Effect of *Phyllanthus amarus* Schum. & Thonn. extract on the Pharmacokinetics of Midazolam in Rabbits

Putthaporn Kaewmeesri

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Pharmacology

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

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ชื่อวิทยานิพนธ์ ผลของสารสกัดลูกใต้ใบต่อเภสัชจลนศาสตร์ของยามิดาโซแลมในกระต่าย

ผู้เขียน นางสาวพุทธพร แก้วมีศรี

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ปีการศึกษา 2556

บทคัดย่อ

ลูกใต้ใบเป็นสมุนไพรพื้นบ้านที่ใช้รักษาโรคตับที่มีรายงานว่ามีฤทธิ์ยับยั้งการทำงานของเอนไซม์ CYP3A4 ในหลอดทดลอง วัตถุประสงค์ของการศึกษานี้เพื่อประเมินผลของสารสกัดลูกใต้ใบต่อเภสัชจลนศาสตร์ของยามิดาโซแลมในกระต่าย การศึกษาแบ่งเป็น 2 ช่วง ห่างกัน 7 วัน ใช้กระต่ายจำนวน 9 ตัว โดยในช่วงที่ 1 กระต่ายได้รับยามิดาโซแลม ขนาด 10 มก./กก. ทางปาก เพียงครั้งเดียว และในช่วงที่ 2 ให้สารสกัดลูกใต้ใบขนาด 500 มก./กก. วันละ 1 ครั้ง ทางปากเป็นเวลา 7 วัน และครั้งสุดท้าย 1 ชั่วโมงก่อนได้รับยามิดาโซแลม จากการทดลองพบว่า กระต่ายที่ได้รับสารสกัดลูกใต้ใบ มีค่าความเข้มข้นของยามิดาโซแลม ในพลาสมาสูงสุด (C_{max}), เวลาที่ระดับยาในพลาสมาสูงสุด (T_{max}), พื้นที่ใต้กราฟระหว่างความเข้มข้นของยากับเวลาที่ให้ยา (AUC_{0-8}) และค่าครึ่งชีวิต ($T_{1/2}$) ของยามิดาโซแลม เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) เมื่อเปรียบเทียบกับกระต่ายที่ได้รับยามิดาโซแลมเพียงอย่างเดียว แสดงว่าสารสกัดลูกใต้ใบอาจมีฤทธิ์ยับยั้งการทำงานของ CYP3A ซึ่งเป็นเอนไซม์ที่ใช้ในการแปรรูปยามิดาโซแลมในกระต่ายได้ ดังนั้น การรับประทานลูกใต้ใบร่วมกับยาแผนปัจจุบันบางชนิดที่ถูกแปรรูปโดย CYP3A4 อาจทำให้ระดับยาในเลือดเพิ่มสูงขึ้นจนเกิดอาการข้างเคียงที่รุนแรงได้ จึงควรระวังการเกิดปฏิกิริยาระหว่างยากับสมุนไพรในผู้ป่วย

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Major Program Pharmacology

Academic Year 2013

ABSTRACT

Phyllanthus amarus Schum. & Thonn. has been used for the treatment of liver disease. Previous *in vitro* study demonstrated the inhibitory activity of *P. amarus* extract on CYP3A4, an enzyme responsible for various drug metabolism. The objective of this study was to evaluate the effect of *P. amarus* ethanolic extract on the pharmacokinetics of midazolam, a CYP3A4 probe drug, in rabbits. The study was an open-label, randomized, two-phase design with one week washout period. Nine male rabbits received multiple-dose of *P. amarus* extract 500 mg/kg once a day orally for 7 days and the last dose at 1 h before midazolam administration. Midazolam plasma concentration time-profiles were characterized after a single oral dose of 10 mg/kg midazolam on the day before and after *P. amarus* administration. The results showed that pretreatment with *P. amarus* significantly increased the mean maximum plasma concentration (C_{max}), time to reach maximum concentration (T_{max}), area under the drug concentration-time curves (AUC_{0-8}) and half-life ($T_{1/2}$) of midazolam compared with control group receiving a single oral dose of midazolam. The results suggest that *P. amarus* extract inhibits CYP3A, which is the enzyme responsible for midazolam metabolism in rabbits. Thus, coadministration of *P. amarus* and CYP3A4 substrates may increase plasma drug concentration leading to serious side effects. Clinical relevance of herb-drug interaction between *P. amarus* and CYP3A substrates should be warranted. Further human investigation is needed to reveal the clinical significant potential of these herb-drug interactions

ACNOWLEDGEMENTS

I would like to express my sincere grateful thanks and sincere appreciation to my advisor, Associate Professor Malinee Wongnawa, for her helpful suggestions, valuable guidance and comments throughout my study. Sincere appreciation is extended to Associate Professor Werawath Mahatthanatrakul, M.D., my co-advisors, for his valuable contributions and advice relating to this study.

I would like to thank to Associate Professor Wibool Ridtitid, M.D. and Associate Professor Dr. Payom Wongpoowarak, for their comment and advice relating to this study

I would like to express my grateful thank to Assistant Professor Suwit Ruengkittisakul, M.D., for HPLC equipment supported and Mr. Wirachai Samai, M.D., for the valuable suggestions and advice. I am so grateful thank for kindness and support from the staff working at the Forensic Medicine and Toxicology Unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University.

I am very grateful to Mr. Somchai Sriwiriyajan, a lecturer of the department of Pharmacology, for his kindness, valuable suggestions and guidance with a warm encouragement throughout my study. I would like to thank Associate Professor Tanomjit Supavita, for identified of plant material, and also thank to Dr. Wanida Sukketsiri for standard Phyllanthin and Hypophyllanthin.

I would like to express my honest thanks to Miss Potjanee Soontaro for her kindly and helpful advice during my entire study. This research was financial supported by Faculty of Science, Prince of Songkla University (annual budget of 2011) and Faculty of Graduate School, Prince of Songkla University.

Finally, I want to thank my beloved, father and mother, including my brother and all my friends for their support and encouragement.

Putthaporn Kaewmeesri

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LIST OF ABBREVIATIONS AND SYMBOLS

AUC = Area under the concentration-time curve

°C = Degree Celsius

C_{max} = Maximum plasma concentration

CL/F = Apparent clearance

cm = Centimeter

μm = Micrometer

CV = Coefficient of variation

EC_{50} = Median Effective Concentration

g = Gram

h = Hour

IC_{50} = Median Inhibition Concentration

ID_{50} = Median Inhibitory dose

K_i = The inhibition constant

K_m = The affinity of the substrate for the enzyme

Kg = Kilogram

L = Liter

mg = Milligram

min = Minute

mL = Milliliter

mm = Millimeter

MRT = Mean residence time

MW = Molecular weight

μg = Microgram

μL = Micro liter

μm = Micrometer

ng = Nanogram

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

r^2 = Correlation coefficient

SD = Standard diviation

$T_{1/2}$ = Half-life

T_{max} = Time to maximum concentration

V_z /F = Apparent volume of distribution

v/v/v = Volume by volume by volume

λ_z = Elimination rate constant

∞ = Infinite

CHAPTER 1

INTRODUCTION

Millions of people today use medicinal plants along with prescription and nonprescription drugs. Although considered natural, many of these herb can interact with other drugs, causing either potentially dangerous side effects and/or reduced benefits from the medications. Currently, there is very little information published on herb-drug interactions whilst the use of herbs is progressively growing across the world. In Thailand, the interests in using herbal medicines and traditional Thai medicines have been increasing during the past decade. The Thai government began to support an attempt to develop herbal medicines and Thai herbal drugs systematically (Satyapon, 2010), but the report concerning about Thai herb-drug interaction are still lacking. Thus, it is necessary to study the interaction between herbs and drugs to prevent possible serious adverse effects or failure of therapy with conventional medicines.

As with conventional medicines, herbal medicines interact with drugs in two general ways: pharmacokinetically and pharmacodynamically. Pharmacokinetic interactions result in alterations of the drug's or herb absorption, distribution, metabolism or elimination. These interactions affect drug action by quantitative alterations, either increasing or decreasing the amount of drug available to have an effect. The interfering drug may act as an inducer, inhibitor and/or substrate of the cytochrome P450 enzyme that is responsible for the metabolism of the respective drugs. This is the most important mechanism for interactions between herbal therapies and drugs (Roger, 2004). St John’s wort is a clear sample for CYP inducer herb, widely prescribed for various psychopathologic conditions involving depression and anxiety (Cupp, 1999). It is reported to lower serum concentrations of cyclosporine (Rey and Welter, 1998), theophyllin (Nebel *et al*., 1999), warfarin, oral

contraceptives (Hall *et al*., 2003), digoxin (Scott and Elmer, 2002), indinavir (Pisctelli *et al*., 2000) and clopidogrel (Lau et al., 2005) by inducing CYP 3A4, CYP 2C9 and CYP1A2 (Cozza *et al*., 2003).

Phyllanthus amarus Schum. & Thonn., belonging to the family Euphorbiaceae, is a medicinal plant that has been used in traditional Thai medicine for treatment of fever, jaundice, ascites, hemorrhoid and diabetes (Pongboonrod, 1976). Several pharmacological activities of *P. amarus* have been reported including antiamnesic, antibacterial, antifungal, antiviral, anticancer, anti-diarrheal, gastroprotective, antiulcer, analgesic, anti-inflammatory, antioxidant, diuretic, antiplasmodial, aphrodisiac, contraceptive, antihypertensive, hypoglycemic, hypocholesterolemic, immunomodulatory, nephroprotective, radioprotective and spasmolytic activities (Patel *et al*., 2011), hepatoprotective against ethanol-, paracetamol- and carbon-tetrachloride-toxicity (Pramyothin *et al*., 2007, Wongnawa *et al*., 2006, Krithika and Verma 2009). The secondary metabolites present in *P. amarus* are alkaloids, flavonoids, hydrolysable tannins (ellagitannins), major lignans, polyphenols, triterpines, sterols and volatile oil (Patel *et al*., 2011). An alcoholic extract of *P. amarus* was found to inhibit some cytochrome P450 isozymes (CYP 1A1, 1A2, 2B1/2, 2E1) which are responsible for activation of various procarcinogens both *in vivo* as well as *in vitro* suggesting its inhibitory mechanisms of action on carcinogenesis (Kumar and Kuttan, 2006). Moreover, Taesotikul *et al*. (2011) have reported the inhibitory potency of the ethanolic and aqueous extract of *P. amarus* on CYP3A4 activity *in vitro* in human liver microsome which was about 2-3 orders of magnitude stronger than the known CYP3A4 inhibitors such as erythromycin and clarithromycin suggesting its potential to cause herb-drug interaction since CYP 3A4 is an enzyme responsible for metabolism of various drugs. Previous studies have demonstrated that inhibition of CYP3A4 increased plasma concentration of drugs which are CYP 3A4 substrate leading to serious side effects or toxicity (Lilja *et al*, 2000). However, studies involving interaction between *P. amarus* and drugs metabolized with CYP3A4 *in vivo* are rarely reported.

Midazolam, a short-acting benzodiazepine used as a sedative-hypnotic drug, is a common CYP3A probe drug recommended by the US Food and Drug Administration (FDA). It has been extensively employed in the *in vitro* and *in vivo* CYP3A enzyme inhibition and/or induction assays (Bjornsson *et al*., 2003). The drug is not a substrate of P-glycoprotein (Kim *et al*., 1999) and is metabolized to a comparable extent by both intestinal and hepatic CYP3A, making it possible to study effects of CYP3A in both the intestine and the liver (Chung *et al*., 2006).

Therefore, this study was aimed to investigate the effect of *P. amarus* on the pharmacokinetics of midazolam, a CYP3A substrate, in rabbits in order to reveal the possibility of herb-drug interaction between *P. amarus* and midazolam *in vivo*.

CHAPTER 2

LITERATURE REVIEWS

2.1 *Phyllanthus amarus* Schum. & Thonn.

1.1 Description of the plant

Phyllanthus amarus Schum. & Thonn. (Fig. 1), is an annual, glabrous herb grows upto 10-60 cm high. It has an erect, stem terete, younger parts rough, cataphylls 1.5-1.9 mm long, deltoid acuminate. Leaves are green, 3.0-11.0 x 1.5-6.0 mm in size, elliptic oblong to obvate, obtuse or minutely apiculate at apex, obtuse or slightly inequilateral at base. Flowers axillary, proximal 2-3 axils with unisexual 1-3 male flowers and all succeeding axils with bisexual cymules. Male flowers - pedicel 1 mm long, calyx 5, sub equal 0.7 x 0.3 mm, oblong, elliptic, apex acute, hyaline with unbranched mid rib; disc segments 5, rounded, stamens 3, filaments connate. Female flowers-pedicel 0.8-1.0 mm long, calyx lobes 5, 0.6 x 0.25 mm, ovate-oblong, acute at apex; disc flat deeply 5 lobed, lobes often toothed at apex, styles 3, free, shallowly bifid at apex. Capsule 1.8 mm in diameter, oblate and rounded, seeds about 0.9 mm long, triangular with 6-7 longitudinal ribs and many transverse striations on the back (Bagchi *et al*., 1992). It is widespread throughout the tropics and subtropics in sandy regions as a weed in cultivated and wasteland (Ross, 1999).

