The result suggested a slow rate of elimination of PM in rat receiving a multiple doses of AE causing prolonged elimination half-life of PM.

The onset of PM on ASR in phase II for rats receiving a single dose of MG and AE were significantly delayed compared with those in phase I. This may be caused by the concentration of PM in blood sample decreased. The previous study, reported by Chittrakarn and colleagues (2008), Kratom extract (50-100 mg/kg) was shown to decreased gastrointestinal motility.

The duration of action of PM on ASR in phase II for rats receiving a single dose of MG and AE were significantly prolonged compared with that in phase I. This results was attributable to MG and AE decreased elimination of PM.

The acoustic startle response (ASR) of PM presented a pharmacodynamic interaction *in vivo.* MG and AE delayed onset and duration of action ASR of PM. The results indicate toxicity of PM increased which combination between PM and MG or AE.

Conclusion

The present study shows a pharmacokinetic interaction between the single and multiple co-administration of PM and MG or AE. A single and multiple dose(s) administration of MG or AE reduction of PMR and elimination rate of PM caused by MG and AE indicated that an interference the hydrolysis of PM to PBA. It may be likely that MG, which is also metabolized via hydrolysis at the ester bond, compete with PM for the same catalytic esterase resulting to a decreased metabolism and a delayed elimination rate of PM. Both of a single and multiple dose(s) of MG or AE, decreased metabolism of PM was not significantly different. Decreased metabolism after given a single dose of MG was significantly greater than that given a single dose of AE. Similar results were observed in rats pretreated with multiple doses. All groups of pretreatment was significantly delayed elimination rate of PM. Additionally, AE multiple doses pretreatment delayed elimination rate constants of PM highest cause accumulation of alkaloids in the body after multiple doses administration. After pretreatment with a single and multiple dose(s) of MG and a single dose of AE was significantly prolonged elimination half-life of PM for 2-folds. A pharmacodynamic interaction is also needed to describe by ASR test. MG and AE delayed onset and duration of action ASR of PM. The finding suggested that toxicity of PM increased which combination between PM and MG or AE.

Deceleration of metabolism and elimination of PM may lead to an increase in risk of PM neurotoxicity among individuals using kratom cocktail formulas containing synthetic pyrethroids.

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Appendix-1

Method validation for analysis of trans-permethrin and phenoxybenzyl alcohol in plasma

Table A1.1 Data for determination of linearity and lower limit of quantification of the method for analyzing trans-permethrin and phenoxybenzyl alcohol in rat plasma (n = 4)

Concentration (µg/mL)	Peak area (AU)								Measured concentration (µg/mL)		
Phenoxybenzyl alcohol	**n_1**	**n_2**	**n_3**	**n_4**	**Mean**	**Normalized**	**SEM**	**%RSD**	**Mean**	**SEM**	**%DEV**
0.1	3037	3287	2992	3041	3089.25	30892.50	66.85	4.33	0.06	0.00	-15.63
0.8	24344	24848	27446	27498	26034.00	32542.50	836.65	6.43	0.73	0.02	-8.53
6.4	229892	230317	224857	240165	231307.75	36141.84	3202.20	2.77	6.70	0.09	4.73
51.2	1745392	1757497	1774687	1699460	1744259.00	34067.56	16096.95	1.85	50.71	0.47	-0.95
102.4	3599060	3586046	3583520	3364806	3533358.00	34505.45	56287.05	3.19	102.75	1.64	0.35
204.8	7186915	7201510	7229239	6539860	7039381.00	34371.98	166738.16	4.74	204.74	4.85	-0.03
trans-permethrin											
0.1	2373	2444	2241	2402	2365.00	23650.00	43.83	3.71	0.12	0.00	16.01
0.2	4223	4142	4039	4236	4160.00	20800.00	45.38	2.18	0.20	0.00	-2.09
0.4	8520	8466	8577	8141	8426.00	21065.00	97.67	2.32	0.39	0.00	-3.63
0.8	17344	18384	17243	17919	17722.50	22153.13	266.05	3.00	0.80	0.01	-0.15
1.6	36520	36584	34210	36018	35833.00	22395.63	555.60	3.10	1.60	0.02	0.25
3.2	72389	70456	71143	72918	71726.50	22414.53	563.71	1.57	3.20	0.03	-0.01

Table A1.2 Data for determination of intra-day precision of the method for analyzing phenoxybenzyl alcohol in rat plasma (n = 5)

Concentration of phenoxybenzyl alcohol (µg/mL)	N	Intra-day precision			
		Peak area (AU)	Mean	SEM	%RSD
0.1	1	3801	3800.80	39.28	2.31
	2	3862			
	3	3663			
	4	3788			
	5	3890			
0.8	1	27027	26512.20	433.64	3.66
	2	27761			
	3	26052			
	4	26522			
	5	25199			
51.2	1	1773073	1749292.80	9220.56	1.18
	2	1722704			
	3	1733635			
	4	1761000			
	5	1756052			
204.8	1	7120211	7166820.80	55450.14	1.73
	2	7191101			
	3	7367901			
	4	7043363			
	5	7111528			

Table A1.3 Data for determination of intra-day precision of the method for analyzing trans-permethrin in rat plasma (n = 5)

Concentration of trans-permethrin (µg/mL)	N	Intra-day precision			
		Peak area (AU)	**Mean**	**SEM**	**%RSD**
0.1	1	2078	2252.60	76.90	7.63
	2	2473			
	3	2130			
	4	2394			
	5	2188			
0.2	1	4259	4337.00	98.04	5.05
	2	4677			
	3	4237			
	4	4410			
	5	4102			
0.8	1	16944	16661.40	195.06	2.62
	2	16562			
	3	16790			
	4	15955			
	5	17056			
3.2	1	69958	69935.40	701.28	2.24
	2	71867			
	3	70095			
	4	70265			
	5	67492			

Table A1.4 Data for determination of inter-day precision of the method for analyzing phenoxybenzyl alcohol in rat plasma (n = 5)

Concentration of phenoxybenzyl alcohol (µg/mL)	Day	Inter-day precision			
		Peak area (AU)	**Mean**	**SEM**	**%RSD**
0.1	1	3763	3834.40	47.36	2.76
	2	3732			
	3	3912			
	4	3979			
	5	3786			
0.8	1	25199	25548.40	303.75	2.66
	2	25164			
	3	25018			
	4	26678			
	5	25683			
51.2	1	1703635	1739634.40	15753.35	2.02
	2	1751829			
	3	1739501			
	4	1791804			
	5	1711403			
204.8	1	7120211	7026246.80	108655.19	3.46
	2	7041447			
	3	7215188			
	4	7148235			
	5	6606153			

Table A1.5 Data for determination of inter-day precision of the method for analyzing trans-permethrin in rat plasma (n = 5)

Concentration of trans-permethrin (µg/mL)	Day	Inter-day precision			
		Peak area (AU)	Mean	SEM	%RSD
0.1	1	2078	2084.60	62.90	6.75
	2	2172			
	3	1964			
	4	2272			
	5	1937			
0.2	1	4659	4339.20	128.61	6.63
	2	4344			
	3	3892			
	4	4298			
	5	4503			
0.8	1	16944	16321.00	227.65	3.12
	2	16784			
	3	16095			
	4	15810			
	5	15972			
3.2	1	67492	68451.60	1256.48	4.10
	2	63958			
	3	70806			
	4	69759			
	5	70243			

Table A1.6 Data for determination of intra-day accuracy of the method for analyzing phenoxybenzyl alcohol in rat plasma (n = 5)

Concentration of phenoxybenzyl alcohol (µg/mL)	N	Intra-day accuracy			
		measured concentration (µg/mL)	**Mean**	**SEM**	**%DEV**
0.1	1	0.09	0.09	0.00	-12.89
	2	0.09			
	3	0.08			
	4	0.09			
	5	0.09			
0.8	1	0.76	0.75	0.01	-6.52
	2	0.78			
	3	0.73			
	4	0.75			
	5	0.71			
51.2	1	51.56	50.87	0.27	-0.65
	2	50.09			
	3	50.41			
	4	51.21			
	5	51.06			
204.8	1	207.11	208.47	1.61	1.79
	2	209.17			
	3	214.32			
	4	204.87			
	5	206.86			

