Influence of Phenethyl Isothiocyanate (PEITC) on N-Demethylation of Caffeine in Rats

Angkana Wongsakul

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacology

Prince of Songkla University

2011

Copyright of Prince of Songkla University

i

Thesis Title   Influence of phenethyl isothiocyanate (PEITC) on N-demethylation of caffeine in rats

Author   Miss Angkana Wongsakul

Major Program   Pharmacology

Major Advisor:

....

(Assoc. Prof. Dr. Benjamas Janchawee)

Co-advisor:

....

(Dr. Sathaporn Prutipanlai)

Examining Committee:

....Chairperson

(Assoc. Prof. Dr. Krongtong Yoovathaworn)

....

(Assoc. Prof. Dr. Benjamas Janchawee)

....

(Dr. Sathaporn Prutipanlai)

....

(Assist. Prof. Dr. Kitja Sawangjaroen)

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

....

(Prof. Dr. Amornrat Phongdara)

Dean of Graduate School

ชื่อวิทยานิพนธ์	อิทธิพลของเฟนเอทิลไอโซไธโอไซยาเนทต่อการเกิด เอ็น-ดีเมทิเลชั่นของคาเฟอีนในหนูขาว
ผู้เขียน	นางสาวอังคณา วงษ์สกุล
สาขาวิชา	เภสัชวิทยา
ปีการศึกษา	2553

บทคัดย่อ

เฟนเอทิลไอโซไธโอไซยาเนท (PEITC) พบในพืชตระกูลกะหล่ำ เช่น บร็อคโคลี กะหล่ำปม และผักน้ำ PEITC มีผลป้องกันการเกิดมะเร็งปอดและมะเร็งหลอดอาหาร PEITC มีฤทธิ์ต้านสารเคมีก่อมะเร็งโดยอาจเกิดจากการรบกวนการสร้างสารก่อมะเร็ง วัตถุประสงค์หลักเพื่อศึกษาผลของ PEITC ต่อการเกิดเอ็น-ดีเมทิเลชั่นของคาเฟอีนในหนูขาวโดยใช้คาเฟอีนเป็นตัวชี้วัดและวัดอัตราส่วนเมแทบอลิค เนื่องจากผักน้ำมีสาร PEITC และผักน้ำเบตงเป็นผักน้ำชนิดหนึ่งที่พบในประเทศไทย จึงศึกษาผลของผักน้ำเบตงต่อการต่อการเกิดเอ็น-ดีเมทิเลชั่นของคาเฟอีน การศึกษาจะแบ่งสัตว์ทดลองออกเป็น 9 กลุ่ม (กลุ่มละ 6 ตัว) ตามการได้รับยา กลุ่มที่ 1 สัตว์ทดลองได้รับฟลูโวซามีนโดยฉีดทางช่องท้องครั้งเดียว ขนาด 10 มิลลิกรัม/กิโลกรัม กลุ่มที่ 2, 3, และ 4 ให้ PEITC ทางปากครั้งเดียว ขนาด 2, 10, และ 20 มิลลิกรัม/กิโลกรัมตามลำดับ กลุ่มที่ 5, 6, และ 7 ให้ PEITC ทางปากหลายครั้ง (ขนาด 2, 10, และ 20 มิลลิกรัม/กิโลกรัม ตามลำดับ) เป็นเวลา 5 วัน กลุ่ม 8 และ 9 ให้ผักน้ำเบตงทางปากครั้งเดียว (ขนาด 800 มิลลิกรัม/กิโลกรัม) และหลายครั้ง (ขนาด 800 มิลลิกรัม/กิโลกรัม) เป็นเวลา 5 วัน การทดลองของแต่ละกลุ่มจะประกอบด้วย 2 ระยะ ระยะที่ 1 สัตว์ทดลองได้รับคาเฟอีนอย่างเดียว ครั้งเดียวทางปากขนาด 10 มิลลิกรัม/กิโลกรัม ระยะที่ 2 สัตว์ทดลองตัวเดียวกันได้รับฟลูโวซามีน, PEITC หรือ ผงแห้งของน้ำคั้นผักน้ำเบตงแล้วตามด้วยคาเฟอีน วัดความเข้มข้นของคาเฟอีนและเมแทบอไลท์ในซีรั่มโดยเทคนิคโครมาโทกราฟีแบบของเหลวสมรรถนะสูง หาค่าและเปรียบเทียบอัตราส่วน เมแทบอลิค (TB/CF, PX/CF, TP/CF, and TB+PX+TP/CF) การศึกษานี้ได้วัด PEITC ในผงแห้งของน้ำคั้นผักน้ำเบตงด้วย

ผลการศึกษาแสดงว่าอัตราส่วนเมแทบอลิคของคาเฟอีนจะลดลงอย่างมีนัยสำคัญจากการได้รับ ฟลูโวซามีน, PEITC (2-20 มิลลิกรัม/กิโลกรัม) และผักน้ำเบตง ($p < 0.001$) การให้ PEITC ขนาด 10 และ 20 มิลลิกรัม/กิโลกรัม ลดอัตราส่วนเมแทบอลิคได้มากกว่าฟลูโวซามีนอย่างมีนัยสำคัญ อัตราส่วนเมแทบอลิคของคาเฟอีนก็ลดลงอย่างมีนัยสำคัญเช่นกันหลังจากได้รับ

PEITC ทุกขนาดและผักน้ำเบตง ($p<0.005$) อัตราส่วนเมทาบอลิคของคาเฟอีนหลังจากได้ให้ PEITC หลายครั้ง ไม่แตกต่างอย่างมีนัยสำคัญจากการได้รับแบบครั้งเดียว เปอร์เซ็นต์การลดลงในอัตราส่วนเมทาบอลิคที่เกิดจากการได้รับผักน้ำเบตงครั้งเดียวน้อยกว่าการได้รับ PEITC ครั้งเดียว การให้ผักน้ำเบตง 5 วันลดอัตราส่วนเมทาบอลิคมากกว่าการได้รับแบบครั้งเดียวอย่างมีนัยสำคัญ ผงแห้งของน้ำคั้นผักน้ำเบตง 1 มิลลิกรัมมีปริมาณสาร PEITC 2.5 ไมโครกรัม

สรุปได้ว่า PEITC (2-20 มิลลิกรัม/กิโลกรัม) ยับยั้งการเกิดเอ็น-ดีเมทิเลชั่นของคาเฟอีนในกายหลังจากได้รับแบบครั้งเดียวและหลายครั้งซึ่งแสดงโดยการลดลงของอัตราส่วนเมทาบอลิคของคาเฟอีน PEITC ขนาดสูง (20 มิลลิกรัม/กิโลกรัม) ยับยั้งอัตราส่วนเมทาบอลิคได้มากกว่าฟลูโวซามีน (10 มิลลิกรัม/กิโลกรัม) ผงแห้งของน้ำคั้นของผักน้ำเบตง (800 มิลลิกรัม/กิโลกรัม) มีผลยับยั้งการเกิดเอ็น-ดีเมทิเลชั่นของคาเฟอีนได้เช่นกัน แต่น้อยกว่าเมื่อเปรียบเทียบกับ PEITC ผลเหล่านี้ชี้ให้เห็นว่า PEITC และน้ำคั้นผักน้ำเบตงอาจมีผลยับยั้งแอคติวิตี้ของ CYP1A2 ได้ซึ่งอาจสนับสนุนการอธิบายกลไกสำหรับการป้องกันการเกิดมะเร็งของ PEITC ได้

Thesis Title   Influence of phenethyl isothiocyanate (PEITC) on

N-demethylation of caffeine in rats

Author   Miss Angkana Wongsakul

Major Program   Pharmacology

Academic Year   2010

Abstract

Phenethyl isothiocyanate (PEITC) is found in cruciferous vegetables such as broccoli, brussel sprout, and watercress. PEITC has chemoprotective effect against lung- and esophageal cancers. Its anticarcinogenicity is probably due to interfering with chemical bioactivation. The main objective of this study was to investigate the effect of PEITC on N-demethylation of caffeine in rats by using caffeine as a probe substrate and measuring its metabolic ratios. Since a variety of watercress is found in Betong District, Yala Province, the effect of Betong watercress on N-demethylation of caffeine was also investigated. The animals were divided into nine groups (n=6) based on the pre-treatment regimen. Group 1, the animals were intraperitoneally pre-treated with a single dose of fluvoxamine (10 mg/kg). Group 2, 3, and 4, the animals were orally pre-treated with a single dose of PEITC (2, 10, and 20 mg/kg, respectively). Group 5, 6, and 7, the animals were orally pre-treated with multiple doses of PEITC (2, 10, and 20 mg/kg, respectively) for five days. Group 8 and 9, the animals were orally pre-treated with a single dose of dry power of Betong watercress juice (800 mg/kg) and multiple doses (800 mg/kg) for five days. Experiment for each group consisted of two phases. Phase I, the animal was given a single oral dose of 10 mg/kg of caffeine alone. Phase II, the same individual was pre-treated with fluvoxamine, PEITC or dry powers of Betong watercress juice followed by caffeine. Serum concentration of caffeine and its metabolites were determined by a validated HPLC method. Metabolic ratios (TB/CF, PX/CF, TP/CF, and

(TB+PX+TP)/CF were determined and compared. The contents of PEITC in dry powers of Betong watercress juice were also determined.

The results showed that metabolic ratios of caffeine were significantly decreased by single pretreatment of fluvoxamine, PEITC (2-20 mg/kg), and Betong watercress ($p<0.02$). The decreases caused by 10 and 20 mg/kg of PEITC were significantly greater than those resulted from fluvoxamine. Caffeine metabolic ratios were also significantly reduced after five days pretreatment with all doses of PEITC and Betong watercress ($p<0.025$). The percentage of decrease in caffeine metabolic ratios was not significant with increasing doses of PEITC. The percentage of decrease in caffeine metabolic ratios after multiple doses of PEITC was not significantly different from that after a single dose. The percentage of decrease in metabolic ratios caused a single pretreatment of Betong watercress smaller than that produced by a single dose of PEITC. The percentage of decrease in metabolic ratios after five day pretreatment of Betong watercress was significantly larger than after its single pretreatment. Quantification of PEITC contents showed that one milligram of dry powders of Betong watercress juice contained 2.5 µg of PEITC.

In conclusion, PEITC (2-20 mg/kg) inhibited N-demethylation of caffeine *in vivo* after single- and multiple dose(s) as shown by a reduction of caffeine metabolic ratios. Magnitude of inhibition was higher at the higher dose of PEITC (20 mg/kg) and greater than that produced by fluvoxamine (10 mg/kg). Dry powder of Betong watercress juice (800 mg/kg) also inhibited N-demethylation of caffeine but with a lesser extent compared with PEITC. These findings could be a supportive explaination for chemopreventive mechanism of PEITC.

Acknowledgements

The completion of this thesis would be impossible without the help of many people, whom I would like to thank. I would like to express my gratitude and sincere appreciation to Associate Professor Dr. Benjamas Janchawee, my major advisor, for helpful suggestion, valuable guidance and comments throughout this study. I would like to thank Dr. Sathaporn Prutipanlai, my co-advisor, for valuable contributions and advices related to this study. I would also like to thank Associate Professor Dr. Krongtong Yoovathaworn, Associate Professor Dr. Benjamas Janchawee, Dr. Sathaporn Prutipanlai, and Assistant Professor Dr. Kitja Sawangjaroen for the examination committee of this thesis for their valuable time. I am grateful to Professor Dr. Supayang Voravutthikunchai, Director of Natural Products Research Center for supporting scholarships throughout this study.

Thanks are also sincerely expressed to:

Dr. Pritsana Raungrut for her advices related to this study.

Associate Professor Dr. Benjamas Janchawee, Head of the Department of Pharmacology, Faculty of Science, for supporting all facilities needed for the success of this study.

Princess Maha Chakri Sirindhorn Natural History Museum, Prince of Songkla University, especially Associate Professor Dr. Kitichate Sridith and Mr. Charoensak Saewei for their kind assistance in authenticating plant.

Amphoe Betong Aqricultural Office, Yala province for the information about Betong watercress and plant samples.

Scientific Equipment Center (SEC), Prince of Songkla University and Pharmaceutical Laboratory Service Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University for helpful preparation of dry powder of Betong watercress juice by lyophilized.

Graduate School, Prince of Songkla University and Faculty of Science, Prince of Songkla University for partial financial support to this study.

Finally, I would like to express my thanks to my parents and sister, including all of my good friends for their understanding, supporting and encouragement

Contents

List of Tables

Table   Page

List of Figures

ANOVA; α $p<0.02$, compared with single dose

List of Abbreviations

AITC	Allyl isothiocyanate
AM	Axozymethane
AP	Acetaminophen
AFMU	5-acetylamino-6-formylamino-3-methyluracil
BaP	benzo(a)pyrene
BITC	Benzyl isothiocyanate
DEV	Standard deviation
DMBA	7, 12-dimethylbenz(a)anthracene
ESP	Epithiospecifier protein
etc	Et cetera
FDA	Food and drug administration
GC	Gas chromatography
HPLC	High performance liquid chromatography
IS	Internal standard
ITCs	Isothiocyanates
LC-MS	Liquid chromatography coupled with mass spectrometry
LLOQ	Lower limit of quantification
NMBA	N-nitrosobenzylmethylamine
NNK	4-(methyl nitrosamino)-1-(3-pyridyl)-butanone
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
PBITC	4-phenylbutyl isothiocyanate
PEITC	Phenethyl isothiocyanate
PHITC	6-phenylhexyl isothiocyanate
PITC	Phenyl isothiocyanate

List of Abbreviations (cont.)

QC	Quality control
r	Correlation coefficient
RSD	Relative standard deviation
SD	Standard deviation
SF	Sulforaphane
S/N	Signal to noise
137X, CF	Caffeine
17X, PX	Paraxanthine
37X, TB	Theobromine
13X, TP	Theophylline
137U	1,3,7- trimethyluric acid

CHAPTER 1

Introduction

1.1 Background and rationale

Cytochrome P450 1A2 is an important enzyme responsible for metabolism of caffeine (95%) by N-demethylation reaction. CYP1A2 converts hydrophobic from xenobiotics to hydrophilic form in order to excretion. In addition, environmental pollutants such as aromatic amines, polycyclic aromatic hydrocarbon are metabolized by CYP1A2 to highly reactive carcinogenic metabolite that can cause cancer in human body. For example, 4-(methyl nitrosamino)-1-(3-pyridyl)-butanone (NNK), a procacinogen, is converted by CYP1A2 into 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), which acts as a lung carcinogen (Hecht, 1997).

Phenethyl isothiocyanate (PEITC) is a dietary compound classified into isothiocyanates (ITCs) found in the genus *Brassica* of cruciferous vegetables (e.g. cabbage, cauliflower, brussel sprouts, watercress, broccoli, and kale) and the genus *Raphanus* (radishes and daikons). PEITC is produced from glucosinolate precursors by hydrolysis reaction via myrosinase enzyme released from plant cells during chopping or chewing (Morse *et al.* 1997). PEITC and other ITCs have cancer preventive property in animals treated with chemical carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines. It has been reported that PEITC inhibited 4-(methyl nitrosamino)-1-(3-pyridyl)-butanone (NNK)-induced lung tumors by interfering the metabolic activation of NNK in rats and mice (Chung *et al.* 1996). PEITC also blocked tumors of the mammary gland and esophagus induced by *N*-nitrosobenzylmethylamine (NBMA) and 7, 12-dimethylbenz(*a*)anthracene (DMBA) in rats (Stoner *et al.* 1991; Wattenberg, 1977). Thus, PEITC may inhibit enzyme involving in bioactivation of procarcinogen. Moreover, consumption of watercress which contains gluconasturtiin (a precursor of PEITC) has been shown to toxic metabolite (NAPQI) of acetaminophen. It is likely that production of PEITC may influence the activity of enzyme in drug metabolism (Chen *et al.*, 1996).

1

The effect of PEITC on N-demethylation of caffeine *in vivo* has also been investigated in earlier study but such effect is uncertain and not concordant with *in vitro* data. Therefore, the inhibition property of PEITC on N-demethylation of caffeine should be carefully done in animal model. Since a variety of watercress is cultivated at Betong District, Yala provine, Thailand. The effect of Betong watercress on N-demethylation of caffeine should also investigate. To evaluate N-demethylation of caffeine *in vivo*, caffeine is used as a marker because it is metabolized by CYP1A2 to paraxanthine (N-3-demethylation), theobromine (N-1-demethylation) and theophylline (N-7-demethylation). Wistar rat was used in this study because demethylation of caffeine by CYP1A2 is also occurred in rat (Jorritsma *et al.*, 2000; Caubet *et al.*, 2002). The outcomes of the present study may provide the information for supporting the explaination of mechanism of PEITC anticarcinogenesis in humans and animals.

CHAPTER 2

Literature review

2.1 Phenethyl isothiocyanate (PEITC)

2.1.1 Source and phytochemical classification

Phenethyl isothiocyanate (PEITC) is a dietary compound classified into isothiocyanates (ITCs) which are characterized by the presence of an N=C=S group whose central carbon is highly electrophilic. ITCs are found in the order Capparales, which include the large family *Brassicaceae* of cruciferous vegetables. *Brassicaceae* (syn. Cruciferae) is a plant family containing a large number of economically important crops generally referred to as crucifers. The name crucifer comes from the shape of the flowers, with four diagonally opposite petals in the form of a cross (Higdon, 2005). Occurring in the wild in Western Europe, the Mediterranean and temperate regions of Asia, this remarkable genus contains more important agricultural and horticultural crops than any other genus. *Brassica* vegetables, such as cabbage, broccoli, watercress, brussels sprout, cauliflower, kohlrabi, chinese cabbage, turnips, and rutabaga, are rich sources of glucosinolate precursors of isothiocyanates (Pedras *et al.*, 1998). It is one of the healthiest and most nutritious vegetable groups.

The Brassicaceae, which currently includes 3,709 species and 338 genera (Warwick *et al.*, 2005), is one of the ten most economically important plant families (Rich, 1991). *Brassica* vegetables have been cultivated for several thousands of years. Most *Brassica* vegetables are good sources of oil that may be used for edible and industrial purposes. *Brassica* vegetables also contribute almost exclusively to our intake of a very powerful group of phytochemicals called 'glucosinolates', which can have important implications for human health. More than 100 glucosinolates have been identified, mainly in vegetables of the family Cruciferae (Verhoeven *et al.*, 1997; Tookey *et al.*, 1980). Common vegetables of this family are summarized in Table 2.1 (from Internation Agency for Research on Cancer, 2004). Hydrolysis of the glucosinolates is catalyzed by multiple forms of the enzyme myrosinase

3

(thioglucoside glucohydrolase, EC 3.2.3.1), which occur in the same plants, separated cellularly from the glucosinolates. When the plant is macerated or chewed, myrosinase mixes with the glucosinolate and affects the hydrolysis. Myrosinase catalyzes hydrolysis of the glucosinolate S-sugar bond leading to an unstable thiohydroxamic acid that undergoes a lossen rearrangement yielding the isothiocyanate. Depending on the nature of the R group and the conditions, other products such as nitriles and thiocyanates may also form. A large number of glucosinolates with many different R groups occur in substantial quantities in cruciferous plants and crops.

Table 2.1 Scientific and common names of cruciferous vegetables in human diet

Genus	Species and variety	Common name
Brassica	*B. oleracea* var. *botrytis*	Cauliflowers
	B. oleracea var. *capitata*	Cabbage, white cabbage
	B. oleracea var. *costata*	Portuguese cabbage
	B. oleracea var. *gemmitera*	Brussels sprouts
	B. oleracea var. *gongyloides*	Kohlrabi, turnip cabbage, stem turnip
	B. oleracea var. *italica*	Broccoli
	B. oleracea var. *rubra*	Red cabbage
	B. oleracea var. *sabauda*	Savoy cabbage
	B. oleracea var. *sabelica*	Curly kale
	B. oleracea var. *viridis*	Kale, collards
	B. oleracea var. *albogtabra*	Kai tan, Chinese kale
Brassica	*B. rapa* var. *chinensis*	Chinese cabbage, pak-choi, bok choi
	B. rapa var. *oleifera*	Turnip rape
	B. rapa var. *pekinensis*	Chinese cabbage, pe-tsai, celery
	B. rapa var. *rapa*	Turnip
	B. rapa var. *aparachinensis*	Choi sum
Brassica	*B. napus* var. *napobrassica*	Swede, Swedish turnip, rutabaga
	B. napus var. *oleifera*	Rape, canola, colza
Brassica	*B. alba*	White mustard
	B. juncea	Indian mustard, brown mustard

Genus	Species and variety	Common name
	B. juncea var. *rugosa*	Kai choi
	B. nigra	Black mustard
Rhaphanus	*R. sativus*	radish
Armoracia	*A. rusticana*	Horseradish
Nasturtium	*N. officinale*	Watercress
Lepidium	*L. sativum*	Cress, garden cress
Eruca	*E. vesicaria*	Arugula, rocket, Italian cress
Wasabia	*W. japonica*	Wasabi
Beta	*B. vulgaris flavescens*	Swiss chard
Crambe	*C. abyssinica*	Crambe

(from Internation Agency for Research on Cancer, 2004)

Crucifers also contain many other bioactive components including flavonoids such as quercetin (Chiriboga *et al.*, 1998), minerals such as selenium (Se) (Lasch and Naumann, 1998), S-methyl cysteine sulfoxide, and 1,2-dithiole-3-thione (McIntosh *et al.*, 1999). Cruciferous vegetables are the principle dietary source of ITCs, but the types of crucifers frequently consumed by humans are limited. Although about 100 different isothiocyanate glucosinolates have been identified, only a few is present frequently in the cruciferous vegetables commonly eaten, including phenyl isothiocyanate (PITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), 4-phenylbutyl isothiocyanate (PBITC), 6-phenylhexyl isothiocyanate (PHITC), allyl isothiocyanate (AITC), sulforaphane (SF), erucin, dehydroericin, and iberin (Zhang *et al.*, 1992; Fahey *et al.*, 1997) (Figure 2.1).

⬡—N=C=S	PITC
⬡—CH$_2$—N=C=S	BITC
⬡—CH$_2$—CH$_2$—N=C=S	PEITC
⬡—CH$_2$—CH$_2$—CH$_2$—CH$_2$—N=C=S	PBITC
⬡—CH$_2$—CH$_2$—CH$_2$—CH$_2$—CH$_2$—CH$_2$—N=C=S	PHITC
H$_2$C=CH—CH$_2$—N=C=S	AITC
CH$_3$—S—CH$_2$—CH$_2$—CH$_2$—CH$_2$—N=C=S	Erucin
CH$_3$—S—CH—CH—CH$_2$—CH$_2$—N=C=S	Dehydroerucin
CH$_3$—S(=O)—CH$_2$—CH$_2$—CH$_2$—N=C=S	Iberin
CH$_3$—S(=O)—CH$_2$—CH$_2$—CH$_2$—CH$_2$—N=C=S	Sulforaphane
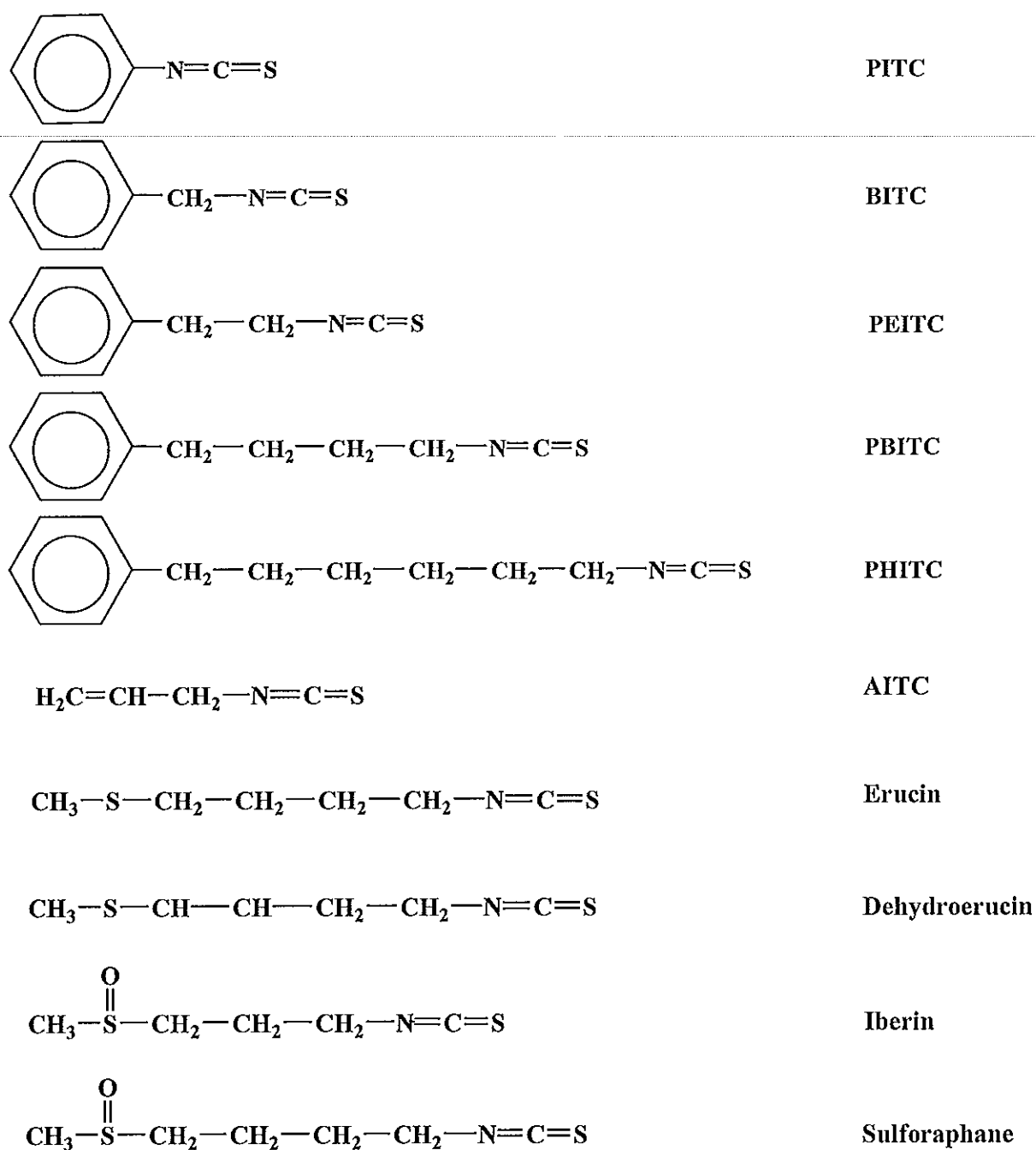
Figure 2.1 Chemical structures of ITCs (from Zhang, 2004).

Abbreviations: PITC, phenyl isothiocyanate; BITC, benzyl isothiocyanate; PEITC, phenethyl isothiocyanate; PBITC, 4-phenylbutyl isothiocyanate; PHITC, 6-phenylhexyl isothiocyanate; AITC, allyl isothiocyanate; SF, sulforaphane

2.1.2 Precursor and bioactivation

PEITC is a product of degradation of glucosinolate in plant. Glucosinolates are a family of about 120 plant compounds and are secondary metabolites in the Brassicaceae and related plant families. The basic structure of glucosinolates shares a core structure containing a β-D-glucopyranose residue linked via a sulfur atom to a (Z)-N-hydroximino sulfate ester, and is distinguished from each other by a variable R group, derived from one of several amino acids (Fahey *et al.*, 2001) (Figure 2.2). Depending on whether they are derived from aliphatic amino acids, phenylalanine, tyrosine, or tryptophan, glucosinolates are grouped into three categories, aliphatic, aromatic, or indolyl (heterocyclic) glucosinolates, respectively (Halkier and Du, 1997)
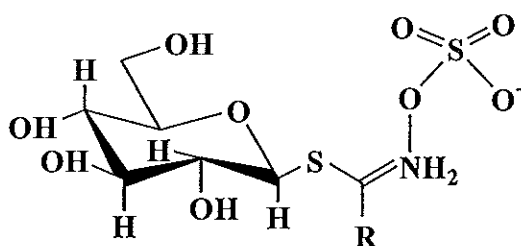
Figure 2.2 Glucosinolate basic structures (from Rowan *et al.*, 1991).

Glucosinolates are present in all plant parts and are physically separated from the enzyme myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1). Injury of plant tissues during processing or ingestion facilitates the contact between glucosinolates and myrosinase enzyme. The glucosinolates are then hydrolysed by both myrosinase enzyme present in plant and myrosinase produced by intestinal microflora, to release a range of breakdown products (Figure 2.3) (Bernardi *et al.*, 2003; Fenwick *et al.*, 1983; Finiguerra *et al.*, 2001; Cheng *et al.*, 2004). The conversion of glucosinolates by myrosinase is pH dependent. When crushed plant tissue or seeds containing glucosinolates are added to water, myrosinases catalyse the hydrolytic cleavage of the thioglucosidic bond, giving D-glucose and a thiohydroximate-O-sulphonate (aglycone). Isothiocyanates are usually produced at neutral pH while nitrile production occurs at lower pH. Isothiocyanates with a hydroxyl group in the 2 position spontaneously cyclise to give oxazolidine-2-thiones

(Labague *et al.*, 1991). The addition of ferrous ions to reaction mixtures promotes the formation of the nitrile hydrolysis product. At low pH, a proton may block the lossen rearrangement of the aglycone, thus promoting formation of the nitrile. Epithiospecifier protein, ESP, is a small protein of molecular weight 30 to 40 kDa, which co-occurs with myrosinase. ESP does not have thioglucosidase activity, but interacts with myrosinase to promote the transfer of sulphur from the S-glucose moiety of terminally unsaturated glucosinolates to the alkenyl moiety, resulting in the formation of epithionitriles. The presences of ferrous ions are essential for ESP function. Enzyme characteristics (substrate affinity, temperature and pH optima) may alter relative proportions of products by causing some glucosinolates to be hydrolyzed at different rates. Thiocyanate production is controlled by the presence of specific R group.
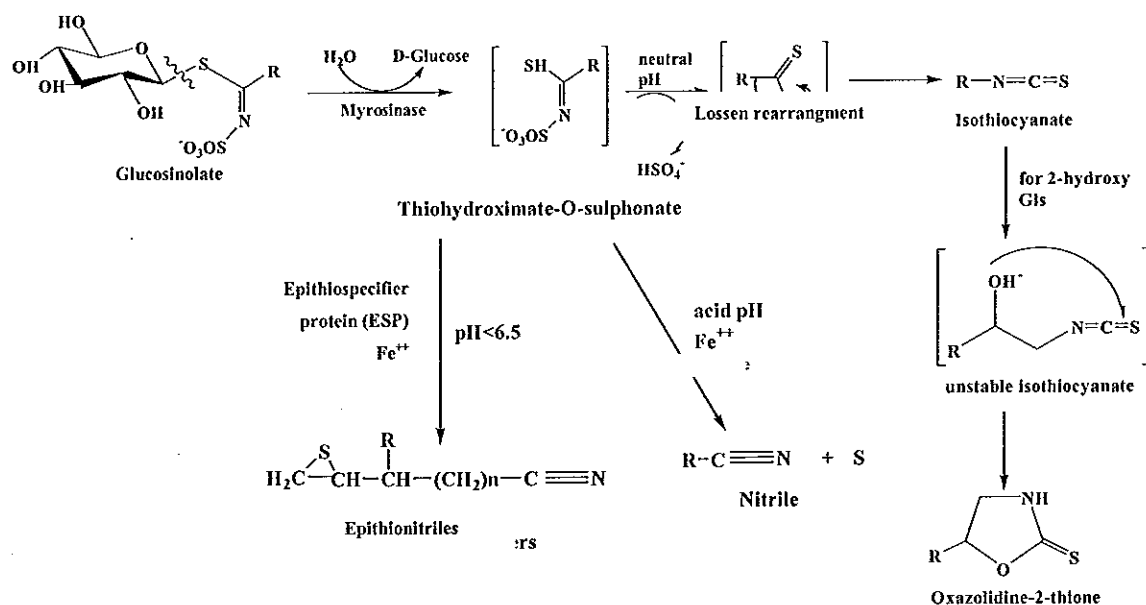
Figure 2.3 Bioactivation of glucosinolates: Hydrolysis of glucosinolates by endogenous enzyme myrosinase, to form nitriles, oxazolidine-2-thione, isothiocyanates, and thiocyanates (from Tripathi and Mishra, 2007).

2.1.3 Physicochemical properties

PEITC ($C_6H_5CH_2CH_2NCS$; m.w. 163.24 Figure 2.1) as its pure compound is light yellow liquid. Synonyms are 2-phentlethyl isothiocyanate, 2-isothiocyanatoethylbenzene, 2-phenethylisothiocyanate, 1-(2-isothiocyanatoethyl) benzene. It has a boiling point of 75 °C and a density of 1.094 g/mL at 25 °C. It is insoluble in water (Chemicalbook, 2008).

2.1.4 Pharmacokinetics

Pharmacokinetic data of PEITC in animals (male F344 rats) have been established in several studies. Conaway and colleaques (1999) have reported that peak level of plasma PEITC was achieved within 2.9 h. The elimination half-life was about 21.7 h after a single gavage dose of 50 μmol/kg (8.15 mg/kg). Within 48 h PEITC appeared in the urine and feces about 89% and 10%, respectively. Distribution of PEITC is rapid in liver, kidneys and lungs.

Ji and Morris (2003) reported pharmacokinetic data of PEITC in plasma and urine obtained from four healtly volunteers after consumption of 100 g of watercress (approximately 25 mg PEITC). Elimination half-life was 4.9 h. The t_{max} value was 2.6 h. Absorption rate constant (k_a) was 1.3 h.

Ji and colleaques (2005) have studied that pharmacokinetics of PEITC in male Sprague-Dawley rats administered PEITC at doses of 2, 10, 100, and 400 mmol/kg intravenously, or 10 and 100 mmol/kg orally. PEITC had high oral bioavailability (115 and 93% at doses of 10 and 100 mmol/kg, respectively), low clearance (0.70 ± 0.17 L/h/kg at the lowest dose of 2 μmol/kg), and high plasma protein binding (86 %). Nonlinear elimination and distribution were occured following the administration of high doses.

Little or no PEITC was detected in most of the plasma samples from volunteers who ingested 40 g of watercress (releasing 6-12 mg PEITC) and from subjects after 40 mg oral PEITC administration (Liebes *et al.*, 2001). Additionally, PEITC was not detected in urine samples in subjects following watercress consumption using HPLC (Chung *et al.*, 1992).

2.1.5 Cytotoxic effect

Several *in vitro* studies showed cell growth inhibitory effect of ITCs. Inhibition of cell growth and modulation of the cell cycle have been reported by Hasegawa and colleaques (1993) for allyl isothiocyanate (AITC) (10 µM), benzyl isothiocyanate (BITC) (5 µM) and PEITC (2.5 µM) in HeLa cells, and similar effects have been reported for sulforaphane (SF) (15 µM) in HT-29 cells. Similar results have been reported in other cell lines including human leukemia HL60 and prostate cancer cells lines LNCaP and DU-145 (reviewed by Conaway and colleaques (2002)). A comprehensive study of the effect of ITCs on the growth of different cancer cells reported that AITC, BITC and PEITC were able to inhibit cell growth (Zhang *et al.*, 2003). The cytotoxicity of PEITC, BITC, NITC and SF, as well as the cytotoxicity of the chemotherapeutic agent daunomycin (DNM) and vinblastine (VBL), has been evaluated in human breast cancer MCF-7 and human mammary epithelial MCF-12A cells (Tseng *et al.*, 2004). BITC and PEITC can inhibit the growth of human breast cancer cells as well as human mammary epithelium cells.

While some studies have demonstrated similar cytotoxicity in cancer and normal cells (Fimognari *et al.*, 2003; Tseng *et al.*, 2004), others have reported differences. A synthetic ITC 4-(methylthio) butyl ITC has shown selective action against human leukemia cells, and almost no effect on lymphocytes (Fimognari *et al.*, 2003). AITC also has an inhibitory effect in the human prostate cancer cell lines, while normal cell line PrEC remained unaffected by the same exposure (Xiao *et al.*, 2003).

The cytotoxic effects of ITCs are apparent even after shorter exposures. Comprehensive time-dependent studies of the effect of ITCs on the growth of different cell lines reported that a number of ITCs, including AITC, BITC, PEITC and NITC, were able to inhibit cell growth after 2 or 3 h exposures. That produced IC_{50} similar to those for exposure for 48-72 h, and indicated that short-term exposure is sufficient to produce observable effect *in vitro* (Zhang *et al.*, 2003; Tseng *et al.*, 2004).

Animal studies demonstrated the anticarcinogenic activities of ITCs in a number of carcinogen-induced cancer models *in vivo*. Over 20 natural and synthetic ITCs have demonstrated cancer preventive properties in animals treated with chemical

carcinogens, including polycyclic aromatic hydrocarbons and nitrosamines. For example, ITCs can inhibit NNK-induced carcinogenesis by inhibiting the microsomal metabolism of NNK to reactive species that form methyl and pyridyloxobutyl adducts in DNA (Hecht, 1999; Guo et al., 1992). Carcinogen inhibition was seen in a number of different organs such as lung, liver, forestomach, mammary gland and colon. BITC has shown effectiveness in mammary gland cancer, while PEITC has been effective in carcinogen induced models of mammary, lung, oral as well as esophageal cancer in rats and mice (Morse et al., 1989; Stoner et al., 1991). Studies have shown that SF is effective in prevention of azoxymethane-induced colonic cancer (Chung et al., 2000). Table 2.2 summarizes the results of several studies on carcinogen-induced cancer models in rodents.

A prospective case-control study in male subjects reported that the section of the population with the highest intake of cruciferous vegetables had a lower incidence of bladder cancer. Detailed studies into the risks associated with individual cruciferous vegetables showed that cabbage and broccoli were able to reduce the risk, independent of the intake of other crucifers (Michaud et al., 1999). Extensive epidemiological evidence supports the contention that high fruit and vegetable consumption is associated with a reduction in the incidence of cancer. It is well established that consumption of cruciferous vegetables is inversely associated with the risk of cancer of the lung, colon, stomach and prostate (Lin et al., 1998; van Poppel et al., 1999; Kristal and Lampe, 2002).

Although an inverse correlation between the intake of Brassica vegetables and the risk of cancers in humans cannot be attributed solely to ITCs, as other constituents such as vitamins, folic acid and fiber may also play a role in the reduction of cancer risk, newer studies quantifying the amount of ITCs in the biological samples have provided evidence that ITCs are important in cancer prevention. Epidemiological studies in lung cancer risk have shown associations between consumption of ITCs and reduced risk after the smoking status of the subject is taken into account. A case-control study in Chinese women reported an odds ratio (OR) of 0.31 for smokers with high ITC intake, and an OR of 0.70 for non smokers with comparable ITC intake (Zhao et al., 2001).

A breast cancer study with 720 cases and 810 controls reported that the consumption of broccoli, a vegetable rich in SF, was inversely associated with breast cancer risk in premenopausal women and that the association was not significant in post-menopausal women. When they measured cancer risk and total cruciferous vegetable intake, the correlation was not significant (Ambrosone *et al.*, 2004). A study in Chinese women evaluated the correlation between urinary ITC levels (determined by a cyclocondensation derivatization of the total ITCs and dithiocarbamates in the urine and breast cancer risk (Liebes *et al.*, 2001). The study, performed on 337 cases and matched controls, reported a 50% reduction in the cancer risk for the highest quartile of ITC consumption (Fowke *et al.*, 2003).