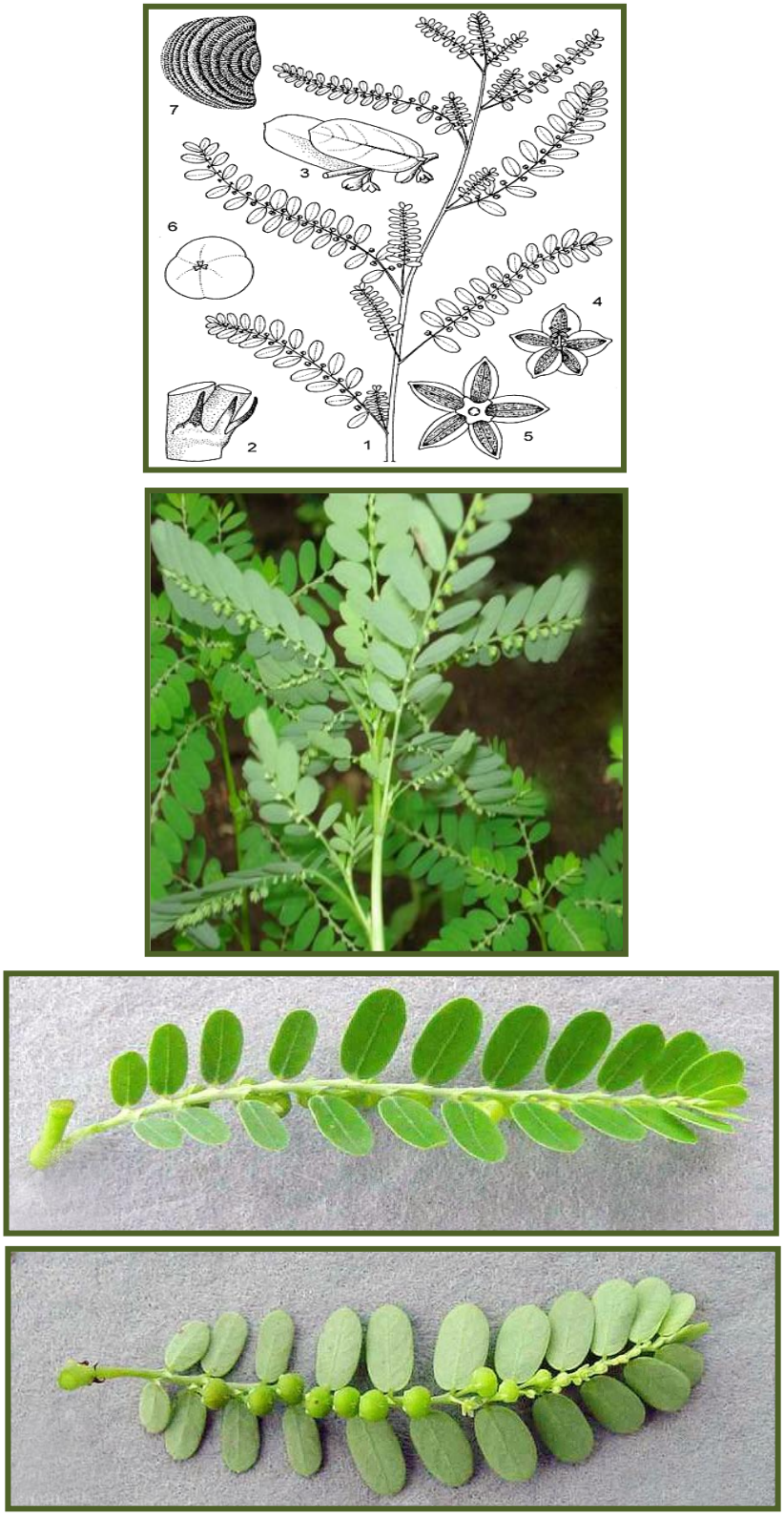


Fig. 1 Morphology of *Phyllanthus amarus* Schum. & Thonn. (from: http://www.tramil.net/fototeca/imageDisplay.php?id_elem=275; accessed August15, 2013)

1.2 Chemical constituents

P. amarus contains lignans such as phyllanthin and hypophyllanthin; major compounds (Sharma *et al*., 1993, Somanabandhu *et al*., 1993), geraniin and 5 flavoniods (quercertin, astralgin, quercertrin, isoquercitrin and rutin). It also contains minor compounds which are hydrolysable tannins such as phyllanthusiin D (Houghton *et al*., 1996), amariin (Foo, 1993) amarulone (Rao and Bramley, 1971), amarinic acid and alkaloids such as ent-norsecurinine, sobubbialine, epibubbialine; diarylbutane, nyrphyllin and a neolignan, phyllnirurin. The structures of major lignans founded in *P. amarus* are shown in Fig. 2.

The phyllanthin (bitter constituent) and hypophyllanthin (non-bitter compound) are soluble in methanol, ethanol and chloroform but slightly dissolved in water. Phyllanthin has a melting point of 196 - 198°C and a molecular weight of 418.53. Hypophyllanthin has a melting point of 128°C - 129°C and a molecular weight of 430.40 (Nair and Abraham 2008). There are many general techniques to analyze phyllanthin, hypophyllanthin such as thin-layer chromatography (Tripathi *et al.*, 2006), NMR characterization technique (Kaiser, 2007) and column chromatography.

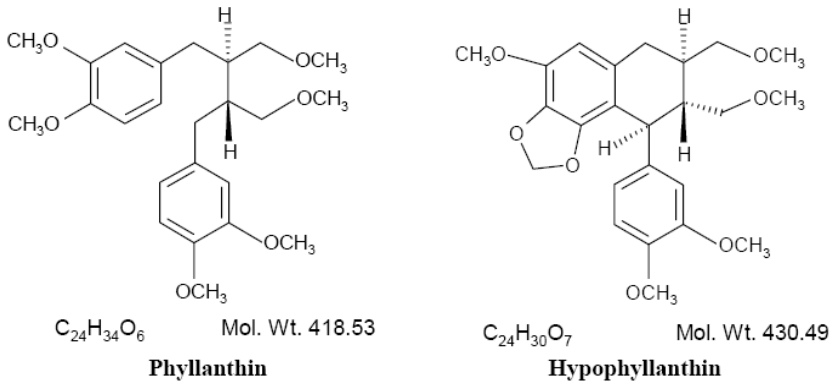


Fig. 2 Chemical structures of the major lignans in *P. amarus*

1.3 Pharmacological activities

1.3.1 Hepatoprotective

Phyllanthin has protective effect on ethanol induced rat liver cell injury. Primary cultures of rat hepatocytes (24 h culturing) were pretreated with phyllanthin (1- 4 µg/mL) for 24 h. After 24 h pretreatment, cells were treated with ethanol (80µL/mL) for 2 h. Ethanol decreased %MTT, increased the release of ALT and AST, the production of intracellular reactive oxygen species and lipid peroxidation. Phyllanthin demonstrated its role in protection by antagonizing the above effect induced by ethanol and also restored the antioxidant capability of rat hepatocytes including level of total glutathione, and activities of superoxide dismutases and glutathione reductase which were reduced by ethanol (Chirdchupunseree and Pramyothin, 2010).

The methanol extract of *P. amarus* leaves (50-800 mg/kg) total cholesterol, AST, ALT, urea, uric acid, total protein, prostatic alkaline and acid phosphatase. The highest reduction effect was obtained with uric acid at 400 mg/kg of *P. amarus* extract while the least effect was observed in total cholesterol. These effects were dose- and time-dependent. This shows that the leaves of *P. amarus* have hepatoprotective, nephroprotective and cardioprotective properties (Obianime and Uche, 2008). Hot water extracts of *P. amarus* (0.8, 1.6 or 3.2 g/kg) were orally administered b.i.d. for 7 days prior, 2 days after, or 7 days prior and 2 days after single oral dose of paracetamol (3 g/kg). The extract at 1.6 and 3.2 g/kg decreased the paracetamol-induced hepatotoxicity as indicated by the decrease in SGOT and SGPT, bilirubin and histopathological score while the ALP did not change. It was evident that the hepatoprotective mechanism of this plant was neither related to inhibition on cytochrome P450, nor induction on sulfate and glucuronide conjugation pathways of paracetamol, but partly due to the antioxidant activity and the protective effect on the decrease of hepatic reduced glutathione (Wongnawa *et al.*, 2005).

P. amarus powder (200 mg/rat/day) given for 45 days after 30 days pretreatment with ethanol in rats reduced the increased deposition of triglyceride, phospholipids are reduced in liver, brain, kidney and heart. (Tripathi *et al*., 1992).

Administration of whole plant powder for 7 days at dosage of 35 and 70 mg/kg body weight helped in restoring the levels of biochemical parameter within 48 h. in calves (Sane *et al*., 1995).

1.3.2 Antioxidant activity

The total phenolic content and antioxidant activity of fresh and dried *P. amarus* were evaluated by Flin-Ciocalteau method, 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and ferric radical antioxidant power assays. Different drying treatments led to significant reducing in antioxidant properties of *P. amarus* methanolic extracts, with microwave drying causing the highest decrease in total phenolic content and antioxidant activities, the boiling water extracts appeared to exhibit stronger antioxidant potentials. These provide for its strong free radical scavenging activity (Lim and Murtijaya, 2007).

The antioxidant activity of methanolic extracts of five species including *P. debilis, P. urinaria, P. virgatus, P. maderaspatensis, P. amarus* from the genus Phyllanthus was evaluated by various antioxidant assays. All the extracts at the concentration of 50 µg/mL showed strong antioxidant activity in all the tested methods. Among the five plants, *P. debilis* has been found to possess the highest activity and *P. amarus* posses the lowest activity in all tested models (Kumaran and Karunakaran, 2007).

Methanolic extract of leaves and stems of *P. amarus* was found to have potential antioxidant activity as it could inhibit lipid peroxidation, and scavenge hydroxyl and superoxide radicals *in vitro*. The amount required for 50% inhibition of lipid peroxide formation was 104 µg/mL and the concentrations needed to scavenge hydroxyl and superoxide radicals were 117 and 19 µg/mL respectively (Raphael *et al*., 2002). Amariin, repandusinic acid, phyllanthusiin D, phyllanthin and phenolic compouds isolated from *P. amarus* showed remarkable high antioxidant activity (Patel *et al*., 2011).

1.3.3 Analgesic, anti-edematogenic and anti-inflammatory activity

The aqueous leaves extract of *P. amarus* was investigated for analgesic and anti-inflammatory activities using both thermal and chemical models of pain assessment in rats. The extract caused a significant ($p < 0.05$) dose related increased inhibition of the carrageenan-induced paw edema in rats. The inhibition produced by 200 mg/kg aqueous extract of *P. amarus* (70.2%) was significantly higher than that of the reference drug (acetylsalicylic acid). The extract produced a marked analgesic activity by inhibiting both early and late phases of pain stimulus in formalin induced paw licking in rats and also produced a significant and dose related increase in inhibition of the mean tail immersion duration at varying water bath temperature (50, 55 and 60 $^{\circ}$C) (Iranloye *et al*., 2011).

The local administration of nirtetralin, phyltetralin or niranthin (30 nmol/paw), similar to WEB2170 (platelet activity factor receptor antagonist, 30 nmol/paw), significantly inhibited platelet activity factor-induced paw edema formation in mice. The extracts of *P. amarus* (100 µg/mL) and niranthin (30 µM), but not nirtetralin or phyltetralin (30 µM), decreased the specific binding of [3H]- platelet activity factor in mouse cerebral cortex membranes. The mean IC_{50} values from these effects were 6.5 and 0.3 µM, respectively. Both niranthin and WEB2170 (30 nMol/paw) inhibited the increase of myeloperoxidase activity induced by platelet activity factor injection in the mouse paw (Kassuya *et al*., 2006). The hexane extract, The lignan-rich fraction or the lignans phyltetralin, nirtetralin, niranthin of *P. amarus* when given orally inhibited carrageenan-induced paw oedema and neutrophils influx. the hexane extract, the lignan-rich fraction or nirtetralin also inhibited the increase of IL-1β tissue levels induced by carrageenan. Bradykinin-, platelet activity factor- and endothelin-1 induced paw edema were significantly inhibited by hexane extract, the lignan-rich fraction. Finally, nirtetralin caused inhibited of paw edema induced by platelet activity factor or endothelin-1. These results show that hexane extract, The lignan-rich fraction and lignin niranthin, phyltetralin and nirtetralin exhibited marked anti-inflammatory properties (Kassuya *et al*., 2005).