Table A1.7 Data for determination of intra-day accuracy of the method for analyzing trans-permethrin in rat plasma (n = 5)

Concentration of trans-permethrin (µg/mL)	N	Intra-day accuracy			
		measured concentration (µg/mL)	**Mean**	**SEM**	**%DEV**
0.1	1	0.10	0.11	0.00	10.92
	2	0.12			
	3	0.11			
	4	0.12			
	5	0.11			
0.2	1	0.20	0.20	0.00	1.86
	2	0.22			
	3	0.20			
	4	0.21			
	5	0.19			
0.8	1	0.76	0.75	0.01	-5.95
	2	0.75			
	3	0.76			
	4	0.72			
	5	0.77			
3.2	1	3.13	3.12	0.03	-2.37
	2	3.21			
	3	3.13			
	4	3.14			
	5	3.02			

Table A1.8 Data for determination of inter-day accuracy of the method for analyzing phenoxybenzyl alcohol in rat plasma (n = 5)

Concentration of phenoxybenzyl alcohol (µg/mL)	Day	Inter-day accuracy			
		measured concentration (µg/mL)	Mean	SEM	%DEV
0.1	1	0.09	0.09	0.00	-11.91
	2	0.09			
	3	0.09			
	4	0.09			
	5	0.09			
0.8	1	0.71	0.72	0.01	-10.03
	2	0.71			
	3	0.70			
	4	0.75			
	5	0.72			
51.2	1	49.54	50.58	0.46	-1.20
	2	50.94			
	3	50.58			
	4	52.10			
	5	49.76			
204.8	1	207.11	204.38	3.16	-0.21
	2	204.82			
	3	209.87			
	4	207.93			
	5	192.16			

Table A1.9 Data for determination of inter-day accuracy of the method for analyzing trans-permethrin in rat plasma (n = 5)

Concentration of trans-permethrin (µg/mL)	Day	Inter-day accuracy			
		measured concentration (µg/mL)	Mean	SEM	%DEV
0.1	1	0.10	0.10	0.00	3.44
	2	0.11			
	3	0.10			
	4	0.11			
	5	0.10			
0.2	1	0.22	0.20	0.01	1.91
	2	0.20			
	3	0.18			
	4	0.20			
	5	0.21			
0.8	1	0.76	0.74	0.01	-7.85
	2	0.76			
	3	0.73			
	4	0.71			
	5	0.72			
3.2	1	3.02	3.06	0.06	-4.44
	2	2.86			
	3	3.16			
	4	3.12			
	5	3.14			

Table A1.10 Data for determination of recovery of the method for analyzing phenoxybenzyl alcohol in rat plasma (n = 5)

Concentration of phenoxybenzyl alcohol (µg/mL)	N	Peak area (AU)		% Recovery		
		Direct injection	After extraction	%Recovery	Mean	SEM
0.1	1	3922	3801	96.91	95.07	1.10
	2	4056	3862	95.22		
	3	4022	3663	91.07		
	4	3991	3788	94.91		
	5	4001	3890	97.23		
0.8	1	28876	27027	93.60	91.72	1.54
	2	28865	27761	96.18		
	3	28961	26052	89.96		
	4	28913	26522	91.73		
	5	28913	25199	87.15		
51.2	1	1911202	1773073	92.77	91.53	0.48
	2	1911273	1722704	90.13		
	3	1911298	1733635	90.70		
	4	1911316	1761000	92.14		
	5	1911256	1756052	91.88		
204.8	1	7549114	7120211	94.32	94.93	0.73
	2	7549178	7191101	95.26		
	3	7549212	7367901	97.60		
	4	7549338	7043363	93.30		
	5	7549162	7111528	94.20		

Table A1.11 Data for determination of recovery of the method for analyzing trans-permethrin in rat plasma (n = 5)

Concentration of trans-permethrin (µg/mL)	N	Peak area (AU)		% Recovery		
		Direct injection	After extraction	%Recovery	Mean	SEM
0.1	1	2711	2078	76.65	82.34	2.66
	2	2783	2473	88.86		
	3	2711	2130	78.57		
	4	2699	2394	88.70		
	5	2773	2188	78.90		
0.2	1	5385	4259	79.09	81.07	1.96
	2	5327	4677	87.80		
	3	5384	4237	78.70		
	4	5314	4410	82.99		
	5	5342	4102	76.79		
0.8	1	20739	16944	81.70	80.43	0.96
	2	20758	16562	79.79		
	3	20687	16790	81.16		
	4	20716	15955	77.02		
	5	20680	17056	82.48		
3.2	1	85180	69958	82.13	82.12	0.84
	2	85118	71867	84.43		
	3	85155	70095	82.31		
	4	85172	70265	82.50		
	5	85207	67492	79.21		

Table A1.12 Data for determination of lower limit of quantification of the method analyzing phenoxybenzyl alcohol in rat plasma (n = 5)

Concentration (µg/mL)	N	Peak area (AU)		S/N
		Phenoxybenzyl alcohol	**Niose**	
0.1	1	3595	663	5.42
	2	3854	668	5.77
	3	3551	679	5.23
	4	3679	672	5.47
	5	3786	684	5.54
mean		3693.00	673.20	5.49

Table A13 Data for determination of lower limit of quantification of the method analyzing trans-permethrin in rat plasma (n = 5)

Concentration (µg/mL)	N	Peak area (AU)		S/N
		Trans-permethrin	**Noise**	
0.1	1	2078	309	6.72
	2	2172	387	5.61
	3	1964	343	5.73
	4	2272	358	6.35
	5	1937	344	5.63
mean		2084.60	348.20	5.99

Appendix-2

Body weight of rats and time of sample collection for a single dose of mitragynine (MG) and alkaloid extract (AE) pretreatment

Table A2.1 Body weight of rats and time of blood collection for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg)

	Phase I								Phase II							
Rat	**Weight (g)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**	**Wash-out**	**Weight (g)**	**MG (p.o.)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**
n_1	208	7.30	8.00	16.00	22.05	2.05	6.00		220	8.00	9.30	10.00	18.00	24.05	4.00	8.05
n_2	206	7.45	8.15	16.15	22.20	2.20	6.20		239	8.00	9.30	10.00	18.05	24.00	4.05	8.00
n_3	210	7.35	8.00	16.05	22.00	2.00	6.05		248	8.10	9.30	10.10	18.10	24.10	4.10	8.10
n_4	209	7.45	8.15	16.15	22.20	2.20	6.20		231	8.20	9.30	10.20	18.20	24.20	4.20	8.20
n_5	220	7.40	8.20	16.20	22.20	2.20	6.20		243	8.15	9.30	10.15	18.20	24.25	4.25	8.20
n_6	216	7.50	8.30	16.35	22.30	2.30	6.30		227	8.10	9.30	10.10	18.15	24.10	4.10	8.10

Table A2.2 Body weight of rats and time of blood collection for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg)

Phase I								Wash-out	Phase II							
Rat	**Weight (g)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**		**Weight (g)**	**AE (p.o.)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**
n_1	205	7.50	8.20	16.20	22.20	2.20	6.20		220	8.10	9.45	10.10	18.10	24.15	4.10	8.15
n_2	193	8.00	8.25	16.30	22.35	2.30	6.35		200	8.10	9.45	10.10	18.15	24.15	4.15	8.10
n_3	210	7.45	8.15	16.20	22.20	2.20	6.20		221	8.20	9.45	10.20	18.25	24.25	4.20	8.20
n_4	210	7.50	8.30	16.30	22.35	2.30	6.35		225	8.30	9.45	10.30	18.30	24.30	4.35	8.35
n_5	208	8.00	8.30	16.30	22.35	2.30	6.30		223	8.30	9.45	10.30	18.30	24.35	4.35	8.30
n_6	221	8.10	8.40	16.45	22.50	2.45	6.45		242	8.20	9.45	10.20	18.25	24.20	4.25	8.20