Table 2.2 Lists of studies on the effect of ITCs on carcinogen-induced cancer in animals

ITC treatment	Animal	Tissue	Carcinogen (dose)	Administration	% Rats with tumors (test vs. control)	Reference
BITC						
50 mg	Sprague-dawley rat (female)	Mammary	DMBA (12 mg)	Oral: single dose 4 h before DMBA	8/77	Wattenberg, 1977
0.017 mmol/g diet	ICR/Ha mice (male)	Forestomach	DMBA (0.05 mg/g diet)	Diet: DMBA+BITC 10 weeks	63/100	Wattenberg, 1977
5.0 mg/g diet				Diet: DMBA+BITC 4 weeks	5/87	Wattenberg, 1977
PEITC						
55 mg	Sprague-dawley rats (female)	Mammary	DMBA (12 mg)	Oral: single dose 4 h before DMBA	43/100	Wattenberg, 1977
5.5 mg/g diet	ICR/Ha mice (female)	Forestomach	DMBA (12 mg)	Diet: DMBA+PEITC 4 weeks	12/93	1977
3 µmol/g diet	F344 rats (male)	Lung	NNK (1.76 mg/kg Body wt)	Diet: PEITC 21 weeks +s.c. NNK at end of week 1	43/80	Morse et al., 1989

ITC treatment	Animal	Tissue	Carcinogen (dose)	Administration	% Rats with tumors (test vs. control)	Reference
5 µmol	A/J mice (female)	Buccal pouch	NNK (10 mmol)	Oral: PEITC 2 h before NNK i.p.	62/100 (tumor multiplicity in each rat)	Jiao et al., 1994
50 mM	Syrian hamster (male)		NMBA (50 mM)	Topical 24 weeks	6%	Solt et al., 2003
SF						
20 µmol		Colon	AM (15 mg/kg body wt)	Oral: 3 times a week for 8 weeks, weekly s.c.	40/100	Chung et al., 2000
5 µmol	F344 rats (male)			Am weekly for 2 weeks	42/100	

Note: BITC: benzyl isothiocyanate; PEITC: phenethyl isothiocyanate; SF: sulforaphane; DMBA: 7, 12-dimethyl-benz(a)anthracene; NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1butanone; AM: axozymethane; NMBA: N-nitrosomethylbenzyla

2.1.6 Mechanisms of anticarcinogenesis

More than 20 natural and synthetic ITCs have been shown to block carcinogenesis. Recent studies describing the effect of ITCs *in vitro*, *in vivo* and in clinical studies are described earlier in section 2.1.5. Current mechanisms proposed for the anticarcinogenic effects of ITCs include: (1) inhibition of phase I enzymes converting procarcinogens to highly reactive electrophilic carcinogens; (2) induction of phase II enzymes inactivating carcinogens and promoting their excretion; and (3) induction of apoptosis of cancer cells (Talalay and Fahey, 2001; Thornalley, 2002).

1) Inhibition of phase I enzymes

ITCs have been shown to inhibit rat and human CYP isoforms. Human and rodent studies have demonstrated that PEITC blocks metabolic activation of NNK and benzo(*a*)pyrene (BaP), major lung carcinogens in tobacco smoke, via CYPs, resulting in increased urinary excretion of detoxified metabolites, suggesting inhibitory effects on CYP1A1, CYP1A2 and CYP2B1 (Smits *et al.*, 1993; Hecht, 1999). Recently, using microsomes from baculovirus-infected insect cells expressing human CYP isoforms, PEITC was found to competitively inhibit CYP1A2 and CYP2A6, noncompetitively inhibit CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1, and inhibit CYP3A4 following a mixed-type of competitive and noncompetitive inhibition (Nakajima *et al.*, 2001).

2) Induction of phase II enzymes

Phase II enzymes are generally referred to as detoxifying enzymes, transferring hydrophilic endogenous substances such as glucuronic acid, sulfate or glutathione to phase I metabolites, or parent molecules. The hydrophilic molecules formed are more easily cleared from the body than their lipophilic parent compounds. ITCs have been classified as monofunctional inducers of phase II enzymes, i.e. they induce phase II enzymes without inducing phase I enzymes. A number of studies have demonstrated the phase II inducing properties of ITCs both *in vivo* and *in vitro* (Munday and Munday, 2004). A mixture indole-3-carbinol, PEITC and 1-isothiocyanato 3-(methylsulfinyl)-propane (a glucosinolate breakdown product) administration to F344 mice at dietary doses showed an increase in the pancreatic

mRNA levels for the enzymes quinone reductase (QR) and glutathione S transferase (GST) by 3.1 and 7.1 fold respectively. All of the other isothiocyanates, however, increased GST and QR activities in the duodenum, forestomach, and/or the urinary bladder of the animals, with the greatest effects being seen in the urinary bladder. Phase II enzymes are known to protect against chemical carcinogenesis, and the selectivity of isothiocyanates in inducing such enzymes in the bladder is of interest in view of recent epidemiological studies showing a decreased incidence of cancer of this organ in individuals with a high dietary intake of *Brassica* vegetables.

Other ITCs, 7-methylsulfinylheptyl ITC and 8-methylsulfinyloctyl ITC present in watercress have also been reported to have potent inducing activity towards QR (Rose *et al.*, 2000). BITC induced GSTP1 isoform in rat liver epithelial cells RL34 cells and increased in NQO1 activity in LS-174 human colon cell line (Nakamura *et al.*, 2000). PEITC increases the activities of UDP-glucuronosyltransferase (UGT), GST and NQO1 (Rose *et al.*, 2000). PEITC treatment of LS-174 human colon cells produced an increased protein expression of NQO1 and γ-glutamylcysteine synthetase (Bonnesen *et al.*, 2001). Following watercress ingestion in smokers, there was an increased in urinary elimination of the glucuronides of cotinine, suggesting the increasing of glucuronidation (Hecht, 1999).

3) Induction of apoptosis of cancer cells

Most ITCs inhibit cell growth by arresting the G_2/M or the G_1 cycle. The induction of apoptosis occurs via the activation of intracellular signaling pathways such as the MAPK and the caspase pathway. The MAPKs (ERK, JNK and p38) convert various extracellular signals to intracellular responses through a series of phosphorylation pathways. Caspases are cysteine proteases, which are activated as a result of binding of death ligands to death receptors. The balance of anti-apoptotic (Bcl-2 and Bcl-XL) versus pro-apoptotic (Bad and Bax) proteins on the cell is also an important determinant of stress required to initiate apoptosis.

Studies into the mechanism of *in vitro* apoptosis induction have shown that while the action of SF and AITC is linked to an increase in caspase activity and the expression of pro-apoptotic proteins in the Bcl-2 family (Xiao *et al.*, 2003; Singh *et al.*, 2004), PEITC may cause the activation of the MAPK pathways, with JNK

strongly activated. Components such as p53 dependent pathways have also been reported to be involved in the process (Chen *et al.*, 1998; Huang *et al.*, 1998).

2.2 CYP1A2

2.2.1 Cytochrome P450 (CYP)

CYPs are a superfamily of enzymes crucial for the oxidative, peroxidative, and reductive metabolism (phase I reaction) of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins, and leukotrienes, and xenobiotics, including most of the therapeutic drugs and environmental pollutants (Nelson *et al.*, 1996; Bertz and Granneman, 1997).CYPs are mostly localized to the endoplasmic reticulum, but some CYPs are also present in mitochondria inner membranes. In order to function, CYPs require an electron transfer chain. In the endoplasmic reticulum, this source is NADPH-CYP reductase, previously called NADPH-cytochrome c reductase (Omura *et al.*, 1999). In mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to CYP (Gonzalez and Gelboin, 1994).

Humans have been estimated more than 2,400 CYP sequences known and this gene can be grouped into 17 families of CYP genes and 42 subfamilies (Table 2.3) (Nelson, 1996). Mainly enzymes from the CYP1, CYP2, and CYP3 families metabolise xenobiotics in humans. The CYP1 family includes 3 subfamilies (3 genes and 1 pseudogene); CYP2 family, 13 subfamilies (16 genes and 16 pseudogenes), and CYP3 family, 1 subfamily (4 genes and 2 pseudogenes). CYP enzymes are divided into families based on amino acid sequence similarities and each family can be further separated into subfamilies which are designated by capital letters following the family designation (e.g., CYP3A) (Smith *et al.*, 1991). Individual enzymes are subsequently indicated by arabic numerals (e.g., CYP3A4). An enzyme belongs to the same family when the amino acid seauence possesses more than 40% homology, enzymes with more than 55% homology belong to a subfamily and individual enzymes can have up to 97% homology between the sequences (Vercruysse and Claerebout, 1997). An individual CYP enzyme may be able to metabolize many different drugs and a given drug may be primarily metabolized by a single enzyme (Meyer and Rodvold, 1996).

Table 2.3 Human CYP families, gene and their subfamilies (from Nelson *et al.*, 1996).

CYP family	Gene and their subfamilies
CYP1	3 subfamilies, 3 genes, 1 pseudogene
CYP2	13 subfamilies, 16 genes, 16 pseudogenes
CYP3	1 subfamily, 4 genes, 2 pseudogenes
CYP4	5 subfamilies, 11 genes, 10 pseudogenes
CYP5	1 subfamily, 1 gene
CYP7	2 subfamilies
CYP8	2 subfamilies
CYP11	2 subfamilies, 3 genes
CYP17	1 subfamily, 1 gene
CYP19	1 subfamily, 1 gene
CYP21	1 subfamily, 1 gene, 1 pseudogene
CYP24	1 subfamily, 1 gene
CYP26	3 subfamilies
CYP27	3 subfamilies
CYP39	1 subfamily
CYP46	1 subfamily
CYP51	1 subfamily, 1 gene, 3 pseudogenes

The enzymes in the families 1-3 are mostly active in the metabolism of xenobiotics, whereas the other families have important endogenous functions (Table 2.4). Inactivating mutations in the CYPs with physiological functions often lead to serious diseases, whereas similar mutations in xenobiotic-metabolizing CYPs rarely do, although they affect the host's drug metabolism and susceptibility to some diseases, without directly causing disease (Nelson, 1999).

Table 2.4 Human CYP families and their main functions. Data adapted from (Gonzalez, 1992; Nelson *et al.*, 1996; White *et al.*, 1997; Nelson, 1999)

CYP family	Main functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7α-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage Steroid 11β -hydroxylation Aldosterone synthesis
CYP17	Steroid 17α-hydroxylation
CYP19	Androgen aromatization
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP39	Unknown
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

The CYP1A2 gene is found on chromosome 15 mainly in the liver (Sesardic *et al.*, 1990). CYP1A2 plays an important role in the metabolism of several clinically used drugs. It is one of the major P450 enzymes and accounts for approximately 13% of the total content of this enzyme group in the human liver (Shimada *et al.*, 1994). There are two members of the CYP1A subfamily in human: CYP1A1 and CYP1A2 (Guengerich *et al.*, 1995). CYP1A2 is mainly a hepatic enzyme with substrates as aromatic amines, polycyclic aromatic hydrocarbons; among drugs metabolized preferentially by this enzyme are acetaminophen, caffeine, clozapine, phenacetin, tacrine, and theophylline. Most of the drugs are also substrates of other CYP enzymes, as the metabolic pathways are in most cases complicated, involving the formation of more products or alternative routes. This is why CYP1A2 substrates include drugs such as fluvoxamine, imipramine, clomipramine, ondansetron, propafenone, terbinafine, and verapamil. Levels of CYP1A2 are known to be variable depending on induction by known factors or diet. For example, brassica vegetables increase and apiaceous vegetables decrease CYP1A2 activity (Lampe *et*

al., 2000). A role of CYP1A2 in chemical carcinogenesis is recognized heterocyclic and aromatic amines, certain nitroaromatic compounds or aflatoxin B_1 (which may, however, also be present in contaminated food) belong to substances activated by this enzyme (Eaton *et al.* 1995).

The CYP1A2 enzyme is known to be genetically polymorphic; one allele has been shown to be connected with decreased *in vivo* activity. However, detailed study of CYP1A2 phenotyping is needed, because a functionally significant polymorphism has not yet been confirmed (Welfare *et al.*, 1999). This CYP protein is not only conserved among species, but is also rather stable against denaturation (Anzenbacher *et al.*, 1998). Its active site is able to accommodate planar molecules of moderate volume and basicity (Lewis, 2000).

2.2.2 Inhibition of CYP1A2 and other isoforms

ITCs have been shown to inhibit rat and human CYP isoforms. CYPs 1A1, 1A2, 2B1, 2E1 and 3A4 are inhibited by ITCs, via competitive, noncompetitive or mixed inhibition (Nakajima *et al.*, 2001). Of these isoforms, 1A1 and 2E1 play the most important role in the activation of carcinogens. Structural activity relationship studies have shown that aryl alkyl isothiocyanates with 6 carbon chains can cause maximum inhibition of CYP enzymes in rat liver microsomes (Conaway *et al.*, 1996). In rodent study, PEITC blocks metabolic activation of NNK and benzo(*a*)pyrene (BaP), major lung carcinogens in tobacco smoke, via CYPs, resulting in increased urinary excretion of detoxified metabolites, suggesting inhibitory effects on CYP1A1, 1A2 and 2B1 (Smits *et al.*, 1993; Conaway *et al.*, 1996; Hecht, 1999).

In addition, CYP1A2 plays a major role in bioactivation of procarcinogens. Becquemont and colleaques (1996) have studied the influence of the CYP1A2 inhibitor fluvoxamine administration on the disposition kinetics of single-dose tacrine administration in humans. Tacrine is extensively metabolized by CYP1A2 and fluvoxamine is a potent CYP1A2 inhibitor. Thirteen healthy volunteers participated in this double-blind, randomized crossover study, which compared the effects of fluvoxamine (100 mg/day during 6 days) and placebo on the pharmacokinetics of a single oral dose of tacrine (40 mg). From study was found Fluvoxamine caused a decrease in the apparent oral clearance of tacrine from 1,683 ±

802 to 200 ± 106 L/hr, which was explained by a decrease in its nonrenal clearance. Fluvoxamine administration was associated with significant increases in the plasma AUC values of three monohydroxylated tacrine metabolites and in the total urinary recovery measurements of tacrine and its metabolites (9.1% ± 4.6% versus 24.0% ± 2.6% of recovery). These results may be attributable to fluvoxamine-dependent inhibition of CYP1A, which is responsible of the biotransformation of tacrine into its monohydroxylated metabolites and further into dihydroxylated and reactive metabolites. These results suggest that fluvoxamine inhibits the metabolism of tacrine. CYP1A2 may be the target of this inhibition.

Chen and colleaques (1996) have studied effect of the consumption of watercress on acetaminophen metabolism, the pharmacokinetics of acetaminophen and its metabolites were studied in a crossover trial of human volunteers. A single oral dose of acetaminophen (1 gm) was given 10 h after ingestion of watercress homogenates (50 gm) in comparison with acetaminophen alone. From study was found watercress ingestion resulted in a significant reduction in the area under the plasma cys-acetaminophen concentration-time curve and in the peak plasma Cys-acetaminophen concentration by 28% ± 3% and by 21% ± 4%, respectively. Correspondingly, the cys-acetaminophen formation rate constant and cys-acetaminophen formation fraction were decreased by 55% ± 9% and 52% ± 7%, respectively. These results suggest that the consumption of watercress causes a decrease in the levels of oxidative metabolites of acetaminophen, probably due to inhibition of oxidative metabolism of this drug.

Leclercq and colleaques (1998) studied effect of watercress on the metabolism of chlorzoxazone; an *in vivo* probe for CYP2E1, the oral pharmacokinetics of chlorzoxazone was studied in 10 healthy volunteers before and after a single ingestion of a watercress homogenate (50 gm). Have a reported the area under the chlorzoxazone plasma concentration-time curve was significantly increased by 56% after watercress ingestion. Similarly, chlorzoxazone elimination half-life was prolonged after watercress (53%) administration. These results show that a single ingestion of watercress inhibits the hydroxylation of chlorzoxazone, an in vivo probe for CYP2E1

Nakajima and colleaques (2001) have studied the inhibition and mechanism-based inactivation potencies of PEITC for human CYP activities were investigated using microsomes from baculovirus-infected insect cells expressing specific human CYP isoforms, including CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4. PEITC was found to competitively inhibit CYP1A2 and 2A6, non-competitively inhibit CYP2B6, 2C9, 2C19, 2D6, and 2E1 and inhibit CYP3A4 following a mixed-type of competitive and non-competitively inhibition. Furthermore, PEITC is a mechanism-based inactivator of human CYP2E1. Human CYP1A2, CYP2A6, CYP2B6, CYP2D6, and CYP3A4 were not inactivated. These present study directly proved that the chemopreventive effects of PEITC for nitrosamine-induced carcinogenesis are due to the inhibition of CYP by an *in vitro* study. The possibility that PEITC would affect the pharmacokinetics of clinically used drugs that are metabolized by these CYP isoforms was also suggested.

2.3 Betong watercress

Watercress (*Nasturtium officinale*) (Figure 2.4) is a member of the Cruciferae (or *Brassicaceae*) family, and therefore related to broccoli, cabbage, brussel sprouts, cauliflower, and watercress. Watercress is the richest source of glucosinolate nasturtiin, which on hydrolysis by myrosinase, an enzyme that is activated upon crushing the vegetables by chopping or chewing, produces PEITC (Getahun and Chung, 1999). Watercress is fairly easy to cultivate, it prefers to grow in cool, flowing, water about 2 to 3 inches deep, in a partially shaded area. Being semi-aquatic, watercress is well-suited to hydroponic cultivation, thriving best in water that is slightly alkaline.

In Thailand, watercress is widely grown for commercial purpose in Betong District, Yala province. It is so called Phak num Betong in Thai or Betong watercress. Watercress is originally from France and has been brought to grow in China. In Thailand, watercress was grown in north and spread to the south by Chinese (Education, Municipality of Betong, 2009).
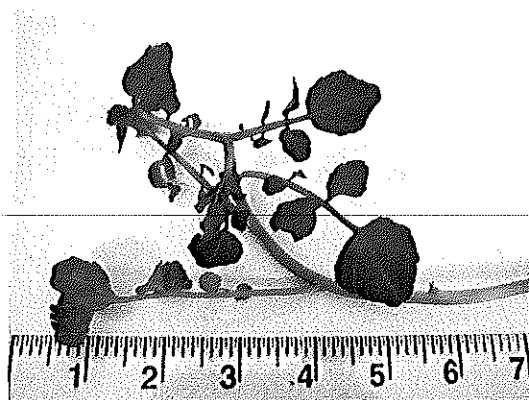
Figure 2.4 Betong watercress collected from watercress farm at Betong, Yala

(Photographed by Angkana Wongsakul at 10/05/2553)

Watercress is rich in vitamin A (from beta-carotene) and vitamin C, and is a source of folate, calcium, iron, and vitamin E. It also contains useful amounts of vitamin K, thiamin, vitamin B_6, potassium, and iodine and is naturally low in sodium. Due to its high water content (93%) it is low in calories. It contains very little carbohydrate and fat but provides some protein (Lindel *et al.*, 2007). In addition to nutrients, watercress is a rich source of a variety of phytochemicals. The most clearly identified compounds include glucosinolate derivatives such as PEITC and methylsulphinylakyl isothiocyanates (MEITCs), flavonoids such as; quercetin, hydroxycinnamic acids, and carotenoids such as; betacarotene and lutein. PEITC and MEITC have been shown to play a role in the suppression of carcinogenesis in a number of *in vitro* studies. Flavonoids and carotenoids have a potent antioxidant activity (Lampe *et al.*, 2000).

Jiao and colleaques, (1998) studied ITC contents in the nine types of cruciferous vegetables namely broccoli, cabbage, cauliflower, choi sum (*Brassica chinensis* var. *parachinensis*, also known as Chinese flowering cabbage), kai choi (*Brassica juncea* var. *rugosa*, also known as mustard cabbage or Chinese mustard), kai lan (*Brassica alboglabra*, also known as Chinese kale), bok choi (*Brassica chinensis*, also known as Chinese white cabbage), watercress, and wong nga pak (*Brassica pekinensis* var. *cylindrica*, also known as celery cabbage). After treatment of cooked vegetables with myrosinase, the highest level of ITCs was found in watercress (81.3 µmol/100 g wet weight; Table 2.5)

Table 2.5 Cruciferous vegetables and their total ITC cotent (Jiao *et al.*, 1998).

Vegetables (n = 9)	ITC contents, mean and range (μmol/100 g wet weight)
Watercress (*Nasturtium officinale*)	81.3 (17.1 - 144.6)
Kai choi (*Brassica juncea* var. *rugosa*)	71.2 (25.6 - 138.4)
Broccoli (*Brassica oleracea* var. *italica*)	38.6 (10.1 - 62.0)
Cabbage (*Brassica oleracea* var. *capitata*)	27.5 (11.9 – 62.7)
Kai lan (*Brassica* var. *alboglabra*)	15.4 (3.1 - 35.9)
Caulifliwer (*Brassica oleracea* var. *botrytis*)	11.6 (2.7 - 24.0)
Choi sum (*Brassica chinensis* var. *parachinensis*)	11.1 (3.5 - 23.4)
Wong nga pak (*Brassica pekinensis* var. *cylindrica*)	5.6 (2.5 - 8.8)
Bok choi (*Brassica chinensis*)	4.9 (2.0 - 7.5)

Population studies show an association between an increased intake of cruciferous vegetables with reduced risk of cancers at several sites (Talalay and Fahey, 2001).

Wattenberg (1977) study has been reported that BITC and PEITC inhibit DMBA-induced mammary tumor formation in female Sprague-Dawley rats when administered 4 h prior to the DMBA. Comparable studies in which BITC was administered 24 h before or 4 h after DMBA showed almost complete loss of inhibition. Additions of BITC or PEITC to a diet containing DMBA inhibited formation of neoplasms of the forestomach and pulmonary adenomas in female ICR/Ha mice.

Hecht and colleaques (1995) studied observed significant benefits of PEITC for cancer protection in animals and wanted to assess its potential in humans. By used watercress, the richest known food source. Eleven smokers volunteered to expose themselves to the lung-specific tobacco carcinogen NNK for 3 days, while consuming 2 ounces (56.8 g) of watercress at each meal. From the study watercress was found a highly significant increase in urinary NNK breakdown products during days 2 and 3 which correlated with the intake of PEITC from watercress during this

period. This linked PEITC and watercress consumption to the metabolism and secretion/neutralization of the lung carcinogen NNK.

Chiao and colleaques (2004) studied *in vivo* affects of a PEITC-NAC (N-acetylcysteine conjugate of PEITC) supplemented diet versus a nonsupplemente diet on tumours of human prostate cancer cells grafted on to mice. After a 9-week treatment period, there was a significant reduction in tumour size in 100% of the mice on the supplemented diet. Tumour weight was reduced by 50% compared with mice on the diet without PEITC-NAC. Mechanisms identified in this study suggest that PEITC-NAC may reduce tumour growth by inhibiting proliferation (uncontrolled cell growth) via cell cycle regulators, and inducing apoptosis (externally triggered cell death).

2.3.1 History of watercress

The correct name for watercress is *nasturtium officinale* and it belongs to the family *Cruciferea*. Cress is native to Europe and Asia, common in Great Britain and widely naturalized in the United States and Canada. It has also been introduced into the West Indies and South America. *Nasturtium officinale* is called watercress in Great Britain and America, Brunnenkress in Germany, Crescione in Italy, and Nerokarthamon in Greece. Watercress is the most ancient of green vegetables known to man and its use can be traced back to the persians, Greeks and Romans. In fact, a famous persian chronicler advised persians to feed cress to their children to improve bodily growth. He also strongly recommended its use to the Greek and Persians soldiers of that time. Although these eminent rulers knew nothing of such matters as mineral content and vitamins, they did observe that their soldiers were in better condition when this plant was made part of will and diet.

In the middle of the 16th century. English cultivation started in early 1800, when a farmer near London began to give cress attention as a product of agriculture to be used in salads. It was not long before its popularity spread and it became increasingly difficult to meet the rather sudden increase in demand for watercress. Recent chemical analysis of watercress reveals that the beneficial effects are due to its generous content of vitamins A and C, and the minerals calcium and iron. Watercress also contains credible amounts of folic acid and lucien. It is also considered an excellent functional food for the prevention of cancer and related

diseases. The United States, with its abundance pure running water, proper soils and atmospheric conditions is the most highly favored country in the world for raising aquatic plants like watercress. These favorable conditions had made it possible for large-scale commercial watercress cultivations in the United States. Wherever watercress has been reported in history, it has been common to eat the crisp green sprigs out of hand, combined with other tender greens in salads and as a garnish on hot and cold dishes. In addition, certain nationalities have made a particular watercress use famous in their country (B&W Quality Growers, 2008).

2.3.2 Health benefits of watercress

Watercress is among the most highly nutritious vegetable, many of the great herbalists wrote of the revitalizing power of watercress. The health benefits of watercress are attributed to its nutrient content. Watercress is an excellent source of vitamins B1, B2, B6, C, E, manganese, and carotenes. It is also a good source of calcium, fiber, iron and copper. The health benefits of watercress include preventing of cancer, protecting the eyes as it contains a high level of two carotenoids, lutein and zeaxanthine, normalizing cholesterol and blood pressure, increasing sexual energy and enhances fertility, improving memory, mental function and to retard ageing, cleansing the body, and increasing breast milk production.

Watercress livens up raw salad and it also makes a valuable juice. Watercress juice is a green juice and must not be consumed alone. It also tastes bitter and is much easier to swallow if mixed with carrot, potato, and a little parsley or carrot, spinach, and turnip leaves. For optimal health benefits, eat watercress raw and as fresh as possible (Hubpages Inc., 2010).

2.4 Caffeine

2.4.1 Physicochemical properties of caffeine and its primary metabolites

Caffeine or 1,3,7-trimethylxanthine ($C_8H_{10}N_4O_2$; m.w. 194.19)(Figure 2.5) is an alkaloid of the methylxanthine family, which also includes the similar compounds 1,3-dimethylxanthine (theophylline; Figure 2.5), 3,7-dimethylxanthine (theobromine; Figure 2.5) and 1,7-dimethylxanthine (paraxanthine; Figure 2.5). Pure caffeine is white, odorless and has slight bitter taste, with a taste threshold in water of 0.2-1.8 mM. Caffeine is a weak base and the ionization constant (pK_a) is 14 and the lipid partition coefficient (log P) is 0.85 (Scott *et al.*, 1999). It is a boiling point of 178°C and a melting point of 238°C. It is a density of 1.2 g/cmÂ³ and 1 gm dissolves in 46 mls water, 5.5 ml water at 80°C, alcohol, chloroform, slightly in ether.
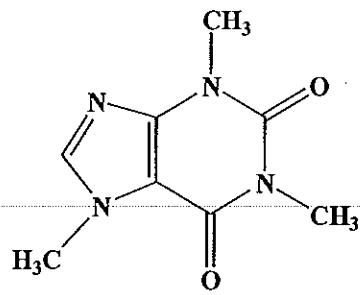
1,3,7-Trimethylxanthine (caffeine)
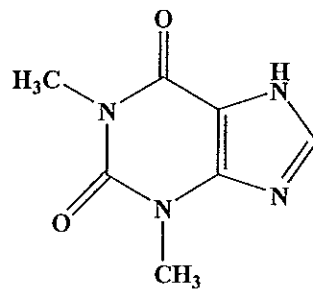
3,7-Dimethylxanthine (theobromine)
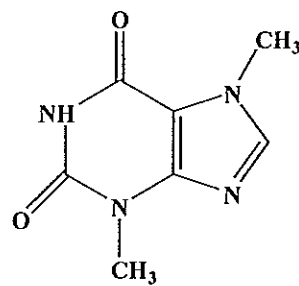
1,3-Dimethylxanthine (theophylline)
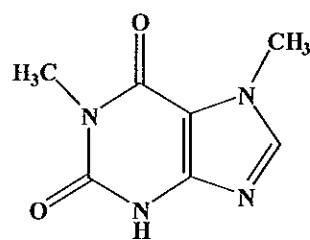
1,7-Dimethylxanthine (paraxanthine)

Figure 2.5 Chemical structures of caffeine, theobromine, theophylline and paraxanthine

3,7-Dimethylxanthine ($C_7H_8N_4O_2$ m.w. 180.16) or theobromine as its pure state it is an odorless white crystalline powder, the colour has been listed as either white or colourless. Theobromine is a weak base and the ionization constant (pK_a) is 10 (Gennaro *et al.*, 1992). It has a boiling point of -295°C and a melting point of 290-295°C. One gram is soluble in approximately 2000 mL of water and 150 mL of boiling water, 0.5M NAOH, insoluble in benzene, ether, chloroform, and carbon tetrachloride. Theobromine affects humans similarly to caffeine, but on a much smaller scale.

1,3-Dimethylxanthine ($C_7H_8N_4O_2$ m.w. 180.16) or theophylline occurs as a white crystalline powder with a bitter taste. Theophylline is a weak acid and the ionization constant (pK_a) is 8.8 (Gennaro *et al.*, 1992). It has a melting point of 270-274°C and clear colorless solution at 50 mg/1M ammonium hydroxide, soluble in hot water, alkaline solution.

1,7-Dimethylxanthine ($C_7H_8N_4O_2$ m.w. 180.16) or paraxanthine is the most abundant of the dimethylxanthines, accounting for approximate 80% of the hepatic degradation of caffeine in humans (Lelo *et al.*, 1986). It is a white to off-white powder. Paraxanthine is a weak acid and the ionization constant (pK_a) is 8.65. It has a melting point of 351-352°C and clear colorless to faint yellow solution at 50 mg/ml in 1M ammonium hydroxide.

2.4.2 Pharmacokinetics

1) Absorption

Caffeine is rapidly absorbed from gastrointestinal tract, and reaches 99% bioavailability in human about 45 min after ingestion (Bolton and Null, 1981), depending on variations in gastric emptying and the presence of dietary constituent (Pekthong, 2000). Orally administered caffeine is mainly absorbed from the small intestine although 20% has been reported to be absorbed from the stomach (Garattini, 1993). Pharmacokinetics of caffeine is independent on the route of administration. Its pharmacokinetics is comparable after oral or intravenous administration of caffeine in humans and animals, leading to superimposable plasma curves (Garattini, 1993). Sweating does not play a significant role in the flux of caffeine. Absorption is, however, not complete when the substance is taken as caffeine (Fredholm *et al.*,

1999). Caffeine absorption is also complete in animals the peak plasma caffeine concentration is reached 15-120 min after oral ingestion in human (Bonati *et al.*, 1982). The caffeine elimination half-life ranges from 0.7-1.2 h in rats and mice (Bonati *et al.*, 1982), 2.5-4.5 h in young and elderly men increasing to 80 h in newborn infants (Arnaud, 1976; Le Guennec and Billon, 1987) and can be over 100 hours in premature infants (Parsons and Neims, 1981). The elimination half-life of caffeine is reduced by 30-50% in smokers, doubled in women taking oral contraceptives and raised to 15 h in the last trimester of pregnancy (Barone and Roberts, 1996).

2) Distribution

Caffeine is rather poorly bound to plasma albumin (10-30%) (Blanchard and Sawers, 1983). It seems that no physiological "barriers" limit the passage of caffeine through out tissues; consequently, easy and rapid equilibrium is reached between mother and fetus, blood and all tissues, including the brain (Burg, 1975). Caffeine may enter the brain by simple diffusion and carrier-mediated transport (McCall *et al.*, 1982). It has been found that caffeine and its metabolites are strong inhibitors of the human organic anion transporter (Sugawara *et al.*, 2005) which is expressed in human lungs, kidneys, and testes (Lee *et al.*, 2001), as well as along the blood brain-barrier, as shown in cultured human brain endothelial cells (Gao *et al.*, 2000).

3) Metabolism

Caffeine has been applied as a metabolic probe for determining CYP1A2 activity. This enzyme is involved in the liver biotransformation of many drugs and xenobiotics (Berthou *et al.*, 1992; Rostami-Hodjegan *et al.*, 1996). As shown in Figure 2.6, CYP1A2 catalyses the N-1, N-3, and N-7 demethylations of caffeine (137X) to form theobromine (37X), paraxanthine (17X), and theophylline (13X) respectively, accounting for about 11, 80, and 4% of caffeine metabolism (Miners and Birkett, 1996). Each of these xanthines undergoes an N-mono dimethylation reaction to give the corresponding monomethylxanthine, 1-methylxanthine (1X), 3-methylxanthine (3X), and 7-methylxan-thine (7X). Caffeine

and xanthines are hydroxylated into their corresponding uric acids, 1,3,7-trimethyluric acid (137U), 1,3-dimethyluric acid (13U); 1,7-dimethyluric acid (17U); 3,7-dimethyluric acid (37U); 1-methyluric acid (1U); 3-methyluric acid; (3U), and 7-methyluricacid (7U). Other non-microsomal enzymes participate in caffeine metabolism (Tang *et al.*, 1991). The polymorphic N-acetyl-transferase, NAT2, catalyses the C8-N9 bond scission and the acetylation of paraxanthine to produce 5-acetylamino-6-formylamino-3-methyluracil (AFMU) which is then spontaneously transformed into 5-acetylamino-6-amino-3-methyluracil (AAMU) in urine. Xanthine oxidase (XO) is responsible for the conversion of 1X into 1U.

Metabolism of caffeine in rats similar to human is shown in Figure 2.7, CYP1A2 catalyses the N-1, N-3, N-7 demethylations to form theobromine (37X), paraxanthine (17X), and theophylline (13X), respectively. Metabolism of caffeine in rat was shown that CYP1A2 catalyse the C-8-hydroxylation of caffeine (137X) to form 1,3,7- trimethyluric acid (137U) (70%) which is the major metabolic reaction compared to N-1, N-3, and N-7 demethylations (Kot and Daniel, 2008). Then three dimethylxanthines may be demethylated to form dimethyluric acids. Characteristic of caffeine biotransformation in rats is the formation of 6-amino-5-(*N*-formylmethylamino)-1,3-dimethyluracil (1,3,7-DAU), a metabolite with an opened imidazole ring, in amounts of quantitative importance that was not found in human (Arnaud, 1976; Bienvenu *et al.*, 1993).
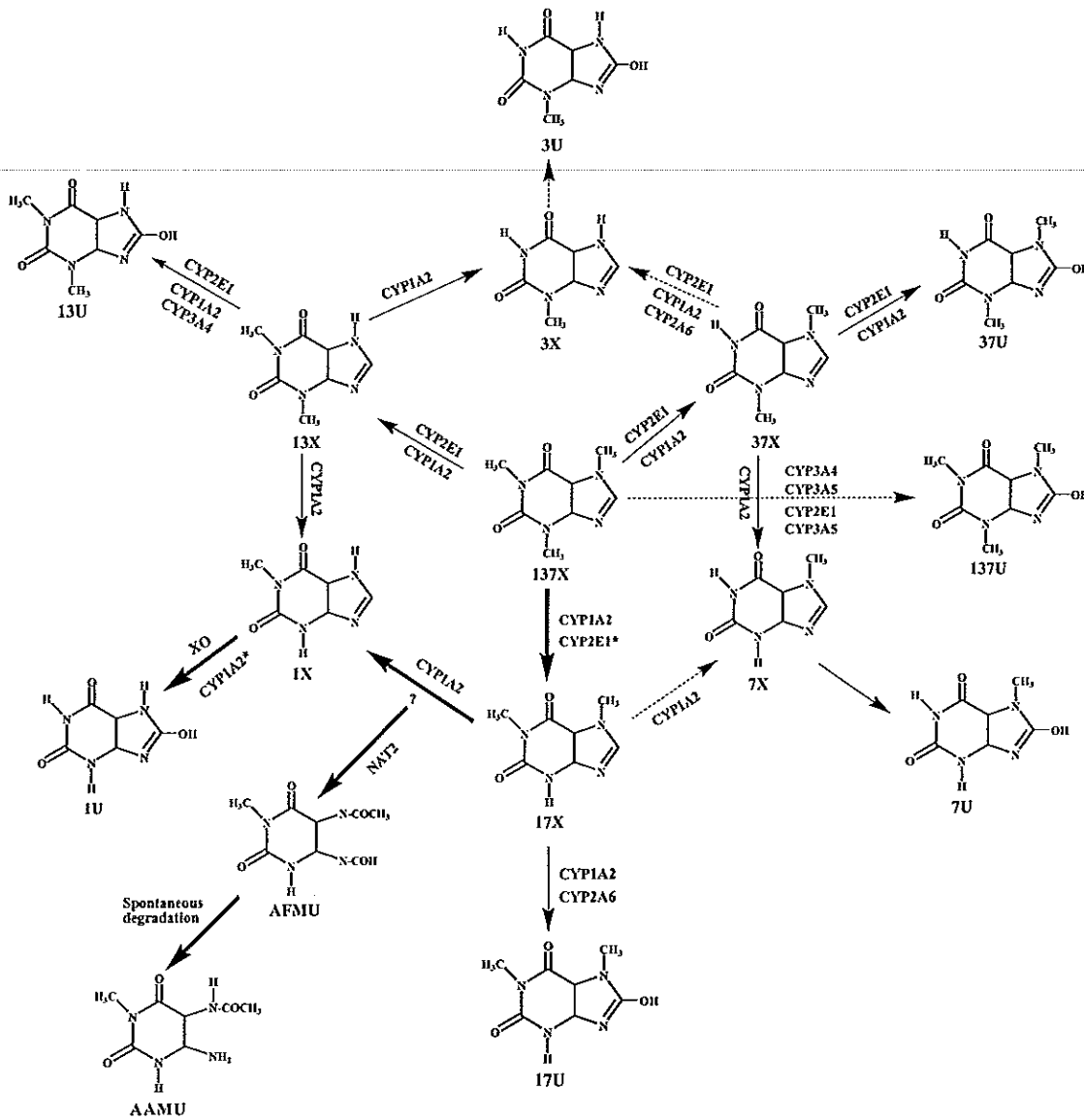
Figure 2.6 Caffeine metabolic pathways in human liver (from Caubet *et al*. 2002).
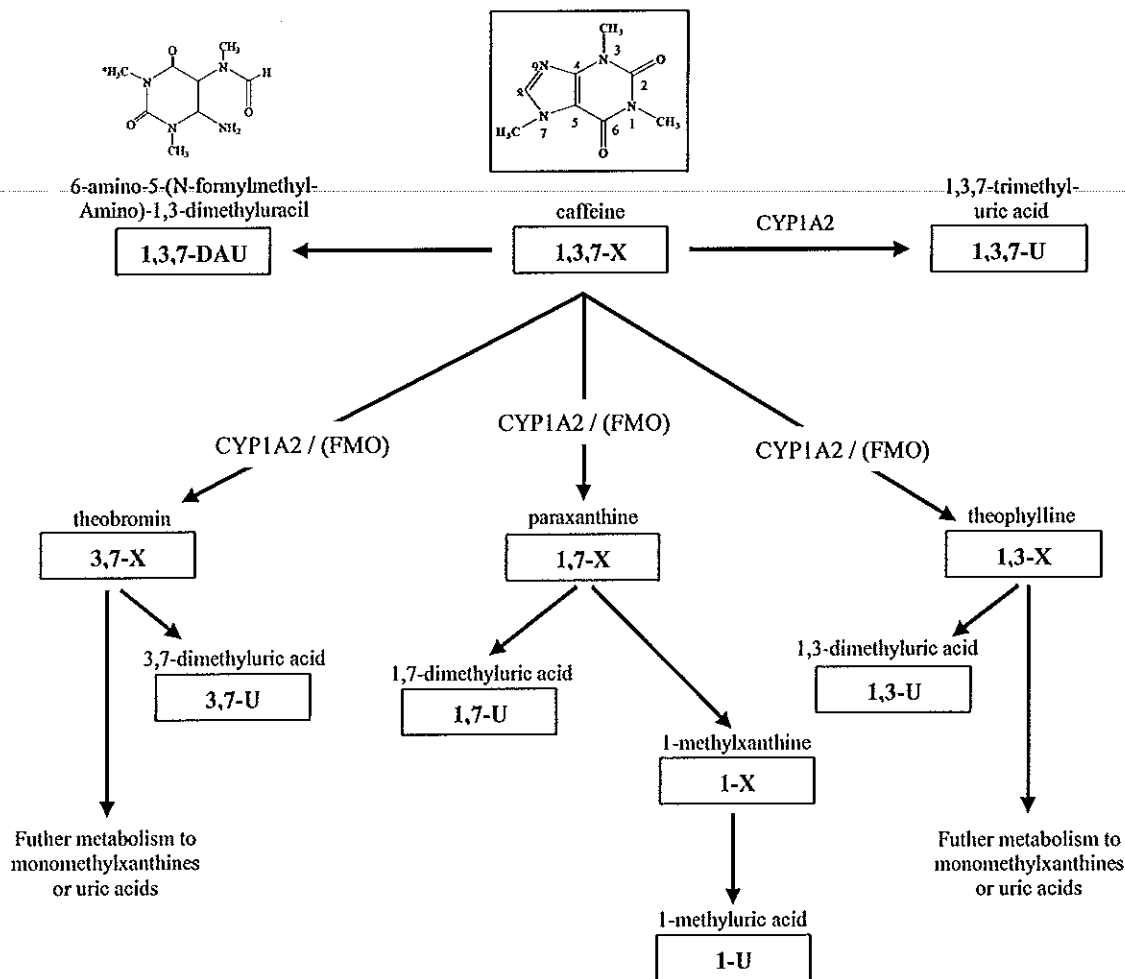
Figure 2.7 Caffeine metabolic pathways in rat liver (from Jorritsma *et al.* 2000).