In antinoceceptive activity study, the hydroalcoholic extract of 4 *Phyllanthus* species, given intraperitoneally, produced significant inhibition of acetic acid-induced abdominal constrictions, with mean ID_{50} value of 0.3, 1.8, 7.4 and 26.5 mg/kg for *P. amarus*, *P. orbiculatus*, *P. fratermus* and *P. stipulates*, repectively. In the formalin test, the 4 species of Phyllanthus produced graded inhibition in both phase. The hydroalcoholic extract of the plants elicited significant inhibition of the capsaicin-induced neurogenic pain. Orally, all hydroalcoholic extract of the *Phyllanthus* species were less potent and efficacious than when given by intraperitoneally. (Santos *et al*., 2000).

1.3.4 Antitumor and anticarcinogenic activity

Aqueous extract of *P. amarus* exhibited potent anticarcinogenic activity against 20-methylcholanthrene induced sarcoma and increased the survival of tumor harboring mice. The extract administration was also found to prolong the life span of Dalton's Lymphoma Ascites and Ehrlich Ascites Carinoma bearing mice and reduced the volume of transplanted solid tumor. The extract inhibited aniline hydroxylase. The concentration required for 50% inhibition (IC_{50}) was found to be 540 µg/mL. The extract also inhibits DNA topoisomerase II of *Saccharomyces cerevisiae* mutant cell cultures and inhibited cell cycle regulatory enzyme tyrosine phosphatase (IC_{50} = 25 µg/mL). Antitumor and anticancer activity of *P. amarus* may be evident by the inhibition of metabolic activation of carcinogen as well as the inhibition of cell cycle regulator (Rajeshkumar *et al*., 2002).

1.3.5 Immunostimulant activity

Albino rats treated with *P. amarus* aqueous extract showed decreased in erythrocyte sedimentation rate increased in red blood cell count and packed cell volume but *P. amarus* did not affect the haemoglobin concentration, total and differential count increased the number of circulating leucocytes and neutrophil, respectively, especially with 100 mg/kg of extracts. The aminotransferases; (ALT) and (AST) were higher in treated rats. It was suggested that the plant can serve as an immunostimulant (Taiwo *et al*., 2009).

1.3.6 Antihyperglycemic and hypolipidemic activities

The antihyperglycemic and hypolipidemic activities of aqueous extract of whole plant of *P. amarus* were evaluated in streptozotocin induced diabetic male Wistar albino rats. Aqueous extract of *P. amarus* was administered at 200 mg/kg /day to normal treated and streptozotocin induced diabetic rats by gavage for 8 weeks. During the experimental period, blood was collected from fasted rats at 10 days intervals and plasma glucose level was estimated. The plasma lipid profile was estimated at the end of experimental period. After the treatment period, kidney lipid peroxidation, protein oxidation and glutathione were estimated. The significant decreased in the body weight, hyperglycemia and hyperlipidemia was observed in streptozotocin induced diabetic rats with the treatment of aqueous extract of *P. amarus* in diabetic treated group. Streptozotocin induced diabetic rats showed increased renal oxidative stress with increased lipid peroxidation and protein oxidation (Karuna *et al*., 2011).

In a clinical trial conducted on nine mild hypertensive patients associated with diabetes mellitus, the patients were treated with a preparation of the *P.amarus* water extract for 10 days. The observations indicated that *P. amarus* as potential diuretic hypotensive and hypoglycaemic drug for human. Blood glucose was significantly reduced in treated group (Srividya and Periwal, 1995). In another trial, 25 patients in the age group of 35-55 with moderate and severe diabetic blood sugar level (250-400 mg/100 mL) showed statistically significant ($p<0.05$) lowering of blood sugar level at a dose of 1 g of the water extract thrice daily for a period of 3 month (Sivaprakasam *et al*., 1995).

1.3.7 Antiviral activity

In vitro, study on 25 compounds isolated from *P. amarus*, *P. multiflorus*, *P. tenellus* and *P. virgatus*, found that niranthin, nirtetralin, hinokinin and geraniin at the non-cytotoxic concentration of 50 µM, suppressed effectively both Hepatitis B surface antigen (HBsAg) and hepatitis B effective antigen (HbeAg) expression, of these, niranthin showed the best anti-HBsAg activity, while the most potent anti-HBeAg activity was observed with hinokinin (Huang *et al*., 2003).

A single dose (1 mg/mL) of an aqueous extract affected on human hepatocellular carcinoma cell line. Inhibition of secretion of HBsAg for a period of 48 h was observed (Jayaram and Thyagarajan, 1996; Yeh *et al*., 1993). Disruption of hepatitis B virus polymerase activity, mRNA transcription and replication supported the role of *P. amarus* being used as an antiviral agent (Lee *et al*., 1996; Ott *et al*., 1997). Alcoholic, hexane, chloroform, butanol and water extract of *P. amarus* were tested for *in vitro* effects on HBsAg, HBeAg and HBV-DNA in serum samples positive for HBV antigen followed by the screening of the respective antigen by ELISA. The extracts against HBV antigen, the butanol extract are the most potent (Mehrotra *et al*., 1991).

In mice infected with wood chuck hepatitis virus, administration with extract was effective in three animal in reducing the virus within 3-6 weeks, eliminating both the surface antigen titer and DNA polymerase activity in serum (Vankateswaran *et al*., 1987).

Water-alcohol extract of leaves of *P. amarus in vitro* blocks HIV-1 attachment and the HIV-1 enzymes integrase, reverse transcriptase and protease to different degrees. A gallotannin containing fraction and the isolated ellagitannins geraniin and corilagin were shown to be the most potent mediators of these antiviral activities. The *P. amarus* extract, blocked the interaction of HIV-1 gp120 with its primary cellular receptor $CD4^+$ at IC_{50} 2.65 (water-alcohol extract) and 0.48 µg/mL (geraniin). Inhibition was also evident for the HIV-1 enzymes integrase (0.48-0.16 µg/mL), reverse transcriptase (8.17-2.53µg/mL) and protease (21.80-6.28µg/mL) (Notka *et al*., 2004). Aqueous as well as alcohol-based *P. amarus* extracts potently inhibited HIV-1 replication in HeLa $CD4^+$ cells with EC_{50} values ranging from 0.9 to 7.6 µg/mL. A gallotannin enriched fraction showed enhanced activity of multicellular activation of galactosidase indicator cell (0.4 µg/mL), and the purified gallotannins geraniin and corilagin were most active (0.24 µg/mL). HIV-1 replication was also blocked in $CD4^+$ lymphoid cells with comparable EC_{50} values of atazanavir, HIV-RT inhibitor. Applying a cell-based internalization assay, it was demonstrated 70-75% inhibition of virus uptake at concentrations of 2.5 µg/mL for the water-alcohol extract and geraniin. In addition, a concentration- dependent inhibition of HIV-1 reverse

transcriptase was demonstrated *in vitro*. The IC_{50} values varied from 1.8 to 14.6 µg/mL (Notka *et al*., 2003).

1.3.8 Antibacterial activity

The antibacterial activity of the *P. amarus* ethanolic extract from root and leaf was assessed to against Extended Spectrum β-Lactamases (ESBL) producing *Escherichia coli* isolate from the stool sample of HIV sero-positive patients using disc diffusion method. The strains isolated from both HIV sero-positive patients were susceptible to various concentrations of extracts (5, 10, 20, 40 and 80 mg/mL). This proves the antibacterial activity of the extract (Akinjogunla *et al*., 2010).

1.3.9 Antiamnesic activity

The effect of aqueous extract of leaves and stems of *P. amarus* was evaluated on cognitive functions and brain cholinesterase activity in male Swiss albino mice. *P. amarus* extract (50, 100 and 200 mg/kg) produced a dose-dependent improvement in memory scores of young and older mice. *P. amarus* also reversed successfully the amnesia induced by scopolamine (0.4 mg/kg, i.p.) and diazepam (1 mg/kg, i.p.). Interestingly, brain cholinesterase activity was also reduced. Piracetam 400 mg/kg, i.p. was used as positive control (Joshi and Parle, 2007). Nootropic activity of [6]-gingerol and phyllanthin was studied in mice using elevated plus maze and passive avoidance paradigm. [6]-gingerol (25 and 50 mg/kg, p.o.) and phyllanthin (7.5 and 15 mg/kg, p.o.) significantly attenuated amnestic deficits induced by diazepam, scopolamine (0.4 mg/kg, i.p.) and natural aging. [6]-gingerol and phyllanthin increased step down latencies significantly in the aged mice, diazepam and scopolamine induced amnesic mice as compared with piracetam (200 mg/kg, i.p.). [6]-gingerol and phyllanthin significantly decreased whole brain acetyl cholinesterase activity (Joshi and Parle, 2006).

1.3.10 Anti-diarrheal, gastroprotective and antiulcer activity

Oral administration of absolute ethanol (1 mL/200 g body weight) induced multiple, elongated, reddish bands of hemorrhagic erosions in rat gastric mucosa. The ethanol control group had the highest ulcer index of 45.20 ± 2.39.

Pretreatment with *P. amarus* leaves aqueous extract (500 mg/kg) and cimetidine (100 mg/kg) significantly inhibited ($p < 0.001$) ulceration by 59.3 and 41.2% respectively. The acetone extract yielded a dose-dependent percentage ulcer inhibition and the lower dosage of the aqueous extract had a higher ulcer inhibition (Shokunbi and Odetola, 2008). Methanolic extract of leaves and stems of *P. amarus* at the dose of 50, 200, and 1000 mg/kg body weight p.o., on adult male Wistar rats significantly inhibited gastric lesions, induced by intragastric administration of absolute ethanol (8 mL/kg body weight). Mortality, increased stomach weight, ulcer index, and intraluminal bleeding were reduced significantly by *P. amarus.* Biochemical analysis indicated that reduced glutathione of gastric mucosa produced by ethanol administration was significantly elevated by treatment with *P. amarus* (Raphael and Khuttan, 2003). Graded doses of the aqueous extract of *P. amarus* (100-800 mg/kg) administered orally produced a dose related inhibition of gut meal travel distance in normal mice. The highest intestinal transit inhibition of 31.65% was obtained with 400 mg/kg. In castor oil induced diarrhoea in mice, *P. amarus* extract (400 mg/kg) delayed the onset of diarrhoea, reduced frequency of defecation and reduced gut meal travel distance significantly resulting in intestinal transit inhibition of 79.94% compared to 86.92% produced by morphine (100 mg/kg). In addition, the activities of some intestinal mucosa enzymes (maltase, sucrase, lactase and alkaline phosphatase) in mice pretreated with extract before castor oil were not as severely depressed as those in castor oil treated mice (control) (Odetola and Akojenu, 2000).

1.3.11 Antifungal activity

The effect of nor-securinine, an alkaloid isolated from *P. amarus* was studied against spore germination of some fungi (*Alternaria brassicae*, *Alternaria solani*, *Curvularia pennisetti*, *Curvularia* sp., *Erysiphe* pisi, *Helminthosporium frumentacei*) as well as pea powdery mildew (*E. pisi*) under glasshouse conditions. The sensitivity of fungi to nor-securinine varied considerably. Nor-securinine was effective against most of the fungi. *H. frumentacei* was more sensitive even at the lowest concentration (1000 µg/mL). Conidia of *E. pisi* were also inhibited in partially or completely appressorium formation. Pre-inoculation treatment showed greater efficacy than post-inoculation in inhibiting powdery mildew development on pea

plants in a glasshouse. Maximum inhibition occurred at 2000 µg/mL (Sahni *et al*., 2005). Antifungal bioassay in terms of reduction in weight, colony diameter and sporulation of the target fungal colony was carried out using Broth Dilution method. Root part of the *P. amarus*, extracted in various organic solvents have not shown any noticeable antifungal activity. The percentage inhibition observed in different solvent extracts of aerial part was found as reduction in weight. It was concluded that chloroform fraction of the aerial part of the plant *P. amarus* have showed significant inhibitory effect against dermatophytic fungi *M. gypseum* (Agrawal *et al*., 2004).

1.3.12 Contraceptive effect

Oral administration of *P. amarus* alcoholic extract, at the dose of 100 mg/kg body weight, affected their cyclicity, when cohabited females with normal male mice they can not be pregnant. These factors are related to a change in the hormonal milieu that governs female reproductive function. Upon withdrawal of feeding for 45 days, these effects were reversible. Thus this extract manifests a definite contraceptive effect in female mice (Rao and Alice, 2001).