Appendix-3

Body weight of rats and time of sample collection for a multiple doses of mitragynine (MG) and alkaloid extract (AE) pretreatment

Table A3.1 Body weight of rats and time of blood collection for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg)

Phase I								
Rat	**Weight (g)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**	**Wash-out**
n_1	202	7.40	8.10	16.10	22.05	2.00	6.00	
n_2	200	7.30	8.00	16.05	22.00	2.10	6.10	
n_3	210	7.30	8.00	16.00	22.00	2.05	6.00	
n_4	206	7.45	8.15	16.15	22.20	2.20	6.20	
n_5	198	7.20	8.00	16.00	22.05	2.10	6.20	
n_6	213	7.35	8.10	16.10	22.15	2.20	6.30	

Phase II														
	Day1		**Day2**		**Day3**		**Day4**							
Rat	**Weight (g)**	**MG (p.o.)**	**Weight (g)**	**MG (p.o.)**	**Weight (g)**	**MG (p.o.)**	**Weight (g)**	**MG (p.o.)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**
n_1	225	8.10	230	8.00	241	8.00	250	8.10	9.30	10.00	18.00	24.05	4.00	8.05
n_2	227	8.20	232	8.10	239	8.10	253	8.15	9.30	10.05	18.05	24.00	4.05	8.00
n_3	234	8.00	243	8.05	242	8.10	248	8.10	9.30	10.00	18.05	24.00	4.05	8.00
n_4	227	8.15	231	8.10	236	8.20	245	8.00	9.30	10.10	18.15	24.10	4.05	8.10
n_5	224	8.10	233	8.15	240	8.00	249	8.10	9.30	10.05	18.00	24.10	4.10	8.05
n_6	237	8.20	242	8.00	248	8.10	256	8.00	9.30	10.10	18.15	24.10	4.10	8.10

Table A3.2 Body weight of rats and time of blood collection for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg)

Phase I								
Rat	**Weight (g)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**	**Wash-out**
n_1	200	7.50	8.20	16.20	22.20	2.20	6.30	
n_2	208	7.45	8.20	16.25	22.30	2.30	6.3	
n_3	210	7.45	8.15	16.20	22.20	2.25	6.20	
n_4	210	8.00	8.25	16.30	22.35	2.30	6.35	
n_5	206	7.30	8.20	16.20	22.20	2.25	6.30	
n_6	208	7.50	8.30	16.30	22.35	2.30	6.30	

Phase II														
	Day1		**Day2**		**Day3**		**Day4**							
Rat	**Weight (g)**	**AE (p.o.)**	**Weight (g)**	**AE (p.o.)**	**Weight (g)**	**AE (p.o.)**	**Weight (g)**	**AE (p.o.)**	**T_0**	**PM (p.o.)**	**T_8**	**T_{14}**	**T_{18}**	**T_{22}**
n_1	228	8.15	224	8.10	220	8.10	225	8.15	9.45	10.10	18.10	24.15	4.10	8.15
n_2	220	8.25	222	8.15	200	8.10	208	8.20	9.45	10.10	18.15	24.15	4.10	8.20
n_3	220	8.10	218	8.15	221	8.20	224	8.20	9.45	10.15	18.20	24.20	4.20	8.20
n_4	228	8.20	230	8.20	225	8.30	231	8.10	9.45	10.20	18.20	24.25	4.20	8.30
n_5	226	8.20	235	8.25	223	8.30	231	8.20	9.45	10.20	18.25	24.30	4.20	8.30
n_6	219	8.30	225	8.10	232	8.20	245	8.10	9.45	10.20	18.25	24.20	4.25	8.20

Appendix-4

Data of metabolic ratio of sample collection for single dose

Table A4.1 Data of peak area for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg)

Gr.1	Time (h)	Peak area PBAlc (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	3384512	3405217	3305112	3291876	3350812	3251856	3331564.17	58697.45	23963.13	1.76
	14	2843561	2903125	3002562	2769324	2798856	2834967	2858732.50	83703.84	34171.95	2.93
	18	2493251	2410436	2298467	2512053	2398021	2598785	2451835.50	104945.09	42843.65	4.28
	22	2200134	2003213	1928980	1990432	2000789	2027890	2025239.67	91821.09	37485.80	4.53
		Peak area trans-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	9526	8281	9842	8912	9078	8956	9099.17	540.25	220.56	5.94
	14	4946	4923	6356	4892	6202	5089	5401.33	684.92	279.62	12.68
	18	3298	3543	3085	3812	3605	3492	3472.50	252.50	103.08	7.27
	22	2621	2698	2512	2289	2103	2549	2462.00	223.54	91.26	9.08
		Peak area cis-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	10832	10910	13733	12561	12909	10276	11870.17	1383.30	564.73	11.65
	14	5444	7399	9230	8437	8643	7440	7765.50	1341.29	547.58	17.27
	18	4812	5321	5149	5390	5025	5623	5220.00	286.72	117.05	5.49
	22	3819	4135	3689	3960	3561	4470	3939.00	328.82	134.24	8.35

Table A4.1 Data of concentration for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.1	Time (h)	Concentration PBAlc (µg/mL)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	98.42	99.03	96.11	95.73	97.44	94.57	96.88	1.71	0.70	1.76
	14	82.69	84.42	87.31	80.53	81.39	82.44	83.13	2.43	0.99	2.93
	18	72.50	70.09	66.83	73.05	69.73	75.57	71.29	3.05	1.25	4.28
	22	63.97	58.24	56.09	57.87	58.17	58.96	58.89	2.67	1.09	4.54
		Concentration trans-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	0.43	0.38	0.45	0.41	0.41	0.41	0.42	0.02	0.01	5.79
	14	0.23	0.23	0.29	0.23	0.29	0.24	0.25	0.03	0.01	12.14
	18	0.16	0.17	0.15	0.18	0.17	0.17	0.17	0.01	0.00	6.80
	22	0.13	0.13	0.12	0.11	0.10	0.12	0.12	0.01	0.00	8.28
		Concentration cis-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	1.13	1.14	1.42	1.31	1.34	1.08	1.24	0.14	0.06	11.28
	14	0.59	0.79	0.97	0.89	0.91	0.79	0.82	0.14	0.06	16.45
	18	0.52	0.58	0.56	0.58	0.55	0.61	0.57	0.03	0.01	5.11
	22	0.42	0.46	0.41	0.44	0.40	0.49	0.44	0.03	0.01	7.60

Table A4.1 Data of metabolic ratio for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.1	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
PM alone	8	226.69	261.41	214.43	235.28	235.23	231.31	234.06	15.48	6.32	6.61
	14	358.68	367.82	297.76	352.98	284.19	347.99	334.91	34.92	14.26	10.43
	18	461.00	416.81	452.20	405.54	407.97	455.53	433.17	25.70	10.49	5.93
	22	503.07	446.02	458.52	514.87	558.65	475.64	492.79	41.44	16.92	8.41
	Time (h)	**Metabolic ratio PBAlc/cis-PM**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	86.96	86.89	67.48	73.29	72.65	87.91	79.19	9.06	3.70	11.44
	14	140.55	107.47	89.99	90.46	89.34	104.40	103.70	19.71	8.05	19.00
	18	138.21	121.70	119.65	125.32	127.70	124.62	126.20	6.52	2.66	5.17
	22	150.74	127.65	136.36	131.94	146.03	120.31	135.51	11.39	4.65	8.40
	Time (h)	**Metabolic ratio PBAlc/trans+cis-PM**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	62.85	65.21	51.32	55.88	55.51	63.70	59.08	5.59	2.28	9.46
	14	100.98	83.17	69.11	72.01	67.97	80.31	78.92	12.41	5.07	15.73
	18	106.33	94.20	94.62	95.74	97.25	97.85	97.66	4.48	1.83	4.59
	22	115.98	99.25	105.11	105.03	115.77	96.02	106.19	8.27	3.37	7.78