4) Excretion

Mainly the kidney excretes caffeine and its metabolites. It is submitted to 98% renal tubular reabsorption, only a small percentage 0.5-2% of the ingested dose in humans is excreted unchanged in urine (Garattini, 1993). The elimination half-life of caffeine is highly variable with a mean value of 5 h but with a range of 2-12 h for most individuals. In neonates, the elimination half-life may range up to 100 h. Since CYP1A2 can be both induced and inhibited, various physiological and environmental factors can influence the disposition of caffeine. For example, prior to the onset of menstruation, caffeine elimination may be slowed in the late luteal phase, potentially leading to exaggerated pharmacological effects during this time (Denaro *et*

al., 1990). Caffeine's elimination was decreased in subjects with cirrhosis compared with healthy control subjects (Denaro *et al.*, 1996).

2.4.3 Assessment of CYP1A2 activity using a marker substrate

A marker substrate is devised to provide information on the *in vivo* CYP isoform activity (phenotyping), its level of induction, inhibition or drug interactions. It must be characterized by safety, wide availability, and assay reliability in bodily fluids and, of course, a metabolic pathway specific for one CYP isoform. Currently, different marker substrates for various isoforms of CYP are widely used to assess genetic, ethnic, and environmental differences in the *in vivo* metabolism of drugs. CYP1A2 is one of the major phase I enzymes in the liver, accounting for about 13% of the total liver CYP content (Shimada *et al.*, 1994). This enzyme is important in the metabolism of widely used drugs such as theophylline, clozapine, and imipramine. It is the most important catalyst in the metabolism of caffeine (Berthou *et al.*, 1991; Gu *et al.*, 1992) and has been involved in the metabolic activation of environmental carcinogens, including heterocyclic amines, arylamine, and nitrosamine, and in the biotransformation of drugs (Eaton *et al.*, 1995).

Interestingly, caffeine is the first-choice substrate for assessment of CYP1A2 activity. It is extensively metabolized in human and at least 14 metabolites have been identified. About 80% of caffeine is N3-demethylated to paraxanthine (1,7X) and the major enzyme catalyzing this reaction is CYP1A2 (Campbell *et al.*, 1987; Berthou *et al.*, 1991; Tassaneeyakul *et al.*, 1992). In addition, caffeine has proven to be useful for simultaneous estimation of N-acetyltransferase-2 (NAT-2) and xanthine oxidase (XO) activities (Kalow and Tang, 1993; Rostami-Hodjegan, 1996). This is not surprising since caffeine is relatively safe, low toxic, and possesses many favorable pharmacokinetic characteristics for a marker substrate. Several caffeine-based approaches for assessing CYP1A2 activity *in vivo* have been described. These approaches have utilized caffeine concentrations in plasma or saliva (caffeine clearance), as well as plasma, saliva, or urinary caffeine metabolite ratios.

A number of parameters based on caffeine metabolism have been selected as marker of CYP 1A2 activity, including caffeine breath test, urinary caffeine metabolites ratio and plasma or serum caffeine metabolites ratio (Kalow and

Tang, 1993; Fuhr and Rost, 1994). The first is tested by the measurement of $^{13}CO_2$ or $^{14}CO_2$ following a dose of caffeine labeled in the N3-methyl group (Rost and Root, 1994). The requirement for isotope labeled and specialized equipment is needed to measure the labeled carbondioxide in exhaled air. The second is the determination of caffeine and its metabolites in urine after caffeine intake (Rasmussen *et al.*, 1998). This method based on either secondary or tertiary metabolites are not ideal indices of CYP1A2 activity because these metabolites are not exclusively formed by CYP1A2. These methods might vary substantially with factors such as urinary flow, inter-ethnic differences in renal function and sampling time of urine collection (Ou-Yang *et al.*, 2000). The last one is the determination of caffeine and its metabolites in plasma or serum. Paraxanthine/caffeine ratio has been recognized as a good index for CYP1A2 activity because paraxanthine is a major metabolite and catalyzed by CYP1A2 (Fuhr *et al.*, 1996).

The studies using caffeine as a probe to assess CYP1A2 enzyme activity are as follows. Krul and Hageman (1998) reported an analysis of five urinary metabolites of caffeine; caffeine or (137X), paraxanthine (17X), 1,7-dimethylurate (17U), 1 methylxanthine (1X), 1-methylurate (1U) and 5-acetylamino-6-formylamino-3-methyluracil (AFMU). A standardized procedure was used for oral intake of caffeine and for urine collection. CYP1A2 activity was calculated from the molar ratio (AFMU+1X+1U)/17U, *N*-acetyltransferase (NAT) from the ratio AFMU/1X, xanthine oxidase (XO) from the ratio 1U/1X+1U and CYP2A6 from the ratio 17U/(17U+17X+1U+1X+AFMU). Using this procedure, metabolic ratios were determined for four groups of subjects; healthy, non-smoking females using oral contraceptives (n=55) and non-users (n=55), healthy nonsmoking males (n=59) and children (n=57). A higher CYP1A2 ratio was found for males compared to females. For the other enzyme activities, no significant differences were found between the groups of subjects in this study.

Jodynis-Liebert and Matuszewska (1999) determined levels of caffeine and its primary metabolites in plasma of rats pretreated with toluidine at doses: 1, 10, and 60 mg/kg/b.w (p.o.) and dinitrotoluene (DNT) at doses: 100 and 200 mg/kg/b.w (p.o.). Caffeine metabolite ratios in plasma: TB/CF, PX/CF, TP/CF, and (TB+PX+TP)/CF were calculated and compared to those of control rats group which

received olive oil and caffeine alone. Administration of toluidines resulted in a 2–20 fold increase of the concentration ratios of metabolites to caffeine. All toluidines seem to be inducers of CYP1A2. Two out of the four tested dinitrotoluenes slightly affected CYP1A2 activity; 2,3- and 3,4-DNT increased estimated parameters 2–6 fold. Two others, 2,4- and 2,6-DNT can be considered as moderate hepatotoxic agents decreasing CA metabolic ratios to 4–70% of the control values.

Bozikas and colleaques (2004) study was to assess the impact of smoking on the metabolism of psychotropic drugs in a group of patients with schizophrenia, by measuring CYP1A2 activity. This activity was assessed by the molar ratio (MR) of caffeine metabolites in urine (AFMU + 1U+ 1X)/17U and saliva (17X/137X). Participants were 40 patients with schizophrenia: 30 current cigarette smokers and 10 nonsmokers. The two groups (smokers and nonsmokers) differed significantly in their ratio of men to women (83% men and 17% women were among smokers compared with 50% men and 50% women nonsmokers). Smokers had significant higher MR in urine as well as in saliva than nonsmokers, suggesting a higher activity of CYP1A2 dependent on smoking. When gender was used as a covariate, the differences between the two groups remained significant for MR. Cigarette smoking may be a factor influencing the plasma levels of antipsychotics that are metabolized through CYP1A2.

2.4.4 Mechanism of action

Caffeine acts through multiple mechanisms, the most important of which is the antagonism of adenosine receptors (A_1 and A_{2A}). Caffeine blocks adenosine receptors and competitively inhibits the action of adenosine at the therapeutic concentrations of caffeine (10-100 M), with K_i of 29 and 48 M at the A_1 and A_{2A} adenosine receptors, respectively. As an adenosine receptor antagonist, caffeine increases the release of various neurotransmitters (Fredholm et al. 1999). Moreover, due to a negative interaction between adenosine and dopaminergic receptors, caffeine increases responses from dopaminergic receptors (Fredholm and Svenningsson, 2003). Because of its ability to affect neurotransmission in different regions of the brain, caffeine displays psychomotor stimulant properties and promotes

behavioral functions such as vigilance, attention, mood, and arousal (Fisone *et al.*, 2004).

2.4.5 Pharmacological effects

1) Effects on central nervous system

Caffeine has CNS stimulant properties. In humans, the manifestration is as followa; increased arousal and vigilance, decreased fatigue, increased capacity for work, decreased motor reaction time for some tasks, and elevated mood (Benowitz, 1990; Serafin, 1996). Caffeine also has substantial effects on sleep. Ingesting caffeine 30-60 min before sleep will increase sleep latency, decrease total sleep time, and substantially worsen subjective estimations of sleep quality (Regestein, 1980). Caffeine also stimulates the medullary respiratory center. This effect is due to an increased sensitization to carbon dioxide but needs large doses to elicit this effect, 150-250 mg, parenterally. The spinal cord is stimulated at higher doses and convulsions and death may result. A caffeine withdrawal syndrome which is well described includes headache and fatigue most prominently; anxiety, impaired psychomotor performance, nausea, and vomiting, or an intense desire for coffee occur less commonly (Griffiths and Woodson, 1988). Headache is usually preceded by a feeling of fullness in the head that occur about 18 h after the last caffeine intake.

2) Effects on cardiovascular system

Caffeine has an effect on the cardiovascular system by stimulating the heart and often increasing contraction rate (pulse). Its direct stimulatory effect on the heart may be neutralized by its central vagus stimulation. The direct effect predominates at very large doses with tachycardia and, eventually, arrythmias resulting (Ritchie *et al*, 1975). Although caffeine dilates blood vessels by a direct action, its central effect is one of constriction. At higher doses, the dilating effect is apparent (Peach, 1972; Poisner, 1973). Similarly, because its direct and central effects are antagonistic, the resultant effect of caffeine on blood pressure is unpredictable. The net effect is usually of less than 10 mm of Hg in blood pressure (Ritchie *et al.*, 1975).

3) Effects on respiratory system

In the respiratory system, one of the most common disorders of breathing is asthma, which is greatly relieved by the stimulating effects of caffeine and theophylline. Caffeine expands these passages as well as increasing the breathing rate. It remains uncertain to what extent caffeine and theophylline exert anti-asthmatic effects through blockade of adenosine receptors, and to what extent through phosphodiesterases inhibition (Onken, 2000). In patients with asthma, caffeine functions as a bronchodilator. Bronchodilation is only about 40% of the potency of theophylline at comparable concentration (Gong *et al.*, 1986).

4) Effects on gastrointestinal system

Caffeine stimulates the small intestine to secrete water and sodium. Caffeine doses greater than 100 mg enhance the production of gastric acid secretions and cause gastric irritation. Interestingly, decaffeinated coffee also stimulates gastric acid production, suggesting that caffeine is not the only compound in coffee that influences gastric juice flow. In the distal colon, coffee stimulates motility and promotes defecation (Sawynok, 1995).

5) Effect on renal system

The effects of caffeine on renal function, namely diuresis, increase blood flow, and stimulation of rennin release, appear to be linked to blockade of adenosine receptors-adenosine analogs having the opposite effects to xanthines (Grattini, 1992). Xanthines, including theophylline and caffeine, reverse adenosine-mediated reduction in glomerular filtration, vasoconstriction, and inhibition of renin release. Caffeine has a diuretic effect, and the pattern of enhanced excretion of water and electrolytes is similar to that produced by the thiazide drugs. It is believed that this effect results from direct action on the renal tubule. Caffeine causes an increased rate of sodium and chloride excretion (Serafin, 1996).

2.4.6 Withdrawal symptoms

In human, caffeine withdrawal translates into typical symptoms. The most often reported are headaches, feelings of weariness, weakness, and drowsiness, impaired concentration, fatigue, and work difficulty, depression, anxiety, irrritability, increased muscle tension, occasionally tremor, and nausea and vomiting, as well as withdrawal feelings (Hughes *et al.*, 1993; Nehlig *et al.*, 1994; Richardson *et al.*, 1995). Withdrawal symptoms generally begin about 12-24 h after sudden cessation of caffeine consumption and reach a peak after 20-48 h. However, in some individuals, these symptoms can appear within only 3-6 h and last for one week (Barone and Roberts, 1984; Lane, 1998).

In animal, caffeine withdrawal signs have been reported in rats, cats and monkeys. Those include decreases in locomotor activity (Finn and Holtzman, 1986) operant behavior (Mumford *et al.*, 1988), and in the reinforcement threshold for electrical brain stimulation. The severity of caffeine withdrawal depends on the dose, and decreases in locomotor activity do not appear when caffeine doses lower than 67 mg/kg/day are substituted by water. The length of the decrease in locomotor activity depends also upon the dose of caffeine and the duration of the treatment before the substitution by water. The latency to the onset of caffeine withdrawal effects usually occurs within 24 h, and peaks around 24-48 h (Finn and Holtzman, 1986). The caffeine withdrawal-induced behavioral changes usually last a few days (Griffiths and Mumford, 1996), except for the sleep-related signs that have been shown to last up to 30 days after the onset of caffeine withdrawal (Sinton and Petitjean, 1989).

2.4.7 Toxicity

Acute toxicity

The signs of acute caffeine toxicity include nausea, vomiting, diarrhea, cramps, and muscle facoiculations. Central nervous system effects are manifested as restlessness, agitation, and irritability. In severe cases, seizures may occur (Massachusetts Poison Control, 1994).

Animal studies have demonstrated teratogenecity at extremely high doses, but not at physiologic or even moderately toxic doses. Clinical trials in humans have shown heavy coffee drinkers (>600 mg caffeine/day) to have an increased

incidence of miscarriages and premature birth, however these differences lose significance when adjusted for other factors such as smoking and demographics (Massachuseet Poison Control, 1994). In addition, caffeine is reported as a teratogen causing developmental abnormalities of the craniofacial and musculoskeletal systems, abortion, and stillbirth (Duke University Medical Center, 2000; Massachuseet Poison Control, 1994).

2.5 Analytical methods for caffeine and its metabolites

Various analytical methods have been available for initial screening, confirmation, and quantification of caffeine and metabolites (as shown in Table 2.6) such as gas chromatography (GC) (Hirai and Kondo, 1985), high performance liquid chromatography (HPLC) (Ventura *et al.*, 2003), an liquid chromatography coupled with mass spectrometry (LC-MS) (Arinobu *et al.*, 2009)

Table 2.6 Methods for the determination of caffeine and metabolites and their analogues from 1979-presnt

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (µg/mL)	Ref.
1979	HPLC-UV	CF and metabolite	LLE	urine	µ Bondapak C18 (250×4.6 mm i.d.) 5 µm particle size	(1.5-7.5%) Acetronitrile in 0.5% acetic acid Flow rate 2 min/mL	280	-	-	Aldridge *et al.*
1983	HPLC-UV	CF, TB, PX, TP	LLE	urine	Ultraphere ODS (25×4.6 mm i.d.) 5 µm particle size	0.05% acetic acid:MeOH (88:15 v/v) Flow rate 1.2 min/mL	280	100%	-	Grant *et al.*
1985	GC-FID	CF	LLE	plasma and urine	OV-17 (2 mm i.d.×6 ft)	-	-	-	0.01	Hirai and Kondo
1990	HPLC-UV	CF, TB, PX, TP	Protein precipitate	breast milk	C18 ODS (250×4.6 mm i.d.) 5 µm particle size	0-16% MeOH in 10 mM sodium acetate and 5 mM tetrabutyl-ammonium-hydrogen sulfate Flow rate 1.5 min/mL	272	-	-	Blanchard *et al.*

Table 2.6 Methods for the determination of caffeine and metabolites and their analogues from 1979-presnt (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (µg/mL)	Ref.
1991	HPLC-UV	CF,TB, PX, TP	LLE	serum	Ultrashere C18 (150×2 mm i.d.) 5 µm particle size	MeOH 1% acetic acid: 1.4 mM tetrabutyl-ammonium-phosphate: 40% phosphoric acid (12:52:36 v/v/v) Flow rate 0.3 min/mL	230	63.6-91.5%	0.05	Lau and Falk
1991	HPLC-UV	CF, TB PX, TP	LLE	plasma	C18 reverse phase (150×4.6 mm i.d.) 5 µm particle size	MeOH : Phosphate buffer (0.1 M-NaH$_2$PO$_4$) Flow rate 0.8 min/mL	274	-	TB & CA 0.05 PX & TP 0.01	Tanaka et al.
1993	HPLC-UV	TP	Protein precipitate	serum	LiChrosorb RP-18 ODS (250×4 mm i.d.) 5 µm particle size	0.05 M Ammonium acetate: MeOH (58:42 v/v) Flow rate 1 min/mL	273	-	0.025	Papado-yannis et al.

Table 2.6 Methods for the determination of caffeine and metabolites and their analogues from 1979-presnt (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (µg/mL)	Ref.
1996	HPLC-UV	CF, TP	Protein precipitate	plasma	reverse phase ODS 2 (150×4.6 mm i.d.) 5 µm particle size	acetonitrile:tetrahydrofuran:acetic acid :distilled water (20:20:5:955 v/v/v/v) Flow rate 1 min/mL	273	-	-	Schreiber-Deturmeny and Brugure-rolle
1998	HPLC-UV	CF, TP	Protein precipitate	serum	C18 bonded silica ODS (250×4.6 mm i.d.) 5 µm particle size	buffer (H_3PO_4 pH 7.4) (100%) Flow rate 0.7 min/mL	273	-	TP 0.2 CF 0.5	Tomanari et al.
1999	HPLC-UV	CF	Protein precipitate	urine	C18 reverse phase (250×4.6 mm i.d.) 5 µm particle size	Eluent A: 10 mM sodium acetate buffer (pH 5.5) MeOH-dimethylformamide (99.0:0.5:0.5v/v/v) Eluent B: :methanol Eluent A (50:50 v/v) Flow rate 1 min/mL	280	-	-	Scharder et al.

Table 2.6 Methods for the determination of caffeine and metabolites and their analogues from 1979-presnt (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelength (nm)	%Recovery	Limit of detection (µg/m)	Ref.
1999	HPLC-UV	CF	LLE	urine	Novapak C18 reverse-phase (250×4.6 mm i.d.) 5 µm particle size	acetic acid:MeOH: water (0.5:90: 905.5 v/v/v) Flow rate 1 min/mL	280	-	-	Simues et al.
1999	HPLC-MS /MS	CF	LLE	urine	Bondapak C18 (2×300 mm i.d.) 10 µm particle size	methanol-acetonitrile aqueous buffer solvent system Flow rate 0.5 min/mL	280	-	-	Tuomi et al.
2000	HPLC-UV	CF,TB, PX, TP	LLE	urine	Rexchrom S5-100-ODS (25×34.6 mm i.d.) 5 µm	0.05% acetic acid-:methylalcohol 92.5:7.5 v/v) Flow rate 1 min/mL particle size	280	-	CF 0.5 PX 0.2	Bendriss et al.
2002	HPLC-DAD	CF,TB, PX, TP	SPE	urine	Eclipse XDB C18 reverse-phase (250×4.6 mm i.d.) 5 µm particle size	mixture distilled water: acetic acid: teteahydrofuran (996.5:1:2.5 v/v/v) :acetronitrile	270	-	-	Caubet et al.

Table 2.6 Methods for the determination of caffeine and metabolites and their analogues from 1979-present (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelenght (nm)	%Recovery	Limit of detection (µg/mL)	Ref.
2002	HPLC-UV	CF	SPE	plasma	C18 Supelco (15×4.6 mm i.d.) 5 µm particle size	mixture MeOH 0.05 M aqueous CH_3COONH_4 (20:80 v/v) Flow rate (20:80 v/v) 0.8 min/mL	272	-	-	Theodoridis and Manesiotis
2003	HPLC-UV	CF	LLE	urine	C18 Ultrashere ODS (7.5×0.46 mm i.d.) 3 µm particle size	water:acetronitrile (90:10 v/v) gradient elution Flow rate 1 min/mL	280	81.4-87.3%	0.7	Ventura et al.
2005	HPLC-DAD	CF,TB, PX, TP	LLE	milk	Supelcosil LC-18-DB (250×4.6 mm i.d.) 5 µm particle size	methanol:buffer (pH 5.8) (20:80 v/v) Flow rate 1 min/mL	230-280	60.2-98.6%	-	Aresta et al.
2008	HPLC-UV	CF	LLE	saliva	Beckman Ultrasphere ODS (25×4.6 mm i.d.) 5 µm particle size	acetic acid containing 9% MeOH (A) and 100% MeOH (B) (gradient) Flow rate 1.2 min/mL	280	-	0.05	Boylan et al.

Table 2.6 Methods for the determination of caffeine and metabolites and their analogues from 1979-presnt (cont.)

Year	Technique	Drugs	Extraction method	Specimens	Chromatographic column	Mobile phase & Flow rate	Detection wavelenght (nm)	%Recovery	Limit of detection (µg/mL)	Ref.
2009	HPLC-MS	TP, CF	SPE	serum	Chromolith Perfo-rmance RP-18e (100×4.6 mm i.d.) 5 µm particle size	0.1% formic acid/ ultra pure water Flow rate 4.0 min/mL	280	more than 75%	TP 1.0 CF 0.5	Arinobu et al.

2.5.1 High performance liquid chromatography (HPLC) and
Liquid chromatography coupling mass spectrometry (LC-MS)

HPLC is an important analytical tool for separating and quantifying components in complex liquid mixtures. HPLC is a separation technique utilizing differences in distribution of compounds to two phases, called stationary phase and mobile phase. The stationary phase designates a thin layer created on the surface of fine particles and the mobile phase designates the liquid flowing over the particles. Under a certain dynamic condition, each component in a sample has different distribution equilibrium depending on solubility in the phases and/or molecular size. As a result, the components move at different speeds over the stationary phase and are thereby separated from each other. Mobile phase is constantly fed into the column inlet at a constant rate by a liquid pump. A sample is injected from a sample injector, located near the column inlet. The injected sample enters the column with the mobile phase and the components in the sample migrate through it, passing between the stationary and mobile phases. Compounds move in the column only when is in the mobile phase. Compounds that tend to be distributed in the mobile phase therefore migrate faster through the column while compounds that tend to be distributed in the stationary phase migrate slower. In this way, each component is separated on the column and sequentially elutes from the outlet. Each compound eluting from the column is detected by a detector connected to the outlet of the column.

When the separation process is monitored by the recorder starting at the time the sample is injected, a graph is obtained. This graph is called a chromatogram. The time required for a compound to elute (called retention time) and the relationship between compound concentration (amount) and peak area depend on the characteristics of the compound. Retention time is therefore used as an index for qualitative determination and peak surface area (or height) as an index for quantitative determination. The retention time of the target compounds and the concentration for each unit of peak area are based on data obtained in advance by analyzing a sample with known quantities of the reference standards. Normally, the reference standards are highly purified target compounds. A HPLC system is basically consist of a pump, an injector, a column, a column oven, and detector (Global Centre Environmental foundation, 1997).

There are several types of HPLC defined by the type of mobile phase and stationary phase that is used. One of the most commonly used types of HPLC is referred to as "reversed phase." In this type, consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. Water, methanol (methyl alcohol), ethanol (ethyl alcohol), and acetone are examples of polar solvents. The stationary phase in reverse phase HPLC is a nonpolar material such as a long chain hydrocarbon molecule.

HPLC is widely used for caffeine and metabolites analysis. The separation of caffeine and its metabolites are usually performed with silica-based reverse phase column, mainly C18 (Lau and Falk, 1991; Tanaka *et al.*, 1991; Scharder *et al.*, 1999; Sinues *et al.*, 1999; Tuomi *et al.*, 1999; Caubet *et al.*, 2002; Theodoridis and Manesiotis, 2002; Ventura *et al.*, 2003).

Caubet and colleaques (2002) described the development and validation of analytical methodology for the determination caffeine and metabolites in human urine by HPLC-DAD detection. The chromatographic separation was achieved on an Eclipse XDB C18 reverse-phase column (250 × 4.6 mm i.d., 5 μm). The mobile phase was a mixture of distilled water:acetic acid:tetrahydrofuran (996.5:1:2.5 v/v/v) :acetronitrile in gradient mode and the extraction is solid phase extraction. The results showed that the method allows to separates and quantify rapidly (23 min) the metabolites of caffeine, with high precision and accuracy.

Liquid chromatography coupling mass spectrometry (LC-MS) is a technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity. An analysis of the mass and pattern of fragmentation provides information on composition and structure.

2.5.2 Gas chromatography (GC)

GC is an instrumental technique used in drug analysis. A gas chromatography consists of a flowing mobile phase, an injection port, a separation column containing the stationary phase, and a detector. GC is a physical method of separation based on the distribution of a solute (i.e., analyte) between a mobile gaseous phase (or carrier gas) is inert gas helium (He), hydrogen (H_2), or nitrogen (N_2), and a stationary liquid phase that are coated on a solid support material found within the chromatographic column. When the sample is injected onto the column, the individual molecules continuously transfer between the stationary and mobile phases as the injected solute band moves down the column. Each solute moves at a rate determined by its partition between the gas and liquid phase and the flow rate of carrier gas. Selective partitioning between the mobile and stationary phases is highly dependent on the analyte molecule's solubility in each phase. Thus, solutes that are less soluble in the stationary phase will elute from the column first while solutes that are more soluble will elute later. Because solubility depends on the physical and chemical properties of a compound, a series of compounds exhibiting a systematic variation in structure and thus, chemical properties, would be expected to partition between the mobile and stationary phases to different extents (Skoog et al., 1998). A number of different detectors are available for use in gas chromatography. Caffeine and its metabolites determination have been performed using GC couple with different systems such as mass spectrometry (often abbreviated as 'MSD' for mass selective detector), flame ionization (FID), and nitrogen-phosphorus (NPD)).

The MSD is the most common of the three, principally because it can provide definitive identification of compounds along with quantitative information. The FID is used in arson cases because of its sensitivity to hydrocarbons, the primary ingredient in most accelerants. The NPD is used in drug analysis and toxicology. One of the advantages of chromatographic systems such as GC is the ability to provide both qualitative information (identification of individual components) and quantitative information (concentrations of individual components). If the instrument is properly calibrated, it can be used to determine quantities of materials present in samples, and this is commonly done in drug analysis. For example, the purity of a drug sample seized as evidence can provide important information and may be used as part of the

prosecution. Similarly, when a sample of plastic or a fiber is analyzed by pyrolysis GC, the relative abundance of the individual components can be useful in creating a chemical signature or fingerprint of that particular sample.

In the determination of caffeine GC technique, Hirai and Kondo (1985) study determination of caffeine in human plasma and urine by using a nitrogen phosphorus-detector (NPD). The glass column (2 mm i.d. × 6 ft.) was packed with 1.8% OV-17. The column temperature was 240°C and the injector and detector temperatures were 260°C and 270°C, respectively. Helium was used as carrier gas, at a flow rate of 30 mL/min. The result showed this method seems thoroughly satisfactory as a convenient, rapid and highly sensitive analytical method for routine uses.

2.6 Sample preparation for determination of caffeine and metabolites

Sample preparation and clean-up procedures are important prior to drug analysis. The major goal of sample preparation is to isolate and concentrate an analyte from matrix components. The extent of isolate, purification, and concentration of the analyte is determined by the complexity and composition of the matrix, the selectivity and sensitivity required for the subsequent analysis (Diana, 1997). The various different methods have commonly been used to isolate caffeine and metabolites and other drugs, including liquid-liquid extraction (Caubet et al., 2000; Ventura et al., 2003), solid phase extraction (Theodoridis and Manesiotis, 2002; Arinobu et al., 2009; Tuomi et al., 1999) and protein precipitation (Scharder et al., 1999).

2.6.1 Liquid-liquid extraction (LLE)

Traditional liquid-liquid extraction (LLE) is one of the most useful techniques that are being used for selective extraction of caffeine and metabolites from aqueous solution (Chung, 2004). LLE, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is of a substance from one liquid phase into another liquid phase. LLE is a basic technique in chemical laboratories, where it is performed using a separatory funnel. In other words,

this is the separation of a substance from a mixture by preferentially dissolving that substance in a suitable solvent. By this process a soluble compound is usually separated from an insoluble compound. Solvent extraction is used in nuclear reprocessing, ore processing, the production of fine organic compounds, the processing of perfumes and other industries (http://en.academic.ru/). Extraction of caffeine and metabolites by LLE has been reported by several approaches which wide variety of solvents have been used, including dichloromethane (Tanaka *et al.*, 1991), chloroform:ethanol (82.5:17.5 v/v) (Lau and Falk, 1991), and chloroform:isopropanol (80:20 v/v) (Boylan *et al.*, 2008). The more polar solvents are more effective for recovering the polar hydrolyzed metabolites.

Several studies have reported using LLE for isolating caffeine and its metabolites from samples. In a previous study, Grant and colleaques (1983) used the chloroform:isopropanol (85:15 v/v) as organic solvent to extracting of caffeine and metabolites compound form human urine samples where recovery of 90-100% was obtained from sixty-eight subjects from the Toronto population when taken drug daily.

Tanaka and colleaques (1991) used the dichloromethane (3 mL) as organic solvent to extracting of caffeine and its three primary metabolites in human plasma samples. The organic layer was evaporated to dryness and analyzed with HPLC.

In addition, Lau and Falk (1991) used the chloroform:ethanol (82.5:17.5 v/v) as organic solvent to extract caffeine and metabolites from rat serum samples. The percentage recoveries were obtained in the range of 63.6-91.5%.

2.6.2 Solid phase extraction (SPE)

SPE is the most widely used technique for sample preparation. The isolation of selected analytes is based on the partition equilibrium between sorbent and eluting solvent. SPE is used most often to prepare liquid samples and extract semi-volatile or nonvolatile analytes, but also can be used with solids that are pre-extracted into solvents. SPE products are excellent for sample extraction, concentration, and clean-up sample and transfer from the sample matrix to a different solvent. Selecting the most suitable product for each application and sample is

important. The SPE designs frequently used are in the form of cartridge, syringe or disk. SPE cartridges and disks are available with a variety of stationary phases, each of which can separate analytes according to different chemical properties. Most stationary phases are based on silica that has been bonded to a specific functional group. Sorbents can be classified into four categories depending on their primary retention mechnism; reverse phase, normal phase, ion exchange, and adsorption (Simpson, 2000). Reversed phase separations involve a polar or moderately polar sample matrix (mobile phase) and a nonpolar stationary phase, such as LC-18, LC-8, ENVI-8, LC-4, and LC-Ph. Normal phase SPE procedures typically involve a polar analyte, a mid- to nonpolar matrix (e.g. acetone, chlorinated solvents, and hexane), and a polar stationary phase, such as LC-Si, LC-Florisil, and LC-Alumina. Ion exchange SPE can be used for compounds that are charged when in a solution. The sorbents can be classified as either anionic or cationic; Anionic (negatively charged) compounds can be isolated on LC-SAX or LC-NH$_2$. Cationic (positively charged) compounds are isolated by using LC-SCX or LC-WCX.

The SPE method is popular to isolate caffeine and its metabolites from simple biological matrices such as urine, plasma, and serum. Many reports evaluated the different types of SPE procedure for isolation of caffeine and its metabolites. Caubet and colleaques (2002) used SPE to those compounds from human urine. The sample was first acidified with 1 N HCl before loading onto a SPE column Oasis C18 preconditioned with methanol, 0.02 N HCl and water. The analytical method yielded high recovery of extraction (83-99%).

Theodoridis and Manesiotis (2002) used SPE to extract with human plasma. The sample was loaded on the MIP-SPE cartridge (molecularly imprinted polymer) using different loading conditions (solvents, pH value). Washing and eluting of the caffeine bound to the MIP was studied utilizing different protocols. The analytical method showed high recovery of about 80%.

In another work, Arinobu and colleaques (2009) have used Oasis HLB cartridge used as a precolumn for extraction from human serum. The analytical method showed the recovery of more than 75%. SPE method has been preferred over LLE because of the practical advantages of no emulsions, better recoveries, cleaner extracts achievable, and the ability to remove many interferences and matrix

components selectively, and so on. Of course, SPE was also prized because of the much lower quantities of solvents required, and the corresponding decrease in the volume of waste solvents produced. In addition, the choices of different sorbents and tunable selectivity of the organic solvent mixtures used as eluents, provides SPE with a very high degree of versatility compared with LLE (Agilent, 2000)

2.6.3 Protein precipitation

Protein precipitation achieves separation by the conversion of soluble proteins to an insoluble state, which subsequently can be removed by various means. Precipitation is usually induced by addition of a salt or an organic solvent or by changing the pH to alter the nature of the solution (Technical Bulletin, 2006). The properties of the solvent can also be modified by addition of high concentrations of certain salts or of miscible organic solvents. Addition of specific metal cations such as Zn^{2+}, Cd^{2+}, and Ba^{2+}, or of compounds with bulky anions such as picrate, tannate, tungstate, molbdate, trichloroacetate, perchlorate and sulfosalicylate, can also precipitate proteins at appraparite pH vale. Protein precipitates reagen inclued acetronitrile, methanol, acetone, ethanol, perchloric acid and ammonium sulfate.

Schreiber-Deturmeny and Bruguerolle, (1996) determined caffeine and its metabolites in human plasma using HPLC. The sample was prepared by using protein precipitation with 20% perchloric acid. After centrifugation (2000 × g, 5 min), the supernatant was transferred to autosampler vial and injected into HPLC system. The extraction procedure is limited to a deproteinization with perchloric acid, which allows rapid processing.

The main advantage of precipitation is the relative ease of use. In addition, precipitating agents can be chosen that provide a more stable product than found in the soluble form. A protein is made insoluble by changing its surface, charge characteristics or changing the solvent characteristics; the latter being preferred (Asenjo, 1990). However, some proteins may denature.

2.7 Objectives

The objectives of this work are to study the influence of PEITC on N-demethylation of caffeine in rat serum. The effect of different doses of PEITC on N-demethylation of caffeine was investigated. In addition, the effect of dry powders of Betong watercress juice on N-demethylation of caffeine in rat serum was investigated which studied the following subtopics:

2.7.1 Investigate the effect of a single dose of PEITC and dry powders of Betong watercress juice

2.7.2 Investigate the effect of multiple dose of PEITC and dry powders of Betong watercress juice

2.7.3 Determination of PEITC content in dry powders of Betong watercress Juice

CHAPTER 3

Methodology

3.1 Chemicals and apparatus

Caffeine ($C_8H_{10}N_4O_2$), theobromine ($C_7H_8N_4O_2$), theophylline ($C_7H_8N_4O_2$) phenethyl isothiocyanate (PEITC), and paraxanthine ($C_7H_8N_4O_2$) (purity >98%) which were used as a reference standard, and acetaminophen ($C_8H_9NO_2$) which was used as an internal standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fluvoxamine, 1,2-benzenedithiol and myrosinase enzyme (thioglucosidase) were also purchased from Sigma-Aldrich. Methanol HPLC grade, and ammonium hydroxide (NH_4OH) (J.T. Baker) were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Ammonium sulphate ((NH_4)$_2SO_4$) was purchased from Okhla Industrial Area (Delhi, New Delhi, India). Potassium phosphate (KH_2PO_4) was purchased from Ajax Finechem (Botany, Auckland, New Zealand). Acetic acid (CH_3COOH), isopropanol (C_3H_7OH), and sodium hydroxide (NaOH) AR grade were purchased from Merck (Darmstadt, Frankfurter, Germany.). Acetronitrile HPLC grade was purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). Chloroform ($CHCl_3$) AR grade was purchased from VWR international (Zl de Vaugereau, Briare, France.). Tetrahydrofuran HPLC grade was purchased from Fisher Scientific UK Limited (Loughborough, Leicestershire, UK).

Centrivap concentrator was purchased from Becthai Bangkok Equipment & Chemical Company Limited (LABCONCO, Bangkok, Thailand.). Vortex-2 genie was purchased from Scientific Industries Inc. (Bohemia, New York, USA). Waterbath shaker was purchased from Grant Instrument (Barrington, Cambridge, England). Ultrasonic cleaner was purchased from KODO Tech Research (Hwaseong-City, Gyeonggi-Do, South korea). Hettich centrifuge was purchased from Andrease Hettich Gmbh & Co.KG (Tuttlingen, Baden-Wuerttmberg, Germany)

3.2 Overview of the study protocol

This study was aimed to investigate the effect of a single and multiple oral doses of PEITC (2, 10, and 20 mg/kg) on CYP1A2 activity in rats. The CYP1A2 activity induced by PEITC was also compared with that resulted from a selective CYP1A2 inhibitor, fluvoxamine. The scope of the study protocol is shown in Figure 3.1. Fresh Betong watercress purchased from Betong district, Yala province was identified botanical species. Betong watercress in a form of dry powder was prepared by lyophilization. The effect of a single and multiple dose(s) of PEITC and Betong watercress on CYP1A2 activity in rats were investigated by using caffeine as a marker substrate. Then, serum caffeine and its metabolites were measured for the calculation of metabolic ratio before and after receiving the study regimens.