Effect of *P. amarus* aqueous extract leaves was studied on implantation and pregnancy rats. *P. amarus* aqueous extract at the dose 0.2 mg/100g body weight from day 1 of pregnancy compared with control, received distilled water equal volume, the results revealed, *P. amarus* aqueous extract reduced the time frame for implantation in the treated rats and caused abortion of pregnant rats. Flavonoids, found in *P.amarus* have been shown to have an antispasmodic action (Robaki et al 1988). It is therefore possible that the extract act directly on the uterine muscle to cause foetal abortion. Thus the extract may have an abortifiacent property which does not support the traditional claim that it can treat sterility but may ease difficult child birth, (Iranloye *et al*., 2010). Consistent with, Malan and Neuba (2011), reported in the use of plants during pregnancy is a common practice in Africa; Anyi-Ndenye Women, was found that, *P. amarus* used to facilitate childbirth (Schmelzer *et al*., 2009) by anal route using enema bag in the last trimesters (Malan and Neuba, 2011).

1.4 Pharmacokinetics

Research reported the pharmacokinetics of phyllanthin and hypophyllanthin, both *in vitro* and *in vivo* after pretreatment with *P. amarus* extract.

- *In vitro* study in human liver microsomes reported the ethanolic extract of *P. amarus* and its major lignans; phyllanthin, hypophyllanthin were potent inhibitors of CYP 3A4 (Taesotikul *et al*., 2011).

- *In vivo* study in rats revealed that, single dose (800 mg/kg/day) *P. amarus* ethanolic extract increased oral bioavailability of midazolam (increased the C_{max} and $AUC_{0-\infty}$ of midazolam by 3.9- and 9.6-fold and decrease the clearance by 12%) through inhibited intestinal CYP 3A. Repeated administration of the extract (200, 800 mg/kg/day/15 days) slightly induced hepatic CYP 3A (Taesotikul *et al*., 2012).

1.5 Toxicological assessment

Acute oral toxicity assay in mice when treated with aqueous and hydroalcoholic extract of *P. amarus* at a dose of 5 g/kg, not show any sign of toxicity and no mortality during two weeks observation. (Evi *et al*., 2008). *P. amarus* had reported to be safe and nontoxic to mice in a dose of 10 g/kg body weight (Mokkhasmit *et al*., 1971).

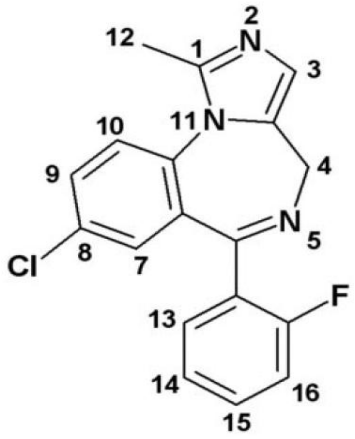
Sub acute toxicity was evaluated in rats after 28 days, the results revealed that it not decrease body weight gain compared to control, except in females when treated with aqueous and hydroalcoholic extract of *P. amarus* at dose 1 g and 3 g/kg,. The extracts have no effect on fasting blood glucose level, AST and ALT, *Y*-glutamyltransferase, creatine phosphokinase, creatinine. Histopathological studies examination of liver, kidney and pancreas excised from rats showed no pathological change (Evi *et al*., 2008).

2.2 Midazolam

Midazolam is commonly used in the emergency department to provide sedation prior to procedures such as for hypnosis, preoperative, sedation, induction and maintenance of anesthesia and sedation of patients in intensive care units. Midazolam is also effective in the treatment of generalized seizures, status epilepticus, and behavioral emergencies, particularly when intravenous access is not available. Midazolam is often employed as an induction agent for rapid sequence endotracheal intubation.

2.2.1 Structure and property

Midazolam is a short-acting benzodiazepine, known as the imidazobenzodiazepines, the chemical structure is 8-chloro-6-(2-fluorophenyl)-1-methyl-4*H*-imidazo[1,5-*a*] [1,4] benzodiazepine (Fig. 3). It is basic and very stable in aqueous solution. The nitrogen in the 2-position provides sufficient basicity (pk_a = 6.15). The parenteral preparation of midazolam used in clinical practice is buffered to an acidic pH (3.5). Clinical studies of midazolam have been performed with the drug prepared either as hydrochloride or maleate salt. In acidic aqueous media, midazolam is water soluble, in strong acidic solutions the diazepine ring reversibly opens between the positions 4 and 5 producing a polar water-soluble primary amine derivative (Gerecke, 1983). It is highly lipid soluble at physiologic pH. This allows for rapid entry into brain tissue and a correspondingly rapid onset of action (Joseph and Giovannitti, 1987).



MW = 325.78

Fig. 3 The chemical structure of midazolam (Klieber *et al*., 2008)

2.2.2 Mechanism of action

Midazolam acts on the benzodiazepine binding site of $GABA_A$ receptors (Fig. 4). This receptor, which functions as a chloride ion channel, result in inhibitory effects on the CNS (Skerritt *et al*., 1983). The mechanism is shown in Fig. 5. Midazolam bind to the gamma (γ) sub-unit of the $GABA_A$ receptor. Their binding causes an allosteric (structural) modification of the receptor that result in an increase in $GABA_A$ receptor activity. Midazolam do not substitute for GABA (gamma amino butyric acid), which bind at the alpha (α) sub-unit, but increase the frequency of channel opening events which leads to an increase in chloride ion conductance with resulting membrane hyperpolarization and inhibition of the action potential (Trevor and Way, 2007).

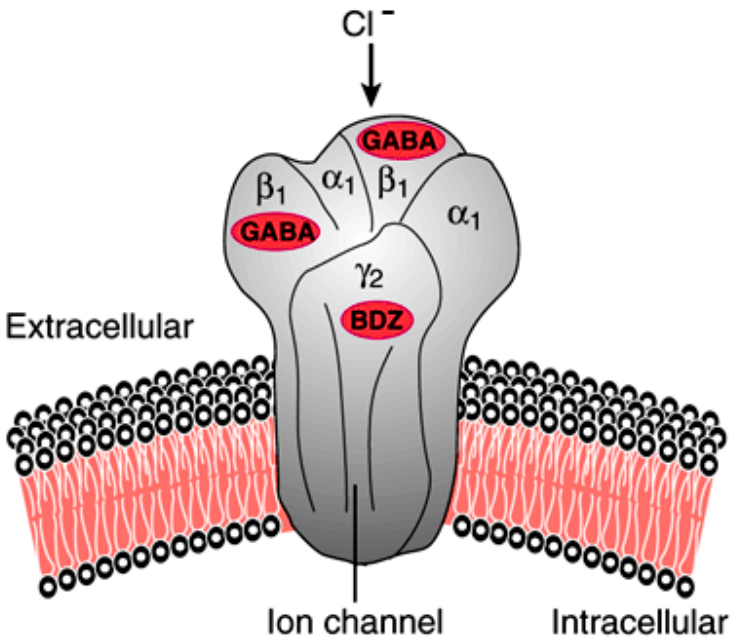


Fig. 4 A model of the $GABA_A$ receptor-chloride ion channel macromolecular complex (Modified from Zorumski and Isenberg,1991).

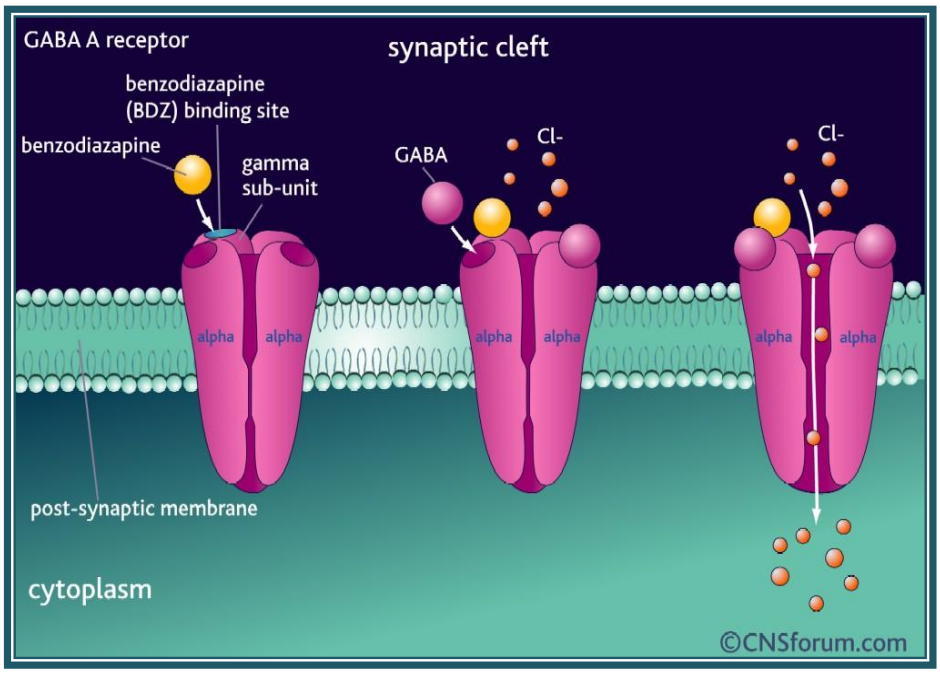


Fig. 5 Mechanism of action of midazolam (http://www.cnsforum.com/imagebank/item/drug_benzo/default.aspx; accessed August 16, 2013)

The subset of $GABA_A$ receptors that also bind benzodiazepines are referred to as benzodiazepine receptors. The $GABA_A$ receptor is composed of five subunits, most commonly two α, two β and one γ ($\alpha_2\beta_2\gamma$). $GABA_A$ receptors that are made up of different combinations of subunit have different properties, different distributions within the brain and different activities relative to pharmacological and clinical effects (Johnston, 1996).

2.2.3 Pharmacology

Midazolam has the hypnotic, sedative, anxiolytic, amnestic, anticonvulsant and muscle relaxant effects. Midazolam has a high affinity for the benzodiazepine receptor in the CNS, with *in vitro* data demonstrating approximately twice the affinity of diazepam (Kanto, 1985; Reves *et al*., 1985).

Hypnotic effect

The hypnotic effect of midazolam is related to GABA-benzodiazepine receptor. The most widely accepted hypothesis for the hypnotics effect of benzodiazepines is that there are separate benzodiazepine and GABA receptors coupled to a common ionophore (chloride) channel (Gallagher, 1982). Occupation of both receptors produces membrane hyperpolarization and neuronal inhibition. Midazolam interferes with reuptake of GABA, thereby causing accumulation of GABA. This is consistent with the benzodiazepine-GABA interaction hypothesis, as well as the general hypothesis that anesthesia involves excess GABA at neuronal synapses (Dundee, 1979).

Anxiolytic effect

In an anxiolytic effect study, in rats and squirrel monkeys, midazolam diminishes punished behavior (a laboratory measure of anxiety) less than diazepam, apparently because of a more pronounced hypnotic component (Pieri *et al*., 1981). The dorsal hippocampus and the dorsal raphé nucleus are two brain sites that mediate the anxiolytic effects of midazolam. In the social interaction test of anxiety, microinjections of midazolam (2-8 μg) into the dorsal hippocampus or dorsal raphé

nucleus significantly increased the time spent in active social interaction, without changing locomotor activity, thus indicating specific anxiolytic effects (Irvine *et al*., 2001).

Amnesic effect

Midazolam, like other benzodiazepines produce anterograde amnesia. The incidence and duration appear to be related directly to the dose of midazolam (Ruiz *et al*., 1983). Its site and mechanism of the amnesic action are not known. The degree of amnesia often, but not always, parallels to the degree of drowsiness produced by midazolam (Dundee and Wilson, 1980), and the drowsiness seems to outlast amnesia (Forster, 1981). The amnesic effect of an intravenous dose of 5 mg midazolam, ranges from 20-30 min. Intramuscular administration may prolong the amnesic effect (Fragen *et al*., 1983). The amnesic effect of midazolam may be more intense than diazepam but shorter lasting than lorazepam (Kothary *et al*., 1981). Prolonged amnesia could be a problem in outpatients by interfering with their ability to recall oral instruction; however, in this setting, amnesia with midazolam lasts no longer than with thiopental (Crawford *et al*., 1984).

Anticonvulsant effect

Mechanism of midazolam's anticonvulsant activity is the enhanced action of GABA on motor circuits in the brain (Richer, 1981). Anticonvulsant effects of misazolam are demonstrated in mice by electroshock and antipentetrazole (pentylenetetrazole) tests (Pieri *et al*., 1981). Midazolam injected (intramuscular) in mice reduces the incidence of convulsions and death from overdose of local anesthetic and is more effective anticonvulsant than either diazepam or lorazepam (Dejong and Bonin, 1981).

Muscle relaxant

Midazolam has been used effectively in the treatment of muscle spasms in the emergency department and as an agent to perform rapid sequence induction prior to endotracheal incubation (Nordt and Clark, 1997). Midazolam impairs motor performance in experimented animal and probably exhibits a muscle

relaxant effect similar to the other benzodiazepines (Pieri *et al*., 1981). *In vitro* studies have demonstrated airway smooth muscle relaxation following the administration of midazolam. The mechanism of smooth muscle relaxation is unclear, however, the presence of peripheral benzodiazepine receptors on smooth muscle or a direct effect on smooth muscle may be possible (Koga *et al*., 1992).