Table A4.1 Data of peak area for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.1	**Time (h)**	**Peak area PBAlc (AU)**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
Treat	8	1185886	1429045	1492895	1315868	1484832	1476597	1397520.50	122819.19	50140.73	8.79
	14	607191	832145	954666	856929	826938	979259	842854.67	132069.23	53917.04	15.67
	18	428638	621027	780972	681579	674822	781607	661440.83	130493.41	53273.71	19.73
	22	291352	405324	665625	406666	417895	680506	477894.67	158134.24	64558.03	33.09
		Peak area trans-PM (AU)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	3388	3561	4934	3607	4849	4304	4107.17	683.87	279.19	16.65
	14	2405	2708	3761	2580	2989	2861	2884.00	476.13	194.38	16.51
	18	2177	2535	2825	2644	2513	2445	2523.17	215.35	87.92	8.54
	22	1964	2021	2659	2164	2383	2226	2236.17	255.35	104.25	11.42
		Peak area cis-PM (AU)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	4066	5547	8471	5306	5069	8048	6084.50	1763.59	719.98	28.98
	14	2947	4456	5624	4327	4120	5089	4427.17	912.86	372.67	20.62
	18	2314	3448	4245	3654	3817	4023	3583.50	681.21	278.10	19.01
	22	2001	2504	3937	2952	3388	3582	3060.67	719.93	293.91	23.52

Table A4.1 Data of concentration for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.1	**Time (h)**	**Concentration PBAlc (µg/mL)**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
Treat	8	54.47	41.54	43.40	58.25	43.17	42.93	56.63	3.57	1.46	8.79
	14	37.64	44.18	37.74	34.90	34.03	24.46	40.49	3.84	1.57	15.69
	18	22.44	28.04	22.69	29.80	19.60	22.71	28.21	3.80	1.55	19.75
	22	18.45	11.76	19.34	11.80	12.13	19.77	15.88	4.60	1.88	33.15
		Concentration trans-PM (µg/mL)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	0.26	0.17	0.23	0.27	0.23	0.20	0.27	0.03	0.01	15.73
	14	0.22	0.15	0.18	0.23	0.21	0.17	0.22	0.02	0.01	15.25
	18	0.17	0.12	0.14	0.19	0.12	0.15	0.18	0.01	0.00	7.80
	22	0.14	0.10	0.13	0.11	0.12	0.13	0.13	0.01	0.00	10.32
		Concentration cis-PM (µg/mL)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	0.45	0.60	0.89	0.57	0.55	0.85	0.65	0.18	0.07	27.25
	14	0.34	0.49	0.61	0.48	0.45	0.55	0.49	0.09	0.04	18.96
	18	0.27	0.39	0.47	0.41	0.42	0.44	0.40	0.07	0.03	17.15
	22	0.24	0.29	0.44	0.34	0.38	0.40	0.35	0.07	0.03	20.87

Table A4.1 Data of metabolic ratio for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.1	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
Treat	8	213.74	245.88	188.69	223.68	190.81	212.51	212.55	21.34	8.71	10.04
	14	150.02	184.53	156.00	198.66	167.42	206.47	177.18	23.05	9.41	13.01
	18	115.83	146.25	166.56	154.46	160.21	190.30	155.60	24.56	10.03	15.78
	22	86.26	117.08	150.06	110.47	104.05	180.37	124.72	34.37	14.03	27.56
	Time (h)	**Metabolic ratio PBAlc/cis-PM**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	76.72	69.39	48.56	66.59	78.41	50.44	65.02	12.81	5.23	19.71
	14	52.42	49.48	45.75	52.35	52.84	51.51	50.72	2.72	1.11	5.36
	18	45.65	46.61	48.55	48.56	46.21	51.04	47.77	2.01	0.82	4.20
	22	35.06	40.32	44.32	35.03	31.84	49.36	39.32	6.62	2.70	16.84
	Time (h)	**Metabolic ratio PBAlc/trans+cis-PM**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	56.45	54.12	38.62	51.32	55.57	40.77	49.47	7.80	3.19	15.77
	14	38.85	39.02	35.37	41.43	40.16	41.22	39.34	2.22	0.91	5.65
	18	32.74	35.35	37.59	36.94	35.87	40.24	36.46	2.50	1.02	6.86
	22	24.93	29.99	34.21	26.60	24.38	38.76	29.81	5.71	2.33	19.16

Table A4.2 Data of peak area for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg)

Gr.2	Time (h)	Peak area PBAlc (AU)									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
PM alone	8	3218983	3578903	3389076	3498567	3106789	3489789	3380351.17	182387.78	74459.50	5.40
	14	2589803	2900831	2746321	2995672	2593561	2904782	2788495.00	172232.85	70313.77	6.18
	18	2324672	2598321	2459832	2398012	2298673	2478929	2426406.50	110365.28	45056.44	4.55
	22	2095871	2289842	1980467	2109453	2009856	1996732	2080370.17	115682.99	47227.38	5.56
		Peak area trans-PM (AU)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	9567	9019	8967	9505	9189	9243	9248.33	246.06	100.45	2.66
	14	4845	5012	4967	5974	5378	4948	5187.33	426.37	174.07	8.22
	18	3045	3967	3198	3356	3876	3349	3465.17	372.55	152.09	10.75
	22	2212	2693	2321	2287	2312	2634	2409.83	201.06	82.08	8.34
		Peak area cis-PM (AU)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	12311	12247	10283	11507	12008	11278	11605.67	766.45	312.90	6.60
	14	6904	6461	5179	6321	6032	6137	6172.33	574.18	234.41	9.30
	18	4223	3620	3398	4055	4636	3898	3971.67	440.37	179.78	11.09
	22	3623	2799	2909	2848	2817	2941	2989.50	315.03	128.61	10.54

Table A4.2 Data of concentration for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.2	Time (h)	Concentration PBAlc (µg/mL)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	93.61	104.08	98.56	101.74	90.35	101.49	98.30	5.31	2.17	5.40
	14	75.31	84.35	79.86	87.11	75.42	84.47	81.09	5.01	2.05	6.18
	18	67.60	75.56	71.53	69.73	66.84	72.08	70.55	3.21	1.31	4.55
	22	60.94	66.58	57.58	61.34	58.44	58.06	60.49	3.37	1.37	5.56
		Concentration trans-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	0.44	0.41	0.41	0.43	0.42	0.42	0.42	0.01	0.00	2.59
	14	0.23	0.23	0.23	0.28	0.25	0.23	0.24	0.02	0.01	7.86
	18	0.15	0.19	0.15	0.16	0.18	0.16	0.16	0.02	0.01	10.06
	22	0.11	0.13	0.11	0.11	0.11	0.13	0.12	0.01	0.00	7.59
		Concentration cis-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	1.28	1.27	1.08	1.20	1.25	1.18	1.21	0.08	0.03	6.39
	14	0.74	0.69	0.56	0.68	0.65	0.66	0.66	0.06	0.02	8.75
	18	0.47	0.40	0.38	0.45	0.51	0.43	0.44	0.04	0.02	10.10
	22	0.40	0.32	0.33	0.33	0.32	0.34	0.34	0.03	0.01	9.33

Table A4.2 Data of metabolic ratio for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.2	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
PM alone	8	214.70	252.84	240.78	234.84	215.52	240.72	233.23	15.21	6.21	6.52
	14	333.15	361.30	345.01	315.35	301.97	366.26	337.17	25.38	10.36	7.53
	18	462.93	404.01	468.05	436.23	365.31	451.83	431.39	39.74	16.22	9.21
	22	559.18	510.73	505.88	546.10	515.20	454.47	515.26	36.56	14.93	7.10
		Metabolic ratio PBAlc/cis-PM									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
	8	73.07	81.66	91.56	84.79	72.25	86.24	81.60	7.63	3.11	9.35
	14	102.38	122.09	142.20	128.72	116.45	128.33	123.36	13.40	5.47	10.86
	18	145.32	186.86	187.27	155.57	131.88	166.71	162.27	22.39	9.14	13.80
	22	150.60	207.09	173.12	187.88	180.74	172.87	178.72	18.70	7.63	10.46
		Metabolic ratio PBAlc/trans+cis-PM									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
	8	54.52	61.72	66.33	62.30	54.11	63.49	60.41	4.99	2.04	8.25
	14	78.31	91.26	100.70	91.41	84.04	95.03	90.12	7.94	3.24	8.81
	18	110.60	127.77	133.76	114.67	96.90	121.78	117.58	13.18	5.38	11.21
	22	118.65	147.34	128.98	139.79	133.80	125.23	132.30	10.31	4.21	7.80