Betong watercress → Preparation of dry powder by lyophilization, Botanical identification, Determination of PEITC & Experiments in animal

Standard PEITC → Determination of PEITC & Experiments in animal

Gr.1 Fluvoxamine + caffeine

Gr.2 PEITC (single low dose) + caffeine

Gr.3 PEITC (single medium dose) + caffeine

Gr.4 PEITC (single high dose) + caffeine

Gr.5 PEITC (multiple low doses) + caffeine

Gr.6 PEITC (multiple medium doses) + caffeine

Gr.7 PEITC (multiple high doses) + caffeine

Gr.8 Dry watercress (single dose) + caffeine

Gr.9 Dry watercress (multiple doses) + caffeine

Figure 3.1 Scope of the study

3.3 Betong watercress

3.3.1 Botanical identification

Fresh Betong watercress was obtained from a local farm in Betong district, Yala province. The specimen was identified botanically for its scientific name at Maha Chakri Sirindhorn Natural History Museum, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

3.3.2 Preparation of dry powder of Betong watercress juice

Fresh Betong watercress (10 kg) was cleaned thoroughly and divided into portions. A portion of approximately 150 g of betong watercress was chopped and added with 100 mL of deionized water. The mixture was blended for 1 min into a fine paste using a kitchen miniblender. The vegetable paste was filtered with cheesecloth and squeezed to release the juice into a large beaker. The residue was mixed with 125 mL deionized water and squeezed again (Jiao *et al.*, 1998). The vegetable juice was lyophilized at Scientific Equipment Center (SEC) and Pharmaceutical Laboratory Service Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

3.4 PEITC analysis

3.4.1 Preparation of standard solutions

1) Stock solution

A stock solution of 1000 µg/mL of PEITC was prepared in acetronitrile (6 mL) in a 15 mL-centrifuge tube and stored at -5°C until analysis.

2) Working standard solutions

Working standards of PEITC were prepared by serial diluting a stock standard solution with acetronitrile to concentrations of 0.1, 0.3, 1, 5, 10, 15, 20 and 30 µg/mL.

3) Calibration standard solutions

Calibration standards solutions (0.3, 1, and 5 µg/mL) were prepared by adding working standard of PEITC at the concentrations 0.3, 1, and 5 µg/mL to derivertization reaction of PEITC (100 µL).

3.4.2 Chromatographic instrument and condition of PEITC

The HPLC system consisted of a Waters 2695 Separation Module and a Water 5487 Dual λ Absorbance detector (Milford, MA, U.S.A.). Data were collected and processed using the Empower[TM] Software System.

Concentration of PEITC was determined using method reported by Jiao and collaques with some modifications (Jiao *et al.*, 1998). The HPLC column was Fortis® C18 (150 × 4.6 mm i.d., 5 μm particle size). It was connected to the guard-sunfire (20 × 4.6 mm) precolumn module inserted with C18 packing materials. A mobile phase was a mixture of methanol:water (90:10, v/v) and flowed at 1.1 mL/min. The column temperature was controlled at 25°C. The sample injection volume was 20 μL. The mobile phase was freshly prepared each day and was filtered through 0.22 μm nylon filtered paper and degassed (30 min) before using. Detection was made at a wavelength of 365 nm.

3.4.3 Derivatization of PEITC in standard solutions

PEITC was derivatized via cyclocondensation reaction of ITC substrate with 1,2-benzenedithiol to yield 1,3-benzenedithiole-2-thione as shown in Figure 3.2. To analyze one hundred microliter of PEITC standard solution was mixed with 600 μL of 1,2-benzenedithiol in isopropanol (10 mM) and 500 μL of 0.1 M phosphate buffer (pH 8.5) in a 10 mL-glass tube. The mixture was incubated for 2 h at 65°C in a waterbath shaker (Jiao *et al.*, 1998) (Figure 3.4).

ITC substrate   1,2-benzenedithiol   1,3-benzenedithiol-2-thione
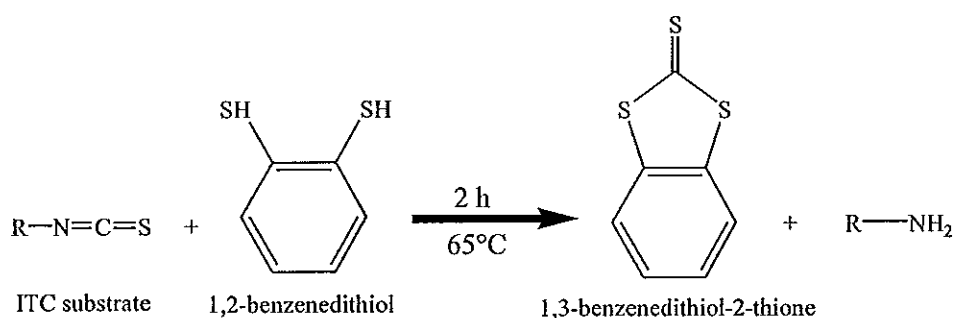
Figure 3.2 Cyclocondensation of isothiocyanates with 1,2-benzenedithiol producing a derivative (1,3-benzenedithiole-2-thione) (from Liebes *et al.*, 2001)

```
┌─────────────────────────────────────────┐
│   100 µL of PEITC standard solution      │
└─────────────────────────────────────────┘
                  │   - reaction with 1,2-benzenedithiol 600 µL
                  ▼   - vortex for 1 min
┌─────────────────────────────────────────┐
│   Add 500 µL of phosphate buffer (pH 8.5) │
└─────────────────────────────────────────┘
                  │   - vortex for 1 min
                  ▼   - incubation at 65°C for 2 h
┌─────────────────────────────────────────┐
│   1,3-benzenedithiole-2-thione           │
└─────────────────────────────────────────┘
                  │
                  ▼
┌─────────────────────────────────────────┐
│   Analysis by HPLC                       │
└─────────────────────────────────────────┘
```
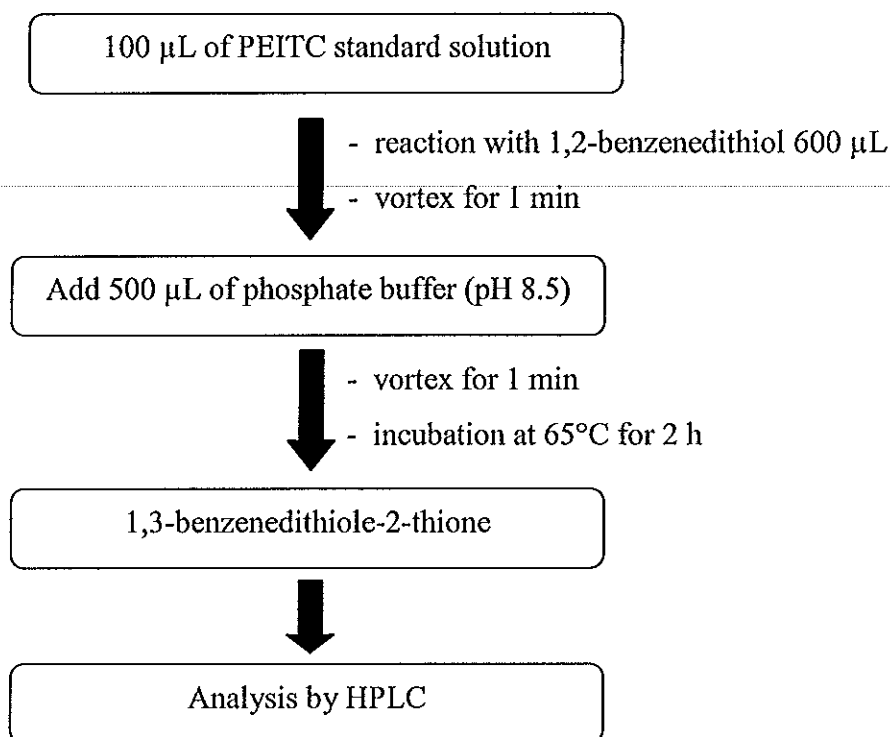
Figure 3.3 Sample preparations by derivatization of PEITC via cyclocondensation reaction (from Jiao *et al.*, 1998)

3.4.4 Method validation

The method of analysis was validated in accordance with the Guidance for Industry: Bioanalytical Method Validation (FDA, 2001). Parameters included linearity and range, intra-day and inter-day precisions and accuracies, and lower limit of quantification (LLOQ).

1) Linearity and range

Linearity was evaluated by preparing eight standard samples with different concentrations of PEITC in five replicates of each concentration; 0.1, 0.3, 1, 5, 10, 15, 20 and 30 µg/mL. Calibration curve was constructed by plotting peak area (Y) of 1,3-benzenedithiole-2-thione versus the concentrations of PEITC (X). Regression analysis was performed to obtain the calibration equation and correlation coefficient (r).

2) Precision

Precision of analysis was determined by using four quality controls (QC) samples obtained by preparing different concentrations of PEITC; 0.1, 3, 10 and 25 µg/mL (five replicates of each concentration). Intra-day precision was determined by assaying five samples of each concentration on the same day under the same experimental condition while the inter-day precision was evaluated by assaying of the samples for five consecutive days. The precision was expressed as the relative standard deviation (RSD) and calculated by the formula:

$$\text{Relative standard deviation}: \text{RSD (\%)} = \frac{\text{standard deviation}}{\text{mean}} \times 100.$$

The level of acceptance for precision is within 15% RSD except at the LLOQ where 20% RSD is an accepted (FDA, 2001).

3) Accuracy

Accuracy of analysis was determined by using the same QC samples (five replicates). Intra-day accuracy was determined by assaying five samples of each concentration on the same day while the inter-day accuracy was evaluated by assaying of the samples for five consecutive days. The accuracy was expressed as the deviation (DEV), which was derived from the formula:

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

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

4) Lower limit of quantification (LLOQ)

Lower limit of quantification were determined as the lowest concentration on the calibration curve that could be determined based on signal to noise ratio (S/N). For LLOQ, S/N is usually required to be equal to or greater than 5 (FDA, 2001).

3.4.5 Quantification of PEITC in dry powders of Betong watercress juice

Analysis of PEITC content in dry powder of Betong watercress juice followed the method described by Jiao and colleaques with some modifications (Jiao *et al.*, 1998). The procedure consisted of 2 steps; 1) glucosinolate conversion to PEITC and 2) PEITC derivatization via cyclocondensation (Figure 3.4). Prior to starting the real experiment, a pilot study to determine optimum concentration of myrosinase used for glucosinolate conversion was carried out. Different concentrations of myrosinase at 0, 2, 3, and 4 mg/mL in 0.1 M potassium phosphate buffer (pH 6.6) were added into 2 mg of dry powder of Betong watercress juice dissolved in 2 mL of deionized water prior to the next step.

To quantify PEITC, the following procedure was done. Two milligrams of dry powder was placed in a 10 mL-glass tube. Two milliliters of deionized water was added and the mixture was sonicated for 10 min at 37°C to facilitate dissolution. One milliliter of myrosinase solution (4 mg/mL in 0.1 M potassium phosphate buffer, pH 6.6) was added before mixing by vortex for 1 min. The mixture was incubated for 2 h at 37°C in a waterbath shaker. After finishing the incubation, the mixture was immediately centrifuged at 875 xg for 15 min. One hundred microliter of a clear fraction of the reaction mixture which contained PEITC derived from glucosinolate conversion was transferred to another clean 10 mL-glass tube. A 600 µL-aliquot of derivertizing agent 1,2-benzenedithiol (10 mM) and 500 µL-aliquot of 0.1 M phosphate buffer (pH 8.5, degasses) were added. The mixture was incubated for 2 h at 65°C in a waterbath shaker. Then, the sample (20 µL) was analyzed by HPLC.

PEITC contents were calculated by the following expression:

$$\text{Amount of PEITC/mg of dry powders} = \frac{\left(\dfrac{\text{Measured PEITC conc. by HPLC} \times \text{Vol. of cyclocondensation reaction mixture}}{\text{Vol. of aliquot of enzyme reaction mixture}} \right) \times \text{Total vol. of enzyme reaction mixture}}{\text{Weight of dry powders}}$$
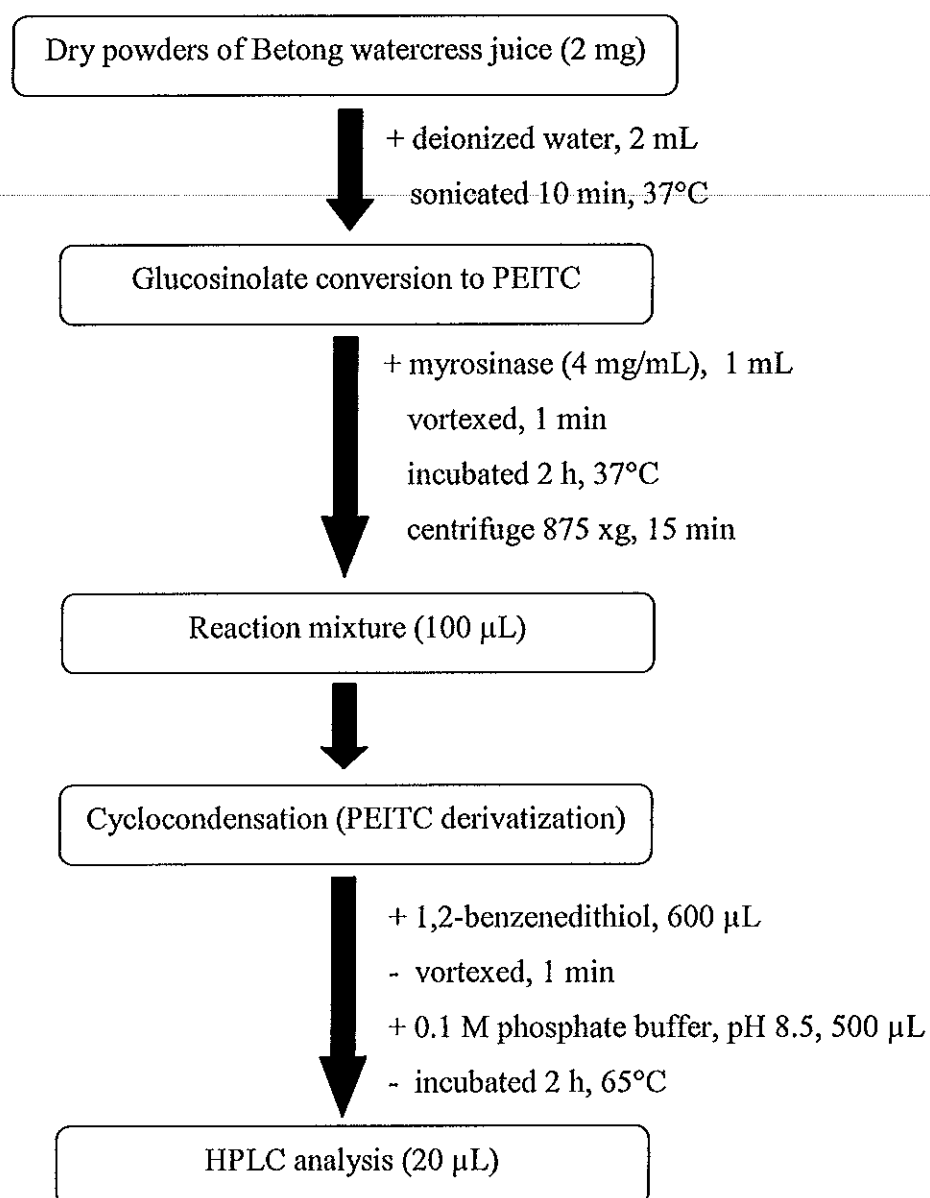
Figure 3.4 Sample preparations for determination of PEITC in dry powders of Betong watercress juice (modified from Jiao *et al.*, 1998)

3.5 Animals

Male Wistar rats weighing between 195 and 220 g were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The experimental protocol was approved by the Animal Ethic Committee, Prince of Songkla University (Ref. 17/51). During the experimental period, the animals were maintained under a controlled environment (temperature of 25 ± 2 °C, light/dark cycle of 12/12 h) with food and water *ad libitum*. Before dosing, the animals were fasted overnight (12 h) but freely access to water.

3.6 Experimental design

The animals were divided into nine groups (six animals each) based on drug pre-treatments. Group 1, the animals were intraperitoneally pre-treated with fluvoxamine at a single dose of 10 mg/kg (dissolved in water, (10:1). Group 2, 3, and 4, the animals were orally pre-treated with PEITC at a single dose of 2, 10, and 20 mg/kg, respectively. Group 5, 6, and 7, the animals were orally pre-treated with PEITC at multiple doses of 2, 10, and 20 mg/kg, respectively. PEITC at the doses 2, 10, and 20 mg/kg was dissolved in corn oil (1:9, 1:4, and 1:1, respectively). Group 8 and 9, the animals were orally pre-treated with dry powder of Betong watercress which suspended in 20% acacia (1:15) at a single dose and multiple doses of 800 mg/kg, respectively.

Experiment for each group consisted of two phases, i.e. phase I and phase II (Figure 3.5). In phase I, the animals were given a single oral dose of 10 mg/kg of caffeine (dissolved in water, (10:1). In phase II, the same individuals were pre-treated with the study drugs, i.e. fluvoxamine, PEITC, and dry watercress, and received the same dose of caffeine as in phase I. There was a two-day period between phase I and phase II to completely wash out caffeine. Blood samples were collected at 0 and 3 h after caffeine administration in phase I and phase II of each group.

3.6.1 Animal handling

Blood samples (2 mL) were taken the orbital sinus of rat. After allowing blood samples to clot for 30 min, serum was separated by centrifugation at 1000 xg for 20 min at room temperature (ca. 25°C). Serum samples were kept in a 2 mL microcentrifuge tube at -70°C until analysis.

Single dose

Gr.1 Fluvoxamine 10 mg/kg

Gr.2 PEITC 2 mg/kg

Gr.3 PEITC 10 mg/kg

Gr.4 PEITC 20 mg/kg

Gr.5 Dry watercress 800 mg/kg

Blood collection followed by CF 10 mg/kg

Blood collection

CF 10 mg/kg

Blood collection

3 h T_{0h} T_{3h} Wash-out (2 days)   30 min   3 h T_{3h}

Phase I   Phase II

Multiple doses

Gr.6 PEITC 2 mg/kg

Gr.7 PEITC 10 mg/kg

Gr.8 PEITC 20 mg/kg

Gr.9 Dry watercress 800 mg/kg

Blood collection followed by CF 10 mg/kg

Blood collection

CF 10 mg/kg

Blood collection

3 h T_{0h} T_{3h} Wash-out (2 days)   1 2 3 4 5 (Days)   30 min   3 h T_{3h}

Phase I   Phase II

Figure 3.5 Experimental design

3.7 Analysis of caffeine and it metabolites

3.7.1 Preparation of standard solutions

1) Stock solutions

Stock solutions of caffeine and acetaminophen (internal standard) were separately prepared in deionized water. Those of paraxanthine and theophylline were also separately prepared in 0.1 M ammonium hydroxide, while that of theobromine was prepared in 0.1 M sodium hydroxide. The concentrations were 1000 µg/mL

2) Working standard solutions

Working standard solutions of caffeine, paraxanthine, theobromine, and theophylline were prepared as a mixture by diluting their stock solutions with deionized water to different concentrations of 0.08, 0.16, 0.32, 0.4, 2, 4, 10, 20, and 40 µg/mL. A stock solution of 1000 µg/mL of internal standard (acetaminophen) was prepared in deionized water. It was also diluted further with deionized water to a final concentration of 100 µg/mL.

3) Calibration standard solutions

Calibration standard solutions were prepared by adding working standard mixture of caffeine, paraxanthine, theobromine, and theophylline at the concentrations of 0.02, 0.04, 0.08, 0.1, 0.5, 1, 2.5, 5, and 10 µg/mL and 50 µL of internal standard acetaminophen (final concentration of 25 µg/mL) to rat serum blank samples (final volume 200 µL).

3.7.2 Chromatographic instrument and condition of caffeine and it metabolites

The HPLC system consisted of a Waters 2695 Separation Module and a Water 5487 Dual λ Absorbance detector (Milford, MA, U.S.A.). Data were collected and processed using the Empower [TM] Software System.

Serum concentrations of caffeine and its metabolites (theobromine, paraxanthine, and theophylline) were determined using the method described by Caubet and colleaques with some modifications (Caubet *et al.* 2002). A HPLC column was Symmetry® C18 (250 × 4.6 mm i.d., 5 µm particle size). It was connected

to the guard-symmetry (20 × 3.9 mm) precolumn module inserted with C18 packing materials. A mobile phase consisted of a mixture of water:acetic acid:tetrahydrofuran (996.5:1:2.5 v/v/v) as a solvent A and acetronitrile as a solvent B. Separation was performed using the following gradient elution: B was set at 6% for 9 min, then increased to 15% from 9 to 10 min under curve 10 and then decrease to 6% in 1 min under curve 6; with a post run of 3 min in order to equilibrate the column between injection. The flow rate of the mobile phase was maintained at 1.3 ml/min and column compartment was controlled at 32°C. The mobile phase was freshly prepared each day and was filtered through 0.22 μm nylon filtered paper and degassed before using (30 min). Detection was made at a wavelength of 274 nm.

3.7.3 Sample extraction of caffeine and it metabolites

Serum samples were extracted following the technique described by Grant and colleaques with some modifications (Grant et al., 1982). A serum sample (200 μL) was combined with 50 μL of acetaminophen (100 μg/mL) followed by adding of 60 mg of ammonium sulphate and mixed by vortex for 30 s. Then, 6 mL of a mixture of chloroform:isopropanol (85:15 v/v) was added. After 30 s mixing by vortex, the extracted sample mixture was centrifuged at 875 xg for 15 min and the aqueous (upper) phase was removed by aspirated. The organic (lower) phase was transferred to a clean conical centrifuge tube and evaporated to dry under vacuum at 40°C using a centrivap concentrator (LABCONCO, Bangkok, Thailand). Finally, the residue was reconstituted in 100 μL of the mobile phase and a 20 μL aliquot was injected into the HPLC system for analysis as follows (Figure 3.6).
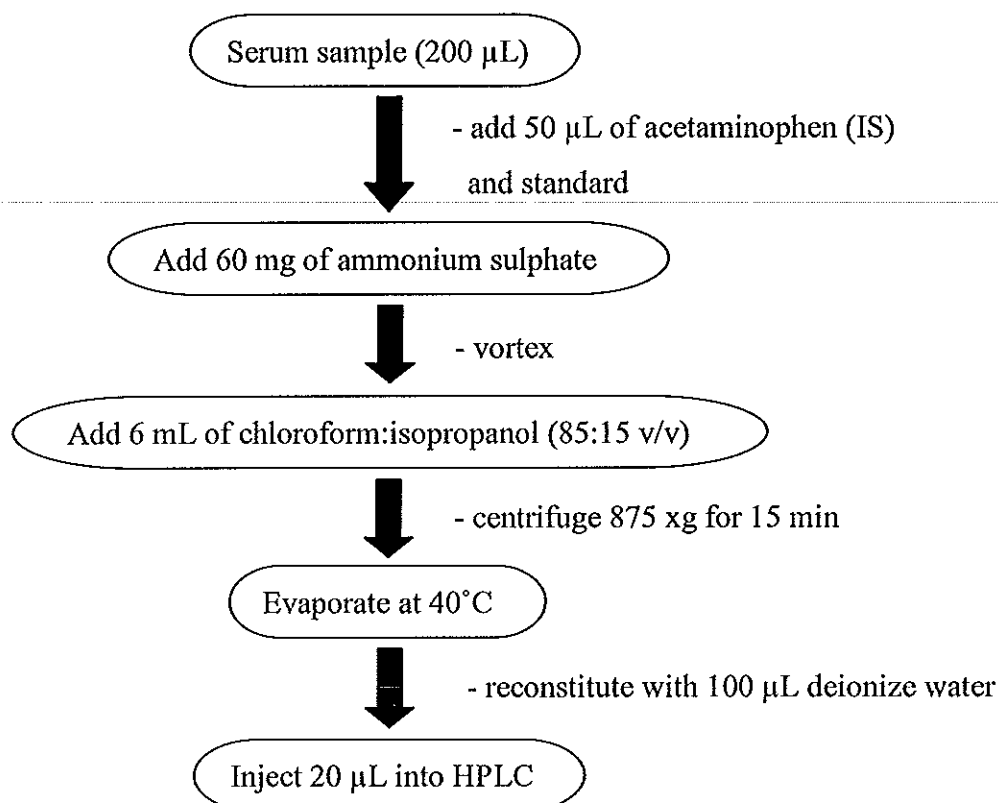
Figure 3.6 Serum sample preparation for determination of caffeine and it metabolites (modified from Grant *et al.*, 1982).

3.7.4 Method validation for caffeine and metabolites in serum analysis

The method of analysis was validated in accordance with the Guidance for Industry: Bioanalytical Method Validation (FDA, 2001). Parameters included linearity and range, intra-day and inter-day precisions, accuracy, recovery and lower limit of quantification (LLOQ).

1) Linearity and range

Linearity was evaluated by preparing standard serum samples with different concentrations of caffeine and its metabolites in five replicates of each concentration; 0.02, 0.04, 0.08, 0.1, 0.5, 1, 2.5, 5, and 10 µg/mL for caffeine and paraxanthine; 0.04, 0.08, 0.1, 0.5, 1, 2.5, 5, and 10 µg/mL for theophylline, and 0.08, 0.1, 0.5, 1, 2.5, 5, and 10 µg/mL for theobromine, and 25 µg/mL of acetaminophen into serum. The calibration curve was constructed by plotting the ratio of the peak

area of the analyte to that of the internal standard (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 of analysis was determined by using four quality controls (QC) samples obtained by preparing of mixture of caffeine and its metabolites; 0.02, 0.45, 4.5, and 9 μg/mL for caffeine and paraxanthine, 0.04, 0.45, 4.5, and 9 μg/mL for theophylline and 0.08, 0.45, 4.5, and 9 μg/mL for theobromine; five replicates of each concentration, and 25 μg/mL of acetaminophen in serum blank were used. Intra-day precision was determined by assaying five samples of each concentration on the same day under the same experimental condition while the inter-day precision was evaluated by assaying of the samples for five consecutive days. The precision was expressed as the relative standard deviation (RSD) and calculated by the formula:

$$\text{Relative standard deviation}: \text{RSD (\%)} = \frac{\text{standard deviation}}{\text{mean}} \times 100.$$

The level of acceptance for precision is within 15% RSD except at the LLOQ where 20% RSD is accepted (FDA, 2001).

3) Accuracy

Accuracy of analysis was determined by using four quality controls (QC) samples obtained by preparing of mixture of caffeine and its metabolites; 0.02, 0.45, 4.5, and 9 μg/mL for caffeine and paraxanthine, 0.04, 0.45, 4.5, and 9 μg/mL for theophylline and 0.08, 0.45, 4.5, and 9 μg/mL for theobromine; five replicates of each concentration, and 25 μg/mL of acetaminophen in serum blank were used. 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 evaluated by assaying the samples for five consecutive days. The accuracy was expressed as the deviation (DEV), which was derived from the formula:

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

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

4) Recovery

The recovery of mixture caffeine and its metabolites after extracting from serum samples was determined at LLOQ (0.02 µg/mL for caffeine and paraxanthine, 0.04 µg/mL for theophylline, and 0.08 µg/mL for theobromine), low (0.45 µg/mL), medium (4.5 µg/mL), and high (9 µg/mL); five replicates of each concentration, and 25 µg/mL of acetaminophen by comparing with the response obtained after direct injection of standard mixture caffeine and its metabolites and acetaminophen. The recovery for the determination of all analytes was from the 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 precision of 20% and accuracy of 80-120% (FDA, 2001).

3.8 Data analysis

Metabolic ratios (TB/CF, PX/CF, TP/CF and (TB+PX+TP)/CF were calculated by determining the ratios of serum concentrations of each metabolite, i.e. TB, PX, TP and summation of those of all metabolites, i.e. TB+PX+TP. The data were analyzed using SPSS software version 15.0. Statistical significant were compared between groups using a Student-paired t-test and ANOVA followed by LSD test. p values less than 0.05 ($p < 0.05$) were used as the significant level.

CHAPTER 4

Results

4.1 Botanical identification of Betong watercress

The whole plant was identified at the Princess Maha Chakri Sirindhorn Natural History Musem, Prince of Songkla University, Hat Yai, Songkhla where the voucher specimens (B. Janchawee 1) have been deposited. The watercress is member of the family *Brassicaceae*, Vernacula name is Phak Num Betong, and Sciencific name is *Nasturtium officinale* R.Br. (Figure 4.1B). In Thailand, watercress is a local plant grown in Betong District, Yala Province, so called Phaknum Betong in Thai or Betong watercress (Figure 4.1A). Watercress supply of grown widely in France then, watercress was grown in China. In Thailand, watercress was grown the firstly in north and spreaded to the south by Chinese. Watercress is grown mostly in the south of Thailand at Betong, Yala (Education, Municipality of Betong, 2009).
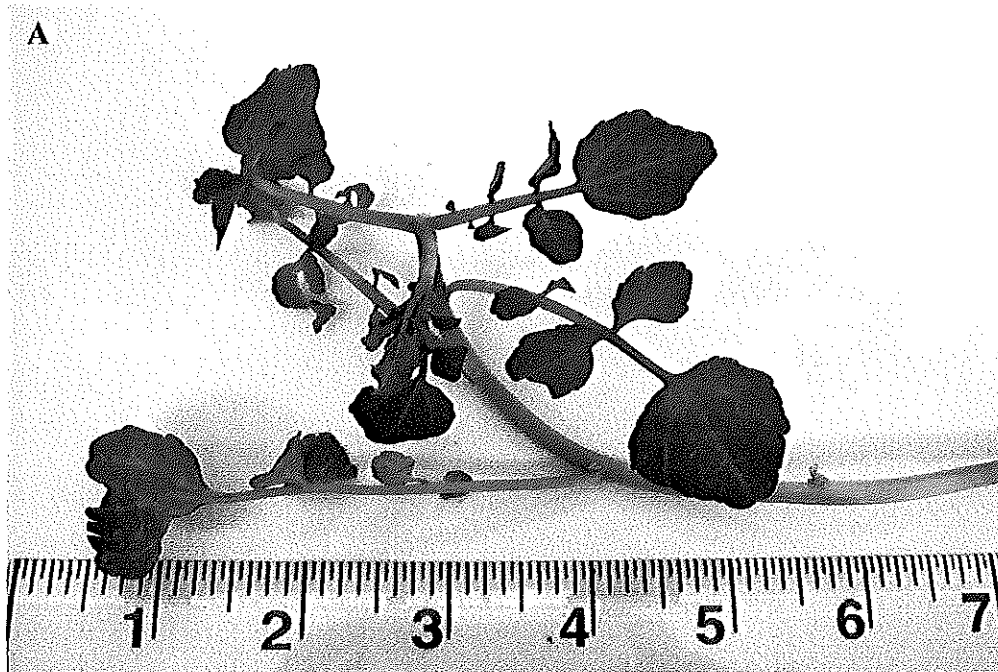
Figure 4.1 Betong watercress collected from watercress farm at Betong district, Yala province; (A) Fresh watercress and (B) Watercress was identified at the Princess Maha Chakri Sirindhorn Natural History Museum

70

Figure 4.1 (cont.) Betong watercress collected from watercress farm at Betong district, Yala province; (A) Fresh watercress and (B) Watercress identified at the Princess Maha Chakri Sirindhorn Natural History Museum

4.2 Yield of dry powder of Betong watercress

The samples of fresh Betong watercress (10 kg) were made the vegetable juice. Then, the vegetable juice was lyophilized at Scientific Equipment Center (SEC) and Pharmaceutical Laboratory Service Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Dry powder of Betong watercress juice is a green coarse powder. All samples (dry powder of Betong watercress) were collected in a plastic bag. Then, all samples were weighed. The total weights are 146 g and keep at -20°C until analysis.

4.3 Chromatographic profile for PEITC analysis

Analysis of PEITC using HPLC was performed by detecting a derivative product obtained from cyclocondensation reaction of PEITC with 1,2-benzenedithiol (derivertizing agent). Theoretically, the product is 1,3-benzenedithiole-2-thione. Since standard 1,3-benzenedithiole-2-thione was not commercially available so indirect method was performed to confirm the presence of derivatization product. The reaction mixture with varying concentration of PEITC were prepared and added to 600 μL of 10 mM of derivertizing agent. The mixture after derivatization was analyzed by HPLC and chromatograms of separation were shown in Figure 4.2. Peak appeared at 3.89-3.95 min after adding the different concentration of PEITC (1, 3, 5, and 50 μg/mL) in the mixture. Their intensity was increasing as the concentrations of PEITC increased while the intensity of peaks at 8.09-8.18 min decreased. It is mostly likely that peaks at 3.89-3.95 min represent a derivative 1,3-benzenedithiole-2-thione. Chromatographic profile of separation of PEITC product in Figure 4.2 provided peak area of the derivative used for quantification of PEITC concentration by using standard curve of PEITC concentrations against peak response of the derivative.

Chromatographic analysis of PEITC in dry powders of Betong watercress juice was shown in Figure 4.3. The retention times of the derivative which is 1,3-benzenedithile-2-thione were 3.91-3.97 min. Peak response shown in Figure 4.3 B was back calculated to PEITC concentration of 0.15 μg/mL.
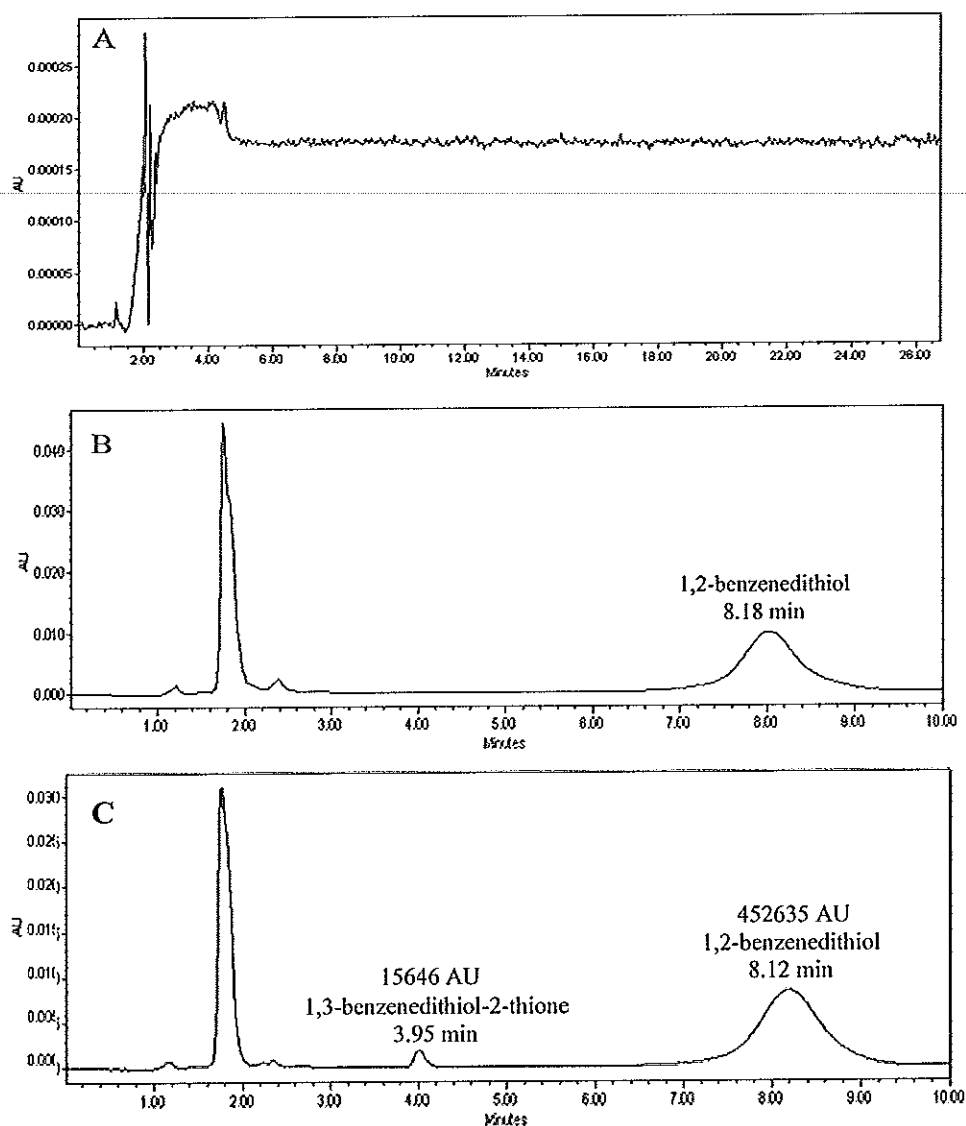
Figure 4.2 Representative chromatograms for separation of derivative product of PEITC cyclocondensation reaction with varying concentrations of PEITC; 1, 3, 5, and 50 µg/mL (A) acetronotrile blank; (B) derivatizing agent (1,2-benzenedithiol at 10 mM) blank and (C) PEITC 1 µg/mL
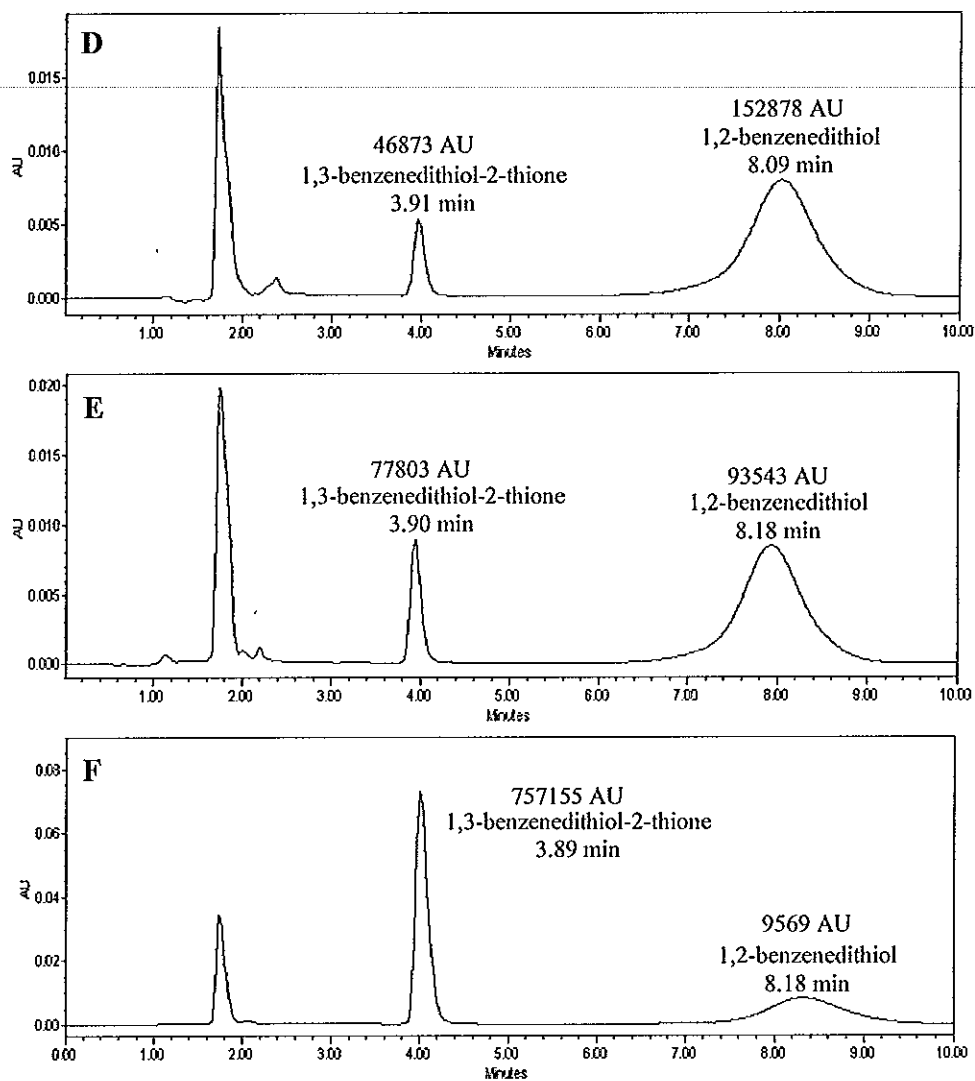
Figure 4.2 (cont.) Representative chromatograms for separation of derivative product of PEITC cyclocondensation reaction with varying concentration of PEITC; 1, 3, 5, and 50 μg/mL (D) PEITC 3 μg/mL; (E) PEITC 5 μg/mL and (F) PEITC 50 μg/mL
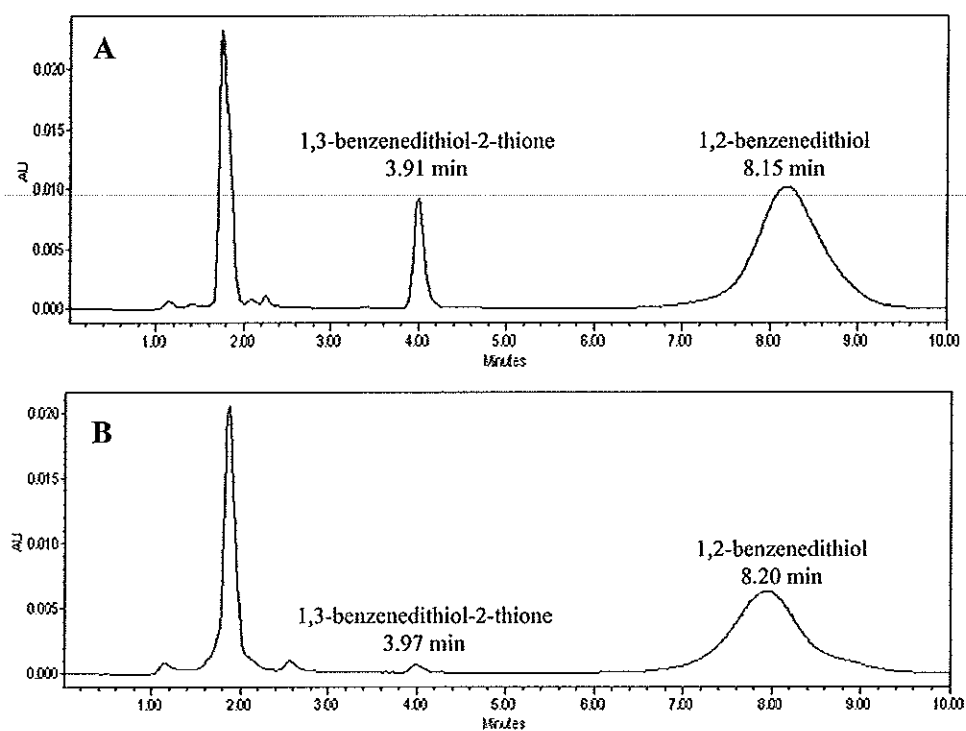
Figure 4.3 Representative chromatograms for analyzing PEITC in dry powders of Betong watercress juice (A) reaction mixture obtained from derivatization of standard PEITC (5 µg/mL) and (B) reaction mixture of derivatization of PEITC producted from treatment of dry powders of Betong watercress juice with myrosinase (4 mg/mL)