2.2.4 Pharmacokinetics

(1) Absorption

Midazolam is rapidly absorbed from the gastrointestinal tract following oral administration. Drowsiness has been observed 15 min after an oral dose. The C_{max} of a single 15 mg midazolam tablet in healthy volunteers has been reported to be 0.05 mg/L at 0.5-1.5 h. The bioavailability of oral midazolam was 49% (Langlois *et al*., 1987). The duration of action following intranasal administration is similar to that after oral dosing, although the onset of sedation may be earlier (Baraff, 1994). Rectally administered midazolam has been shown to be safe and effective, with peak plasma concentrations of 9-29 min after administration (Hartgraves, 1994). Unlike diazepam and chlordiazepoxide, which have erratic and incomplete intramuscular absorption, midazolam is rapidly absorbed following intramuscular injection, with an absolute bioavailabilty of more than 90%. The onset of sedation following intramuscular injection is early as 5 min, with peak effects seen within 15-30 min (Taylor and Simon, 1990). The onset of sedation is quite rapid after intravenous infusion, with clinical effects seen in approximately 3 min (Kanto, 1985).

(2) Distribution

Midazolam has a volume of distribution of about 1-2.5 L/kg in normal healthy adults but is increased in obesity and pregnancy (Reves *et al.*, 1985; Dundee *et al.*, 1984). Midazolam crosses the placenta and is distributed into breast milk. An average of 0.005% of the maternal midazolam dose excreted into milk within 24 h of induction of anesthesia (Nitsun *et al*., 2006). Midazolam was 94-98% bound to plasma proteins, primarily albumin (Reves *et al.*, 1985; Greenblatt *et al.*, 1984). Small changes in protein binding of midazolam may produce large changes in the unbound fraction, which may have significant consequences in clinical practice. In

patients with chronic renal failure, the unbound fraction is increased (Dundee *et al.*, 1984). The pharmacological effect of midazolam changes from 1 to 4 h. The duration of effect is determined primarily by the rate of movement from the central to the peripheral compartment (Kanto, 1985).

(3) Elimination

Midazolam is extensively first-pass metabolism in both the liver and intestine (Thummel *et al*., 1996). The mean of apparent oral clearance (CL/F) after 15 mg midazolam was 0.52 L/h/kg (Langlois *et al*., 1987). The biotransformation of midazolam is catalyzed by at least three different cytochrome P450 3A (CYP3A) isoenzymes: CYP3A4, CYP3A5 and CYP3A7 (Gorski *et al*., 1994). CYP3A7 is principally expressed in fetal tissues, CYP3A4 and CYP3A5 represent the main cytochrome P450 (CYPs) isoforms in adult liver and intestine. Hydroxylation of midazolam produces its major metabolite, mainly to 1′-hydroxymidazolam, which is an active metabolite with the $T_{1/2}$ of 2 h, and to an inactive secondary metabolite 4-hydroxymidazolam. Hydroxy derivatives of midazolam undergo rapid conjugation with glucuronic acid and then are excreted into urine within 24 h. Up to 80% of midazolam are excreted in the urine as 1′- hydroxymidazolam glucuronide. Less than 1% of the administered midazolam are excreted in urine over 24 h as unchanged drug (Lin *et al*., 2001). Midazolam has the $T_{1/2}$ of 1.5-3 h (Stanski and Hudson, 1985) and has been associated with accumulation and prolonged sedation in patients with renal dysfunction (Baurer *et al*., 1995). Metabolic pathway of midazolam is shown in Fig. 6.

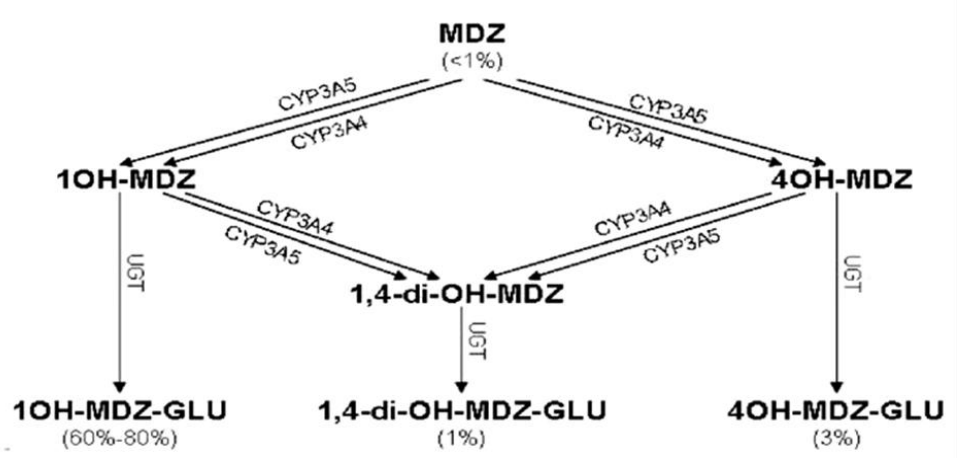


Fig. 6 Schematic representation of the CYP3A4-, CYP3A5- and UGT-catalyzed metabolic pathway for Midazolam (MDZ), the percentages of the administered MDZ dose which are excreted in the urine within 24 hours are given below the compounds. Abbreviations: MDZ, midazolam; 1OH-MDZ, 1-hydroxymidazolam; 4OH-MDZ, 4-hydroxymidazolam; 1,4-di-OH-MDZ, 1,4-dihydroxymidazolam; 1OH-MDZ-Glu, 1-hydroxymidazolam glucuronide; 1,4-di-OH-MDZ-Glu, 1,4-dihydroxymidazolam glucuronide; 4-OH-MDZ-Glu, 4-hydroxymidazolam glucuronide; UGT, uridine diphosphate-glucuronosyl-transferase. (Vossen *et al*., 2007)

2.2.5 Clinical use

Midazolam has a multiplicity of uses. It has been used for induction and maintenance of general anesthesia, sedative and hypnotic and status epilepticus (Nordt *et al*., 1997).

Premedication

Midazolam, like diazepam and the other benzodiazepines, is well-suited for premedication, since it has both anxiolytic and hypnotic properties. Studies of its hypnotic effect show that the onset of sleep is relatively rapid and the duration of the hypnotic effects and sleep stages are highly variable and dose related. Performance on tests of mental function returns to normal 4 h after the administration of midazolam. When midazolam, 5 mg was given as intravenous premedication, the hypnotic and anxiolytic effects appeared within 1-2 min. This effect persisted for 30 min. The only prominent side effect from this dose was dizziness or lightheadedness. Midazolam appears to be an excellent drug for the intravenous sedation of anxious patients and for supplementation of inadequate premedication (Reves *et al*., 1985).

Sedation

Midazolam is a useful intravenous adjuvant to local or regional anesthesia for a variety of therapeutic and diagnostic procedures. Midazolam produce mild sedation and amnesia in patients for diagnostic and therapeutic procedures. The average dose required is 0.1 mg/kg. Midazolam can be used for the sedation of healthy patients receiving subarachnoid or epidural anesthesia. Sedation occurs without loss of airway reflexes or significant cardiovascular changes. Compare with diazepam, midazolam produces less postoperative drowsiness and more amnesia but the time to complete recovery is not shorter (Driessen *et al*., 1981: McClure *et al*., 1983). Midazolam is also useful for intravenous sedation for endoscopic procedure, including gastroscopy, esophagoscopy and cystoscopy. The onset of sedation is more rapid with midazolam than diazepam, but time of recovery is similar (Cole *et al*., 1983).

Induction of anesthesia

Midazolam may be used intravenously for the induction of anesthesia. Induction is accomplished when there is unresponsiveness to command and loss of eyelash reflex. As induction drug, midazolam produces sleep and amnesia but it does not have a great analgesic effect. Midazolam is not as rapid acting as thiopental. The differences in induction times as well as the interpatient variability in response make the induction of anesthesia less predictable, but it is well accepted by patients (Freuchen *et al*., 1983).

Maintenance of anesthesia

Midazolam is a useful hypnotics-amnesic during maintenance of general anesthesia. Midazolam proved superior to thiopental because there were fewer adjuvant anesthetics required to maintain an acceptable depth of anesthesia. Midazolam also confers more amnesia and evokes better patient and physician acceptance and fewer emergence complications. Midazolam cannot be used alone to maintain adequate anesthesia, fentanyl and nitrous oxide are effective supplement (Reves *et al*., 1985).

2.2.6 Dosage and drug administration

Trade name: Dormicum®, Hypnovel®, Midacum® and Versed®

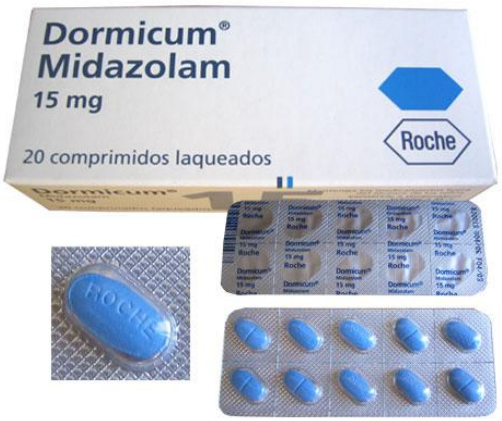


Fig. 7 Midazolam; Dormicum® Tablet 15 mg
(http://www.nonbenzodiazepines.org.uk/benzodiazepinenames.html;
accessed August 16, 2013)

Table 1. Dosage regimen of midazolam (Wildschut *et al*., 2010)

Indication	Adults < 60 yrs of age	Adults > 60 yrs, of age and debilitated or chronically ill patients	Children
- Conscious sedation	i.v. Starting dose: 2-2.5 mg Titration dose: 1 mg Total dose: 3.5-7.5 mg	i.v. Starting dose: 0.5-1 mg Titration dose: 0.5-1 mg Total dose: < 3.5 mg	i.v. 6 months - 5 years: Starting dose: 0.05-0.1 mg/kg Total dose: < 6 mg i.v. 6-12 years: Starting dose: 0.025-0.05 mg/kg Total dose: < 10 mg rectal > 6 months 0.3-0.5 mg/kg
- Amnesia induction - Anxiety - Preoperative sedation	i.v. 1-2 mg titrated i.m. 0.07-0.1 mg/kg	i.v. Starting dose: 0.5 mg, slowly titrate towards effect i.m. 0.025-0.05 mg/kg	rectal > 6 months 0.3-0.5 mg/kg i.m. 1-15 years 0.08-0.2 mg/kg oral > 6 months 0.25-1 mg/kg

2.2.7 Side effects

The incidences of side effects are the most commonly reported; nausea and vomiting, coughing and hiccoughs. Respiratory depression with prolonged somnolence may also occur infrequently with midazolam. However, these effects have been rapidly and effectively reversed with the specific benzodiazepine antagonist, flumazenil (Olkkola and Ahonen, 2008). Midazolam impairs the ability to recall lists memorized after drug administration. This specific effect on memory acquisition has been termed anterograde amnesia. In one study, it was shown that children undergoing general anesthesia and surgery completed preoperative baseline memory testing using a series of picture cards, after receiving oral midazolam (0.5 mg/kg) produces significant anterograde amnesia when given as early as 10 min before a surgical procedure (Zeev *et al*., 2000)

2.2.8 Overdosage

The manifestations of midazolam overdosage are similar to those observed with other benzodiazepines, including sedation, somnolence, ataxia, dysarthria, nystagmus, somnolence, mental confusion, impaired motor functions, hypotension, respiratory arrest, coma and death. No evidence of specific organ toxicity from midazolam overdosage has been reported (Darius and Michael, 2005).

Adults: Flumazenil, a specific benzodiazepine antagonist at central GABA-ergic receptors is available. Although it effectively reverses the CNS effects of benzodiazepine overdose, its use in clinical practice is rarely indicated. Use of flumazenil is specifically contraindicated when there is history of co-ingestion of tricyclic antidepressants or other drugs capable of producing seizures (including aminophylline and cocaine), benzodiazepine dependence, or in patients taking benzodiazepines as an anticonvulsant agent. In such situations, administration of flumazenil may precipitate seizures (Lopez, 1990; Mordel *et al*., 1992). Adverse effects associated with flumazenil include hypertension, tachycardia, anxiety, nausea, vomiting and benzodiazepine withdrawal syndrome. The initial intravenous dose of 0.3 to 1.0 mg may be followed by further doses if necessary. The absence of clinical response to 2 mg of flumazenil within 5 to 10 minutes indicates that benzodiazepine poisoning is not the major cause of CNS depression or coma. The patient regains consciousness within 15 to 30 seconds after injection of flumazenil, but since it is metabolised more rapidly than the benzodiazepines, recurrence of toxicity and CNS depression can occur and the patient should be carefully monitored after initial response to flumazenil therapy. If toxicity recurs, further bolus doses may be administered or an infusion commenced at a dose of 0.3 to 1.0 mg/h (Meredith *et al*., 1993).