Table A4.2 Data of peak area for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.2	Time (h)	Peak area PBAlc (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
Treat	8	1907995	1897400	1885168	1821020	1850947	1798490	1860170.00	44091.75	18000.38	2.37
	14	1828835	1675395	1785564	1797596	1729408	1631417	1741369.17	76615.99	31278.35	4.40
	18	1614468	1475426	1529935	1525348	1535922	1576137	1542872.67	47548.44	19411.57	3.08
	22	1361241	1261532	1125986	1281776	1169665	1175532	1229288.67	87546.76	35740.81	7.12
		Peak area trans-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	6075	5785	5933	6166	5837	5889	5947.50	145.77	59.51	2.45
	14	4766	4676	4558	5226	4960	4574	4793.33	258.06	105.35	5.38
	18	4348	4099	4124	4139	4434	4285	4238.17	137.73	56.23	3.25
	22	3997	3401	3443	3216	3318	3260	3439.17	286.09	116.80	8.32
		Peak area cis-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	21075	18214	15430	15890	15306	15503	16903.00	2316.49	945.70	13.70
	14	10766	8873	8914	8957	8468	7712	8948.33	1006.98	411.10	11.25
	18	7348	4544	6198	6044	6122	5520	5962.67	918.16	374.84	15.40
	22	4297	4027	5554	5193	5706	4396	4862.17	711.95	290.65	14.64

Table A4.2 Data of concentration for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.2	Time (h)	Concentration PBAlc (μg/mL)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
Treat	8	55.47	55.17	54.81	52.95	53.82	52.29	54.08	1.28	0.52	2.37
	14	53.17	48.71	51.91	52.26	50.28	47.43	50.63	2.23	0.91	4.40
	18	36.94	42.89	44.48	34.34	44.65	45.82	39.85	1.38	0.56	3.08
	22	31.57	36.67	32.73	31.26	34.00	34.17	31.73	2.55	1.04	7.13
		Concentration trans-PM (μg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	0.28	0.27	0.27	0.28	0.27	0.27	0.28	0.01	0.00	2.36
	14	0.24	0.22	0.24	0.24	0.23	0.21	0.24	0.01	0.00	5.13
	18	0.20	0.19	0.19	0.19	0.21	0.20	0.20	0.01	0.00	3.08
	22	0.19	0.16	0.16	0.15	0.16	0.16	0.16	0.01	0.01	7.78
		Concentration cis-PM (μg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	2.17	1.88	1.60	1.64	1.58	1.60	1.74	0.23	0.10	13.40
	14	1.13	0.93	0.94	0.94	0.89	0.82	0.94	0.10	0.04	10.79
	18	0.78	0.50	0.66	0.65	0.66	0.60	0.64	0.09	0.04	14.46
	22	0.47	0.45	0.60	0.56	0.61	0.48	0.53	0.07	0.03	13.56

Table A4.2 Data of metabolic ratio for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.2	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
Treat	8	197.60	205.97	199.73	185.91	199.20	191.91	196.72	6.95	2.84	1.44
	14	238.94	222.89	243.40	215.09	217.51	221.63	226.58	11.73	4.79	2.11
	18	230.14	222.38	229.28	227.81	214.90	227.80	225.38	5.80	2.37	1.05
	22	210.10	226.58	199.91	242.54	214.97	219.63	218.95	14.64	5.98	2.73
	Time (h)	**Metabolic ratio PBAlc/cis-PM**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	25.62	29.40	34.35	32.24	33.99	32.62	31.37	3.32	1.35	4.32
	14	47.26	52.14	55.32	55.44	56.28	58.05	54.08	3.85	1.57	2.91
	18	60.15	86.21	66.95	68.34	67.99	76.88	71.09	9.12	3.72	5.24
	22	83.73	82.33	54.60	66.18	55.30	70.80	68.82	12.65	5.17	7.51
	Time (h)	**Metabolic ratio PBAlc/trans+cis-PM**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	22.68	25.73	29.31	27.48	29.04	27.88	27.02	2.48	1.01	3.75
	14	39.45	42.25	45.08	44.08	44.71	46.00	43.60	2.38	0.97	2.23
	18	47.68	62.13	51.82	52.57	51.65	57.48	53.89	5.10	2.08	3.87
	22	59.87	60.39	42.89	51.99	43.99	53.54	52.11	7.51	3.07	5.88

Appendix-5

Data of metabolic ratio of sample collection for multiple doses

Table A5.1 Data of peak area for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg)

Gr.3	Time (h)	Peak area PBAlc (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	3390567	3689732	3498426	3309456	3409256	3498365	3465967.00	130821.24	53407.55	3.77
	14	2789364	2906874	2583459	2589354	2795431	2547619	2702016.83	147587.10	60252.18	5.46
	18	2326519	2598673	2398466	2294309	2409678	2390674	2403053.17	105988.74	43269.72	4.41
	22	2045322	2298663	1987565	1995432	2098673	2094576	2086705.17	114020.55	46548.70	5.46
		Peak area trans-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	8965	9256	8951	9012	9798	9054	9172.67	325.62	132.93	3.55
	14	5286	6078	5461	5912	4780	4912	5404.83	521.72	212.99	9.65
	18	3096	3609	3243	3568	3350	3298	3360.67	196.26	80.12	5.84
	22	2230	2498	2318	2593	2209	2385	2372.17	151.35	61.79	6.38
		Peak area cis-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	10335	11709	11844	10921	12326	12176	11551.83	771.70	315.05	6.68
	14	7248	7129	7932	6399	8374	7312	7399.00	683.81	279.16	9.24
	18	4838	4675	4874	5090	5162	5482	5020.17	287.04	117.19	5.72
	22	4414	4023	4448	3961	3961	4070	4146.17	224.67	91.72	5.42

Table A5.1 Data of concentration for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.3	Time (h)	Concentration PBAlc (μg/mL)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	98.60	107.30	101.74	96.24	99.14	101.74	100.79	3.81	1.55	3.78
	14	81.11	84.53	75.12	75.29	81.29	74.08	78.57	4.29	1.75	5.46
	18	67.65	75.57	69.74	66.71	70.07	69.52	69.88	3.08	1.26	4.41
	22	59.47	66.84	57.79	58.02	61.02	60.90	60.67	3.32	1.35	5.47
		Concentration trans-PM (μg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	0.41	0.42	0.41	0.41	0.45	0.41	0.42	0.01	0.01	3.46
	14	0.25	0.28	0.25	0.27	0.22	0.23	0.25	0.02	0.01	9.24
	18	0.15	0.17	0.15	0.17	0.16	0.16	0.16	0.01	0.00	5.45
	22	0.11	0.12	0.11	0.13	0.11	0.12	0.12	0.01	0.00	5.80
		Concentration cis-PM (μg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	1.08	1.22	1.23	1.14	1.28	1.27	1.20	0.08	0.03	6.46
	14	0.77	0.76	0.84	0.68	0.88	0.78	0.79	0.07	0.03	8.78
	18	0.53	0.51	0.53	0.55	0.56	0.59	0.55	0.03	0.01	5.31
	22	0.48	0.44	0.49	0.44	0.44	0.45	0.46	0.02	0.01	4.95