4.4 Method validation for PEITC analysis

1) Linearity and range

Regression analysis results showed that the calibration curve were linear over the concentration range of 0.1-30 µg/mL for PEITC (Figure 4.4) (five replicates of each concentration). The regression equation was y = (15200±172.39) x + (460.02±52.023) (r = 0.9999)
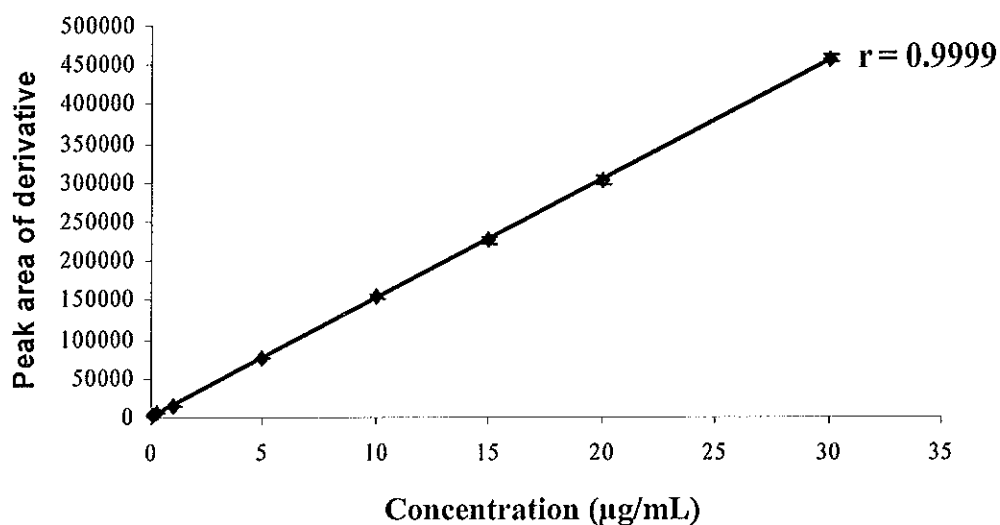
Figure 4.4 Linearity plots of mean peak area ± S.D. against different concentrations of PEITC; (correlation coefficient (r) = 0.9999)

2) Precision

Both intra-day (repeatability) and inter-day (reproducibility) precisions were determined using four quality control (QC) samples prepared by dissolving PEITC (0.1, 3, 10 and 25 µg/mL) in acetronitrile. Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision were determined by analyzing of the samples for five consecutive days. Intra- and inter-day precisions for determining PEITC are shown in Table 3.1. The relative standard deviation (%RSD) of PEITC values ranged from 0.65 to 4.06%. Both values for analytes were found to be within the acceptable value (15%RSD and 20%RSD for the concentration at LLOQ) (FDA, 2001). The results indicated that the method of analysis was precise.

3) Accuracy

The accuracy of an analytical method is defined as the degree to which the determined value of analyte in a sample corresponds to the true value. In this work, the accuracy was determined by using four QC samples of standard PEITC (0.1, 3, 10 and 25 µg/mL) in acetronitrile (five replicates for each concentration). The accuracy (%DEV) ranged from (-) 7.5 to (+) 18.22%. The results for determination of analyte ranged between ±15% and ±20% for the concentration at LLOQ (FDA, 2001).

Table 4.1 Precision and accuracy of the method for determination of PEITC

Concentration	Precision (%RSD)		Accurary (%DEV)	
(µg/mL)	Intra-day	Inter-day	Intra-day	Inter-day
0.1	3.22	4.06	18.04	18.22
3	2.21	3.86	-6.85	-1.15
10	1.18	1.98	-1.32	0.53
25	0.65	1.74	-7.5	-1.4

4) Lower limit of quantification (LLOQ)

For PEITC, the LLOQ was determined based on signal to noise (S/N) ratio which equal to or greater than 5. It was found that the LLOQ for PEITC was 0.1 µg/mL. The sensitivity of the method was in the range of nanograms per component on-column, being quite enough for analysis PEITC. The LLOQ obtained with this method was higher than those shown by Ye and coworker (2002) detecting derivative of PEITC and DTC (Dithiocarbamate) with HPLC.

4.5 Chromatographic profile of caffeine, paraxanthine, theobromine and theophylline

Chromatographic profile of separation of a standard mixture of 4.5 µg/mL of caffeine, paraxanthine, theobromine and theophylline is shown in Figure 4.9. All analytes and the internal standard were well separated with no interference within 14.0 min. The retention times were 6.09, 7.80, 9.28, 9.76 and 12.66 min for theobromine, acetaminophen, paraxanthine, theophylline and caffeine, respectively. Acetaminophen was a good internal standard because it caused no chromatographic interference with the analytes
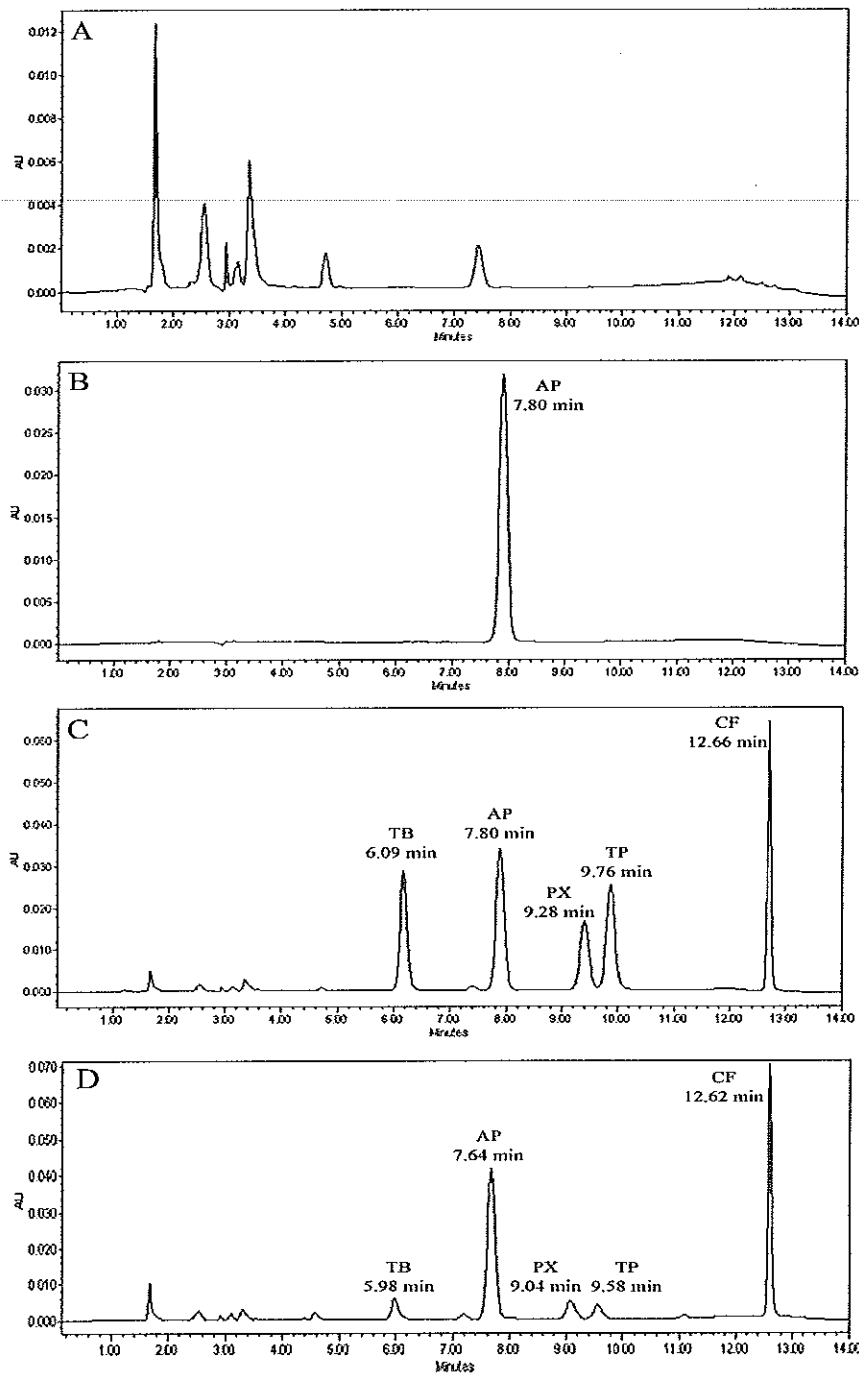
Figure 4.5 Representative chromatograms for separation of caffeine (CF), paraxanthine (PX), theobromine (TB), and theophylline (TP); (A) rat serum blank; (B) rat serum blank containing internal standard (acetaminophen (AP), 25 µg/mL); (C) standard mixture of caffeine, paraxanthine, theobromine, theophylline (4.5 µg/mL) and internal standards spiked in rat serum blank and (D) caffeine, paraxanthine, theobromine, and theophylline detected from serum of a rat receiving caffeine (10 mg/kg, p.o.)

4.6 Method validation for analysis of caffeine, paraxanthine, theobromine and theophylline

1) Linearity and range

For the analysis of caffeine, paraxanthine, theobromine and theophylline in rats serum samples, regression analysis results showed that the calibration curves were linear over the concentration ranges of 0.02-10 μg/mL for caffeine and paraxanthine, 0.04-10 μg/mL for theophylline and 0.08-10 μg/mL for theobromine (Figure 4.6) (five replicates of each concentration). The regression equations were y = (0.195±0.017) x + (0.003±0.002) (r = 1) for caffeine, y = (0.116±0.014) x + (0.006±0.003) (r = 0.9997) for paraxanthine, y = (0.168±0.017) x + (0.005±0.004) (r = 0.9999) for theophylline and y = (0.164±0.020) x + (0.011±0.009) (r = 0.9993) for theobromine (Table 4.2).
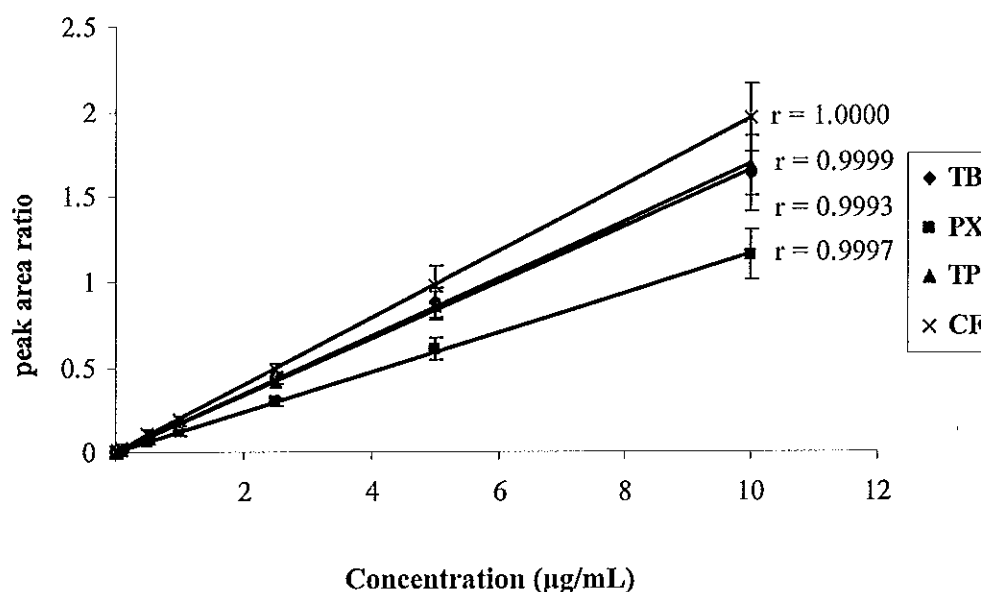
Figure 4.6 Linearity plots of mean peak area ratio±SD against difference concentrations of caffeine (CF), paraxanthine (PX), theophylline (TP) and theobromine (TB) spiked in rat serum; correlation coefficient (r) = 1 for CF, 0.9997 for PX, 0.9999 for TP and 0.9993 for TB (n = 5)

Table 4.2 Linear regression analyses for theobromine, paraxanthine, thephylline, and caffeine

Analyte	Range of linearity (µg/mL)	Calibration equation [a]	Correlation coefficient (r)
Theobromine	0.08-10	$(0.164\pm0.020)x + (0.011\pm0.009)$	0.9993
Paraxanthine	0.02-10	$(0.116\pm0.014)x + (0.006\pm0.003)$	0.9997
Theophylline	0.04-10	$(0.168\pm0.017)x + (0.005\pm0.004)$	0.9999
Caffeine	0.02-10	$(0.195\pm0.017)x + (0.003\pm0.002)$	1.0000

[a] Mean ± SD

2) Precision

Both intra- and inter-day precisions were determined using four (QC) samples prepared by dissolving caffeine and paraxanthine (0.02, 0.45, 4.5 and 9 µg/mL), theophylline (0.04, 0.45, 4.5 and 9 µg/mL) and theobromine (0.08, 0.45, 4.5 and 9 µg/mL) in rat serum blank. Intra-day precision was determined by analyzing five samples of each concentration during the same day under the same instrumental operating condition. Inter-day precision was determined by analyzing of the samples for five consecutive days. Intra- and inter-day precisions for determining caffeine, paraxanthine, theophylline and theobromine are shown in Table 4.3. The relative standard deviation (%RSD) values ranged from 3.46 to 6.46% for theobromine, 2.27 to 9.26% for paraxanthine, 2.09 to 7.35% for theophylline and 2.81 to 13.40% for caffeine. Both intra- and inter-day precisions for all analytes were found to be within the acceptable values (±15%RSD and ±20%RSD for the concentration at LLOQ) (FDA, 2001). The results indicated that the method of analysis was precise.

3) Accuracy

The accuracy (%DEV) ranged from (-) 11.54 to (+) 18.46% for caffeine, (-) 5.74 to (+) 11.32% for paraxanthine, (-) 18.21 to (-) 17.25 for theophylline and (-) 4.57 to (+) 17.90% for theobromine (Table 4.3). The values ranged between ±15% and ±20% for the concentration at LLOQ (FDA, 2001).

Table 4.3 Precision and accuracy of the method for determination of theobromine, paraxanthine, thephylline, and caffeine

Analytes	Concentration (µg/mL)	Precision (%RSD)		Accurary (%DEV)	
		Intra-day	Inter-day	Intra-day	Inter-day
Theobromine	9.00	3.80	4.92	+0.09	-4.57
	4.50	3.80	6.46	+1.55	+6.33
	0.45	4.30	3.46	+10.48	+3.58
	0.08	5.28	3.60	+17.90	+17.23
Paraxanthine	9.00	5.32	3.00	+3.04	+1.65
	4.50	3.68	5.88	+1.11	+5.59
	0.45	2.27	2.60	-5.74	-1.71
	0.02	9.26	6.82	+11.32	+3.50
Theophylline	9.00	6.76	4.34	+2.26	+5.13
	4.50	4.60	6.55	+1.09	+8.26
	0.45	4.54	2.09	-4.61	-1.62
	0.04	7.35	3.08	-17.25	-18.21
Caffeine	9.00	6.55	6.17	-4.03	-5.97
	4.50	5.51	7.33	-3.53	-3.02
	0.45	3.56	2.81	-11.54	-6.80
	0.02	5.91	13.40	+7.08	+18.46

4) Recovery

Results of the recovery of extraction of theobromine, paraxanthine, theophylline, and caffeine from rat serum are shown in Table 4.4. The mean percentages of recovery were 107.77 to 109.77% for theobromine, 108.12 to 118.20% for paraxanthine, 105.11 to 110.54% for theophylline, and 104.82 to 108.97% for caffeine. The percentage of recovery in this study was similar to that reported by other studies. For example, Grant and colleaques (1982), who use LLE method to extract caffeine and metabolites in urine samples prior to detecting with HPLC, reported that the percentages of recovery ranged from 97-101% for caffeine and metabolites. Schrader and colleaques (1999) determining levels of caffeine including eight major metabolites in rat urine by HPLC reported that the percentages of recovery was greater than 70% for all metabolites.

Table 4.4 Extraction recovery for determination of theobromine, paraxanthine, thephylline, and caffeine in rat serum

Compound	Concentration (μg/mL)	Mean peak area ratio		% Recovery of IS	%Recovery[a]
		Direct injection	After extraction		
Theobromine	0.08	0.0114	0.0123	77.03 ± 10.62	107.77 ± 5.41
	0.45	0.0759	0.0831	89.52 ± 10.49	109.51 ± 4.93
	4.5	0.7571	0.8292	85.21 ± 9.53	107.77 ± 5.41
	9	1.5090	1.6424	80.10 ± 6.63	109.51 ± 4.93
Paraxanthine	0.02	0.0021	0.0025	77.40 ± 9.27	118.20 ±17.53
	0.45	0.0473	0.0520	89.52 ± 10.49	110.07 ± 8.05
	4.5	0.4836	0.5229	85.21 ± 9.53	108.12 ± 4.03
	9	0.9577	1.0728	80.10 ± 6.63	112.25 ± 5.29
Theophylline	0.04	0.0063	0.0066	78.52 ± 8.23	104.76 ± 7.64
	0.45	0.0740	0.0816	89.52 ± 10.49	110.27 ± 4.57
	4.5	0.7602	0.8179	85.21 ± 9.53	107.59 ± 4.58
	9	1.5104	1.6697	80.10 ± 6.63	110.54 ± 7.32
Caffeine	0.02	0.0037	0.004	78.40 ± 10.21	108.10 ± 6.59
	0.45	0.0805	0.0847	89.52 ± 10.49	105.21 ± 3.59
	4.5	0.8106	0.8497	85.21 ± 9.53	104.82 ± 5.73
	9	1.1621	1.7093	80.10 ± 6.63	106.01 ± 6.53

[a]Mean (SD)

5) Lower limit of quantification (LLOQ)

For rat serum samples, the LLOQ was determined by using five calibration curves of serum standard theobromine, paraxanthine, theophylline, and caffeine at concentrations of 0.5, 1, 2.5, 5 and 10 μg/mL. It was found that the LLOQs were 0.02 μg/mL for caffeine and paraxanthine, 0.04 μg/mL for theophylline and 0.08 μg/mL for theobromine.

4.5 Optimum concentration of myrosinase

Incubation of mixture of dry powder of Betong watercress (2 mg/2 mL water) with different concentration of myrosinase i.e. 2, 3, and 4 mg/mL result in different peak area of the derivative of PEITC as shown in Figure 4.5. The result showed that increasing of myrosinase from 2 mg/mL to 3 mg/mL increased quantity of derivatization product. At 4 mg/mL of enzyme, the product tended to be the highest. Thus, the concentration at 4 mg/mL was selected as the optimum concentration of myrosinase.
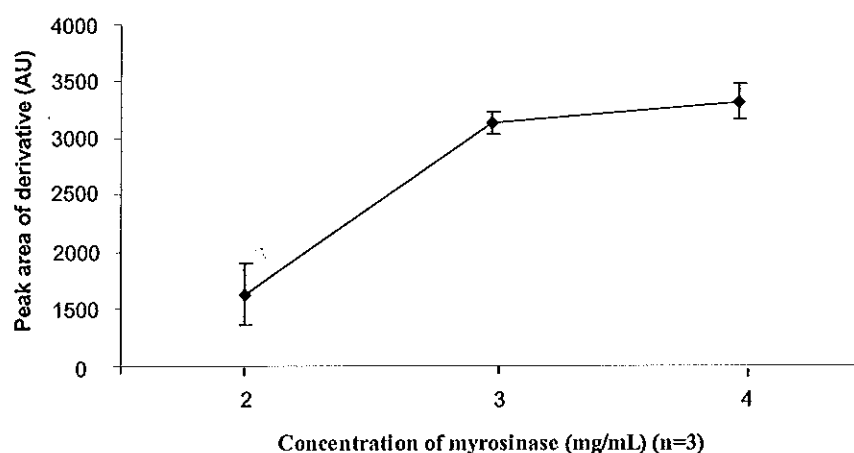
Figure 4.7 Concentration profiles of myrosinase enzyme (at 0, 2, 3, and, 4 mg/mL, respectively).

4.6 Quantity of PEITC in dry powder of Betong watercress juice

The quantification of PEITC after analysis by HPLC peak area was used to determine nominal concentration and calculated by the formula:

$$\text{Amount of PEITC/mg of dry powders} = \frac{\left(\dfrac{\text{Measured PEITC conc. by HPLC} \times \text{Vol. of cyclocondensation reaction mixture}}{\text{Vol. of aliquot of enzyme reaction mixture}}\right)}{\text{Weight of dry powders}} \times \text{Total vol. of enzyme reaction mixture}$$

$$\text{Amount of PEITC/mg of dry powders} = \frac{\dfrac{0.1387\,\mu g/mL \times 1.2\,mL}{0.1\,mL}}{2\,mg} \times 3\,mL$$

$$\text{Amount of PEITC/mg of dry powders} = 1.644\,\mu g/mL$$

$$= 1.6644\,\mu g/mL \times 3\,mL$$

Amount of PEITC/mg of dry powders = 4.9932 µg/ 2 mg of dry

powder Betong watercress

Amount of PEITC/mg of dry powders = 2.5 µg

Table 4.5 Peak area and concentration of dry powder of Betong watercress

Dry powder of Betong watercress (2 mg/2 mL)	Peak area of derivative (AU)	Measured concentration of derivative (µg/mL)	Amount of PEITC (µg)/mg of dry powders
n_1	2538	0.1460	2.6280
n_2	2256	0.1258	2.2644
n_3	2515	0.1443	2.5974
Mean	2436	0.1387	2.4966
S.D.	156.5961	0.0112	0.4022
%RSD	6.4275	8.0824	8.0532

The results were showed peak area and concentration of dry powder of Betong watercress in Table 4.5 and showed quantity of PEITC in dry powder of Betong watercress. The results were calculated amount of PEITC in dry powder of betong watercress equal to 2.5 µg/mg of dry powder of Betong watercress.

4.7 Metabolic ratio of caffeine in rats before (Phase I) and after (Phase II) pretreatment with a single dose of fluvoxamine, PEITC and Betong watercress

Metabolic ratios of caffeine in phase I and phase II for rats receiving a single dose of fluvoxamine (10 mg/kg, i.p.), PEITC (2, 10 and 20 mg/kg, p.o.) and dry powders of Betong watercress juice (800 mg/kg, p.o.) were shown in Figure 4.8. Pretreatment of fluvoxamine before caffeine administration caused a significant reduction of all metabolic ratios in phase II compared with those in phase I (receiving caffeine alone) ($p<0.02$). Similar findings were also observed for pretreatment with different doses of PEITC and Betong watercress. However, TB/CF ratio in phase II after pretreatment with Betong watercress was not significantly decreased compared with those in phase I.

In addition, metabolic ratios of caffeine in phase II for rats receiving a single medium and high doses of PEITC (10 and 20 mg/kg) were significantly lower than those of fluvoxamine ($p<0.03$).
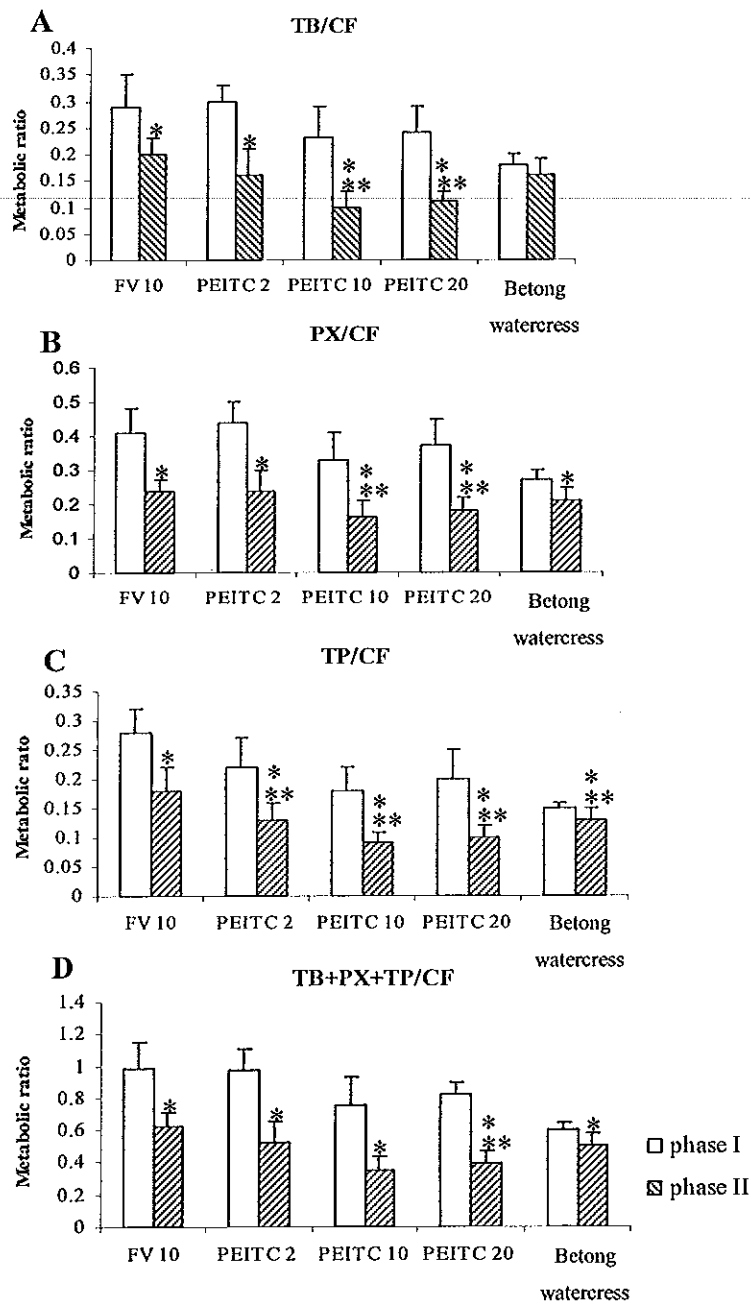
Figure 4.8 Metabolic ratios (mean±SD) of caffeine and its metabolites in rats (n=6) receiving a single dose of caffeine (10 mg/kg, p.o.) alone (phase I) and pretreated with a single dose of fluvoxamine (10 mg/kg, i.p.), phenethyl isothiocyanate (2, 10, 20 mg/kg, p.o.) and Betong watercress (800 mg/kg, p.o.) prior to receiving the same dose of caffeine (phase II); *$p<0.02$, compared with phase I using Student's paired t-test; ** $p<0.03$, compared with phase II of fluvoxamine-pre-treatment using ANOVA

Abbreviations: CF, caffeine; TB, theobromine; PX, paraxanthine; TP, theophylline; FV, fluvoxamine; PEITC, phenethyl isothiocyanate

4.8 Metabolic ratio of caffeine in rats before (Phase I) and after (Phase II) pretreatment with multiple doses of PEITC and Betong watercress

Metabolic ratios of caffeine in phase I and phase II for rats receiving multiple doses of PEITC (2, 10 and 20 mg/kg, p.o.) and Betong watercress (800 mg/kg, p.o.) were shown in Figure 4.9. Pretreatment with different doses of PEITC before caffeine administration caused a significant reduction of all metabolic ratios in phase II compared with those in phase I (receiving caffeine alone) ($p<0.025$). Similar findings were also observed when pretreatment with Betong watercress ($p<0.025$).
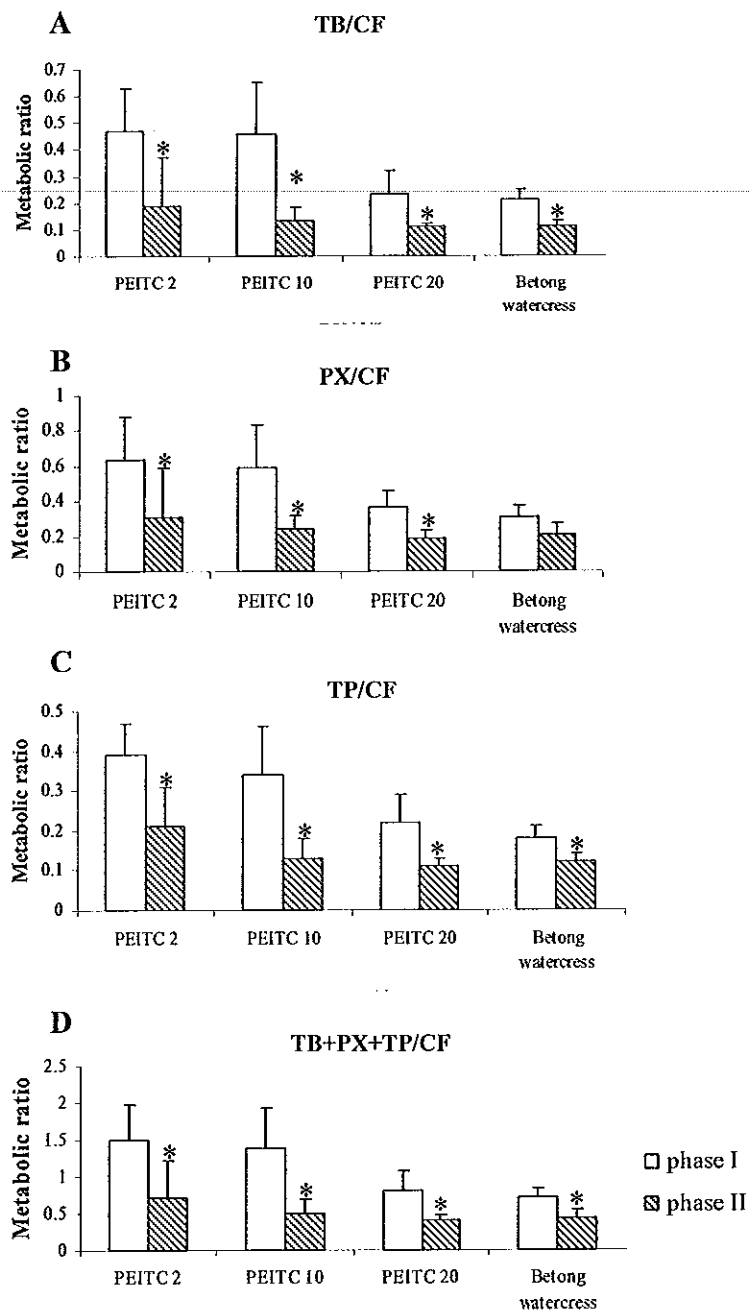
Figure 4.9 Metabolic ratios (mean±SD) of caffeine and its metabolites in rats (n=6) receiving multiple doses of caffeine (10 mg/kg, p.o.) alone (phase I) and 5-day pretreatment with phenethyl isothiocyanate (2, 10, 20 mg/kg, p.o.) and Betong watercress (800 mg/kg, p.o.) prior to receiving the same dose of caffeine (phase II); *p<0.025, compared with phase I using Student's paired t-test

Abbreviations: CF, caffeine; TB, theobromine; PX, paraxanthine; TP, theophylline; PEITC, phenethyl isothiocyanate

4.9 The percentage of decrease in metabolic ratios of caffeine in rats receiving a single- and multiple dose(s) pretreatment with fluvoxamine, PEITC, and Betong watercress

Figure 4.10 shown the percentages of decrease in the metabolic ratios of caffeine in rats after receiving pretreatments with a single dose of fluvoxamine, PEITC, and Betong watercress. The percent reduction of metabolic ratios; TB/CF, TP/CF, and (TB+PX+TP)/CF, were significantly larger in rats receiving PEITC at the dose of 10 and 20 mg/kg, compared with those receiving fluvoxamine at the dose of 10 mg/kg ($p<0.05$). This effect was not significant for PX/CF ratio. The percentages of decrease of metabolic ratios were not significantly different among different doses of PEITC.

The percent reduction of metabolic ratios (TB/CF) in rats receiving multiple doses of PEITC (2-20 mg/kg) was significantly larger than when those receiving a single dose of fluvoxamine at 10 mg/kg ($p<0.05$). The percentages of dedecrease in metabolic ratios after administration of multiple doses of PEITC (2, 10, and 20 mg/kg) were not significantly different from those after single dose administration of the same doses.
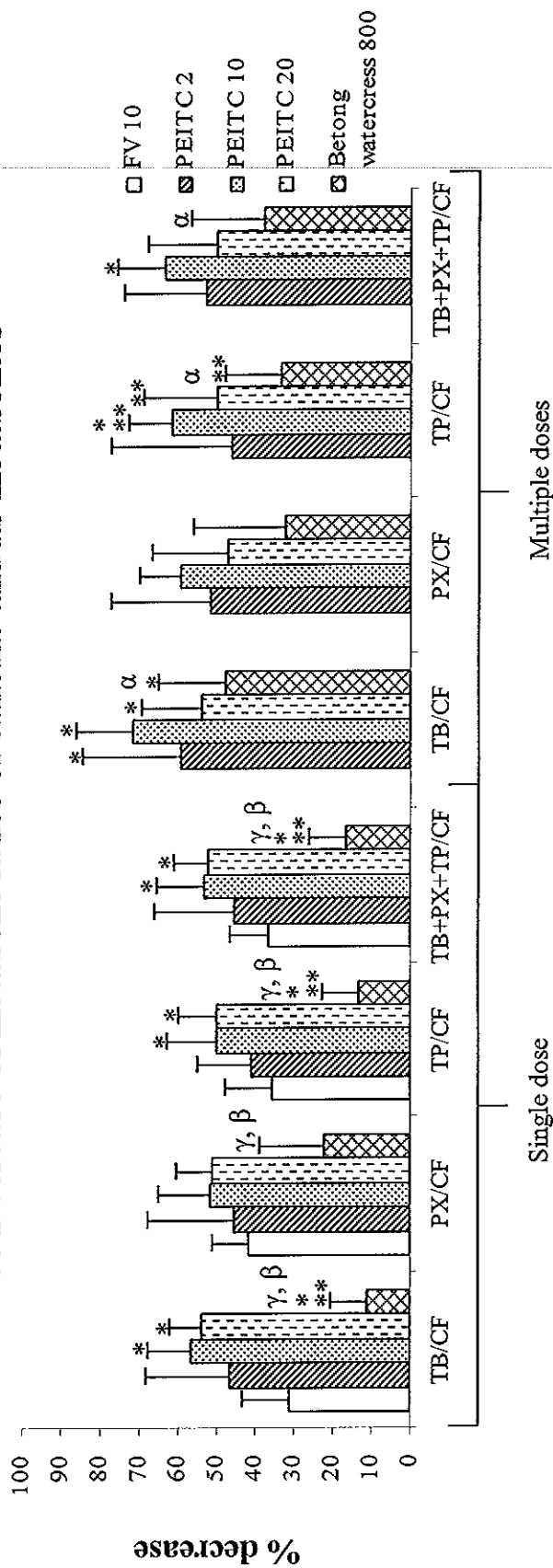
Figure 4.10 Percentage (mean±SD) of the decrease in metabolic ratios of caffeine and its metabolits in rats (n=6) receiving a single dose of fluvoxamine (10 mg/kg, i.p.), phenethyl isothiocyanate (2, 10, 20 mg/kg, p.o.), and Betong watercress (800 mg/kg, p.o.) and multiple doses of phenethyl isothiocyanate (2, 10, 20 mg/kg, p.o.), and Betong watercress (800 mg/kg, p.o.); *$p<0.05$, compared with fluvoxamine-pre-treatment using ANOVA; **$p<0.02$, compared with 2 mg/kg phenethyl isothiocyanate pre-treatment using ANOVA; γ $p<0.015$, compared with 10 mg/kg phenethyl isothiocyanate pre-treatment using ANOVA; β $p<0.015$, compared with 20 mg/kg phenethyl isothiocyanate pre-treatment using ANOVA; α $p<0.02$, compared with single dose

Abbreviations: CF, caffeine; TB, theobromine; PX, paraxanthine; TP, theophylline; FV, fluvoxamine; PEITC, phenethyl isothiocyanate

CHAPTER 5

Discussion and Conclusion

PEITC is widely present in cruciferous vegetables as its glucosinolate precursor, gluconasturtiin, by hydrolysis reaction via myrosinase enzyme released from plant cells during chopping or chewing (Morse *et al.*, 1997). PEITC and other ITCs have cancer preventive property in animals treated with chemical carcinogens such as polycyclic aromatic hydrocarbon and nitrosamines. (Chung *et al.*, 1996; Stoner *et al.*, 1991; Wattenberg, 1977). These carcinogenic compounds are inducers of the xenobiotic metabolizing enzyme, especially CYP1A2 (Degawa *et al.*, 1989; Ayrton *et al.*, 1990). Hence, the inhibition of CYP1A2 activity may play a key role in the elimination of the bioactivation on the certain procarcinogens. Caffeine is an important probe substrate which is capable of revealing activity of CYP1A2 activity since it is almost completely absorbed in gastrointestinal tract is (95%) and primarily metabolized via CYP1A2 mediated N-demethylation to theophylline (TP), paraxanthine (PX) and theobromine (TB) (Liebert and Matuszewska, 1999).