Children: The initial intravenous dose of 0.1 mg should be repeated each minute until the child is awake. Continuous intravenous infusion should be administered at a rate of 0.1 to 0.2 mg/h (Meredith *et al*., 1993).

The duration of treatment with oral midazolam should not be more than 2 weeks. In certain cases extension beyond the maximum treatment period may be necessary.

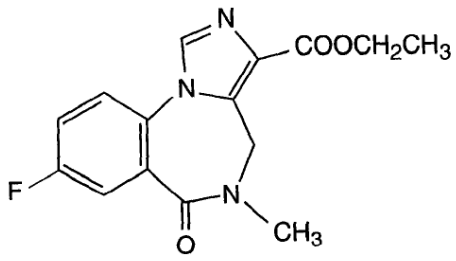


Fig. 8 The chemical structure of Flumazenil (Nordt *et al*., 1997)

2.2.9 Tolerance, dependence and withdrawal

Midazolam can cause a rapid development of drug tolerance, dependence and upon discontinuation a benzodiazepine withdrawal syndrome can occur, including rebound insomnia. Gradual reduction of midazolam after regular use can minimize withdrawal and rebound effects. Tolerance and the resultant withdrawal syndrome may be due to alterations in gene expression which results in long term changes in the function of the GABAergic neuronal system. A study in rats found that midazolam is cross tolerant with barbiturates and is able to effectively substitute for barbiturates and suppress barbiturate withdrawal signs (Yutrzenka *et al*., 1989). Patients who are chronic users of benzodiazepine medication, when given midazolam experience reduced therapeutic effects of midazolam, due to tolerance to benzodiazepines (Potokar *et al*., 1999).

2.2.10 Drug interactions

Midazolam is the preferred *in vivo* and *in vitro* CYP3A probe recommended by the US Food and Drug Administration (FDA) (Bjornsson *et al*., 2003) since CYP3A enzymes are responsible for metabolism of majority of currently used drugs, it is very important to use a standardized CYP3A probe substrate, especially prediction of drug-drug interaction. Some of several characteristics that make midazolam preferred *in vitro* and *in vivo* CYP3A probe are:

- It is exclusively metabolized by CYP3A4/5 to a primary metabolite 1′-hydroxymidazolam (Gorski *et al*., 1994). It is highly sensitive to changes in the activity of the CYP3A4/5 enzymes (Backman *et al*., 1996: Olkkola *et al*., 1994).
- It is not a substrate for P-glycoprotein (Kim *et al*., 1999).

- It is metabolized to a comparable extent by both intestinal and hepatic CYP3A, making it possible to study effects of CYP3A in both the intestine and the liver (Chung *et al*., 2006).

Grapefruit juice

Interaction between midazolam and grapefruit juice was evaluated in 10 patients with liver cirrhosis who were consumed grapefruit juice 60 and 15 min before midazolam (15 mg) was administered orally. Grapefruit juice increased the AUC of midazolam by 106% and the AUC of the metabolite alpha-hydroxymidazolam decreased to 25%. The ratio of the AUC of the metabolite alpha-hydroxymidazolam to midazolam decreased from 0.77 to 0.11. The C_{max} of the metabolite decreased to 30%. Grapefruit juice inhibits intestinal but not liver CYP3A4 or colon levels of CYP3A5. Consequently, intestinal CYP3A4 first pass metabolism can be reduced by inhibition with grapefruit juice. (Andersen *et al*., 2002)

Ketoconazole

The pharmacokinetics of midazolam was assessed in a single oral dose of midazolam (0.075 mg/kg) administered to 19 healthy Caucasian adults (nine male and ten female) and concurrently with ketoconazole (400 mg daily, orally, for 10 days). Coadministered of ketoconazole increased midazolam C_{max} from 23 ng/mL to 55 ng/mL (increased from 41 to 58%), $AUC_{(0-\infty)}$ increased 9.5-fold, from 49 ng.h/mL to 467 ng.h/mL and CL/F decreased from 128.8 to 3.1 (Chen *et al*., 2006). The pharmacokinetics study of midazolam was assessed in nine (six male and three female) healthy volunteers, after single doses of 2 mg intravenous and 6 mg oral midazolam. These pharmacokinetic values were compared with those obtained after single doses of 2 mg intravenous and 6 mg oral midazolam and three doses of 200 mg oral ketoconazole. After ketoconazole therapy, the AUC of midazolam increased 5-fold after intravenous midazolam administration and 16-fold after oral midazolam administration. The CL/F decreased by 84% and total bioavailability increased from 25% to 80% (Tsunoda *et al*., 1999).

Voriconazole

In a randomized, crossover study, ten healthy male volunteers were given either no pretreatment (control phase) or voriconazole (voriconazole phase) orally, 400 mg twice daily on the first day and 200 mg twice daily on the second day. Midazolam was given either 0.05 mg/kg intravenously or 7.5 mg orally, 1 h after the last dose of voriconazole and during the control phase. Voriconazole reduced the CL/F of intravenous midazolam by 72% and increased its $T_{1/2}$ from 2.8 to 8.3 h. The C_{max} and the AUC of oral midazolam were increased by 3.8-fold and 10.3-fold, respectively. The oral bioavailability of midazolam was increased from 31% to 84% (Saari *et al*., 2006).

2.3 Drug metabolism

Pharmacological effects of drugs depended on their ability to pass biological membranes, access site of action and interact with target cells, creating a response. Concentration of a drug at the site of action is determined by its absorption, distribution, metabolism and excretion (pharmacokinetics). In order to be excreted from the body, lipophilic characteristics of drugs promoting their passage through membranes, and transformed to more hydrophilic ones. This is achieved by a series of biotransformation (Xu *et al*., 2005).

Drug metabolizing enzymes play central roles in the metabolism, elimination and/or detoxification of xenobiotics or exogenous compounds introduced into the body (Meyer, 1996). In general, drug metabolizing enzymes protect the body against the potential harmful exposure to xenobiotics from the environment. In order to minimize the potential injury caused by these compounds, most of the tissues and organs are well equipped with diverse and various drug metabolizing enzymes including phase I, phase II metabolizing enzymes. Phase I drug metabolizing enzymes consist primarily of the CYP450s superfamily of microsomal enzymes, which are found abundantly in the liver, gastrointestinal tract, lung and kidney, consisting of families and subfamilies of enzymes that are classified based on their amino acid sequence identities or similarities (Guengerich, 2003). The phase II metabolizing or conjugating enzymes, consisting of many superfamily of enzymes including sulfotransferase (SULT), UDP-glucuronosyltransferase (UGT), glutathione-S-transferase (GST) and *N*-acetyltransferase (NAT). In general, conjugation with phase II DMEs generally increases hydrophilicity and thereby enhances excretion in the bile and/or the urine (Hinson and Forkert, 1995).

The regulation of gene expression of various phase I, phase II DMEs has potential impact on the metabolism, elimination, pharmacokinetics/dynamics, toxicokinetics/dynamics, drug-drug interactions of many therapeutic agents, as well as their ability in the protection of the human body against exposure of environmental xenobiotics (Guengerich, 2003).

Cytochrome P-450 System

Drugs are mainly metabolized by enzymes in the liver, which are found in the endoplasmic reticulum of cells in these tissues and are classified as microsomal enzymes. The cytochrome P450 (CYP) enzyme system consists of a superfamily of heme-containing monooxygenase (Fig. 9) that catalyse the oxidative metabolism of a wide variety of exogenous chemicals including drugs, carcinogens, toxins and endogenous compounds such as steroids, fatty acids and prostaglandins (Shimada *et al*., 1994). There are 2 types of drug-metabolizing enzyme: phase 1, or mixed function oxidase and phase 2 metabolic reactions. Phase 1 reactions convert the parent hydrophobic drug to a more polar metabolite by oxidation, reduction or hydrolysis. These reactions expose or induce a functional group (-OH, -NH_2, -SH, or -CO_2H) and usually result in only a small increase in the hydrophilic of the drug. The CYP enzyme family plays an important role in phase-1 metabolism of many drugs. The broad range of drugs that undergo CYP mediated oxidative biotransformation is responsible for the large number of clinically significant drug interactions during multiple drug therapy (Badyal and Dadhich, 2001).

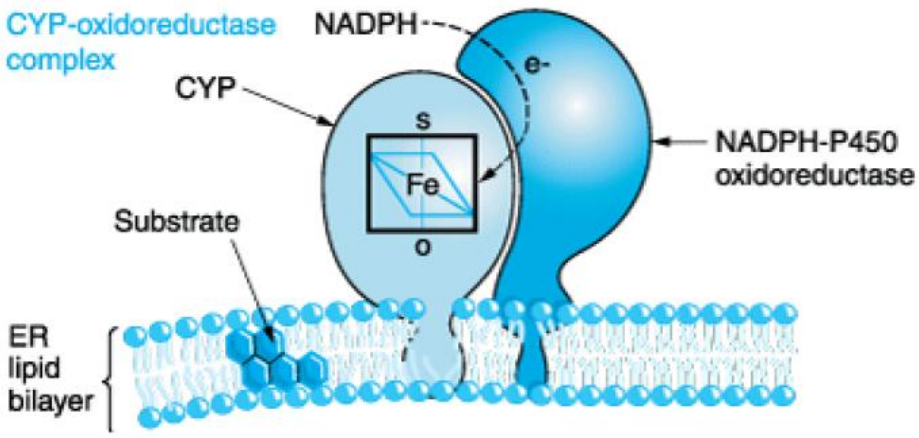


Fig. 9 Cytochrome P450 (CYP) hemoprotein in the lipid bilayer of endoplasmic reticulum (ER); Most part of the CYP enzyme is on the cytosolic surface of ER. The substrate (i.e., drug) often has hydrophobic properties and is dissolved in the membrane (Gonzalez and Tukey, 2006)

Cytochrome P450 catalytic mechanisms

The process of oxidative metabolism of a drug is shown in Fig. 10. The drug (R-H) reacts with the oxidized form of the CYP enzyme (CYP-Fe^{3+}), forming a drug-enzyme complex (CYP-Fe^{3+}-R-H). Cytochrome P450 reductase transfers one electron from NADPH to the CYP-Fe^{3+}-R-H complex, reducing it to CYP-Fe^{2+}-R-H, which in turn, reacts with molecular O_2, followed by transfer of a second electron from NADPH. Finally, one atom of O_2 is released as H_2O and the second atom is transferred to the substrate. In some oxidation reactions catalyzed by CYP3A enzymes, cytochrome b_5 has been shown to support the electron transfer from NADPH to CYP3A via the reductase. In that case, the second of the two electrons donated to the CYP, is coming from cytochrome b_5 (Yamaori *et al*., 2003).

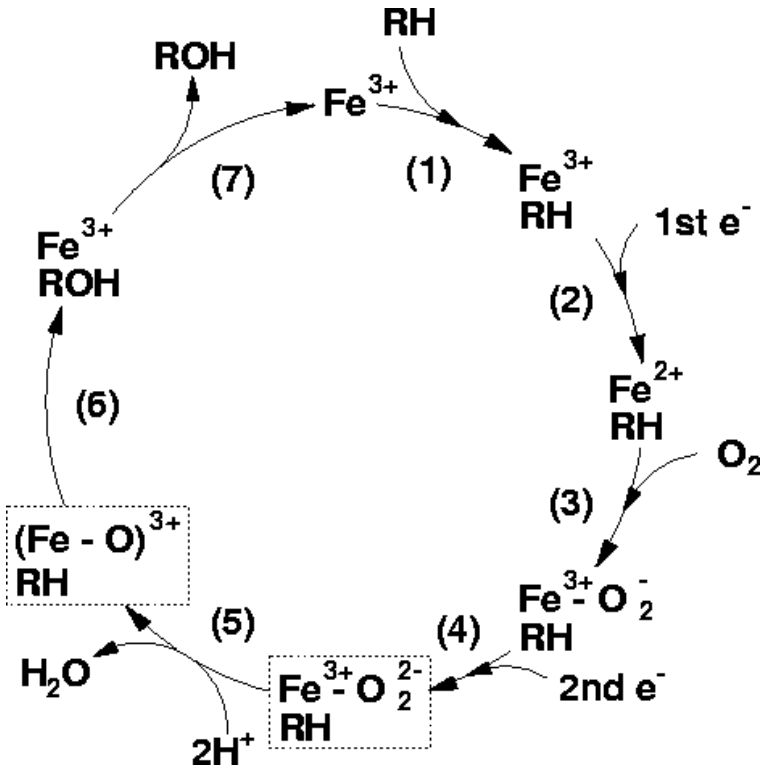


Fig. 10 The catalytic cycle of cytochrome P450
(http://www.tcm.phy.cam.ac.uk/~mds21/thesis/node49.html;
accessed August 18, 2013)

Nomenclature of CYP

Nomenclature of CYP enzyme system has been established by CYP nomenclature committee (Nelson *et al*., 1996). The name cytochrome P450 is derived from the fact that these proteins have a haem group and an unusual spectrum. These enzymes are characterized by a maximum absorption wavelength of 450 nm in the reduced state in the presence of carbon monoxide. Naming a *cytochrome P450 gene* include root symbol "*CYP*" for humans ("*Cyp*" for mouse and Drosophila) an arabic numeral denoting the CYP family (e.g. CYP1, CYP2) letters A, B, C indicating subfamily (e.g. CYP3A, CYP3C) and another arabic numeral representing the individual gene/isoenzyme/isozyme/isoform (e.g. CYP3A4, CYP3A5) (Nelson *et al*., 1996). Of the 74 gene families so far described, 14 exist in all mammals. These 14 families comprise of 26 mammalian subfamilies (Nelson *et al*., 1996). Each isoenzyme of CYP is a specific gene product with characteristic substrate specificity. Isoenzymes in the same family must have >40% homology in their amino acid sequence and members of the same subfamily must have >55% homology (Levy, 1995). In the human liver there are at least 12 distinct CYP enzymes. At present it appears that from about 30 isozymes, only six isoenzymes from the families CYP1, 2 and 3 are involved in the hepatic metabolism of most of the drugs. These include CYP1A2, 3A4, 2C9, 2C19, 2D6 and 2E1 (Levy, 1995).