Table A5.1 Data of metabolic ratio for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.3	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
PM alone	8	240.94	254.17	248.98	233.98	222.16	246.22	241.08	11.56	4.72	4.80
	14	330.19	300.96	296.42	275.31	364.26	323.46	315.10	31.13	12.71	9.88
	18	456.21	441.66	450.47	394.11	439.08	442.02	437.26	22.09	9.02	5.05
	22	541.71	549.23	508.29	460.75	560.62	521.99	523.77	36.17	14.77	6.91
		Metabolic ratio PBAlc/cis-PM									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	**Mean**	**SD**	**SEM**	**%RSD**
	8	91.15	87.93	82.45	84.36	77.30	80.27	83.91	5.06	2.06	6.03
	14	105.30	111.48	89.51	109.97	91.97	95.37	100.60	9.52	3.89	9.46
	18	128.32	147.95	131.38	120.72	125.15	117.39	128.49	10.79	4.40	8.39
	22	122.76	150.21	118.46	132.24	139.09	135.42	133.03	11.45	4.68	8.61
		Metabolic ratio PBAlc/trans+cis-PM									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	**Mean**	**SD**	**SEM**	**%RSD**
	8	66.13	65.33	61.94	62.01	57.35	60.54	62.22	3.22	1.31	5.17
	14	79.84	81.35	68.75	78.58	73.43	73.65	75.93	4.79	1.95	6.31
	18	100.15	110.83	101.72	92.41	97.39	92.76	99.21	6.83	2.79	6.88
	22	100.08	117.95	96.07	102.75	111.44	107.53	105.97	7.99	3.26	7.54

Table A5.1 Data of peak area for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.3	Time (h)	Peak area PBAlc (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
Treat	8	1756712	1728532	1693010	1724903	1712469	1643896	1709920.33	38501.95	15718.36	2.25
	14	1074642	987685	968713	985239	985239	1002468	1000664.33	37792.01	15428.52	3.78
	18	659709	629856	680818	717231	694695	681896	677367.50	29969.72	12235.09	4.42
	22	436350	421240	438078	412312	429636	452789	431734.17	14118.12	5763.70	3.27
		Peak area trans-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	5034	5006	5415	5185	5263	5314	5202.83	160.35	65.46	3.08
	14	3926	4042	3748	4063	4052	3802	3938.83	137.24	56.03	3.48
	18	3126	3202	3067	3598	3322	3167	3247.00	191.99	78.38	5.91
	22	2245	2325	2066	2086	2233	2456	2235.17	146.84	59.95	6.57
		Peak area cis-PM (AU)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	9133	9219	9604	9584	9020	9812	9395.33	314.18	128.26	3.34
	14	6800	6915	6451	6174	6948	6380	6611.33	319.89	130.59	4.84
	18	3940	3873	4038	4365	4693	3756	4110.83	352.27	143.81	8.57
	22	2815	2604	2871	2685	3073	2732	2796.67	164.92	67.33	5.90

Table A5.1 Data of concentration for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.3	**Time (h)**	**Concentration PBAlc (µg/mL)**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
Treat	8	51.07	50.25	49.22	50.15	49.79	47.79	49.71	1.12	0.46	2.25
	14	31.23	38.70	28.15	28.63	28.63	39.13	35.08	1.10	0.45	3.78
	18	19.16	18.30	19.78	24.84	23.18	19.81	22.68	0.87	0.36	4.43
	22	12.67	14.23	13.72	14.97	12.47	13.15	14.53	0.41	0.17	3.28
	Time (h)	**Concentration trans-PM (µg/mL)**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	0.23	0.23	0.25	0.24	0.24	0.25	0.24	0.01	0.00	2.95
	14	0.21	0.21	0.24	0.22	0.23	0.23	0.23	0.01	0.00	3.28
	18	0.17	0.16	0.17	0.17	0.16	0.16	0.17	0.01	0.00	5.51
	22	0.13	0.12	0.13	0.13	0.12	0.12	0.13	0.01	0.00	5.93
	Time (h)	**Concentration cis-PM (µg/mL)**									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	0.96	0.97	1.01	1.01	0.95	1.03	0.99	0.03	0.01	3.21
	14	0.73	0.74	0.69	0.66	0.74	0.68	0.71	0.03	0.01	4.57
	18	0.44	0.43	0.45	0.48	0.51	0.42	0.45	0.04	0.01	7.83
	22	0.32	0.30	0.33	0.31	0.35	0.31	0.32	0.02	0.01	5.18

Table A5.1 Data of metabolic ratio for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.3	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
Treat	8	217.85	215.49	195.79	207.94	203.52	193.57	205.69	9.98	4.08	4.85
	14	168.66	150.80	158.81	149.69	150.08	162.15	156.70	7.81	3.19	4.98
	18	128.09	119.58	134.55	122.14	127.47	130.81	127.11	5.50	2.25	4.33
	22	114.69	107.26	124.09	115.77	113.47	109.70	114.16	5.82	2.38	5.10
		Metabolic ratio PBAlc/cis-PM									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
	8	53.18	51.86	48.83	49.85	52.46	46.45	50.44	2.55	1.04	5.05
	14	43.08	38.96	40.81	43.26	38.69	42.67	41.24	2.07	0.84	5.01
	18	43.89	42.56	44.30	43.46	39.37	47.39	43.50	2.60	1.06	5.98
	22	39.20	40.51	38.68	38.60	35.72	41.76	39.08	2.05	0.84	5.24
		Metabolic ratio PBAlc/trans+cis-PM									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
	8	42.74	41.80	39.09	40.21	41.71	37.46	40.50	1.98	0.81	4.88
	14	34.31	30.96	32.46	33.56	30.76	33.78	32.64	1.50	0.61	4.61
	18	32.69	31.39	33.33	32.05	30.08	34.78	32.39	1.62	0.66	5.01
	22	29.22	29.40	29.49	28.95	27.17	30.25	29.08	1.03	0.42	3.55

Table A5.2 Data of peak area for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg)

Gr.4	Time (h)	Peak area PBAlc (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	3298678	3590452	3553476	3398435	3684534	3298346	3470653.50	162133.94	66190.90	4.67
	14	2795642	2694538	2549830	2893210	2902174	2623451	2743140.83	144655.21	59055.24	5.27
	18	2342918	2209573	2298677	2498540	2609213	2298994	2376319.17	148394.66	60581.87	6.24
	22	2021234	1995684	1980576	2096743	2209563	1980967	2047461.17	90520.21	36954.72	4.42
		Peak area trans-PM (AU)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	9056	9134	8932	9023	9146	8960	9041.83	87.93	35.90	0.97
	14	5194	5685	4976	5523	5803	4962	5357.17	363.82	148.53	6.79
	18	3002	3541	3068	3601	3427	3090	3288.17	264.84	108.12	8.05
	22	2298	2532	2437	2539	2420	2210	2406.00	130.23	53.17	5.41
		Peak area cis-PM (AU)									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**S.D.**	**SEM**	**%RSD**
	8	10705	10953	9890	12507	11728	12422	11367.50	1032.87	421.67	9.09
	14	6081	7489	7391	6914	6578	6078	6755.17	618.46	252.49	9.16
	18	4242	4696	4550	3920	3687	4012	4184.50	386.20	157.67	9.23
	22	2806	3177	3028	2978	2841	3079	2984.83	141.69	57.84	4.75

Table A5.2 Data of concentration for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.4	Time (h)	Concentration PBAlc (µg/mL)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
PM alone	8	95.93	104.41	103.34	98.83	107.15	95.92	100.93	4.72	1.93	4.67
	14	81.30	78.35	74.14	84.13	84.39	76.29	79.77	4.21	1.72	5.28
	18	68.13	64.25	66.84	72.65	75.87	66.85	69.10	4.32	1.76	6.25
	22	58.77	58.03	57.59	60.97	64.25	57.60	59.53	2.63	1.07	4.42
		Concentration trans-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	0.41	0.42	0.41	0.41	0.42	0.41	0.41	0.00	0.00	0.95
	14	0.24	0.26	0.23	0.26	0.27	0.23	0.25	0.02	0.01	6.50
	18	0.14	0.17	0.15	0.17	0.16	0.15	0.16	0.01	0.00	7.51
	22	0.11	0.12	0.12	0.12	0.12	0.11	0.12	0.01	0.00	4.92
		Concentration cis-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	1.12	1.14	1.04	1.30	1.22	1.29	1.19	0.10	0.04	8.79
	14	0.65	0.79	0.78	0.74	0.70	0.65	0.72	0.06	0.03	8.66
	18	0.47	0.51	0.50	0.43	0.41	0.44	0.46	0.04	0.02	8.45
	22	0.32	0.36	0.34	0.34	0.33	0.35	0.34	0.01	0.01	4.20