HPLC method validation confirmed that an HPLC procedure was suitable for PEITC determination. As PEITC is a volatile compound that is sensitive to heat and moisture causing a loss of the analyte during experiment (Ji *et al.*, 2005), derivatization by cyclocondensation reaction was used to determine level of PEITC. PEITC was derivatized with 1,2-benzenedithiol to produce, 1,3-benzenedithiole-2-thione. Although the derivatization is necessary to measure PEITC, it has some disadvantages several when compared with direct analysis. For example, the derivertization involves more complicated preparation steps, requires more time and carries the risk of introducing impurities (Mitrevski and Zdravkovski, 2005). However, due to limitation of PEITC, this step is important and to avoid the impurities from derivertization, samples were filtered through a 0.45 µm syringe filter before injection into HPLC. Retention times of PEITC derivative and 1,2-benzenedithiol were 3.94 and 8.17 min, respectively. The retention time obtained from this study longer than that reported by Getahun and Chung (1999), i.e. 3.2 min.

It may be due to the difference of flow rate, mobile phase, and type of column etc. Regarding PEITC method validation, good linearity was shown for quantification of PEITC derivative. No interference was found in the chromatogram. The inter-day and intra-day precision and accuracy were found within the acceptable values which were ±15%RSD and ±20%DEV, respectively. The LLOQ was determined based on signal to noise ratio (S/N≤5) and the obtained value in this study, 0.1 µg/mL was higher than the LLOQ value reported by other works which were 1.6 ng/mL (Liebes *et al.*, 2001), 1.27 ng/mL (Ji and Morris, 2003), and 0.65 ng/mL (Ji *et al.*, 2005). This is probably due to different analytical techniques i.e. UV-HPLC (274 nm) (Liebes *et al.*, 2001) and LC/MS/MS (Ji and Morris, 2003; Ji *et al.*, 2005). In general, the LC/MS/MS technique is shown to be an analytical technique with higher sensitivity and specificity when compared to HPLC. However, the sensitivity by HPLC technique in this study was sufficiently high for determining PEITC derivative in all samples.

Regarding to caffeine and it metabolites method validation, all analytes were well separated with no interference in blank serum. The retention times of all analytes obtained from this study, i.e. 6.09, 9.28, 9.76 and 12.66 min for TB, PX, TP, and CF, respectively, were shorter than those reported by Caubet and colleaques (2002) which were 14.12, 18.99, 19.31, and 23.08 min, respectively. This could be due to the difference in the column temperature and flow rate. These factors caused the decrease in the retention time of all analytes. The column temperature and flow rate used in this study were 32°C and 1.3 mL/min, respectively, which those reported by Caubet and colleaques (2002) were 23°C and 1 mL/min, respectively.

The inter-day and intra-day precision and accuracy were found within the acceptable values (±15 %RSD and ±20 %DEV for the concentration at LLOQ). Good linearity was shown for quantification of caffeine and its metabolites in the studied range of concentrations. The coefficient of determination (r^2) obtained for the regression line demonstrated the excellent relationship between peak area and concentrations of all analytes. The LLOQ values for caffeine and all metabolites ranged from 0.02 to 0.08 µg/mL. Although the range of LLOQ value for spiked in rat serum was higher than those reported by other works, i.e. 5 ng/mL for all analytes in rat urine (Caubet *et al.*, 2002), less than 8 ng/mL for all analytes in human urine (Schneider *et al.*, 2003), and 24 ng/mL for caffeine in human milk (Aresta *et al.*,

2005). Percentage recovery which was almost 100% showed that this procedure is highly efficient for determining caffeine and its metabolites in rat serum.

Determination of PEITC content in dry powder of Betong watercress consisted of two steps-conversion of glucosinolate to PEITC and derivertization of PEITC to a derivative. To more correctly determine the quantity of PEITC, optimum concentration of myrosinase enzyme was investigated. Increasing of mysosinase enzyme from 2 to 3 mg/ml resulted in increasing of peak area of a derivative. At this concentration myrosinase may entirely convert the glucosinolate compound in dry powder to PEITC. In contrast, increasing of enzyme from 3 to 4 mg/ml showed only slightly increase of peak area of a derivative, probably due to total glucosinolate was completely hydrolysed to PEITC. However, tendency of the peak area of the derivative reached the highest level at the myrosinase concentration of 4 mg/mL. Then, this concentration was considered optimal to convert the glucosinolate compound to PEITC prior to derivatization. Quantity of PEITC in dry powder of Betong watercress was calculated as described in Chapter 2. The amount of PEITC was 2.5/mg of dry powder.

In the present study, caffeine and its primary metabolites were simultaneously determined in rat serum. Ratios of the concentration of each metabolite to that of caffeine were calculated. Additionally, the ratio of total concentrations of all three metabolites recommended to that of caffeine by Tanaka *et al.* (1994) was included. All metabolic ratios of caffeine for rats receiving a single dose of caffeine alone (phase I) were varied. This may be due to a wide range of weight i.e. 168-244 g of animals was included. Similar findings were also observed for multiple doses pretreatment with caffeine. This group induced rats with weight range of 150-200 g.

All metabolic ratios of caffeine for rats receiving pretreatments of a single dose of fluvoxamine before caffeine administration were significantly decreased when compared with those receiving caffeine alone. This result was attributable to fluvoxamine inhibition of N-demethylation of caffeine. *In vitro* studies have shown that fluvoxamine is a potent inhibitor of the metabolism of caffeine in human liver microsomes with the K_i values ranged from 0.12 to 0.24 μM (Brosen *et al.*, 1993), and from 0.08 to 4.4 μM (Rasmussen *et al.*, 1998). In addition, a drug-drug

interaction between fluvoxamine and caffeine has been reported in healthy volunteers. The results indicated that intake of caffeine during fluvoxamine treatment may lead to caffeine intoxication as a result of a potent inhibition of fluvoxamine (Jeppesen *et al.*, 1996). Therefore, fluvoxamine was chosen to use as a reference for inhibition of caffeine metabolism in this study.

All metabolic ratios of caffeine for rats pretreated with a single dose of PEITC (2, 10, and 20 mg/kg) before caffeine administration were significantly decreased when compared with those receiving caffeine alone. Similar findings were also observed for pretreatment with multiple doses of PEITC (2, 10, and 20 mg/kg). The results indicated that a single- and multiple dose(s) of PEITC can inhibit N-demethylation of caffeine in rats.

In addition, all metabolic ratios of caffeine for rats receiving pretreatments of a single- and multiple dose(s) of Betong watercress at the doses 800 mg/kg before caffeine administration were significantly decreased when compared with those receiving caffeine alone. The results indicated that a single- and multiple dose(s) of Betong watercress at the dose 800 mg/kg can also inhibit N-demethylation of caffeine in rats.

The metabolic ratios; TB/CF, TP/CF, and (TB+PX+TP)/CF in rats after receiving pretreatments of a single dose of PEITC at 10 and 20 mg/kg were significantly decreased by approximately 50-60% when compared with fluvoxamine-pretreatment which exhibited the percentage of decrease approximately 30-35%. Similar findings were also observed for the pretreatment with multiple doses of PEITC at 10 and 20 mg/kg which caused the percentages of decrease of metabolic ratios approximately 55-70%. The results indicated that PEITC was able to inhibit the N-demethylation of caffeine *in vivo* and the inhibitory effect was comparing more extensive than fluvoxamine at the dose of 10 mg/kg. It has been reported that PEITC and NNK inhibited activation mediated by human CYP1A2 (Smith 1996). The results reported here in suggested that PEITC itself is a potent inhibitor of CYP1A2 catalyses the N-demethylation of caffeine. Similarly, Nakajima and colleaques (2001) have studied the inhibition and mechanism-based inactivation potencies of PEITC for human CYP activities by using microsomes from baculovirus-infected insect cells expressing specific human CYP isoforms. PEITC was found to competitively inhibit

CYP1A2 with K_i value of 4.5 µM. Thus, it was clearly demonstrated that PEITC, acts as an inhibitor of N-demethylation of caffeine in human.

The percentage of decrease of all metabolic ratios in rats after receiving pretreatments of a single dose of PEITC (2, 10, and 20 mg/kg) was dose-dependent, though not significantly different. In contrast, the percentage of reduction of all metabolic ratios in rats after receiving pretreatments of multiple doses of PEITC in different doses (2, 10, and 20 mg/kg) tended to decrease when rats receiving pretreatments with high dose (20 mg/kg) of PEITC. The results suggested that effects of multiple doses of PEITC at the doses of 2, 10, and 20 mg/kg were dose- and time-independent manner. This result may be due to involve nonlinear pharmacokinetics of PEITC. The previous study, reported by Ji and colleaques (2005), described pharmacokinetics of PEITC in male Sprague-Dawley rats treated with PEITC at doses of 1.63 (low dose) or 16.32 (high dose) mg/kg orally. At higher doses, the clearance (Cl) of PEITC tended to decrease, suggesting that PEITC may be eliminated in a capacity-limited manner. The volume of distribution (V_{ss}) was increased, suggesting that PEITC may permeate into tissues and bind to tissue proteins extensively. Taken together, the data may offer a partial explanation the decreased metabolic ratio induced by PEITC at high dose. In addition, this may be due to animal was prolonged fasting (1-5 days) of animals. This factor caused the increase of activity of enzyme which causes the decreased metabolic ratio. The previous study, reported by Toropila and colleaques (2005), determined the effect of prolonged fasting (0-7 days) on changes in the activity of selected adaptive enzymes: tyrosine aminotransferase (TAT), tryptophan-2-3-dioxygenase (TO), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in Wistar rats. The acitivity of these enzymes in liver of fasting rats were increased ($p < 0.05$) significantly

In the present study, Betong watercress at a single dose of 800 mg/kg which was equivalent with PEITC at the dose of 2 mg/kg caused less reduction in metabolic ratios (i.e. 10-25%) when compared with 2 mg/kg PEITC. Similar finding was also observed after pretreatment with multiple doses of Betong watercress at 800 mg/kg. The percentage of reduction tended to be smaller than that obtained from multiple doses of 2 mg/kg PEITC. The results indicated that Betong watercress was able to inhibit N-demethylation of caffeine but less than PEITC. This may be due to

the extent of conversion from glucosinolate to PEITC. The previous study revealed that the percentage of conversion after ingestion of watercress from glucosinolate to PEITC *in vivo* by the action of hydrolysis was in the range of 30 to 67% (Chung *et al.*, 1992). This reflects the efficiency of glucosinolate conversion in human body resulting in lesser effect in reduction of metabolic induced by 800 mg/kg Betong watercress.

In addition, the percent decrease of all metabolic ratios in rats receiving pretreatments with multiple doses of PEITC at 2 and 10 mg/kg tended to increase when compared with rats receiving pretreatment with a single dose of PEITC at 2 and 10 mg/kg. The result indicated that multiple dose(s) of PEITC at 2 and 10 mg/kg tended to inhibit N-demethylation of caffeine more than a single dose of PEITC at 2 and 10 mg/kg, though not significantly different. The decrease of metabolic ratios; TB/CF, TP/CF, and (TB+PX+TP)/CF in rats receiving pretreatments of multiple doses of Betong waterctess at 800 mg/kg were significantly increased when compared with a single dose of Betong waterctess at 800 mg/kg. Thus, the result indicated that multiple dose(s) of Betong watercress at 800 mg/kg was able to inhibit N-demethylation of caffeine more than a single dose of Betong watercress at 800 mg/kg. Finally, the above data of a single- and multiple dose(s) of PEITC in different doses (2, 10, and 20 mg/kg) and Betong watercress at 800 mg/kg can inhibit N-demethylation of caffeine in rats which seen from the metabolic ratios is decrease.

In fact, metabolism of caffeine in rats is not exactly the same as that in humans. CYP1A2 catalysed the C-8-hydroxylation of caffeine is the major metabolic pathway in rats to form 1,3,7-trimethyluric acid (137U) (70%). In contrast, CYP1A2 catalysed 3-N-demethylation is the main oxidation pathway of caffeine in humans to form paraxanthine (70%) (Kot and Daniel, 2008). Therefore for the future work aiming to investigate CYP1A2 inhibition in *vivo*, measuring of 1,3,7-trimethyluric acid should be performed.

Conclusion

A single- and multiple oral dose(s) administration of PEITC inhibited N-demethylation of caffeine in rats. The effect tended to be more extensive at high doses and was significantly greater than that produced by fluvoxamine (selective CYP1A2 inhibitor). Dry powder of Betong watercress juice also inhibited caffeine N-demethylation but with a lesser extent compared with PEITC. These findings suggested that CYP1A2 activity may be affected and may support the chemopreventive property of PEITC. The HPLC-UV techniques used for the determination of 1,3-benzenedithiole-2-thione (derivative of PEITC), caffeine and its metabolites in was simple, sensitive, precise and accurate. This technique is universal and cost-saving and facilitates routine analytical work.

In addition, the present study described the evaluation of N-demethylation of caffeine in rat by using caffeine as a marker because about 95% of caffeine is metabolized by CYP1A2. Caffeine is relatively safe, low toxic, and possesses many favorable pharmacokinetic characteristics for a marker substrate. Several caffeine-based approaches for assessing N-demethylation of caffeine *in vivo* was calculated from the ratios of the serum concentration of caffeine and its three demethylated metabolites (TB/CF, PX/CF, TP/CF and (TB+PX+TP/CF). Therefore, a method to determine N-demethylation of caffeine *in vivo* is of great pharmacological and toxicological interest.

References

Aldridge, A., Aranda, J.V., Neims, A.H. 1979. Caffeine metabolism in the newborn. *Clinical Pharmacology and Therapeutics*. 25: 447-453.

Ambrosone, C.B., McCann S.E., Freudenheim J.L., Marshall J.R., Zhang Y., Shields P.G. 2004. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. *Journal of Nutrition*. 134: 1134-1138.

Arnaud, M.J. 1976. Metabolism of 1,3,7-trimethyldihydrouric acid in the rat: new metabolic pathway of caffeine. *Experientia*. 32: 1238-1240.

Aresta, A., Palmisano, F., Zambonin, C.G. 2005. Simultaneous determination of caffeine, theobromine, theophylline, paraxanthine and nicotine in human milk by liquid chromatography with diode array UV detection. *Food Chemistry*. 93: 177-181.

Arinobu, T., Hattori, H., Kumazawa, T., Lee, X.P., Mizutani, Y., Katase, T., Kojima, S., Omori, T., Kaneko, R., Ishii, A., Seno, H. 2009. High-throughput determination of theophylline and caffeine in human serum by conventional liquid chromatography-mass spectrometry. *Forensic Toxicology*. 27: 1–6.

Asenjo, J.A. 1990. Separation Processes in Biotechnology. Marcel Dekker, New York.

Ayrton, A.D., McFarlane, M., Walker, R., Neville, S., Ioannides, C. 1990. The induction of P450 I proteins by aromatic amines may be related to their carcinogenic potential. *Journal of Carcinogenesis*. 11: 803-809.

Barone, J.J., Roberts, H.R. 1996. Caffeine consumption. *Food and Chemical Toxicology*. 34: 119-129.

Benowitz, N.L. 1990. Clinical pharmacology of caffeine. *Annual Review of Medicine.* 41:277-288.

Bernardi, R., Finiguerra M.G., Rossi, A.A., Palmieri, S. 2003. Isolation and biochemical characterization of a basic myrosinase from ripe Crambe abyssinica seeds, highly specific for epi-progoitrin. *Journal of Agricultural and Food Chemistry.* 51: 2737-2744.

Berthou, F., Flinois, J.P., Ratanasavanh, D., Beanu, P., Riche, C., Guillouz, A. 1991. Evidence for the involvement of several cytochromes P450 in the first steps of caffeine metabolism by human liver microsomes. *Drug Metabolism Disposition.* 19: 561-567.

Berthou, F., Guillois, B., Riche, C., Dreano, Y., Jacqz-Aigrain, E., Beaune, P.H. 1992. Interspecies variations in caffeine metabolism related to cytochrome P4501A enzymes. *Journal Cover Xenobiotica.* 22: 671-680.

Bertz, R.J., Granneman, G.R. 1997. Use of *in vitro* and *in vivo* data to estimate the likelihood of metabolic pharmacokinetic interactions. *Clinical Pharmacokinetics.* 32: 210-258.

Bienvenu, T., Pons, G., Rey, E., Thiroux, G., Olive, G. 1993. Caffeine metabolism in liver slices during postnatal development in rats. *Drug Metabolism and Disposition.* 21: 178-180.

Blanchard, J., Sawers, S.J. 1983. The absolute bioavailability of caffeine in man. *European Journal of Clinical Pharmacology.* 24: 93-98.

Bolton, S., Null, G. 1981. Caffeine: psychological effects, use and abuse. *Orthomolecular Psychiatry.* 10: 202-211.

Bonati, M., Latini, R., Galletti, F., Young, J.F., Tognoni, G., Garattini, S. 1982. Caffeine disposition after oral doses. *Clinical Pharmacology and Therapeutics*. 32: 98-106.

Bonnesen, C., Eggleston, I.M., Hayes, J.D. 2001. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. *Cancer Research*. 61: 6120-6130.

Bozikas, V.P., Papakosta, M., Niopas, I., Karavatos, A., Mirtsou-Fidani, V. 2004. Smoking impact on CYP1A2 activity in a group of patients with schizophrenia. *European Neuropsychopharmacology*. 14: 39-44.

Brosen, K., Skjelbo, E., Rasmussen, B.B., Poulsen, H.E., Loft, S. 1993. Fluvoxamine is a potent inhibitor of cytochrome P4501A2. *Biochemical Pharmacology*. 45: 1211-1214.

Burg, A.W. 1975. Physiological disposition of caffeine. *Drug Metabolism Reviews*. 4: 199-228.

Campbell, M.E., Spielberg, S.P., Kalow, W. 1987. A urinary metabolite ratio that reflects systemic caffeine clearance. *Clinical Pharmacology and Therapeutics*. 42: 157-165.

Caubet, M.S., Elbast, W., Dubuc, M.C., Brazier, J.L. 2002. Analysis of urinary caffeine metabolites by HPLC-DAD: the use of metabolic ratios to assess CYP1A2 enzyme activity. *Journal of Pharmaceutical and Biomedical Analysis*. 27: 261-270.

Chemicalbook. 2008. Phenylethyl isothiocyanate(2257-09-2). http://www.chemicalbook.com/ProductChemicalPropertiesCB2736659_EN.ht m (accessed 11/10/10).

Chen, L., Mohr, S.N., Yang, C.S. 1996. Decrease of plasma and urinary oxidative metabolites of acetaminophen after consumption of watercress by human volunteers. *Clinical Pharmacology and Therapeutics.* 60: 651-660.

Chen, Y.R., Wang, W., Kong, A.N., Tan, T.H. 1998. Molecular mechanisms of c-Jun N-terminal kinase-mediated apoptosis induced by anticarcinogenic isothiocyanates. *Journal of Biological Chemistry.* 273: 1769-1775.

Cheng, D.L., Hashimoto, K., Uda, Y. 2004. *In vitro* digestion of sinigrin and glucotropaeolin by single strains of bifidobacterium and identification of the digestive products. *Food and Chemical Toxicology.* 42: 351-357.

Chiriboga, L., Xie, P., Yee, H., Zarou, D., Zakim, D., Diem, M. 1998. Infrared spectroscopy of human tissue. IV. Detection of dysplastic and neoplastic changes of human cervical tissue via infrared microscopy. *Cellular and Molecular Biology (Noisy-le-Grand, France).* 44: 219-229.

Chung, F.L., Morse, M.A., Eklind, K.I., Lewis, J. 1992. Quantitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal. *Cancer Epidemiology Biomarkers Prevention.* 1: 383-388.

Chung, F.L., Kelloff, G., Steele, V., Pittman, B., Zang, E., Jiao, D., Rigotty, J., Choi, C.I., Rivenson, A. 1996. Chemopreventive efficacy of arylalkyl isothiocyanates and N-acetylcysteine for lung tumorigenesis in Fischer rats. *Cancer Research.* 56: 772-778.

Chung, F.L., Conaway, C.C., Rao, C.V., Reddy, B.S. 2000. Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. *Journal of Carcinogenesis.* 21: 2287-2291.

Conaway, C.C., Jiao, D., Kohri, T., Liebes, L., Chung, F.L. 1999. Disposition and pharmacokinetics of phenethyl isothiocyanate and 6-phenylhexyl isothiocyanate in F344 rats. *Drug Metabolismand Disposition*. 27: 13-20.

Conaway, C.C., Jiao, D., Chung, F.L. 1996. Inhibition of rat liver cytochrome P450 isozymes by isothiocyanates and their conjugates: a structure-activity relationship study. *Journal of Carcinogenesis*. 17: 2423-2427.

Conaway, C.C., Yang, Y.M., Chung, F.L. 2002. Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. *Current Drug Metabolism*. 3: 233-255.

Daly, A.K., Brockmoller, J., Broly, F., Eichelbaum, M., Evans, W.E., Gonzalez, F.J., Huang, J.D., Idle, J.R., Ingelman-Sundberg, M., Ishizaki, T., Jacqz-Aigrain, E., Meyer, U.A., Nebert, D., Steen, W., Wolf, V.M., Zanger, C.R. 1996. Nomenclature for human CYP2D6 alleles. *Pharmacogenetics*. 6:193-201.

Degawa, M., Tanimura, S., Agatsuma, T., Hashimoto, Y. 1989. Hepatocarcinogenic heterocyclic aromatic amines that induce cytochrome P-448 isozymes, mainly cytochrome P-448H (P-450IA2), responsible for mutagenic activation of the carcinogens in rat liver. *Journal of Carcinogenesis*. 10: 1119-1122.

Denaro, C.P., Brown, C.R., Wilson, M., Jacob, P., Benowitz, N.L. 1990. Dose-dependency of caffeine metabolism with repeated dosing. *Clinical Pharmacology and Therapeutics*. 48: 277-285.

Denaro, C.P., Wilson, M., Jacob, P., Benowitz, N.L. 1996. The effect of liver disease on urine caffeine metabolite ratios. *Clinical Pharmacology and Therapeutics*. 59: 624-635.

Denis M.G., Tang, B.K., Kalow, W. 1982. Variability in caffeine metabolism. *Clinical Pharmacology and Therapeutics*. 33: 591-602.

Duke University Medical Center. 2000. Material safety data sheet. URL: http//www.safety.duke.edu/msds/prodpharmacy/caffeine/pdf (accessed 01/10/ 03).

Eaton, D.L., Gallagher, E.P., Bammler, T.K., Kunze, K.L. 1995. Role of cytochrome P4501A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity. *Pharmacogenetics*. 5: 259-274.

Education, Municipality of Betong. 2009. Food of Betong. http://www.betongtown.com//education (accessed 05/10/10).

Fahey, J.W., Zalcmann, A.T., Talalay, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry*. 56: 5-51.

Fahey, J.W., Zhang, Y., Talalay, P. 1997. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proceedings of the National Academy of Sciences*. 94: 10367-10372.

FDA. 2001. Guidance for industry bioanalytical method validation. http://www.fda.gov/cder/guidance/index.html (acessed 15/05/08).

Fenwick, G.R., Heaney, R.K., Mullin, W.J. 1983. Glucosinolates and their breakdown products in food and food plants. *Critical Reviews in Food Science and Nutrition*. 18: 123-201.

Fimognari, C., Nusse, M., Berti, F., Iori, R., Cantelli-Forti, G., Hrelia, P. 2003. Sulforaphane modulates cell cycle and apoptosis in transformed and non-transformed human T lymphocytes. *Annals of the New York Academy of Sciences*. 1010: 393-398.

Finiguerra, M.G., Iori R., Palmieri S. 2001. Soluble and total myrosinase activity in defatted Crambe abyssinica meal. *Journal of Agricultural and Food Chemistry.* 49: 840-845.

Finn, I.B., Holtzman, S.G. 1986. Tolerance to caffeine-induced stimulation of locomotor activity in rats. *Journal of Pharmacology and Experimental Therapeutics.* 238: 542-546.

Fisone, G., Borgkvist, A., Usiello, A. 2004. Caffeine as a psychomotor stimulant: mechanism of action. *Celular and Molecularl Life Sciences.* 61: 857-872.

Fowke, J.H., Chung, F.L., Jin, F., Qi, D., Cai, Q., Conaway, C.C., Cheng, J.R., Shu, X.O., Gao, Y.T., Zheng, W. 2003. Urinary isothiocyanate levels, brassica, and human breast cancer. *Cancer Research.* 63: 3980-3986.

Fredholm, B.B., Battig, K., Holmen, J., Nehlig, A., Zvartau, E.E. 1999. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacological Reviews.* 51: 83-133.

Fredholm, B.B., Svenningsson, P. 2003. Adenosine-dopamine interactions: development of a concept and some comments on therapeutic possibilities. *Neurology.* 61: 5-9.

Fuhr, U., Rost, K.L. 1994. Simple and reliable CYP1A2 phenotyping by the paraxanthine/caffeine ratio in plasma and in saliva. *Pharmacogenetics.* 4: 109-116.

Fuhr, U., Rost, K.L., Engelhardt, R., Sachs, M., Liermann, D., Belloc, C., Beaune, P., Janezic, S., Grant, D., Meyer, U.A., Staib, A.H. 1996. Evaluation of caffeine as a test drug for CYP1A2, NAT2 and CYP2E1 phenotyping in man by in vivo versus *in vitro* correlations. *Pharmacogenetics.* 6: 159-176.

Gao, B., Hagenbuch, B., Kullak-Ublick, G.A., Benke, D., Aguzzi, A., Meier, P.J. 2000. Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *Journal of Pharmacology and Experimental Therapeutics.* 294: 73-79.

Garattini, S. 1993. Caffeine, coffee and health. Monographs of the Mario Negri Institute for Pharmacological Research, Milan: New York.

Gennaro, M.C., Abrigo, C., Biglino, P. 1992. Quantification of theophylline in human plasma by reversed-phase ion-interaction high-performance liquid chromatography and comparison with the TDx fluorescence polarization immunoassay procedure. *The Analyst.* 117: 1071-1074.

Getahun, S.M., Chung, F.L. 1999. Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. *Cancer Epidemiology Biomarkers and Prevention.* 8: 447-451.

Global Centre Environmental foundation. 1997. Analyzers for Water Pollution Continuous Monitoring. http://www.gec.jp/CTT_DATA/index_wmon.html (acessed 01/08/10).

Gong, H., Simmons, M.S., Tashkin, D.P., Hui, K.K., Lee, E.Y. 1986. Bronchodilator effects of caffeine in coffee. *British Journal of Diseases of the Chest.* 89: 335-342.

Gonzalez, F.J. 1992. Human cytochromes P450: problems and prospects. *Trends in Pharmacological Sciences.* 13: 346-352.

Gonzalez, F.J., Gelboin, H.V. 1994. Role of human cytochromes P450 in the metabolic activation of chemical carcinogens and toxins. *Drug Metabolism Reviews.* 26: 165-183.

Griffiths, R.R., Woodson, P.P. 1988. Reinforcing effects of caffeine in human. *Journal of Pharmacology and Experimental Therapeutics*. 246: 21-29.

Gu, L., Gonzalez, F.J., Kalow, W., Tang, B.K. 1992. Biotransformation of caffeine, paraxanthine, theobromine and theophylline by cDNA-expressed human CYP1A2 and CYP2E1. *Pharmacogenetics*. 2: 73-77.

Guengerich, F.P., Humphrey, W.G., Yun, C.H., Hammons, G.J., Kadlubar, F.F., Seto, Y., Okazaki, O., Martin, M.V. 1995. Mechanisms of cytochrome P450 1A2-mediated formation of N-hydroxy arylamines and heterocyclic amines and their reaction with guanyl residues. *Journal of Investigative Dermatology Symposium Proceedings*. 23: 78-84.

Guo, Z., Smith, T.J., Wang, E., Sadrieh, N., Ma, Q., Thomas, P.E., Yang, C.S. 1992. Effects of phenethyl isothiocyanate, a carcinogenesis inhibitor, on xenobiotic-metabolizing enzymes and nitrosamine metabolism in rats. *Journal of Carcinogenesis*. 13: 2205-2210.

Halkier, B.A., Du, L.C. 1997. The biosynthesis of glucosinolates. *Trends in Plant Science*. 2: 425-431.

Hasegawa, T., Nishino, H., Iwashima, A. 1993. Isothiocyanates inhibit cell cycle progression of HeLa cells at G2/M phase. *Anticancer Drugs*. 4: 273-279.

Hecht, S.S., Chung, F.L., Richie, J.P., Jr Akerkar, S.A., Borukhova, A., Skowronski, L., Carmella, S.G. 1995. Effects of watercress consumption on metabolism of a tobacco-specific lung carcinogen in smokers. *Cancer Epidemiology Biomarkers and Prevention*. 4: 877-874.

Hecht, S.S. 1997. Approaches to chemoprevention of lung cancer based on carcinogens in tobacco smoke. *Environmrntal Health Perspectives*. 4: 955-963.

Hecht, S.S. 1999. Chemoprevention of cancer by isothiocyanates, modifiers of carcinogen metabolism. *Journal of Nutrition*. 129: 768-774.

Higdon, J. 2005. Cruciferous vegetables provided by Linus Pauling Institute. http://lpi.oregonstate.edu/infocenter (acessed 21/07/05).

Hirai, T., Kondo, H. 1985. Determination of caffeine in human plasma and urine by gas chromatography using a nitrogen phosphorus-detector. *Analytical Sciences*. 1: 191-194.

Huang, C., Ma, W.Y., Li, J., Hecht, S.S., Dong, Z. 1998. Essential role of p53 in phenethyl isothiocyanate-induced apoptosis. *Cancer Research*. 58: 4102-4106.

Hubpages Inc. and respective owners. 2010. Health Benefits of Watercress. http://hubpages.com/hub/Health-Benefits-of-Watercress (accessed 11/10/10).

Hughes, J.R., Oliveto, A.H., Bickel, W.K., Higgins, S.T., Badger, G.J. 1993. Caffeine self-administration and withdrawal: incidence, individual differences and interrelationships. *Drug and Alcohol Dependence*. 32: 239-246.

International Agency for Research on Cancer. 2004. Cruciferous Vegetables, Isothiocyanates and Indoles Handbook. Oxford University: USA.

Jeppesen, U., Gram, L.F., Vistisen, K., Loft, S., Poulsen, H.E., Brosen, K. 1996. Dose-dependent inhibition of CYP1A2, CYP2C19 and CYP2D6 by citalopram, fluoxetine, fluvoxamine and paroxetine. *European Journal of Clinical Pharmacology*. 51: 73-78.

Ji, Y., Morris, M.E. 2003. Determination of phenethyl isothiocyanate in human plasma and urine by ammonia derivatization and liquid chromatography-tandem mass spectrometry. *Analytical Biochemistry*. 323: 39-47.

Ji, Y., Kuo, Y., Morris, M.E. 2005. Pharmacokinetics of dietary phenethyl isothiocyanate in rats. *Pharmaceutical Research*. 22: 1658-1666.

Jiao, D., Eklind, K.I., Choi, C.I., Desai, D.H., Amin, S.G., Chung, F.L. 1994. Structure-activity relationships of isothiocyanates as mechanism-based inhibitors of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in A/J mice. *Cancer Research*. 54: 4327-4333.

Jiao, D., Yu, M.C., Hankin, J.H., Low, S.H., Chung, F.L. 1998. Total isothiocyanate contents in cooked vegetables frequently consumed in Singapore. *Journal of Agricultural and Food Chemistry*. 46: 1055-1058.

Jodynis-Liebert, J., Matuszewska, A. 1999. Effect of toluidines and dinitrotoluenes on caffeine metabolic ratio in rat. *Toxicology Letters*. 104: 159-165.

Jorritsma, U., Schrader, E., Klaunick, G., Kapitulnik, J., Hirsch-Ernst, K.I., Kahl, G.F., Foth, H. 2000. Monitoring of cytochrome P-450 1A activity by determination of the urinary pattern of caffeine metabolites in Wistar and hyperbilirubinemic Gunn rats. *Toxicology*. 144: 229-236.

Kalow, W., Tang, B.K. 1993. The use of caffeine for enzyme assays: a critical appraisal. *Clinical Pharmacology and Therapeutics*. 53: 503-514.

Kristal, A.R., Lampe, J.W. 2002. Brassica vegetables and prostate cancer risk: a review of the epidemiological evidence. *Nutrition and Cancer*. 42: 1-9.

Krul, C., Hageman, G. 1998. Analysis of urinary caffeine metabolites to assess biotransformation enzyme activities by reversed-phase high-performance liquid chromatography. *Journal of Chromatography. B, Biomedical Sciences and Applications*. 709: 27-34.

Labague, L., Gardrat, G., Coustille, J.L., Viaud, M.C., Rollin, P. 1991. Identification of enzymatic degradation products from synthesised glucobrassicin by gas chromatography-mass spectrometry. *Journal of Chromatography*. 586: 166-170.

Lampe, J.W., King, I.B., Li, S., Grate, M.T., Barale, K.V., Chen, C., Feng, Z., Potter, J.D. 2000. Brassica vegetables increase and apiaceous vegetables decrease cytochrome P450 1A2 activity in humans: changes in caffeine metabolite ratios in response to controlled vegetable diets. *Carcinogenesis*. 21: 1157-1162.

Lane, J.D., Phillips-Bute, B.G. 1998. Caffeine deprivation affects vigilance performance and mood. *Physiology and Behavior*. 65: 171-175.

Lasch, P., Naumann, D. 1998. FT-IR Microspectroscopic imaging of human carcinoma thin sections based on pattern recognition techniques. *Cellular and Molecular Biology (Noisy-le-Grand, France)*. 44: 189-202.

Lau, C.E., Falk, J.L. 1991. Sustained synergism by chronic caffeine of the motor control deficit produced by midazolam. *Pharmacology Biochemistry and Behavior*. 40: 4723-4731.

Lee, G., Dallas, S., Hong, M., Bendayan, R. 2001. Drug transporters in the central nervous system: brain barriers and brain parenchyma considerations. *Pharmacolgical Reviews*. 53: 569-596.

Lelo, A., Birkett, D.J., Robson, R.A., Miners, J.O. 1986. Comparative pharmacokinetics of caffeine and its primary demethylated metabolites paraxanthine, theobromine and theophylline in man. *British Journal of Clinical Pharmacology*. 22: 177-182.

Lewis, D.F. 2000. On the recognition of mammalian microsomal cytochrome P450 substrates and their characteristics: towards the prediction of human p450 substrate specificity and metabolism. *Biochemical Pharmacology.* 60: 293-306.

Liebes, L., Conaway, C.C., Hochster, H., Mendoza, S., Hecht, S.S., Crowell, J., Chung, F.L. 2001. High-performance liquid chromatography-based determination of total isothiocyanate levels in human plasma: application to studies with 2-phenethyl isothiocyanate. *Analytical Biochemistry.* 291: 279-289.

Lin, H.J., Probst-Hensch, N.M., Louie, A.D., Kau, I.H., Witte, J.S., Ingles, S.A., Frankl, H.D., Lee, E.R., Haile, R.W. 1998. Glutathione transferase null genotype, broccoli, and lower prevalence of colorectal adenomas. *Cancer Epidemiology Biomarkers and Prevention.* 7: 647-652.

Lin, J.H., Lu, A.Y. 1998. Inhibition and induction of cytochrome P450 and the clinical implications. *Clinical Pharmacokinetics.* 35: 361-390.

Lindel, T., Brauchle, L., Golz, G., Bohrer, P. 2007. Total synthesis of flustramine C via dimethylallyl rearrangement. *Organic Letters.* 9: 283-286.

Massachusetts Poison Control. 1994. Clinical toxicology review. URL: http//www.maripoisoncenter.com/ctr/9411caffeine.html (accessed 15/10/03).

Meyer, J.M., Rodvold, K.A. 1996. Drug biotransformation by the cytochrome P-450 enzyme system. *Infection in Medicine.* 13:452,459,463-464,523.

McCall, A.L., Millington, W.R., Wurtman, R.J. 1982. Blood-brain barrier transport of caffeine: dose-related restriction of adenine transport. *Life Sciences.* 31: 2709-2915.

McIntosh, L.M., Jackson, M., Mantsch, H.H., Stranc, M.F., Pilavdzic, D., Crowson, A.N. 1999. Infrared spectra of basal cell carcinomas are distinct from non-tumor-bearing skin components. *The Journal of Investigative Dermatology.* 112: 951-956.

Michaud, D.S., Spiegelman, D., Clinton, S.K., Rimm, E.B., Willett, W.C., Giovannucci, E.L. 1999. Fruit and vegetable intake and incidence of bladder cancer in a male prospective cohort. *Journal of the National Cancer Institute.* 91: 605-613.

Miners, J.O., Birkett, D.J. 1996. The use of caffeine as a metabolic probe for human drug metabolizing enzymes. *General Pharmacology.* 27: 245-249.

Mitrevski, B., Zdravkovski, Z. 2005. Rapid and simple method for direct determination of several amphetamines in seized tablets by GC--FID. *Forensic Science International.* 152: 199-203.

Morse, M.A., Wang, C.X., Stoner, G.D., Mandal, S., Conran, P.B., Amin, S.G., Hecht, S.S., Chung, F.L. 1989. Inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced DNA adduct formation and tumorigenicity in the lung of F344 rats by dietary phenethyl isothiocyanate. *Cancer Research.* 49: 549-553.

Morse, M.A., Lu, J., Gopalakrishnan R., Peterson L.A., D'Ambrosio S.M., Wani G., Stoner. G.D. 1997. Mechanism of enhancement of esophageal tumorigenesis by 6-phenylhexyl isothiocyanate. *Cancer Letters.* 112: 119-125.

Munday, R., Munday, C.M. 2004. Induction of phase II detoxification enzymes in rats by plant-derived isothiocyanates: comparison of allyl isothiocyanate with sulforaphane and related compounds. *Journal of Agricultural and Food Chemistry.* 52: 1867-1871.