The enzymes responsible for most drug metabolism and interactions are listed in more detail.

CYP1A2

Approximately 15% of medications used today are metabolized by CYP1A2, including caffeine, theophylline, tricyclic antidepressants and warfarin. Activity of CYP1A2 can be induced by cigarette smoke, charbroiled foods, and cruciferous vegetables (i.e., broccoli and cabbage). Several medications also affect CYP1A2 activity. Carbamazepine, phenobarbital and rifampin induce CYP1A2 as well as several other enzymes, leading to clinically significant drug interactions. Omeprazole and ritonavir simultaneously induce CYP1A2 and inhibit one or more

other enzymes. Inhibitors of CYP1A2 include erythromycin, ciprofloxacin, fluvoxamine, and grapefruit juice (Manzi and Shannon, 2005).

CYP2C9

CYP2C9 is responsible for the metabolism of several common medications including ibuprofen, phenytoin and warfarin. Rifampin and rifabutin are powerful inducers of CYP2C9 activity and lead to decrease serum concentrations of the above substrates. Other inducers include carbamazepine, ethanol, and phenobarbital. Amiodarone, fluoxetine, and fluconazole are among several drugs known to inhibit CYP2C9 activity. Genetic polymorphisms occur in 1% to 3% of the whites, contributing to abnormally decreased enzyme activity in these individuals (poor metabolizers) (Manzi and Shannon, 2005).

CYP2D6

CYP2D6 comprises a relatively small percentage (2%-6%) of the total CYP450 in the liver but is involved in the metabolism of many medications (up to 25%). Tricyclic antidepressant, β-blockers, haloperidol, sertraline, and thioridazine are metabolized by CYP2D6. The conversion of codeine to the active form, morphine, is catalyzed by CYP2D6, and patients with low activity demonstrate a poor analgesic response (Shannon, 1997). Unlike other CYP450 enzymes, there are no known inducers of this activity. Several medications inhibit CYP2D6, the most potent include cimetidine, fluoxetine, haloperidol, paroxetine, and codeine. Genetic polymorphisms play a significant role in determining CYP2D6 activity, as with CYP2C19. Approximately 1% to 3% of African American and Asian patients and 5% to 10% of whites lack this enzyme, placing them at risk for increased toxicity from medications that are metabolized by CYP2D6 (Manzi and Shannon, 2005).

CYP2E1

Although CYP2E1 metabolizes a relatively small fraction of clinically used medications, this enzyme plays a significant role in the activation and inactivation of toxins. CYP2E1 metabolizes primarily small organic molecules (i.e., ethanol, carbon tetrachloride) as well as acetaminophen and dapsone. Although only a

small percentage of acetaminophen is metabolized by CYP2E1, this hydroxylation produces N-acetyl-p-benzoquinoneimine, a hepatotoxin. Chronic ethanol use can induce CYP2E1 activity leading to a greater percentage of acetaminophen metabolized to N-acetyl-p-benzoquinoneimine, increasing the risk of hepatotoxicity from acetaminophen. The use of inhibitors such as disulfiram to prevent toxicity associated with compounds that form toxic metabolites when metabolized via CYP2E1 is currently being investigated (Hazai et al., 2002).

CYP3A4

The human CYP3A family represents about 30% of the total hepatic P450 content and is considered to be the most important CYP subfamily in the biotransformation of drugs. This family contains one subfamily including three functional proteins: CYP3A4, CYP3A5, and CYP3A7, and one pseudoprotein, CYP3A4 (Ingelman-Sundberg, 2005). These enzymes have overlapping catalytic specificities and their tissue expression patterns differ. CYP3A5 is a minor polymorphic CYP form in human liver (Westlind-Johnsson et al., 2003), but in extrahepatic tissues it is consistently expressed in kidney, lung, colon and esophagus (Burk and Wojnowski, 2004). Despite a few exceptions, the substrate and inhibitor specificity of CYP3A5 seems to be highly similar to CYP3A4, albeit the catalytic capability might be somewhat lower (Williams et al., 2002). CYP3A7 is mainly expressed in embryonic, fetal and newborn livers, where it is the predominant CYP form (Hakkola et al., 2001), whereas in the adult liver, CYP3A7 seems to be a minor form. CYP3A7 has an important role during the fetal period in the hydroxylation of several endogenous substances like retinoic acid and steroid hormones, and therefore it has relevance to normal embryonal development (Hines and McCarver, 2002). In drug metabolism, the role of CYP3A7 is not yet clear. CYP3A4 is the sixth most abundant enzyme in human liver at the mRNA level and constitutes the major CYP form in the liver and the small intestine (Rostami-Hodjegan and Tucker, 2007). CYP3A4 has a pivotal role in xenobiotic metabolism, and it has been estimated to be involved in the metabolism of approximately 50% of the drugs in clinical use. The active site of CYP3A4 is very large and flexible allowing multiple small molecules to be present simultaneously in the active site. The substrate binding is principally based

on hydrophobicity with some steric interactions. A concept where multiple conformations of the enzyme can exist both in the presence and absence of substrate has been proposed (Scott and Halpert, 2005). The kinetic interaction between CYP3A4 and its substrates is often atypical, making the prediction and modeling of CYP3A4-mediated drug-drug interactions troublesome (Houston and Galetin, 2005).

There are several CYP3A4 inducers such as the glucocorticoids, rifampin, carbamazepine, phenobarbital and phenytoin. In addition, there are several CYP3A4 inhibitors such as grapefruit juice, erythromycin, ketoconazole, clarithromycin and verapamil (Manzi and Shannon, 2005). The relative abundance of individual CYP forms in the liver and some examples of substrates, inhibitors and inducers are shown in Fig. 11.

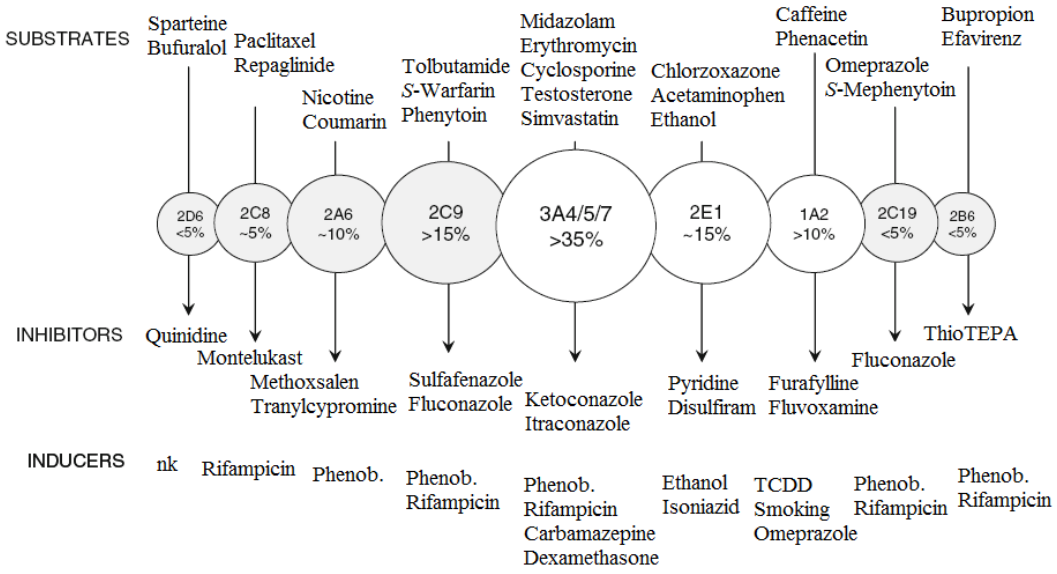


Fig. 11 Relative abundance of individual CYP forms in the liver and some examples of substrates, inhibitors and inducers; *Phenob.*= Phenobarbital, *TCDD* = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *nk* = not known (modified from Pelkonen *et al*., 2008)

Mechanisms of Inhibition of CYP

1) Reversible Inhibition

Reversible inhibition is probably the most common mechanism responsible for the documented drug interactions. In mechanistic terms, reversible interactions arise as a result of competition at the CYP active site and probably involve only the first step of the CYP catalytic cycle. Many of the potent reversible CYP inhibitors are nitrogen-containing drug, including imidazoles, pyridines and quinolones. These compounds can not only bind to the prosthetic haem iron, but also to the lipophilic region of the protein. Inhibitors that simultaneously bind to both regions are inherently more potent inhibitors. The potency of an inhibitors is determined both by is lipophilicity and by the strength of the bond between its nitrogen lone electron pair and the prosthetic haem iron. For example, both itraconazole and cimetidine are imidazole-containing compounds that interact with ferric CYP at its sixth axial ligand position to elicit a type 2 optical difference spectrum. The coordination of a strong ligand to the pentacoordinated iron, or the displacement of a weak ligand from the hexacoordinated haem by strong ligand, gives rise to a "type 2" binding spectrum. However, cimetidine is a relatively weak reversible inhibitor of CYP and apparent result of an intrinsic low binding affinity to microsomal CYP. This later property is most probably because of the low lipophilicity of cimetidine (log P = 0.4). On the other hand, itraconazole, potent CYP inhibitor, has a high lipophilicity (log P = 3.7). The quinolones are another class of nitrogen heterocycles that exhibit potent CYP inhibition. Ellipticine is a quinolone-containing compound that interacts with both ferrous and ferric CYP forms (Lin and Lu, 1998).

2) Quasi-Irriversible Inhibition via Metabolic Intermediate Complexation

A large number of drugs, including methylenedioxybenzenes, alkylamines, macrolide antibiotics and hydrazines undergo metabolic activation by CYP enzymes to form inhibitory metabolites. These metabolites can form stable complexes with the prosthetic haem of CYP, called metabolic intermediate (M1) complex, so that the CYP is sequestered in a functionally inactive state. M1

complexation can be reversed and the catalytic function of ferric CYP can be restored by *in vitro* incubation with highly lipophilic compounds that displace the metabolic intermediate from the active site. Other *in vitro* methods by which the ferrous complex can be disrupted include irradiation at 400-500 nm or oxidation to the ferric state by the addition of potassium ferricyanide. Dissociation or displacement of the M1 complex results in the reactivation of CYP functional activity. However, in *in vivo* situations, the M1 complex is so stable that the CYP involved in the complex is unavailable for drug metabolism and synthesis of new enzymes is the only means by which activity can be restored. The nature of the M1 complexation, therefore, considered to be quasi-irreversible (Lin and Lu, 1998).

3) Irreversible Inhibition of CYP

Drug containing certain functional groups can be oxidized by CYP to reactive intermediates that cause irreversible inactivation of the enzyme prior to its release from the active site. The mechanism-based inactivation of CYP may result from irreversible alteration of heme or protein, or a combination of both. In general, modification of the heme group in variable inactivates the CYP, whereas protein alteration will result in loss of catalytic activity only if essential amino acids, which are vital for substrate binding, electron transfer and oxygen activations are modified.

Drugs containing terminal double-bond (olefins) or triple-bond (acetylenes) can be oxidized by CYP to radical intermediates that alkylate the prosthetic heme group and inactivated the enzyme. The evidence for heme alkylation includes the demonstration of equimolar loss of enzyme and heme, as well as the isolation, structural and characterization of the heme adducts. Heme alkylation is initiated by the addition of activated oxygen to the internal carbon of the double or triple bond and is terminated by binding to heme pyrrole nitrogen.