Table A5.2 Data of metabolic ratio for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.4	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
PM alone	8	232.11	250.55	253.43	239.99	256.78	234.51	244.56	10.40	4.25	4.25
	14	336.53	297.47	319.76	328.40	314.15	329.88	321.03	13.98	5.71	4.35
	18	472.75	382.26	454.56	425.52	465.47	451.62	442.03	33.43	13.65	7.56
	22	520.98	470.96	483.98	493.57	543.41	528.94	506.97	28.33	11.57	5.59
	Time (h)	Metabolic ratio PBAlc/cis-PM									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
	8	85.73	91.27	99.67	75.98	87.67	74.23	85.76	9.55	3.90	11.14
	14	124.58	98.61	94.49	114.22	120.10	116.95	111.49	12.15	4.96	10.89
	18	145.86	125.27	134.18	167.17	184.56	150.60	151.27	21.72	8.87	14.36
	22	182.39	161.34	167.10	179.54	197.23	164.68	175.38	13.59	5.55	7.75
	Time (h)	Metabolic ratio PBAlc/trans+cis-PM									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	SD	SEM	%RSD
	8	62.60	66.90	71.54	57.71	65.36	56.38	63.41	5.74	2.34	9.05
	14	90.92	74.06	72.93	84.74	86.88	86.34	82.65	7.38	3.01	8.93
	18	111.47	94.35	103.60	120.02	132.16	112.94	112.42	13.06	5.33	11.62
	22	135.09	120.17	124.22	131.65	144.71	125.58	130.24	8.88	3.62	6.82

Table A5.2 Data of peak area for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.4	Time (h)	Peak area PBAlc (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
Treat	8	1959364	1855241	1924832	1982508	1982144	1993759	1949641.33	52353.54	21373.24	2.69
	14	1583997	1569772	1450637	1527908	1430333	1575326	1522995.50	67072.99	27382.43	4.40
	18	1115173	1152959	1183049	1001159	1112794	1107539	1112112.17	61711.99	25193.82	5.55
	22	931842	908467	959649	935987	902061	927856	927643.67	20658.37	8433.74	2.23
	Time (h)	Peak area trans-PM (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	6070	6242	6090	6151	6108	6077	6123.00	65.09	26.57	1.06
	14	4939	4775	4218	4763	4242	4849	4631.00	317.00	129.41	6.85
	18	3573	3603	3646	3061	3337	3512	3455.33	221.23	90.32	6.40
	22	2995	2863	2977	2972	2961	2989	2959.50	48.80	19.92	1.65
	Time (h)	Peak area cis-PM (AU)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	9250	9369	9004	10776	9775	9786	9660.00	625.73	255.45	6.48
	14	6377	6761	6682	6831	6534	7018	6700.50	225.48	92.05	3.37
	18	5688	5156	5319	4703	5203	5854	5320.50	410.33	167.52	7.71
	22	4259	4116	4546	4348	4541	4299	4351.50	167.65	68.44	3.85

Table A5.2 Data of concentration for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.4	Time (h)	Concentration PBAlc (µg/mL)									
		n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
Treat	8	56.97	53.94	55.96	57.64	57.63	57.97	56.69	1.52	0.62	2.69
	14	46.05	45.64	42.17	44.42	41.58	45.80	44.28	1.95	0.80	4.41
	18	34.41	33.51	34.39	29.10	34.34	32.19	34.32	1.80	0.73	5.55
	22	27.08	26.40	27.89	27.20	26.21	26.96	25.96	0.60	0.25	2.23
		Concentration trans-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	0.28	0.29	0.28	0.28	0.28	0.28	0.28	0.00	0.00	1.02
	14	0.23	0.22	0.20	0.22	0.20	0.23	0.22	0.01	0.01	6.51
	18	0.17	0.17	0.17	0.17	0.16	0.17	0.17	0.01	0.00	5.99
	22	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.00	0.00	1.53
		Concentration cis-PM (µg/mL)									
	Time (h)	n_1	n_2	n_3	n_4	n_5	n_6	Mean	S.D.	SEM	%RSD
	8	0.97	0.98	0.95	1.13	1.03	1.03	1.01	0.06	0.03	6.23
	14	0.68	0.72	0.71	0.73	0.70	0.75	0.72	0.02	0.01	3.18
	18	0.61	0.56	0.58	0.51	0.56	0.63	0.58	0.04	0.02	7.19
	22	0.47	0.45	0.50	0.48	0.50	0.47	0.48	0.02	0.01	3.54

Table A5.2 Data of metabolic ratio for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg) (cont.)

Gr.4	Time (h)	Metabolic ratio PBAlc/trans-PM									
		n_1	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
Treat	8	203.09	187.19	198.88	202.88	204.22	206.42	200.45	6.94	2.84	3.46
	14	200.02	204.71	212.80	199.72	208.70	202.44	204.73	5.16	2.11	2.52
	18	191.23	196.18	199.07	198.30	203.42	193.01	196.87	4.40	1.80	2.24
	22	188.33	191.41	195.04	190.52	184.24	187.87	189.57	3.66	1.49	1.93
		Metabolic ratio PBAlc/cis-PM									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	58.60	54.80	59.07	51.18	56.22	56.48	56.06	2.87	1.17	5.11
	14	67.48	63.28	59.13	61.00	59.55	61.30	61.96	3.08	1.26	4.97
	18	52.88	59.92	59.73	56.65	57.35	51.12	56.28	3.59	1.47	6.39
	22	57.77	58.10	56.03	56.93	52.72	57.03	56.43	1.96	0.80	3.47
		Metabolic ratio PBAlc/trans+cis-PM									
	Time (h)	**n_1**	**n_2**	**n_3**	**n_4**	**n_5**	**n_6**	**Mean**	**SD**	**SEM**	**%RSD**
	8	45.48	42.39	45.54	40.87	44.08	44.35	43.79	1.83	0.75	4.18
	14	50.46	48.34	46.27	46.73	46.33	47.05	47.53	1.62	0.66	3.41
	18	41.43	45.90	45.94	44.07	44.73	40.42	43.75	2.32	0.95	5.31
	22	44.21	44.57	43.53	43.83	40.99	43.75	43.48	1.27	0.52	2.93

Appendix-6

Data of percentage of decrease in metabolic ratio of PM in sample collection for single dose

Table A6.1 Data of percentage decrease in metabolic ratio of PM for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) and Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg) at 8, 14, 18, and 22 h

Gr.1	n	%decrease of trans-PM				Gr.2	n	%decrease of trans-PM			
		8	**14**	**18**	**22**			**8**	**14**	**18**	**22**
MG+PM	n_1	-5.71	-58.17	-74.87	-82.85	**AE+PM**	n_1	-7.96	-28.28	-50.29	-62.43
	n_2	-5.94	-49.83	-64.91	-73.75		n_2	-18.54	-38.31	-44.96	-55.64
	n_3	-12.00	-47.61	-63.17	-67.27		n_3	-17.05	-29.45	-51.01	-60.48
	n_4	-4.93	-43.72	-61.91	-78.54		n_4	-20.83	-31.79	-47.78	-55.59
	n_5	-18.88	-41.09	-60.73	-81.37		n_5	-7.57	-27.97	-41.17	-58.28
	n_6	-8.13	-40.67	-58.22	-62.08		n_6	-20.28	-39.49	-49.58	-51.67
	Mean	-9.27	-46.85	-63.97	-74.31		**Mean**	-15.37	-32.55	-47.47	-57.35
	S.D.	5.36	6.62	5.80	8.25		**S.D.**	6.04	5.11	3.77	3.87
	SEM	2.19	2.70	2.37	3.37		**SEM**	2.47	2.09	1.54	1.58

Appendix-7

Data of percentage of decrease in metabolic ratio of PM in sample collection for multiple doses

Table A7.1 Data of percentage decrease in metabolic ratio of PM for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg) and Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg) at 8, 14, 18, and 22 h