Mumford, G.K., Neill, D.B., Holtzman, S.G. 1988. Caffeine elevates reinforcement threshold for electrical brain stimulation: tolerance and withdrawal changes. *Brain Research*. 459: 163-167.

Nakajima, M., Yoshida, R., Shimada, N., Yamazaki, H., Yokoi, T. 2001. Inhibition and inactivation of human cytochrome P450 isoforms by phenethyl isothiocyanate. *Drug Metabolism and Disposition*. 29: 1110-1113.

Nakamura, Y., Ohigashi, H., Masuda, S., Murakami, A., Morimitsu, Y., Kawamoto, Y., Osawa T., Imagawa, M., Uchida, K. 2000. Redox regulation of glutathione S-transferase induction by benzyl isothiocyanate: correlation of enzyme induction with the formation of reactive oxygen intermediates. *Cancer Research*. 60: 219-225.

Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*. 6: 1-42.

Nelson, D.R. 1999. Cytochrome P450 and the individuality of species. *Archives of Biochemistry and Biophysics*. 369: 1-10.

Omura, M., Kagami, S., Seki, N., Iizuka, T., Nishikawa, T., Sasano, K. 1999. Case of primary aldosteronism caused by adrenal microadenoma that permitted clinical observation from onset. *Nippon Naika Gakkai Zasshi*. 88: 2474-2475.

Onken, M. 2000. How and why does caffeine effect the pulse rate of a person. URL: http//www.madsci.org/posts/archieves/feb2000/950223638.cb.r.html (accessed 10/09/02).

Papadoyannis, I.N., Samanidou, V.F., Tsoukali, H., Epivatianou, F. 1993. Comparison of a RP-HPLC method with the therapeutic drug monitoring system TD$_x$ for the determination of theophylline in blood serum. *Analytical Letters.* 26: 2127 – 2142.

Parsons, W.D., Neims A.H. 1981. Prolonged half-life of caffeine in healthy tem newborn infants. *Journal of Pediatrics.* 98: 640-641.

Peach, M.J. 1972. Stimulation of Release of Adrenal Catecholamine by Adenosine CMP and Theophylline in the Absence of Extracellular Calcium. *Proceedings of the National Academy of Sciences of the United States of America.* 69: 834-836.

Pedras, M.S., Loukaci, A., Okanga, F.I.. 1998. The cruciferous phytoalexins brassinin and cyclobrassinin are intermediates in the biosynthesis of brassilexin. *Bioorganic and Medicinal Chemistry Letters.* 8: 3037-3038.

Pekthong, D. 2000. Pharmacokinetics and the effect on psychomotor performance of caffeine in energy drinks in Thai healthy volunteers. Master of Pharmacology Thesis, Mahidol University, Bangkok, Thailand.

Poisner, A.M. 1973. Direct Stimulant Effect of Ammophylline on Catecholamine Release from the Adrenal Medulla. *Biochemical Pharmacology.* 22: 469-476.

Rasmussen, B.B., Nielsen, T.L., Brosen, K. 1998. Fluvoxamine is a potent inhibitor of the metabolism of caffeine *in vitro*. *Pharmacology and Toxicology.* 83: 240-245.

Regestein, Q.R., Barbiaz, J.E. 1980. Sleep disorders: recognizing them in patients. *Journal of Practice Nursing.* 30: 21-22.

Rich, T.C.G. 1991. Crucifers of Great Britain and Ireland. Botanical Society of the British Isles, London. Vol. 6, pp 336.

Richardson, N.J., Rogers, P.J., Elliman, N.A., O'dell, R.J. 1995. Mood and performance effects of caffeine in relation to acute and chronic caffeine deprivation. *Pharmacology Biochemistry and Behavior*. 52: 313-320.

Rose, P., Faulkner, K., Williamson, G., Mithen, R. 2000. 7-Methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes. *Carcinogenesis*. 21: 1983-1988.

Rost, K.L., Roots, I. 1994. Accelerated caffeine metabolism after omeprazole treatment is indicated by urinary metabolite ratios: coincidence with plasma clearance and breath test. *Clinical Pharmacology and Therapeutics*. 55: 402-411.

Rostami-Hodjegan, A., Nurminen, S., Jackson, P.R., Tucker, G.T. 1996. Caffeine urinary metabolite ratios as markers of enzyme activity: a theoretical assessment. *Pharmacogenetics*. 6: 121-149.

Rowan, T.G., Lawrence, T.L.J., Kershaw, S.J. 1991. Effects of dietary copper and a probiotic on glucosinolate concentration in ileal digesta and in faeces of growing pigs given diets based on rapeseed meals. *Animal Feed Science and Technology*. 35: 247-258.

Sawynok, J. 1995. Pharmacological rationale for the clinical use of caffeine. *Journal of Drugs*. 49: 37-50.

Serafin, W.E. 1996. Drugs used in the treatment of asthma. In: Hardman, J.G., Limbird, L.E., Molinoff, P.B., *et al.*, editors. Goodman and Gilman's. The Pharmacological Basis of Therapeutics, 9th Ed. New York., Vol. 28, pp 659-682.

Schreiber-Deturmeny, E., Bruguerolle, B. 1996. Simultaneous high-performance liquid chromatographic determination of caffeine and theophylline for routine drug monitoring in human plasma. *Journal of Chromatography B: Biomedical Science Applications.* 677: 305-312.

Schneider, H., Ma, L., Glatt, H. 2003. Extractionless method for the determination of urinary caffeine metabolites using high-performance liquid chromatography coupled with tandem mass spectrometry. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Science.* 789: 227-237.

Scott, R.J., Palmer, J., Lewis, I.A., Pleasance, S. 1999. Determination of a 'GW cocktail' of cytochrome P450 probe substrates and their metabolites in plasma and urine using automated solid phase extraction and fast gradient liquid chromatography tandem mass spectrometry. *Rapid Communication in Mass Spectrometry.* 13: 2305-2319.

Sesardic, D., Boobis, A.R., Murray, B.P., Murray, S., Segura, J., de la Torre R., Davies, D.S. 1990. Furafylline is a potent and selective inhibitor of cytochrome P450IA2 in man. *British Journal of Clinical Pharmacology.* 29: 651-663.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., Guengerich, F.P. 1994. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *Journalof Pharmacology and Experiment Therapeutics.* 270: 414-423.

Smith, W.L., Marnett, L.J., De Witt, D.L. 1991. Prostaglandin and thromboxane biosynthesis. *Pharmacology and Therapeutics.* 49: 153-179.

Smith, P. 1996. Caffeine dependence: an alternative view. *Nature Medecine.* 2: 494

Simpson, N.J.K. 2000. Solid-Phase-Extraction Principles, Technique, and Applications. New York.

Singh, A.V., Xiao, D., Lew, K.L., Dhir, R., Singh, S.V. 2004. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. *Carcinogenesis*. 25: 83-90.

Sinton, C.M., Petitjean, F. 1989. The influence of chronic caffeine administration on sleep parameters in the cat. *Pharmacology Biochemistry and Behavior*. 32: 459-462.

Solt, D.B., Chang, K., Helenowski, I., Rademaker, A.W. 2003. Phenethyl isothiocyanate inhibits nitrosamine carcinogenesis in a model for study of oral cancer chemoprevention. *Cancer Letters*. 202: 147-152.

Skoog, D.A., Holler, F.J., Nieman, T.A. 1998. Principles of Instrumental Analysis, 5th Ed. Saunders College Publishing., pp. 673-724.

Stoner, G.D., Morrissey, D.T., Heur, Y.H., Daniel, E.M., Galati, A.J., Wagner, S.A. 1991. Inhibitory effects of phenethyl isothiocyanate on N-nitrosobenzylmethylamine carcinogenesis in the rat esophagus. *Cancer Research*. 51: 2063-2068.

Sugawara, M., Mochizuki, T., Takekuma, Y., Miyazaki, K. 2005. Structure-affinity relationship in the interactions of human organic anion transporter 1 with caffeine, theophylline, theobromine and their metabolites. *Biochimica et Biophysica Acta*. 1714: 85-92.

Talalay, P., Fahey, J.W. 2001. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *Journal of Nutrition*. 131: 3027-3303.

Tanaka, E., Ishikawa, A., Misawa, S. 1992. Simplified test for determination of drug-oxidizing capacity in rats with chemical-induced liver injury using caffeine and trimethadione as model drugs. *Pharmacology and Toxicology*. 70: 177-180.

Tanaka, E., Ishikawa, A., Misawa, S. 1994. Changes in caffeine, lidocaine and trimethadione metabolism in carbon tetrachloride-intoxicated rats as assessed by a "cocktail" study. *Pharmacology and Toxicology*. 75: 150-153.

Tang, B.K., Kadar, D., Qian, L., Iriah, J., Yip, J., Kalow, W. 1991. Caffeine as a metabolic probe: validation of its use for acetylator phenotyping. *Clinical Pharmacology and Therapeutics*. 49: 648-657.

Tassaneeyakul, W., Mohamed, Z., Birkett, D.J., McManus, M.E., Veronese, M.E., Tukey, R.H., Quattrochi, L.C., Gonzalez, F.J., Miners, J.O. 1992. Caffeine as a probe for human cytochromes P450: validation using cDNA-expression, immunoinhibition and microsomal kinetic and inhibitor techniques. *Pharmacogenetics*. 2: 173-183.

Technical Bulletin. 2006. Protein Purification Techniques. http://www.safcglobal.com/etc/medialib/docs/SAFC/Bulletin/t040.pdf (acessed 13/05/09).

Thornalley, P.J. 2002. Isothiocyanates: mechanism of cancer chemopreventive action. *Anticancer Drugs*. 13: 331-338.

Tookey, H.L., Daxenblicher, M.E., Van Etten, C.H., Kwolek, W.F., Williams, P.H. 1980. Cabbage glucosinolates: correspondence of patterns in seeds and leafy heads. *Journal of the American Society for Horticultural Science. American Society for Horticultural Science*. 105: 714-717.

Tripathi, M.K., Mishra, A.S. 2007. Glucosinolates in animal nutrition: A review. *Animal Feed Science and Technology.* 132: 1-27.

Tseng, E., Scott-Ramsay, E.A., Morris, M.E. 2004. Dietary organic isothiocyanates are cytotoxic in human breast cancer MCF-7 and mammary epithelial MCF-12A cell lines. *Experimental Biology and Medicine.* 229: 835-842.

Van Poppel, G., Verhoeven, D.T., Verhagen, H., Goldbohm, R.A. 1999. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Advances in Experimental Medicine and Biology.* 472: 159-168.

Ventura, R., Jimenez, C., Closas, N., Segura, J., De La Torre, R.2003. Stability studies of selected doping agents in urine: caffeine. *Analytical Technologies in the Biomedical and Life Sciences.* 795: 167-177.

Vercruysse, J., Claerebout, E. 1997. Immunity development against Ostertagia ostertagi and other gastrointestinal nematodes in cattle. *Veterinary Parasitology.* 72: 309-316.

Verhoeven, D.T., Verhagen, H., Goldbohm, R.A., van den Brandt, P.A., van Poppel, G. 1997. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. *Chemio-Biological Interactions.* 103: 79-129.

Warwick, S.I., Sauder, C.A. 2005. Guide to Wild Germplasm of Brassica and Allied Crops (tribe Brassiceae, Brassicaceae) 3rd Ed. Agriculture Canada Research Branch, Technical Bulletin. Canada., Vol. 2, pp 61.

Wattenberg, L.W. 1977. Inhibition of carcinogenic effects of polycyclic hydrocarbons by benzyl isothiocyanate and related compounds. *Journal of the National Cancer Institute.* 58: 395-398.

Welfare, M.R., Aitkin, M., Bassendine, M.F., Daly, A.K. 1999. Detailed modelling of caffeine metabolism and examination of the CYP1A2 gene: lack of a polymorphism in CYP1A2 in Caucasians. *Pharmacogenetics*. 9: 367-375.

White, J.A., Beckett-Jones, B., Guo, Y.D., Dilworth, F.J., Bonasoro, J., Jones, G., Petkovich, M. 1997. cDNA cloning of human retinoic acid-metabolizing enzyme (hP450RAI) identifies a novel family of cytochromes P450. *Journal of Biological Chemistry*. 272: 18538-18541.

Xiao, D., Srivastava, S.K., Lew, K.L., Zeng, Y., Hershberger, P., Johnson, C.S., Trump, D.L., Singh, S.V. 2003. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G2/M arrest and inducing apoptosis. *Journal of Carcinogenesis*. 24: 891-897.

Yaw, C. 2008. Chemical Properties Handbook. McGraw-Hill: USA

Zhang, Y., Talalay, P., Cho, C.G., Posner, G.H. 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proceeding of the National Academy Sciences*. 89: 2399-2403.

Zhang, Y., Tang, L., Gonzalez, V. 2003. Selected isothiocyanates rapidly induce growth inhibition of cancer cells. *Molecular Cancer Therapeutics*. 2: 1045-1052.

Zhang, Y. 2004. Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. *Mutation Research*. 555: 173-190.

Zhao, B., Seow, A., Lee, E.J., Poh, W.T., Teh, M., Eng, P., Wang, Y.T., Tan, W.C., Yu, M.C., Lee, H.P. 2001. Dietary isothiocyanates, glutathione S-transferase - M1, -T1 polymorphisms and lung cancer risk among Chinese women in Singapore. *Cancer Epidemiology, Biomarkers and Prevention*. 10: 1063-1067.

Appendix-1 Data of method validation for PEITC

Table A1.1. Data for determination of linearity and lower limit of quantification of the method for analyzing PEITC in dry powder of Betong watercress juice (n=5)

Concentration of PEITC (µg/mL)	Peak area (AU)								Measured concentration (µg/mL)		
	n_1	n_2	n_3	n_4	n_5	Mean	S.D.	% RSD	Mean	S.D.	% DEV
0.1	2063	1891	1959	1998	1951	1972.40	63.50	3.22	0.12	0.00	18.04
0.3	4618	4510	4636	4612	4634	4602.00	52.44	1.14	0.29	0.04	-0.34
1	15646	15080	15039	15146	15568	15295.80	287.96	1.88	1.00	0.04	0.25
5	76803	76807	76296	76530	76469	76581.00	221.77	0.29	5.03	0.02	0.69
10	151684	150847	157169	153365	154164	153445.80	2461.82	1.60	10.09	0.19	0.92
15	224521	231001	220543	223140	227723	225385.60	4069.31	1.81	14.82	0.32	-1.15
20	312245	299304	302495	299134	299950	302625.60	5543.76	1.83	19.90	0.18	-0.47
30	465600	454529	458714	457817	452168	457765.60	5101.19	1.11	30.11	0.10	0.37

Table A1.2. Data for determination of intra- and inter-day precision of the method for analyzing PEITC in dry powders of Betong watercress juice (n=5)

Concentration of PEITC (µg/mL)	Intra-day precision					Inter-day precision				
	n	Peak area (AU)	Mean	S.D.	% RSD	Day	Peak area (AU)	Mean	S.D.	% RSD
0.1	n₁	2063	1972.4	63.50	3.22	1	2063	2053.40	83.36	4.06
	n₂	1891				2	2147			
	n₃	1959				3	2044			
	n₄	1998				4	1944			
	n₅	1951				5	2069			
3	n₁	39611	40602.00	898.19	2.21	1	44536	45093	1740.21	3.86
	n₂	39952				2	43169			
	n₃	41151				3	46873			
	n₄	41828				4	46520			
	n₅	40468				5	44367			
10	n₁	145721	143239.40	1683.95	1.18	1	148280	151819	3012.05	1.98
	n₂	142380				2	153666			
	n₃	141612				3	153538			
	n₄	142294				4	155160			
	n₅	144190				5	148451			
25	n₁	338736	335605.00	2170.45	0.65	1	374974	372079.2	6488.67	1.74
	n₂	334066				2	365990			
	n₃	336550				3	381789			
	n₄	333205				4	375231			
	n₅	335468				5	362412			

Table A1.3. Data for determination of intra- and inter-day accuracies of the method for analyzing PEITC in dry powders of Betong watercress (n=5)

Concentration (μg/mL)		Intra-day accuracy					Inter-day accuracy			
	n	Measured Concentration (μg/mL)	Mean	S.D.	% DEV	Day	Measured Concentration (μg/mL)	Mean	S.D.	% DEV
0.1	n_1	0.12				1	0.12			
	n_2	0.11				2	0.12			
	n_3	0.12	0.12	0.00	18.04	3	0.13	0.12	0.02	18.22
	n_4	0.12				4	0.09			
	n_5	0.12				5	0.12			
3	n_1	2.73				1	2.92			
	n_2	2.75				2	2.95			
	n_3	2.83	2.79	0.06	-6.85	3	2.99	2.97	0.07	-1.15
	n_4	2.88				4	3.09			
	n_5	2.79				5	2.90			
10	n_1	10.04				1	0.12			
	n_2	9.81				2	0.12			
	n_3	9.76	9.87	0.12	-1.32	3	0.13	10.05	0.39	0.53
	n_4	9.80				4	0.09			
	n_5	9.93				5	0.12			
25	n_1	23.34				1	2.92			
	n_2	23.02				2	2.95			
	n_3	23.19	23.13	0.15	-7.50	3	2.99	24.65	0.37	-1.40
	n_4	22.96				4	3.09			
	n_5	23.12				5	2.90			

Appendix-2 Data of method validation for caffeine and its metabolites

Table A2.1. Data for determination of linearity and lower limit of quantification of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n = 5)

Concentration (µg/mL)	Peak area ratio (AU)								Measured concentration (µg/mL)		
	n_1	n_2	n_3	n_4	n_5	Mean	S.D.	% RSD	Mean	S.D.	% DEV
Theobromine											
0.08	0.0158	0.0127	0.0146	0.0135	0.0145	0.0142	0.0012	8.2923	0.0943	0.0039	17.9027
0.1	0.0199	0.0172	0.0208	0.0205	0.0181	0.0193	0.0016	8.0926	0.0853	0.0840	-14.7024
0.5	0.1006	0.0785	0.0825	0.0906	0.0878	0.0880	0.0085	9.6156	0.4799	0.0435	-4.0111
1	0.1845	0.1597	0.1712	0.1616	0.1770	0.1708	0.0104	6.0969	0.9886	0.0522	-1.1416
2.5	0.4603	0.3926	0.4764	0.4510	0.4205	0.4401	0.0335	7.6120	2.6530	0.3808	6.1184
5	0.9846	0.7694	0.7980	0.9226	0.9123	0.8774	0.0904	10.3077	5.2882	0.3426	5.7643
10	1.7885	1.5479	1.3263	1.5933	1.8969	1.6306	0.2217	13.5994	9.8210	0.2509	-1.7905
Paraxanthine											
0.02	0.0023	0.0029	0.0025	0.0022	0.0021	0.0024	0.0003	12.06	0.0223	0.0020	11.3153
0.04	0.0043	0.0050	0.0051	0.0043	0.0048	0.0047	0.0004	8.3075	0.0350	0.0590	-12.5787
0.08	0.0100	0.0061	0.0091	0.0080	0.0093	0.0085	0.0015	17.6565	0.0671	0.0462	-16.0827
0.1	0.0126	0.0128	0.0133	0.0121	0.0107	0.0123	0.0010	7.9770	0.0866	0.0515	-13.3588
0.5	0.0667	0.0609	0.0542	0.0567	0.0542	0.0586	0.0053	9.1083	0.4842	0.0408	-3.1501
1	0.1189	0.1382	0.1113	0.1017	0.1103	0.1161	0.0138	11.8570	0.9832	0.0758	-1.6831
2.5	0.3132	0.3151	0.3024	0.2800	0.2647	0.2951	0.0220	7.4520	2.5323	0.2111	1.2928
5	0.6821	0.6664	0.5332	0.5680	0.5688	0.6037	0.0662	10.9716	5.1765	0.3089	3.5292
10	1.4154	1.1260	1.0591	1.0538	1.1314	1.1571	0.1488	12.8635	0.0866	0.0515	-13.3588

Table A2.1. Data for determination of linearity and lower limit of quantification of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n = 5) (cont.)

Concentration (µg/mL)	Peak area ratio (AU)								Measured concentration (µg/mL)		
	n_1	n_2	n_3	n_4	n_5	Mean	S.D.	% RSD	Mean	S.D.	% DEV
Theobromine											
0.04	0.0055	0.0078	0.0069	0.0062	0.0076	0.0068	0.0010	14.2182	0.0331	0.0029	-17.2483
0.08	0.0123	0.0132	0.0126	0.0124	0.0133	0.0127	0.0004	3.4824	0.0793	0.0285	-0.9248
0.1	0.0170	0.0174	0.0187	0.0155	0.0159	0.0169	0.0013	7.5202	0.0929	0.0397	-7.1184
0.5	0.0957	0.0833	0.0773	0.0828	0.0801	0.0838	0.0070	8.4066	0.4935	0.0371	-1.2956
1	0.1678	0.1877	0.1593	0.1536	0.1608	0.1658	0.0132	7.9783	0.9853	0.0662	-1.4666
2.5	0.4427	0.4246	0.4249	0.4123	0.3944	0.4197	0.0179	4.2543	2.4984	0.1583	-0.0630
5	0.9700	0.9117	0.7625	0.8285	0.8139	0.8573	0.0827	9.6490	5.0860	0.2464	1.7196
10	1.9894	1.6066	1.5575	1.6053	1.6444	1.6807	0.1753	10.4334	9.9597	0.1338	-0.4035
Caffeine											
0.02	0.0063	0.0050	0.0056	0.0050	0.0045	0.0053	0.0007	13.1345	0.0214	0.0011	7.0824
0.04	0.0114	0.0095	0.0085	0.0081	0.0094	0.0094	0.0013	13.4025	0.0388	0.0164	-3.0572
0.08	0.0174	0.0201	0.0142	0.0162	0.0158	0.0167	0.0022	13.2066	0.0730	0.0133	-8.8077
0.1	0.0291	0.0209	0.0210	0.0219	0.0203	0.0226	0.0037	16.2678	0.1063	0.0208	6.3067
0.5	0.0970	0.0959	0.0827	0.0991	0.0946	0.0939	0.0065	6.8954	0.5160	0.0796	3.1984
1	0.2052	0.2219	0.1771	0.1849	0.1870	0.1952	0.0181	9.2796	0.9866	0.0639	-1.3417
2.5	0.5430	0.4967	0.4769	0.4793	0.4523	0.4896	0.0338	6.8929	2.4949	0.1164	-0.2039
5	1.0986	1.0609	0.8477	0.9444	0.9506	0.9804	0.1142	11.6490	4.9954	0.1875	-0.0930
10	2.2484	1.9795	1.7937	1.8798	1.8970	1.9597	0.1972	10.0649	10.003	0.0842	0.0350

Table A2.2. Data for determination of intra- and inter-day precisions of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n = 5)

Concentration (μg/mL) Theobromine	n	Intra-day precision				Day	Inter-day precision			
		Peak area ratio (AU)	Mean	S.D.	% RSD		Peak area ratio (AU)	Mean	S.D.	% RSD
0.08	n₁	0.0123				1	0.0123			
	n₂	0.0121				2	0.0119			
	n₃	0.0114	0.0123	0.0006	5.2832	3	0.0121	0.0122	0.0006	4.5715
	n₄	0.0132				4	0.0110			
	n₅	0.0123				5	0.0134			
0.45	n₁	0.0798				1	0.0743			
	n₂	0.0891				2	0.0743			
	n₃	0.0814	0.0831	0.0036	4.3017	3	0.0692	0.074	0.003	3.615
	n₄	0.0831				4	0.0764			
	n₅	0.0819				5	0.0736			
4.5	n₁	0.8214				1	0.8569			
	n₂	0.8123				2	0.7478			
	n₃	0.8506	0.8292	0.0315	3.8004	3	0.7263	0.786	0.051	6.488
	n₄	0.7911				4	0.7956			
	n₅	0.8706				5	0.8020			
9	n₁	1.7240				1	1.3259			
	n₂	1.6167				2	1.4520			
	n₃	1.6752	1.6424	0.0625	3.8026	3	1.4519	1.413	0.070	4.934
	n₄	1.6383				4	1.3515			
	n₅	1.5576				5	1.4840			

Table A2.2. Data for determination of intra- and inter-day precisions of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (cont.)

Concentration (µg/mL) Paraxanthine	n	Intra-day precision				Day	Inter-day precision			
		Peak area ratio (AU)	Mean	S.D.	% RSD		Peak area ratio (AU)	Mean	S.D.	% RSD
0.02	n_1	0.0023				1	0.0025			
	n_2	0.0028				2	0.0024			
	n_3	0.0026	0.0025	0.0002	9.2569	3	0.0025	0.0023	0.0002	7.1133
	n_4	0.0023				4	0.0021			
	n_5	0.0023				5	0.0020			
0.45	n_1	0.0798				1	0.0522			
	n_2	0.0562				2	0.0513			
	n_3	0.0506	0.0520	0.0027	5.1800	3	0.0489	0.051	0.001	2.604
	n_4	0.0532				4	0.0520			
	n_5	0.0494				5	0.0513			
4.5	n_1	0.5246				1	0.5954			
	n_2	0.5101				2	0.5302			
	n_3	0.5390	0.5229	0.0193	3.6843	3	0.5100	0.550	0.032	5.879
	n_4	0.4976				4	0.5546			
	n_5	0.5433				5	0.5606			
9	n_1	1.1721				1	1.0854			
	n_2	1.0413				2	1.0436			
	n_3	1.0710	1.0728	0.0571	5.3227	3	1.0222	1.059	0.032	3.002
	n_4	1.0375				4	1.0989			
	n_5	1.0420				5	1.0463			

129

Table A2.2. Data for determination of intra- and inter-day precisions of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (cont.)

Concentration (µg/mL) Theophylline	n	Intra-day precision Peak area ratio (AU)	Mean	S.D.	% RSD	Day	Inter-day precision Peak area ratio (AU)	Mean	S.D.	% RSD
0.04	n1	0.0068				1	0.0066			
	n2	0.0073				2	0.0063			
	n3	0.0065	0.0066	0.0005	7.3450	3	0.0067	0.0065	0.0002	2.6076
	n4	0.0060				4	0.0066			
	n5	0.0064				5	0.0063			
0.45	n1	0.0768				1	0.0763			
	n2	0.0856				2	0.0773			
	n3	0.0791	0.0816	0.0037	4.5447	3	0.0735	0.075	0.002	2.064
	n4	0.0848				4	0.0755			
	n5	0.0818				5	0.0742			
4.5	n1	0.8085				1	0.9092			
	n2	0.7890				2	0.7884			
	n3	0.8262	0.8179	0.0377	4.6043	3	0.7704	0.819	0.054	6.537
	n4	0.7869				4	0.8145			
	n5	0.8789				5	0.8146			
9	n1	1.8682				1	1.6834			
	n2	1.6037				2	1.5517			
	n3	1.6539	1.6697	0.1128	6.7578	3	1.5101	1.591	0.069	4.332
	n4	1.6047				4	1.6357			
	n5	1.6179				5	1.5718			

Table A2.2. Data for determination of intra- and inter-day precisions of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (cont.)

Concentration (µg/mL) Caffeine	n	Intra-day precision Peak area ratio (AU)	Mean	S.D.	% RSD	Day	Inter-day precision Peak area ratio (AU)	Mean	S.D.	% RSD
0.02	n_1	0.0038				1	0.0040			
	n_2	0.0044				2	0.0044			
	n_3	0.0040	0.0040	0.0002	5.9122	3	0.0054	0.0045	0.0006	14.222
	n_4	0.0039				4	0.0042			
	n_5	0.0039				5	0.0043			
0.45	n_1	0.0836				1	0.0947			
	n_2	0.0875				2	0.0957			
	n_3	0.0803	0.0847	0.0030	3.5568	3	0.0908	0.093	0.003	2.830
	n_4	0.0875				4	0.0941			
	n_5	0.0846				5	0.0896			
4.5	n_1	0.8016				1	1.0989			
	n_2	0.8768				2	0.9666			
	n_3	0.8050	0.8497	0.0468	5.5113	3	0.9190	0.975	0.072	7.337
	n_4	0.9106				4	0.9363			
	n_5	1.8972				5	0.9545			
9	n_1	1.6201				1	2.0824			
	n_2	1.7255				2	1.8466			
	n_3	1.6536	1.7093	0.1120	6.5519	3	1.7674	1.891	0.117	6.172
	n_4	1.6499				4	1.8941			
	n_5	0.8016				5	1.8662			

Table A2.3. Data for determination of intra- and inter-day accuracies of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5)

Concentration (µg/mL) Theobromine	n	Intra-day accuracy				Day	Inter-day accuracy			
		Measured Concentration (µg/mL)	Mean	S.D.	% DEV		Measured Concentration (µg/mL)	Mean	S.D.	% DEV
0.08	n_1	0.0944				1	0.0946			
	n_2	0.0937				2	0.0924			
	n_3	0.0890	0.0943	0.0039	17.9027	3	0.0935	0.0938	0.0034	17.2327
	n_4	0.1001				4	0.0870			
	n_5	0.0945				5	0.1014			
0.45	n_1	0.4795				1	0.4708			
	n_2	0.5301				2	0.4703			
	n_3	0.4881	0.4972	0.0195	10.4785	3	0.4396	0.4661	0.0160	3.5751
	n_4	0.4974				4	0.4834			
	n_5	0.4908				5	0.4663			
4.5	n_1	4.5273				1	5.2166			
	n_2	4.4777				2	4.5551			
	n_3	4.6867	4.5700	0.1720	1.5549	3	4.4246	4.7849	0.3091	6.3305
	n_4	4.3620				4	4.8445			
	n_5	4.7961				5	4.8836			
9	n_1	9.4541				1	8.0604			
	n_2	8.8683				2	8.8254			
	n_3	9.1877	9.0085	0.3409	0.0940	3	8.8246	8.5891	0.4228	-4.5650
	n_4	8.9862				4	8.2160			
	n_5	8.5459				5	9.0193			

Table A2.3. Data for determination of intra- and inter-day accuracies of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5) (cont.)

Concentration (µg/mL) Paraxanthine	n	Intra-day accuracy Measured Concentration (µg/mL)	Mean	S.D.	% DEV	Day	Inter-day accuracy Measured Concentration (µg/mL)	Mean	S.D.	% DEV
0.02	n_1	0.0211				1	0.0225			
	n_2	0.0252				2	0.0214			
	n_3	0.0234	0.0223	0.0020	11.3153	3	0.0221	0.0207	0.0014	3.4953
	n_4	0.0207				4	0.0193			
	n_5	0.0209				5	0.0183			
0.45	n_1	0.4795				1	0.4516			
	n_2	0.5400				2	0.4435			
	n_3	0.4927	0.5046	0.0232	12.1372	3	0.4227	0.4423	0.0115	-1.7064
	n_4	0.5150				4	0.4497			
	n_5	0.4819				5	0.4441			
4.5	n_1	4.5648				1	5.1421			
	n_2	4.4396				2	4.5792			
	n_3	4.6883	4.5500	0.1655	1.1102	3	4.4049	4.7517	0.2793	5.5936
	n_4	4.3322				4	4.7903			
	n_5	4.7249				5	4.8421			
9	n_1	10.1270				1	9.3736			
	n_2	9.0036				2	9.0131			
	n_3	9.2586	9.2740	0.4906	3.0440	3	8.8278	9.1482	0.2746	1.6465
	n_4	8.9710				4	9.4906			
	n_5	9.0096				5	9.0359			

Table A2.3. Data for determination of intra- and inter-day accuracies of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5) (cont.)

Concentration (µg/mL) Theophylline	n	Intra-day accuracy				Day	Inter-day accuracy			
		Measured Concentration (µg/mL)	Mean	S.D.	% DEV		Measured Concentration (µg/mL)	Mean	S.D.	% DEV
0.04	n_1	0.0343				1	0.0333			
	n_2	0.0372				2	0.0317			
	n_3	0.0326	0.0331	0.0029	-17.2483	3	0.0341	0.0327	0.0010	-18.2122
	n_4	0.0294				4	0.0331			
	n_5	0.0320				5	0.0313			
0.45	n_1	0.4011				1	0.4483			
	n_2	0.4525				2	0.4544			
	n_3	0.4143	0.4293	0.0219	-4.6098	3	0.4314	0.4427	0.0093	-1.6249
	n_4	0.4482				4	0.4435			
	n_5	0.4301				5	0.4359			
4.5	n_1	4.4980				1	5.4061			
	n_2	4.3914				2	4.6868			
	n_3	4.5944	4.5492	0.2058	1.0924	3	4.5800	4.8715	0.3188	8.2562
	n_4	4.3796				4	4.8421			
	n_5	4.8824				5	4.8426			
9	n_1	10.2886				1	10.0144			
	n_2	8.8430				2	9.2302			
	n_3	9.1172	9.2037	0.6166	2.2630	3	8.9829	9.4615	0.4101	5.1280
	n_4	8.8487				4	9.7301			
	n_5	8.9207				5	9.3500			

Table A2.3. Data for determination of intra- and inter-day accuracies of the method for analyzing caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5) (cont.)

Concentration (μg/mL) Caffeine	n	Intra-day accuracy Measured Concentration (μg/mL)	Mean	S.D.	% DEV	Day	Inter-day accuracy Measured Concentration (μg/mL)	Mean	S.D.	% DEV
0.02	n1	0.0205				1	0.0215			
	n2	0.0232				2	0.0231			
	n3	0.0213	0.0214	0.0011	7.0824	3	0.0279	0.0236	0.0028	17.7723
	n4	0.0212				4	0.0224			
	n5	0.0209				5	0.0229			
0.45	n1	0.3926				1	0.4270			
	n2	0.4119				2	0.4317			
	n3	0.3765	0.3981	0.0148	-11.5366	3	0.4096	0.4194	0.0118	-6.7966
	n4	0.4117				4	0.4244			
	n5	0.3978				5	0.4044			
4.5	n1	4.3650				1	4.9181			
	n2	4.1011				2	4.3265			
	n3	4.4768	4.3413	0.2340	-3.5262	3	4.1135	4.3643	0.3199	-3.0151
	n4	4.1181				4	4.1910			
	n5	4.6457				5	4.2725			
9	n1	9.5763				1	9.3168			
	n2	8.1913				2	8.2622			
	n3	8.7181	8.6370	0.5597	-4.0337	3	7.9079	8.4623	0.5221	-5.9745
	n4	8.3588				4	8.4747			
	n5	8.3404				5	8.3497			

Table A2.4. Data for determination of recoveries of extraction of caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5)

Concentration (µg/mL) Theobromine	n	Peak area ratio (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.08	n_1	0.0110	0.0123	111.2571		
	n_2	0.0116	0.0121	104.3893		
	n_3	0.0113	0.0114	100.4966	107.7744	5.4187
	n_4	0.0116	0.0132	114.1137		
	n_5	0.0113	0.0123	108.6152		
0.45	n_1	0.0767	0.0798	104.1389		
	n_2	0.0765	0.0891	116.5555		
	n_3	0.0769	0.0814	105.8638	109.5109	4.9360
	n_4	0.0762	0.0831	109.1339		
	n_5	0.0732	0.0819	111.8625		
4.5	n_1	0.7548	0.8214	108.8231		
	n_2	0.7516	0.8123	108.0852		
	n_3	0.7584	0.8506	112.1591	107.7744	5.4187
	n_4	0.7565	0.7911	104.5709		
	n_5	0.7642	0.8706	113.9316		
9	n_1	1.5233	1.7240	113.1720		
	n_2	1.5113	1.6167	106.9753		
	n_3	1.5028	1.6752	111.4679	109.5109	4.9360
	n_4	1.5048	1.6383	108.8706		
	n_5	1.5028	1.5576	103.6448		

Table A2.4. Data for determination of recoveries of extraction of caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5) (cont.)

Concentration (μg/mL) Paraxanthine	n	Peak area ratio (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.02	n_1	0.0019	0.0023	120.8585	118.2089	17.5398
	n_2	0.0021	0.0028	134.1547		
	n_3	0.0019	0.0026	135.8453		
	n_4	0.0023	0.0023	99.1247		
	n_5	0.0023	0.0023	101.0613		
0.45	n_1	0.0767	0.0798	104.1389	110.0780	8.0594
	n_2	0.0455	0.0562	123.4137		
	n_3	0.0475	0.0506	106.6195		
	n_4	0.0477	0.0532	111.7191		
	n_5	0.0477	0.0494	103.6172		
4.5	n_1	0.4797	0.5246	109.3626	108.1276	4.0341
	n_2	0.4820	0.5101	105.8223		
	n_3	0.4817	0.5390	111.9023		
	n_4	0.4865	0.4976	102.2836		
	n_5	0.4883	0.5433	111.2670		
9	n_1	0.9673	1.1721	121.1641	111.9955	5.2964
	n_2	0.9553	1.0413	108.9988		
	n_3	0.9561	1.0710	112.0149		
	n_4	0.9527	1.0375	108.9089		
	n_5	0.9569	1.0420	108.8910		

Table A2.4. Data for determination of recoveries of extraction of caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5) (cont.)

Concentration (µg/mL) Theophylline	n	Peak area ratio (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.04	n₁	0.0063	0.0068	107.4669		
	n₂	0.0062	0.0073	116.9896		
	n₃	0.0064	0.0065	102.1875	105.1102	7.6422
	n₄	0.0062	0.0060	96.7699		
	n₅	0.0063	0.0064	102.1373		
0.45	n₁	0.0739	0.0768	103.9248		
	n₂	0.0743	0.0856	115.1682		
	n₃	0.0734	0.0791	107.7780	110.2342	4.5788
	n₄	0.0745	0.0848	113.9299		
	n₅	0.0741	0.0818	110.3701		
4.5	n₁	0.7589	0.8085	106.5449		
	n₂	0.7570	0.7890	104.2244		
	n₃	0.7542	0.8262	109.5370	107.5740	4.5853
	n₄	0.7634	0.7869	103.0787		
	n₅	0.7677	0.8789	114.4849		
9	n₁	1.5161	1.8682	123.2233		
	n₂	1.5247	1.6037	105.1797		
	n₃	1.5002	1.6539	110.2456	110.5426	7.3224
	n₄	1.5034	1.6047	106.7363		
	n₅	1.5074	1.6179	107.3281		

Table A2.4. Data for determination of recoveries of extraction of caffeine, theobromine, paraxanthine, and theophylline in rat serum (n=5) (cont.)