Gr.3	n	%decrease of trans-PM				Gr.4	n	%decrease of trans-PM			
		8	14	18	22			8	14	18	22
MG+PM	n_1	-9.59	-48.92	-71.92	-78.83	AE+PM	n_1	-12.50	-40.56	-59.55	-63.85
	n_2	-15.22	-49.89	-72.92	-80.47		n_2	-25.29	-31.19	-48.68	-59.36
	n_3	-21.36	-46.42	-70.13	-75.59		n_3	-21.53	-33.45	-56.21	-59.70
	n_4	-11.13	-45.63	-69.01	-74.87		n_4	-15.46	-39.18	-53.40	-61.40
	n_5	-8.39	-58.80	-70.97	-79.76		n_5	-20.47	-33.57	-56.30	-66.10
	n_6	-21.38	-49.87	-70.41	-78.98		n_6	-11.98	-38.63	-57.26	-64.48
	Mean	-14.51	-49.92	-70.89	-78.08		**Mean**	-17.87	-36.10	-55.23	-62.48
	S.D.	5.79	4.70	1.38	2.30		**S.D.**	5.38	3.83	3.77	2.74
	SEM	2.37	1.92	0.56	0.94		**SEM**	2.19	1.56	1.54	1.12

Appendix-8

Data of elimination rate constant (k_{el}) and half-life ($t_{1/2el}$) of PBAlc and trans-PM of in sample collection for single dose

Table A8.1 Data of elimination rate constant (kel) and half-life (t1/2 el) of PBAlc and trans-PM for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg)

Gr.1	n	kel		t1/2 el	
		PBAlc	**trans-PM**	**PBAlc**	**trans-PM**
PM alone	n_1	0.034	0.060	20.29	11.55
	n_2	0.027	0.059	25.66	11.74
	n_3	0.050	0.061	13.86	11.36
	n_4	0.026	0.063	26.65	11.00
	n_5	0.038	0.061	18.23	11.36
	n_6	0.035	0.059	19.80	11.74
	Mean	0.035	0.061	20.75	11.46
	S.D	0.009	0.002	4.772	0.282
	SEM	0.004	0.001	1.948	0.115
MG+PM	n_1	0.101	0.031	6.86	22.35
	n_2	0.082	0.029	8.45	23.90
	n_3	0.075	0.036	9.24	19.25
	n_4	0.072	0.036	9.63	19.25
	n_5	0.064	0.040	10.83	17.33
	n_6	0.058	0.039	11.94	17.77
	Mean	0.075	0.035	9.49	19.98
	S.D.	0.015	0.004	1.782	2.606
	SEM	0.006	0.002	0.728	1.064

Table A8.2 Data of elimination rate constant (k_{el}) and half-life ($t_{1/2el}$) of PBAlc and trans-PM for Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg)

Gr.2	n	k_{el}		$t_{1/2el}$	
		PBAlc	**trans-PM**	**PBAlc**	**trans-PM**
PM alone	n_1	0.033	0.055	21.00	12.49
	n_2	0.044	0.062	15.75	11.17
	n_3	0.051	0.059	13.59	11.74
	n_4	0.046	0.062	15.06	11.17
	n_5	0.035	0.059	19.80	11.74
	n_6	0.036	0.061	19.25	11.36
	Mean	0.041	0.060	17.41	11.61
	S.D	0.007	0.003	2.995	0.502
	SEM	0.003	0.001	1.223	0.205
AE+PM	n_1	0.035	0.031	19.80	22.35
	n_2	0.029	0.030	23.89	23.10
	n_3	0.055	0.030	12.60	23.10
	n_4	0.044	0.035	15.75	19.80
	n_5	0.056	0.029	12.38	23.89
	n_6	0.031	0.029	22.35	23.89
	Mean	0.042	0.031	17.80	22.69
	S.D.	0.012	0.002	4.948	1.529
	SEM	0.005	0.001	2.020	0.624

Appendix-9

Data of elimination rate constant (k_{el}) and half-life ($t_{1/2el}$) of PBAlc and trans-PM of in sample collection for multiple doses

Table A9.1 Data of elimination rate constant (kel) and half-life (t1/2 el) of PBAlc and trans-PM for Gr.3 (MG 40 mg/kg+PM dose 460 mg/kg)

Gr.3	n	k_{el}		$t_{1/2\ el}$	
		PBAlc	**trans-PM**	**PBAlc**	**trans-PM**
PM alone	**n_1**	0.040	0.063	19.80	11.00
	n_2	0.032	0.064	21.65	10.83
	n_3	0.031	0.060	22.35	11.55
	n_4	0.031	0.060	22.35	11.55
	n_5	0.036	0.063	19.25	11.00
	n_6	0.040	0.062	17.33	11.18
	Mean	0.035	0.062	20.46	11.19
	S.D	0.004	0.002	2.013	0.304
	SEM	0.002	0.001	0.822	0.124
MG+PM	**n_1**	0.133	0.037	5.21	18.73
	n_2	0.106	0.031	6.54	22.35
	n_3	0.130	0.030	5.33	23.10
	n_4	0.099	0.029	7.00	23.89
	n_5	0.089	0.040	7.78	23.10
	n_6	0.086	0.028	8.05	24.75
	Mean	0.107	0.033	6.65	22.65
	S.D.	0.020	0.005	1.199	2.088
	SEM	0.008	0.002	0.489	0.853

Table A9.2 Data of elimination rate constant (kel) and half-life (t1/2 el) of PBAlc and trans-PM for Gr.4 (AE 100 mg/kg+PM dose 460 mg/kg)

		k_{el}		$t_{1/2\ el}$	
Gr.4	**n**	**PBAlc**	**trans-PM**	**PBAlc**	**trans-PM**
PM alone	**n_1**	0.036	0.056	19.25	12.37
	n_2	0.035	0.060	19.80	11.55
	n_3	0.031	0.059	22.35	11.74
	n_4	0.031	0.065	22.35	10.66
	n_5	0.04	0.056	17.33	12.37
	n_6	0.036	0.060	19.25	11.55
	Mean	0.035	0.059	20.06	11.71
	S.D	0.003	0.003	1.966	0.637
	SEM	0.001	0.001	0.803	0.260
AE+PM	**n_1**	0.057	0.021	12.16	32.23
	n_2	0.051	0.025	13.58	27.72
	n_3	0.058	0.030	11.95	23.10
	n_4	0.063	0.022	11.00	31.50
	n_5	0.068	0.025	10.19	27.72
	n_6	0.056	0.021	12.37	32.23
	Mean	0.059	0.024	11.88	29.08
	S.D.	0.006	0.003	1.170	3.610
	SEM	0.002	0.001	0.478	1.474

Appendix-10

Data of acoustic startle response (ASR) of PM in sample collection for single dose

Table A10.1 Data of acoustic startle response (ASR) of PM for Gr.1 (MG 40 mg/kg+PM dose 460 mg/kg) and Gr.2 (AE 100 mg/kg+PM dose 460 mg/kg)

Group	n	Onset		Duration of action	
		Phase I	Phase II	Phase I	Phase II
MG+PM	n_1	6.50	9.00	11.00	12.00
	n_2	7.00	10.00	11.00	12.50
	n_3	6.50	9.50	10.50	13.50
	n_4	7.00	10.00	11.50	12.50
	n_5	6.50	8.50	10.50	11.50
	Mean	6.70	9.40	10.90	12.40
	S.D	0.27	0.65	0.42	0.74
	SEM	0.12	0.29	0.19	0.33
AE+PM	n_1	7.00	12.00	11.50	13.50
	n_2	6.50	10.50	11.55	14.00
	n_3	7.50	11.50	10.00	13.00
	n_4	7.00	11.00	11.00	13.50
	n_5	6.50	10.50	10.50	12.50
	Mean	6.90	11.10	10.90	13.30
	S.D.	0.42	0.65	0.65	0.57
	SEM	0.19	0.29	0.29	0.25

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List of Publications and Proceedings

Srichana, K., Janchawee, B., Prutipanlai, S. and Raungrut, P. 2013 Effect of mitragynine on elimination of permethrin in rats. Proceeding of the 39th Congress on Science and Technology of Thailand (STT 39), Bangkok, Thailand, 21-23 October 2013.