Concentration (µg/mL) Caffeine	n	Peak area ratio (AU)		% Recovery		
		Direct injection	After extraction	% Recovery	Mean	S.D.
0.02	n_1	0.0036	0.0038	104.3469		
	n_2	0.0036	0.0044	120.2663		
	n_3	0.0036	0.0040	109.1927	108.9777	6.5930
	n_4	0.0037	0.0039	106.3559		
	n_5	0.0037	0.0039	104.7266		
0.45	n_1	0.0814	0.0836	102.6626		
	n_2	0.0799	0.0875	109.5126		
	n_3	0.0796	0.0803	100.8472	105.2154	3.5989
	n_4	0.0810	0.0875	107.9938		
	n_5	0.0806	0.0846	105.0605		
4.5	n_1	0.8093	0.8544	105.5739		
	n_2	0.8077	0.8016	99.2475		
	n_3	0.8026	0.8768	109.2390	104.8272	5.7328
	n_4	0.8156	0.8050	98.7047		
	n_5	0.8176	0.9106	111.3709		
9	n_1	1.6296	1.8972	116.4232		
	n_2	1.6272	1.6201	99.5598		
	n_3	1.5989	1.7255	107.9148	106.0117	6.5393
	n_4	1.5991	1.6536	103.4096		
	n_5	1.6057	1.6499	102.7510		

Appendix-3 Body weight of rats and time of sample collection for a single dose of PEITC pretreatment

Table A3.1. Body weight weight of rats and time of blood collection for Gr. 1 (FV 10 mg/kg+ CF dose 10 mg/kg)

Phase I: Experiment date 20/11/2009; Fasting date/time 19/11/2009/19.30 h					Wash-out	Phase II: Experiment date 23/11/2009; Fasting date/time 22/11/2009/19.30 h			
Rat	Weight (g)	T_0	CF (p.o.)	T_3		Weight (g)	FV (i.p.)	CF (p.o.)	T_3
n_1	193	7.30	8.35	11.35		204	7.45	8.15	11.15
n_2	200	7.35	8.40	11.40		216	7.50	8.20	11.20
n_3	202	7.40	8.45	11.45		204	7.55	8.25	11.25
n_4	196	7.45	8.50	11.50		202	8.00	8.30	11.30
n_5	202	7.50	8.55	11.55		212	8.05	8.35	11.35
n_6	201	7.55	9.00	12.00		207	8.10	8.40	11.40

Table **A3.2.** Body weight weight of rats and time of blood collection for Gr. 2 (PEITC dose 2 mg/kg + CF dose 10 mg/kg)

Rat	**Phase I:** Experiment date 20/11/2009; Fasting date/time 19/11/2009/19.30 h				Wash-out	**Phase II:** Experiment date 23/11/2009; Fasting date/time 22/11/2009/19.30 h			
	Weight (g)	T_0	CF (p.o.)	T_3		Weight (g)	PEITC (p.o.)	CF (p.o.)	T_3
n_1	213	8.00	8.35	9.05		221	8.45	9.15	12.15
n_2	213	8.05	8.40	9.10		222	8.50	9.20	12.20
n_3	212	8.10	9.15	12.35		216	8.55	9.25	12.25
n_4	208	8.15	9.20	12.40		211	9.00	9.30	12.30
n_5	219	8.25	9.25	12.45		222	9.05	9.35	12.35
n_6	208	8.30	9.40	12.50		211	9.10	9.40	12.40

Table A3.3. Body weight weight weight of rats and time of blood collection for Gr. 3 (PEITC dose 10 mg/kg + CF dose 10 mg/kg)

Rat	Phase I: Experiment date 24/11/2009; Fasting date/time 23/11/2009/19.30 h				Wash-out	Phase II: Experiment date 27/11/2009; Fasting date/time 26/11/2009/19.30 h			
	Weight (g)	T_0	CF (p.o.)	T_3		Weight (g)	PEITC (p.o.)	CF (p.o.)	T_3
n_1	168	7.45	8.55	11.55		170	7.35	8.05	11.05
n_2	173	7.50	9.00	12.00		174	7.40	8.10	11.10
n_3	168	7.55	9.05	12.05		166	7.45	8.15	11.15
n_4	174	8.00	9.10	12.10		169	7.50	8.20	11.20
n_5	177	8.05	9.15	12.15		176	7.55	8.25	11.25
n_6	240	8.10	9.20	2.20		247	8.00	8.30	11.30

Table A3.4. Body weight weight of rats and time of blood collection for Gr. 4 (PEITC dose 20 mg/kg + CF dose 10 mg/kg)

Rat	**Phase I**: Experiment date 24/11/2009; Fasting date/time 23/11/2009/19.30 h				Wash-out	**Phase II**: Experiment date 27/11/2009; Fastingdate/time 26/11/2009/19.30 h			
	Weight (g)	T_0	CF (p.o.)	T_3		Weight (g)	PEITC (p.o)	CF (p.o.)	T_3
n_1	237	8.20	9.25	12.25		220	8.35	9.05	12.05
n_2	230	8.25	9.30	12.30		217	8.40	9.10	12.10
n_3	230	8.30	9.35	12.35		214	8.45	9.15	12.15
n_4	240	8.35	9.40	12.40		225	8.50	9.20	12.20
n_5	244	8.45	9.45	12.45		232	8.55	9.25	12.25
n_6	241	8.50	9.50	12.50		233	9.00	9.30	12.30

Table A3.5. Body weight of rats and time of blood collection for Gr. 8 (Dry watercress dose 800 mg/kg + CF dose 10 mg/kg)

Rat	Phase I: Experiment date 20/03/2010; Fasting date/time 19/03/2010/19.30 h				Wash-out	Phase II: Experiment date 23/03/2010; Fasting date/time 22/03/2010/19.30 h			
	Weight (g)	T_0	CF (p.o.)	T_3		Weight (g)	Dry watercress (p.o.)	CF (p.o.)	T_3
n_1	195	7.00	8.15	11.15		205	7.10	9.00	12.00
n_2	205	7.05	8.20	11.20		210	7.20	9.05	12.05
n_3	200	7.10	8.25	11.25		205	7.25	9.10	12.10
n_4	200	7.15	8.30	11.30		200	7.50	9.15	12.15
n_5	200	7.20	8.35	11.35		205	7.55	9.20	12.20
n_6	195	7.25	8.40	11.40		195	8.00	9.25	12.25

Appendix–4 Body weight of rats and time of sample collection for multiple doses of PEITC pretreatment

Table A4.1. Body weight of rats and time of blood collection for Gr. 5 (PEITC dose 2 mg/kg + CF dose 10 mg/kg)

Phase I: Experiment date 14/12/2009; Fasting date/time 13/12/2009/19.00 h

Rat	Weight (g)	Wash-out		
		T_0	CF (p.o.)	T_3
n_1	156	7.05	7.35	10.35
n_2	154	7.10	7.40	10.40
n_3	152	7.15	7.45	10.45
n_4	153	7.20	7.50	10.50
n_5	150	7.25	7.55	10.55
n_6	158	7.30	8.00	11.00

Phase II: Experiment date 17/12/2009; Fasting date/time 16/12/2009/19.00 h

Rat	17/12/2552		18/12/2552		19/12/2552		20/12/2552		21/12/2552			
	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	CF (p.o.)	T_3
n_1	150	7.05	130	7.05	120	7.05	115	7.05	125	7.05	7.35	10.35
n_2	170	7.10	165	7.10	165	7.10	165	7.10	175	7.10	7.40	10.40
n_3	170	7.15	165	7.15	165	7.15	170	7.15	175	7.15	7.45	10.45
n_4	170	7.20	160	7.20	160	7.20	165	7.20	165	7.20	7.50	10.50
n_5	165	7.25	155	7.25	155	7.25	155	7.25	160	7.25	7.55	10.55
n_6	175	7.30	170	7.30	172	7.30	180	7.30	185	7.30	8.00	11.00

Table A4.2. Body weight of rats and time of blood collection for Gr. 6 (PEITC dose 10 mg/kg + CF dose 10 mg/kg)

Phase I: Experiment date 15/12/2009; Fasting date/time 14/12/2009/19.30 h

Rat	Weight (g)	T_0	CF (p.o.)	T_3
				Wash-out
n_1	166	7.35	8.05	11.05
n_2	160	7.40	8.10	11.10
n_3	167	7.45	8.15	11.15
n_4	160	7.50	8.20	11.20
n_5	164	7.55	8.25	11.25
n_6	174	8.00	8.30	11.30

Phase II: Experiment date 18/12/2009; Fasting date/time 17/12/2009/19.00 h

Rat	18/12/2552		19/12/2552		20/12/2552		21/12/2552		22/12/2552			
	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	CF (p.o.)	T_3
n_1	170	7.35	180	7.35	175	7.35	180	7.35	185	7.35	8.05	11.05
n_2	160	7.40	165	7.40	170	7.40	170	7.40	170	7.40	8.10	11.10
n_3	155	7.45	155	7.45	160	7.45	160	7.45	160	7.45	8.15	11.15
n_4	165	7.50	165	7.50	165	7.50	170	7.50	170	7.50	8.20	11.20
n_5	170	7.55	175	7.55	180	7.55	180	7.55	185	7.55	8.25	11.25
n_6	180	8.00	185	8.00	190	8.00	190	8.00	195	8.00	8.30	11.30

Table A4.3. Body weight of rats and time of blood collection for Gr. 7 (PEITC dose 20 mg/kg + CF dose 10 mg/kg)

Phase I: Experiment date 16/12/2009; Fasting date/time 15/12/2009/19.30 h

Rat	Weight (g)	T_0	CF (p.o.)	T_3	Wash-out
n_1	195	8.00	8.30	11.30	
n_2	180	8.05	8.35	11.35	
n_3	205	8.10	8.40	11.40	
n_4	170	8.15	8.45	11.45	
n_5	165	8.20	8.50	11.50	
n_6	170	8.25	8.55	11.55	

Phase II: Experiment date 19/12/2009; Fasting date/time 18/12/2009/19.00 h

Rat	19/12/2552		20/12/2552		21/12/2552		22/12/2552		23/12/2552			
	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	weight (g)	PEITC (p.o.)	CF (p.o.)	T_3
n_1	195	8.05	200	8.05	205	8.05	210	8.05	210	8.05	8.35	11.35
n_2	185	8.10	180	8.10	185	8.10	190	8.10	195	8.10	8.40	11.40
n_3	200	8.15	205	8.15	210	8.15	215	8.15	215	8.15	8.45	11.45
n_4	170	8.20	170	8.20	170	8.20	175	8.20	175	8.20	8.50	11.50
n_5	165	8.25	170	8.25	170	8.25	175	8.25	175	8.25	8.55	11.55
n_6	185	8.30	185	8.30	190	8.30	190	8.30	190	8.30	9.00	12.00

Table A4.4. Body weight of rats and time of blood collection for Gr. 9 (Dry watercress dose 800 mg/kg + CF dose 10 mg/kg)

Phase I: Experiment date 20/03/2010; Fasting date/time 19/03/2010/19.30 h

Rat	Weight (g)	T_0	Dry watercress (p.o.)	T_3	Dry watercress (p.o.)	Wash-out
n_1	195	7.30	8.45	11.45		
n_2	190	7.35	8.50	11.50		
n_3	185	7.40	8.55	11.55		
n_4	190	7.45	9.00	12.00		
n_5	185	7.50	9.05	12.02		
n_6	190	7.55	9.10	12.10		

Phase II: Experiment date 23/03/2010; Fasting date/time 22/03/2010/19.30 h

Rat	23/03/2553		24/03/2553		25/03/2553		26/03/2553		27/03/2553		CF (p.o.)	T_3
	weight (g)	Dry watercress (p.o.)	weight (g)	Dry watercress (p.o.)	weight (g)	Dry watercress (p.o.)	weight (g)	Dry watercress (p.o.)	weight (g)	Dry watercress (p.o.)		
n_1	185	8.05	175	8.05	170	8.05	175	8.05	175	8.05	8.45	11.45
n_2	185	8.10	175	8.10	160	8.10	160	8.10	165	8.10	8.50	11.50
n_3	185	8.20	180	8.20	175	8.20	185	8.20	189	8.20	8.55	11.55
n_4	180	8.30	175	8.30	165	8.30	170	8.30	176	8.30	9.00	12.00
n_5	175	8.35	170	8.35	160	8.35	165	8.35	165	8.35	9.05	12.05
n_6	180	8.40	170	8.40	160	8.40	165	8.40	165	8.40	9.10	12.10

Appendix-5 Data of metabolic ratio of sample collection for single dose

Table A5.1. Data of metabolic ratio for Gr. 1 (FV 10 mg/kg+ CF dose 10 mg/kg)

Gr. 1	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.1199	0.1223	0.1201	0.5994	0.7500	1.1200	0.7700	3.0400	0.2467	0.3684	0.2533	0.8684
	n_2	0.1281	0.1248	0.1231	0.6357	0.8000	1.1400	0.7900	3.2200	0.2484	0.3540	0.2453	0.8478
	n_3	0.1294	0.1315	0.1286	0.5840	0.8000	1.2000	0.8200	2.9600	0.2703	0.4054	0.2770	0.9527
	n_4	0.1270	0.1265	0.1273	0.6592	0.7900	1.1600	0.8100	3.3300	0.2372	0.3483	0.2432	0.8288
	n_5	0.1523	0.1313	0.1280	0.4907	0.9400	1.2000	0.8200	2.5100	0.3745	0.4781	0.3267	1.1793
	n_6	0.1490	0.1410	0.1335	0.5040	0.9200	1.2900	0.8500	2.5700	0.3580	0.5019	0.3307	1.1907
	Mean	0.1343	0.1296	0.1268	0.5788	0.8333	1.1850	0.8100	2.9383	0.2892	0.4094	0.2794	0.9780
	S.D.	0.0131	0.0067	0.0047	0.0686	0.0774	0.0606	0.0276	0.3355	0.0609	0.0660	0.0401	0.1659
	%RSD	9.7744	5.1482	3.6755	11.8469	9.2848	5.1123	3.4035	11.4168	21.0515	16.1186	14.3411	16.9622
Treat	n_1	0.1397	0.1195	0.1047	0.8331	0.8700	1.0900	0.6800	4.1900	0.2076	0.2601	0.1623	0.6301
	n_2	0.1192	0.0977	0.1112	0.7399	0.7400	0.9000	0.7100	3.7300	0.1984	0.2413	0.1903	0.6300
	n_3	0.1254	0.1028	0.1081	0.7361	0.7800	0.9400	0.7000	3.7100	0.2102	0.2534	0.1887	0.6523
	n_4	0.1250	0.1347	0.1026	1.0127	0.7800	1.0300	0.6600	5.0700	0.1538	0.2032	0.1302	0.4872
	n_5	0.1326	0.1091	0.1476	0.7160	0.8200	1.0000	0.9300	3.6100	0.2271	0.2770	0.2576	0.7618
	n_6	0.1367	0.1065	0.1116	0.9077	0.8500	0.9800	0.7200	4.5500	0.1868	0.2154	0.1582	0.5604
	Mean	0.1298	0.1117	0.1143	0.8242	0.8067	0.9900	0.7333	4.1433	0.1973	0.2417	0.1812	0.6203
	S.D.	0.0078	0.0134	0.0167	0.1176	0.0489	0.0669	0.0987	0.5778	0.0252	0.0279	0.0435	0.0922
	%RSD	6.0395	12.0195	14.6057	14.2692	6.0562	6.7609	13.4625	13.9456	12.7481	11.5558	24.0107	14.8688

Table A5.2. Data of metabolic ratio for Gr. 2 (PEITC dose 2 mg/kg + CF dose 10 mg/kg)

Gr. 2	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.0953	0.0846	0.0820	0.4479	0.6000	0.7800	0.4400	2.3000	0.2609	0.3391	0.1913	0.7913
	n_2	0.1298	0.1335	0.1084	0.5811	0.8100	1.2200	0.5700	2.9500	0.2746	0.4136	0.1932	0.8814
	n_3	0.1358	0.1384	0.1478	0.4671	0.8400	1.2600	0.7600	2.3900	0.3515	0.5272	0.3180	1.1967
	n_4	0.1592	0.1583	0.1183	0.6224	0.9900	1.4400	0.6200	3.1500	0.3143	0.4571	0.1968	0.9683
	n_5	0.1326	0.1409	0.1063	0.5380	0.8200	1.2900	0.5600	2.7400	0.2993	0.4708	0.2044	0.9745
	n_6	0.1725	0.1715	0.1496	0.6754	1.0700	1.5600	0.7700	3.4100	0.3138	0.4575	0.2258	0.9971
	Mean	0.1375	0.1379	0.1188	0.5553	0.8550	1.2583	0.6200	2.8233	0.3024	0.4442	0.2216	0.9682
	S.D.	0.0267	0.0297	0.0261	0.0886	0.1631	0.2666	0.1270	0.4326	0.0322	0.0631	0.0489	0.1356
	%RSD	19.4028	21.5420	21.9928	15.9471	19.0719	21.1839	20.4782	15.3208	10.6518	14.2084	22.0556	14.0026
Treat	n_1	0.1438	0.1265	0.1092	0.6944	0.8900	1.1600	0.5700	3.5100	0.2536	0.3305	0.1624	0.7464
	n_2	0.1057	0.1130	0.1076	0.8704	0.6600	1.0400	0.5700	4.3700	0.1510	0.2380	0.1304	0.5195
	n_3	0.0970	0.1101	0.1174	0.9511	0.6100	1.0100	0.6100	4.7700	0.1279	0.2117	0.1279	0.4675
	n_4	0.1077	0.1011	0.1233	0.9590	0.6700	0.9300	0.6400	4.8100	0.1393	0.1933	0.1331	0.4657
	n_5	0.1071	0.1347	0.0994	0.8035	0.6700	1.2300	0.5200	4.0400	0.1658	0.3045	0.1287	0.5990
	n_6	0.1178	0.1267	0.0977	1.2411	0.7300	1.1600	0.5200	6.1900	0.1179	0.1874	0.0840	0.3893
	Mean	0.1132	0.1187	0.1091	0.9199	0.7050	1.0883	0.5717	4.6150	0.1593	0.2442	0.1277	0.5312
	S.D.	0.0164	0.0126	0.0100	0.1857	0.0983	0.1130	0.0479	0.9115	0.0492	0.0600	0.0251	0.1261
	%RSD	14.4804	10.6451	9.1517	20.1915	13.9484	10.3860	8.3831	19.7513	30.8830	24.5530	19.6478	23.7415

Table A5.3. Data of metabolic ratio for Gr. 3 (PEITC dose 10 mg/kg + CF dose 10 mg/kg)

Gr. 3	n	Peak area ratio (AU)				Concentration (μg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.1399	0.1378	0.0980	0.6168	0.8700	1.2600	0.6400	3.1300	0.2780	0.4026	0.2045	0.8850
	n_2	0.1085	0.1075	0.0820	0.9131	0.6800	0.9900	0.5400	4.5800	0.1485	0.2162	0.1179	0.4825
	n_3	0.1391	0.1350	0.1053	0.5423	0.8600	1.2300	0.6800	2.7600	0.3116	0.4457	0.2464	1.0036
	n_4	0.0967	0.0926	0.0811	0.6082	0.6100	0.8500	0.5300	3.0800	0.1981	0.2760	0.1721	0.6461
	n_5	0.1085	0.1089	0.0886	0.5803	0.6800	1.0000	0.5800	2.9500	0.2305	0.3390	0.1966	0.7661
	n_6	0.1193	0.1316	0.0968	0.7295	0.7400	1.2000	0.6300	3.6800	0.2011	0.3261	0.1712	0.6984
	Mean	0.1187	0.1189	0.0920	0.6650	0.7400	1.0883	0.6000	3.3633	0.2279	0.3342	0.1848	0.7470
	S.D.	0.0177	0.0184	0.0097	0.1368	0.1053	0.1651	0.0597	0.6708	0.0591	0.0831	0.0428	0.1832
	%RSD	14.8830	15.4951	10.4954	20.5652	14.2245	15.1696	9.9443	19.9457	25.9063	24.8552	23.1396	24.5217
Treat	n_1	0.0945	0.1075	0.0738	0.9041	0.5900	0.9900	0.4900	4.5400	0.1300	0.2181	0.1079	0.4559
	n_2	0.0767	0.0895	0.0693	1.1558	0.4900	0.8200	0.4600	5.7700	0.0849	0.1421	0.0797	0.3068
	n_3	0.0882	0.0912	0.0733	1.0383	0.5600	0.8400	0.4900	5.2000	0.1077	0.1615	0.0942	0.3635
	n_4	0.0506	0.0565	0.0417	1.0191	0.3300	0.5300	0.3000	5.1000	0.0647	0.1039	0.0588	0.2275
	n_5	0.0718	0.0796	0.0593	0.6733	0.4600	0.7400	0.4000	3.4000	0.1353	0.2176	0.1176	0.4706
	n_6	0.0467	0.0529	0.0405	0.7011	0.3000	0.4900	0.2900	3.5400	0.0847	0.1384	0.0819	0.3051
	Mean	0.0714	0.0795	0.0596	0.9153	0.4550	0.7350	0.4050	4.5917	0.1012	0.1636	0.0900	0.3549
	S.D.	0.0194	0.0213	0.0153	0.1941	0.1184	0.1925	0.0914	0.9535	0.0279	0.0459	0.0212	0.0946
	%RSD	27.2263	26.7300	25.6171	21.2048	26.0326	26.1953	22.5626	20.7656	27.5885	28.0728	23.5403	26.6461

Table A5.4. Data of metabolic ratio for Gr. 4 (PEITC dose 20 mg/kg + CF dose 10 mg/kg)

Gr. 4	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n₁	0.1308	0.1493	0.1096	0.6303	0.8100	1.3600	0.7000	3.1900	0.2539	0.4263	0.2194	0.8997
	n₂	0.1128	0.1200	0.0912	0.6722	0.7000	1.1000	0.5900	3.4000	0.2059	0.3235	0.1735	0.7029
	n₃	0.1221	0.1251	0.0989	0.4866	0.7600	1.1500	0.6400	2.4900	0.3052	0.4618	0.2570	1.0241
	n₄	0.1297	0.1295	0.1042	0.5332	0.8100	1.1800	0.6700	2.7100	0.2989	0.4354	0.2472	0.9815
	n₅	0.1264	0.1190	0.0965	0.6578	0.7900	1.0900	0.6300	3.3300	0.2372	0.3273	0.1892	0.7538
	n₆	0.0929	0.0952	0.0706	0.7063	0.5800	0.8800	0.4700	3.5600	0.1629	0.2472	0.1320	0.5421
	Mean	0.1191	0.1230	0.0952	0.6144	0.7417	1.1267	0.6167	3.1133	0.2440	0.3703	0.2031	0.8174
	S.D.	0.0144	0.0175	0.0136	0.0859	0.0893	0.1554	0.0809	0.4209	0.0546	0.0836	0.0474	0.1840
	%RSD	12.1084	14.2409	14.2600	13.9736	12.0421	13.7922	13.1208	13.5189	22.3956	22.5800	23.3506	22.5170
Treat	n₁	0.0710	0.0877	0.0588	0.6741	0.4500	0.8100	0.4000	3.4100	0.1320	0.2375	0.1173	0.4868
	n₂	0.0559	0.0661	0.0457	0.8645	0.3600	0.6100	0.3200	4.3400	0.0829	0.1406	0.0737	0.2972
	n₃	0.0656	0.0759	0.0554	0.7507	0.4200	0.7000	0.3800	3.7800	0.1111	0.1852	0.1005	0.3968
	n₄	0.0536	0.0541	0.0406	0.5536	0.3500	0.5100	0.2900	2.8200	0.1241	0.1809	0.1028	0.4078
	n₅	0.0600	0.0652	0.0491	0.5730	0.3800	0.6100	0.3400	2.9100	0.1306	0.2096	0.1168	0.4570
	n₆	0.0564	0.0585	0.0444	0.8577	0.3600	0.5500	0.3100	4.3100	0.0835	0.1276	0.0719	0.2831
	Mean	0.0604	0.0679	0.0490	0.7123	0.3867	0.6317	0.3400	3.5950	0.1107	0.1802	0.0972	0.3881
	S.D.	0.0066	0.0122	0.0069	0.1355	0.0398	0.1085	0.0424	0.6640	0.0225	0.0413	0.0201	0.0828
	%RSD	10.9899	17.9747	14.1432	19.0236	10.3016	17.1800	12.4784	18.4704	20.3434	22.8989	20.6909	21.3238

Table A5.5. Data of metabolic ratio for Gr. 8 (dry watercress dose 800 mg/kg + CF dose 10 mg/kg)

Gr. 8	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.1251	0.1296	0.1075	0.8675	0.7784	1.1851	0.6926	4.3565	0.1787	0.2720	0.1590	0.6097
	n_2	0.1025	0.1162	0.0881	0.7992	0.6414	1.0647	0.5753	4.0210	0.1595	0.2648	0.1431	0.5674
	n_3	0.0934	0.0983	0.0808	0.6127	0.5862	0.9036	0.5317	3.1051	0.1888	0.2910	0.1712	0.6510
	n_4	0.0966	0.0962	0.0788	0.6631	0.6058	0.8844	0.5194	3.3527	0.1807	0.2638	0.1549	0.5994
	n_5	0.1030	0.0981	0.0874	0.8196	0.6446	0.9021	0.5713	4.1216	0.1564	0.2189	0.1386	0.5139
	n_6	0.1068	0.1120	0.0844	0.6861	0.6678	1.0269	0.5535	3.4656	0.1927	0.2963	0.1597	0.6487
	Mean	0.1045	0.1084	0.0878	0.7414	0.6540	0.9945	0.5740	3.7371	0.1761	0.2678	0.1544	0.5983
	S.D.	0.0111	0.0133	0.0103	0.1011	0.0676	0.1194	0.0621	0.4966	0.0150	0.0275	0.0119	0.0520
	%RSD	10.6559	12.2356	11.7366	13.6374	10.3299	12.0030	10.8133	13.2879	8.5234	10.2787	7.7199	8.6951
Treat	n_1	0.1257	0.1230	0.0983	0.8652	0.7823	1.1261	0.6367	4.3450	0.1801	0.2592	0.1465	0.5858
	n_2	0.0816	0.0860	0.0643	0.8136	0.5151	0.7929	0.4322	4.0918	0.1259	0.1938	0.1056	0.4253
	n_3	0.1161	0.0973	0.0977	0.9478	0.7240	0.8950	0.6336	4.7511	0.1524	0.1884	0.1334	0.4741
	n_4	0.1094	0.0850	0.0884	0.7531	0.6833	0.7843	0.5776	3.7945	0.1801	0.2067	0.1522	0.5390
	n_5	0.0880	0.0693	0.0659	0.8102	0.5534	0.6422	0.4420	4.0750	0.1358	0.1576	0.1085	0.4019
	n_6	0.1070	0.0898	0.0725	0.6767	0.6691	0.8272	0.4816	3.4195	0.1957	0.2419	0.1409	0.5784
	Mean	0.1046	0.0917	0.0812	0.8111	0.6545	0.8446	0.5340	4.0795	0.1616	0.2079	0.1312	0.5007
	S.D.	0.0168	0.0179	0.0156	0.0928	0.1018	0.1608	0.0937	0.4557	0.0278	0.0371	0.0197	0.0786
	%RSD	16.0477	19.4786	19.1786	11.4403	15.5571	19.0427	17.5567	11.1717	17.2032	17.8637	15.0402	15.6995

Appendix-6 Data of metabolic ratio of sample collection for multiple doses

Table A6.1. Data of metabolic ratio for Gr. 5 (PEITC dose 2 mg/kg + CF dose 10 mg/kg)

Gr. 5	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.1535	0.1351	0.1121	0.4357	0.9510	1.2352	0.7199	2.2360	0.4253	0.5524	0.3220	1.2997
	n_2	0.1143	0.0978	0.1270	0.4982	0.7132	0.8989	0.8096	2.5427	0.2805	0.3535	0.3184	0.9524
	n_3	0.1696	0.1617	0.1183	0.2893	1.0486	1.4739	0.7571	1.5167	0.6914	0.9718	0.4992	2.1623
	n_4	0.1303	0.1218	0.1062	0.3895	0.8104	1.1155	0.6845	2.0088	0.4034	0.5553	0.3408	1.2995
	n_5	0.1097	0.1008	0.1007	0.3358	0.6853	0.9260	0.6512	1.7449	0.3927	0.5307	0.3732	1.2966
	n_6	0.1414	0.1355	0.0971	0.2588	0.8777	1.2388	0.6300	1.3670	0.6421	0.9062	0.4609	2.0091
	Mean	0.1365	0.1254	0.1102	0.3679	0.8477	1.1480	0.7087	1.9027	0.4726	0.6450	0.3857	1.5033
	S.D.	0.0231	0.0241	0.0112	0.0907	0.1399	0.2165	0.0674	0.4456	0.1593	0.2407	0.0765	0.4731
	%RSD	16.8982	19.1774	10.1608	24.6608	16.4993	18.8617	9.5134	23.4194	33.7091	37.3160	19.8425	31.4739
Treat	n_1	0.0625	0.0683	0.2456	0.9691	0.3991	0.6335	1.5239	4.8557	0.0822	0.1305	0.3138	0.5265
	n_2	0.0417	0.0464	0.0381	0.5950	0.2730	0.4361	0.2744	3.0184	0.0905	0.1445	0.0909	0.3259
	n_3	0.0949	0.0929	0.0684	0.6811	0.5956	0.8551	0.4567	3.4408	0.1731	0.2485	0.1327	0.5543
	n_4	0.0492	0.0612	0.1124	0.4639	0.3184	0.5698	0.7219	2.3742	0.1341	0.2400	0.3041	0.6782
	n_5	0.0763	0.0903	0.0744	0.7618	0.4828	0.8316	0.4933	3.8372	0.1258	0.2167	0.1285	0.4711
	n_6	0.1654	0.1723	0.0826	0.3523	1.0228	1.5694	0.5426	1.8260	0.5602	0.8595	0.2972	1.7168
	Mean	0.0817	0.0885	0.1036	0.6372	0.5153	0.8159	0.6688	3.2254	0.1943	0.3066	0.2112	0.7121
	S.D.	0.0452	0.0447	0.0736	0.2194	0.2742	0.4019	0.4430	1.0774	0.1822	0.2753	0.1039	0.5054
	%RSD	55.3717	50.4253	71.0353	34.4275	53.2213	49.2571	66.2393	33.4052	93.7649	89.7783	49.2058	70.9720

Table A6.2. Data of metabolic ratio for Gr. 6 (PEITC dose 10 mg/kg + CF dose 10 mg/kg)

Gr. 6	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.1169	0.0777	0.0636	0.4592	0.7291	0.7186	0.4284	2.3513	0.3101	0.3056	0.1822	0.7979
	n_2	0.0996	0.0887	0.0733	0.2280	0.6242	0.8176	0.4864	1.2155	0.5135	0.6727	0.4001	1.5863
	n_3	0.0866	0.0721	0.0682	0.1914	0.5450	0.6676	0.4559	1.0359	0.5261	0.6445	0.4401	1.6107
	n_4	0.1108	0.1125	0.0891	0.5639	0.6922	1.0315	0.5816	2.8656	0.2416	0.3600	0.2030	0.8045
	n_5	0.1066	0.0945	0.0865	0.3018	0.6664	0.8698	0.5660	1.5782	0.4223	0.5511	0.3587	1.3321
	n_6	0.0744	0.0649	0.0400	0.1054	0.4709	0.6026	0.2863	0.6134	0.7677	0.9824	0.4667	2.2168
	Mean	0.0992	0.0851	0.0701	0.3083	0.6213	0.7846	0.4674	1.6100	0.4635	0.5860	0.3418	1.3914
	S.D.	0.0160	0.0172	0.0178	0.1729	0.0971	0.1552	0.1073	0.8492	0.1863	0.2445	0.1214	0.5418
	%RSD	16.1450	20.2725	25.4188	56.0805	15.6249	19.7841	22.9633	52.7443	40.1819	41.7269	35.5205	38.9382
Treat	n_1	0.0407	0.0530	0.0452	1.0408	0.2667	0.4956	0.3175	5.2079	0.0512	0.0952	0.0610	0.2073
	n_2	0.1050	0.1189	0.0922	0.7626	0.6570	1.0887	0.6000	3.8416	0.1710	0.2834	0.1562	0.6106
	n_3	0.0636	0.1125	0.0799	0.8381	0.4059	1.0314	0.5261	4.2124	0.0964	0.2449	0.1249	0.4661
	n_4	0.0888	0.1038	0.0768	0.8446	0.5587	0.9528	0.5075	4.2441	0.1316	0.2245	0.1196	0.4757
	n_5	0.1125	0.1255	0.0989	0.8979	0.7022	1.1487	0.6408	4.5057	0.1558	0.2549	0.1422	0.5530
	n_6	0.1011	0.1212	0.1052	0.6510	0.6334	1.1096	0.6788	3.2933	0.1923	0.3369	0.2061	0.7354
	Mean	0.0853	0.1058	0.0830	0.8392	0.5373	0.9711	0.5451	4.2175	0.1331	0.2400	0.1350	0.5080
	S.D.	0.0278	0.0270	0.0215	0.1307	0.1683	0.2428	0.1293	0.6419	0.0520	0.0809	0.0477	0.1774
	%RSD	32.5400	25.4930	25.8648	15.5736	31.3281	24.9968	23.7223	15.2199	39.0785	33.7238	35.3380	34.9293

Table A6.3. Data of metabolic ratio for Gr. 7 (PEITC dose 20 mg/kg + CF dose 10 mg/kg)

Gr. 7	n	Peak area ratio (AU)				Concentration (µg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.0870	0.1301	0.1011	0.5044	0.5478	1.1896	0.6536	2.5734	0.2129	0.4623	0.2540	0.9291
	n_2	0.1333	0.1089	0.0960	0.3779	0.8282	0.9995	0.6229	1.9518	0.4243	0.5121	0.3192	1.2556
	n_3	0.0955	0.1084	0.0975	0.5134	0.5991	0.9942	0.6324	2.6173	0.2289	0.3799	0.2416	0.8504
	n_4	0.0861	0.0798	0.0695	0.5346	0.5421	0.7372	0.4633	2.7215	0.1992	0.2709	0.1702	0.6403
	n_5	0.0836	0.0881	0.0678	0.5567	0.5268	0.8119	0.4531	2.8300	0.1861	0.2869	0.1601	0.6331
	n_6	0.0921	0.0985	0.0788	0.6555	0.5785	0.9054	0.5197	3.3152	0.1745	0.2731	0.1568	0.6044
	Mean	0.0963	0.1023	0.0851	0.5237	0.6037	0.9396	0.5575	2.6682	0.2377	0.3642	0.2170	0.8188
	S.D.	0.0186	0.0177	0.0149	0.0898	0.1130	0.1597	0.0898	0.4410	0.0934	0.1046	0.0655	0.2515
	%RSD	19.3656	17.3426	17.5269	17.1438	18.7237	16.9937	16.1073	16.5284	39.3183	28.7237	30.1939	30.7135
Treat	n_1	0.0496	0.0560	0.0370	0.6887	0.3210	0.5226	0.2681	3.4784	0.0923	0.1503	0.0771	0.3196
	n_2	0.0496	0.0511	0.0396	0.5742	0.3208	0.4792	0.2834	2.9160	0.1100	0.1643	0.0972	0.3715
	n_3	0.0560	0.0802	0.0582	0.5787	0.3595	0.7405	0.3955	2.9382	0.1223	0.2520	0.1346	0.5090
	n_4	0.0604	0.0653	0.0495	0.5782	0.3861	0.6068	0.3429	2.9355	0.1315	0.2067	0.1168	0.4551
	n_5	0.0797	0.0795	0.0631	0.8419	0.5033	0.7343	0.4251	4.2309	0.1190	0.1736	0.1005	0.3930
	n_6	0.0530	0.0652	0.0580	0.6571	0.3417	0.6062	0.3945	3.3230	0.1028	0.1824	0.1187	0.4040
	Mean	0.0581	0.0662	0.0509	0.6531	0.3721	0.6149	0.3516	3.3037	0.1130	0.1882	0.1075	0.4087
	S.D.	0.0114	0.0119	0.0107	0.1042	0.0689	0.1069	0.0646	0.5119	0.0142	0.0365	0.0201	0.0660
	%RSD	19.5723	17.9284	21.0799	15.9572	18.5196	17.3773	18.3728	15.4946	12.5603	19.4082	18.7385	16.1511

Table A6.4. Data of metabolic ratio for Gr. 9 (dry watercress dose 800 mg/kg + CF dose 10 mg/kg)

Gr. 9	n	Peak area ratio (AU)				Concentration (μg/mL)				Metabolic ratio			
		TB	PX	TP	CF	TB	PX	TP	CF	TB/CF	PX/CF	TP/CF	TB+PX+TP/CF
CF alone	n_1	0.1030	0.0979	0.0844	0.7415	0.6449	0.9005	0.5533	3.7377	0.1725	0.2409	0.1480	0.5615
	n_2	0.1189	0.1237	0.0916	0.5482	0.7411	1.1319	0.5964	2.7885	0.2658	0.4059	0.2139	0.8856
	n_3	0.0869	0.0905	0.0881	0.6063	0.5472	0.8336	0.5756	3.0737	0.1780	0.2712	0.1873	0.6365
	n_4	0.0949	0.0943	0.0787	0.5168	0.5953	0.8677	0.5188	2.6342	0.2260	0.3294	0.1970	0.7523
	n_5	0.1001	0.0997	0.0804	0.5064	0.6273	0.9167	0.5291	2.5832	0.2428	0.3549	0.2048	0.8025
	n_6	0.0860	0.0796	0.0680	0.5653	0.5413	0.7352	0.4544	2.8723	0.1885	0.2560	0.1582	0.6026
	Mean	0.0983	0.0976	0.0819	0.5808	0.6162	0.8976	0.5379	2.9483	0.2123	0.3097	0.1849	0.7068
	S.D.	0.0122	0.0146	0.0083	0.0865	0.0740	0.1316	0.0500	0.4248	0.0382	0.0645	0.0263	0.1266
	%RSD	12.4046	14.9817	10.1471	14.8917	12.0017	14.6662	9.2953	14.4079	18.0008	20.8180	14.2335	17.9065
Treat	n_1	0.0751	0.0661	0.0513	0.7098	0.4754	0.6142	0.3543	3.5822	0.1327	0.1715	0.0989	0.4031
	n_2	0.0546	0.0550	0.0532	0.9452	0.3514	0.5137	0.3655	4.7381	0.0742	0.1084	0.0771	0.2597
	n_3	0.0671	0.0995	0.0782	0.7580	0.4269	0.9149	0.5161	3.8186	0.1118	0.2396	0.1351	0.4865
	n_4	0.0718	0.0976	0.0624	0.6644	0.4556	0.8973	0.4205	3.3589	0.1356	0.2671	0.1252	0.5280
	n_5	0.0676	0.1074	0.0754	0.7634	0.4297	0.9854	0.4992	3.8451	0.1117	0.2563	0.1298	0.4978
	n_6	0.0633	0.0925	0.0710	0.6985	0.4040	0.8512	0.4727	3.5265	0.1146	0.2414	0.1340	0.4900
	Mean	0.0666	0.0863	0.0653	0.7565	0.4238	0.7961	0.4380	3.8116	0.1134	0.2140	0.1167	0.4442
	S.D.	0.0071	0.0208	0.0114	0.0997	0.0432	0.1877	0.0687	0.4896	0.0220	0.0616	0.0235	0.0995
	%RSD	10.7077	24.1447	17.4926	13.1756	10.2021	23.5715	15.6894	12.8445	19.3662	28.7788	20.1592	22.4017

VITAE

Name   Miss Angkana Wongsakul

Student ID   5010220168

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Prince of Songkla University	2007

List of Publications and Proceedings

Poster Presentation

Wongsakul, A., Janchawee, B., Prutipanlai, S. Effect of a single oral dose administration of phenethyl isothiocyanate (PEITC) on CYP1A2 activity in rats. 36th Congress on Science and Technology of Thailand, Bangkok, Thailand, 2010.