The best known example of inactivation of CYP through protein modification by a suicide inactivator is that of chloramphenical. The dichloroacetamido group is oxidized to an oxamyl moity that acylates a lysine residue in the CYP active center. This acylation event interferes with the transfer of electrons from CYP reductase to the heme group of the CYP and thereby prevents catalytic turnover of the enzyme (Lin and Lu, 1998).

Mechanism of Induction of CYP

The induction of cytochrome P450 (CYP) enzymes by xenobiotics is a major concern due to the enhanced metabolism of pharmaceutical drugs and endogenous substrates. The oxidative metabolism of drugs to more polar metabolites is an important mechanism allowing for the elimination of xenobiotics (Tompkins and Wallace, 2007). In drug therapy, there are 2 major concerns related to CYP induction. First, induction will result in a reduction of pharmacological effects caused by increased drug metabolism. Secondly, induction may create an undesirable imbalance between "toxification" and "detoxification". Like a double-edged sword, induction of drug metabolizing enzymes may lead to a decrease in toxicity through acceleration of detoxification, or to an increase in toxicity caused by increased information of reaction metabolites. Depending upon the delicate balance between detoxification and activation, induction can be a beneficial or harmful response (Lin and Lu, 1998).

Many of the CYPs are induced in humans including CYP1A, CYP2A, CYP2B, CYP2C, CYP2E1, and CYP3A by a diverse array of compounds including drugs, industrial chemicals, natural products and ethanol. The inducible CYPs make up a large percentage of the CYPs in the human liver and are responsible for the metabolism of a large proportion of pharmaceutical drugs. In most cases, induction of CYPs occurs by a process involving de novo RNA and protein synthesis that has been demonstrated in studies using transcription and translation inhibitors. The induction of many CYPs occurs by a similar mechanism, where ligand activation of key receptor transcription factors including pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR) and others, leads to increased transcription. The induction of CYPs is highly conserved and is found not only in humans but also in many other species including rodent models. An alternative mechanism of CYP induction involves compounds that stabilize translation or inhibit the protein degradation pathway (Tompkins and Wallace, 2007).

In the human liver, CYP3A4 is the most highly expressed CYP enzyme and is highly inducible by a wide variety of xenobiotics. The mechanisms by which structurally diverse xenobiotics induce CYP3A4 protein expression have been extensively investigated. Induction of CYP3A4 can also have serious toxicological consequences as a result of increased drug metabolism that contributes to drug–drug

interactions, the bioactivation of xenobiotics to carcinogenic or toxic metabolites and possibly endocrine disruption. The CYP3A4 induction by xenobiotics is now thought to be largely due to xenobiotic binding and activation of this orphan receptor. The Evans group named this protein the steroid and xenobiotic receptor (SXR) for its ability to bind such a wide variety of steroids and xenobiotics.

PXR is a member of the nuclear receptor superfamily and contains modular functional domains, including a DNA-binding domain and a ligand-binding domain. PXR is most closely related to the vitamin D receptor (VDR) and like VDR, binds to DNA elements as a heterodimer with a retinoid X receptor alpha (RXRα). PXR response elements have been well characterized, with the receptor binding to direct repeat with a 3-nucleotide (nt) spacer (DR3), everted repeat with a 6-nt spacer (ER6) and a direct repeat with a 4-nt spacer (DR4). These elements have been identified in the human CYP3A4 and rat CYP3A23 promoters and sites are bound by PXR in the presence of xenobiotics. X-ray crystallography of the ligand-binding domain of PXR determined that PXR has a much larger ligand binding pocket when compared to other receptors; enabling PXR to bind such a wide variety of ligands. Since the initial identification of PXR, a growing list of compounds has been found to bind and activate this receptor including the chemotherapeutic agents, environmental contaminants and antibiotics (Tompkins and Wallace, 2007).

The promiscuous nature of PXR binding to so many xenobiotics led Blumberg and Evans to propose that PXR acts as a nonspecific xenobiotic-sensor. The CYP3A magnitude of induction also varies greatly between individuals in human studies and it has been suggested that polymorphisms in PXR may be important in this wide variability (Tompkins and Wallace, 2007).

2.4 Transporters

Membrane transporters have long been recognized to be an importance class of proteins for regulating cellular and physiologic solute and fluid balance (Ho and Kim, 2005). With the initial sequencing of the human genome, it has been estimated that approximately 500 to 1200 genes encode transport proteins (Venter *et al*., 2001; Lander *et al*., 2001). A number of those transporters appear to facilitate or in some cases prevent drug passage through membrane barriers. These drug transporter proteins tend to be multi-functional and often have normal physiologic role in terms of transporting endogenous substances such as sugar, lipids, amino acids, bile acids, steroids and hormones (Ho and Kim, 2005).

Drug transporter

Drug transporter are expressed in many tissue such as the epithelial cells of the intestine and kidney, hepatocytes, and brain capillary endothelial cells (fig. 12) (Inui *et al*., 2000; Aubel *et al*., 2000; Mizuno *et al*., 2003), play key roles in the absorption, distribution and excretion of drugs and are one of the determinant factors governing drug disposition. Transporters have been classified as primary, secondary or tertiary active transporters. Secondary or tertiary active transporters are driven by an exchange of intracellular ions while the driving force for primary transporters, like ATP-binding cassette transporters, is ATP hydrolysis. The tissue distribution and elimination route of some drugs is determined by the degree of expression of each transporter subtype in each tissue and its corresponding substrate affinity (Mizuno and Sugiyama, 2002). Drug transporter can be generally separated in to 2 major classes- uptake and efflux transporters.

- Uptake transporters act by facilitating the translocation of drug into cells. Included with in this class of transporters are members of the organic anion transporting polypeptide (OATP, *SLCO*) family, organic anion transporter (OAT, *SLC22A*) family, organic cation transporter (OCT, *SLC22A*) family, organic cation/carnitine transporter (OCTN, *SLC22A*) family and peptide transporter (PEPT, *SLC15A*) family (Ho and Kim, 2005).

- Efflux transporters function to export drugs from the intracellular to the extra cellular milieu, often against height concentration gradients. Most efflux transporters are the member of the adenosine triphosphate (ATP)- binding cassette (ABC) superfamily of trans-membrane proteins, which use energy derived from ATP hydrolysis to mediate substrate translocation across biologic membranes (Ho and Kim, 2005).

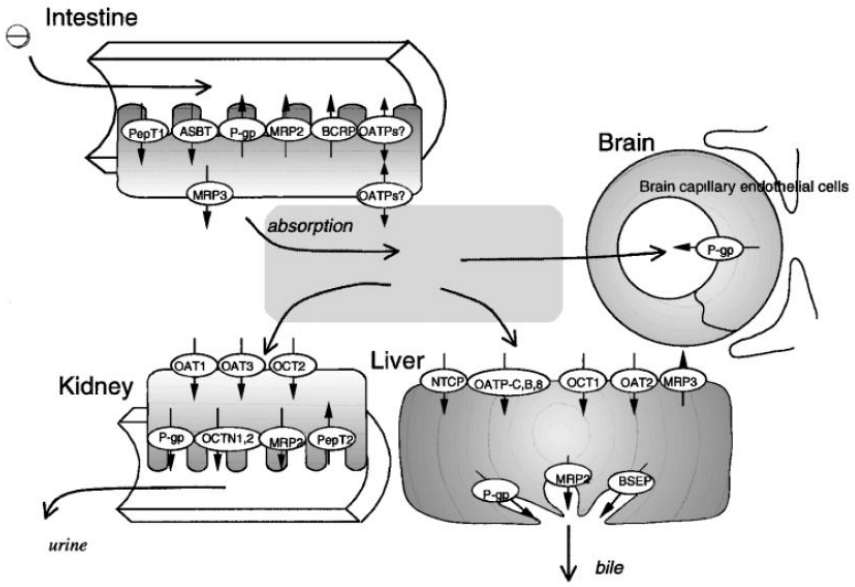


Fig. 12 Major drug transporters in human (Mizuno and Sugiyama, 2002)

Transporters and tissue drug distribution

Targeted locolization of transporters appears to facilitate the intestinal absorption and urinary or biliary excretion of many drugs. In the small intestine, enterocytes possess a number of transporters critical for absorption of dietary constituents and drugs. In the liver, efficient extraction of drug from portal blood into hepatocytes is often madiated by uptake transporters expressed on the sinusoidal (basolateral) membrane and efflux transporters localized on the canalicular (apical) membrane of the hepatocyte are transport of drugs from portal circulation in to bile. In kidney, the coordinate function of uptake and efflux transporters localized to basolateral and apical membranes of proximal tubular cells. In blood brain barrier (BBB), localized to the luminal side of BBB endothelial cells (Ho and Kim, 2005).

Transporters and drug-drug interactions

As the number of newly approved drugs increase, medication errors and drug-drug interactions have become a significant source of patient morbidity and death. Factors such as age, diet, nonprescription drugs, herbal and alternative therapies may also influence the development and extent of drug interactions. In additions, inhibition of drug metabolizing enzymes, as well as transporters, is now recognized as the likely mechanism accounting for many drug-drug interactions, because transporters and CYP enzymes often share overlapping tissue expression and substrate capacities (Ho and Kim, 2005).

2.5 Drug interactions

Drug interactions are a change in the way a drug acts in the body when taken with certain other drugs, herbals, or foods, or when taken with certain medical conditions. Drug interactions may cause the drug to be more or less effective, or cause effects on the body that are not expected. Since, the drug interactions have many reasons to classification, but a major contributor must be extent to which our knowledge of CYP isoforms has burgeoned, since altered drug metabolism is a major mechanism underlying many important drug interactions.

Types of drug interactions can be catagorized as pharmacokinetic and pharmacodynamic.

1) Pharmacokinetic interactions

Pharmacokinetic interactions cause by one drug alters the rate or extent of absorption, distribution, metabolism or excretion of drug. Measured by a change in one or more kinetic parameters, such as maximum concentration, area under the concentration-time curve, half-life, and total amount of drug excreted in urine, etc.

The rate and extent of absorption can be affected by physio-chemical factors such as complexation and non specific absorption of the drug and by physiologic factors such as gastrointestinal motility, gastrointestinal pH, gastrointestinal disease, gastric emptying time, intestinal blood flow, intestinal metabolism and induction or inhibition of transporters.

Altered drug distribution in drug interactions is explained mainly by displacement of drug from plasma proteins or receptor binding sites. Protein binding displacement interactions after oral and intravenous dosing of low hepatically extracted drugs are generally clinically unimportant because unbound drug concentrations at steady state in plasma do not change or are transiently changed by displacement. Protein displacement interactions may cause adverse events if the displaced drug is highly bound (>95%) to plasma proteins at therapeutic concentrations and has a small volume of distribution and narrow therapeutic range.

Most clinically relevant interactions result from changes in drug elimination caused by inhibition or induction of metabolic enzymes present in the liver and extrahepatic tissues. These processes can cause changes in intrinsic clearance of elimination pathways, which result in alteration in unbound plasma concentrations at steady state for orally administration drugs.

The results of these interactions can be a decreased or an increased exposure of both interacting drugs which, in turn, can lead to reduced efficacy or increase toxicity, respectively. Pharmacokinetics drug interactions are manageable using dose adaptation, eventually supported by therapeutic drug monitoring (Byrne, 2003).

2) Pharmacodynamic interactions

Pharmacodynamic interactions are the pharmacological response to a drug, which is directly altered without a change in pharmacokinetic properties. This means that pharmacodynamic interactions are directly related to the desired or undesired drug effects. This can lead to potentiation of effect (including toxicity) in either an additive or synergistic manner, or antagonism (Bruggemann *et al*., 2008).

Additive or synergistic interactions occur when two drugs with similar pharmacological properties are given together. A common example is ethanol combined with other sedative drugs such as benzodiazepine anxiolytics or histamine H_1-receptor antagonists is used for travel sickness. Benzodiazepines alone have a high therapeutic index and while overdoses may cause prolonged sedation they are seldom fatal. In contrast, a combination of benzodiazepine overdose with ethanol is often fatal. Synergy or potentiation is seen when sulphonamide antibiotics are combined with trimethoprim. Both drugs are bacteriostatic when given alone, while the combination is bactericidal. Drugs with similar adverse effects may also be additive particularly with respect to ototoxicity (e.g., ethacrynic acid and streptomycin) or nephrotoxicity (e.g., tobramycin and cephalothin). However, not all combinations of similar drugs cause clinical problems. For example, furosemide and streptomycin combinations do not appear to result in enhanced ototoxicity in practice.

Receptor agonist and antagonist interactions come into this classification. Many of these are useful, such as naloxone reversal of an opioid overdose and flumazenil reversal of benzodiazepine-induced sedation. It also includes functional antagonisms in which two drugs exert opposite effects on different receptor systems and thus physiologically oppose one another. Glucocorticoids cause hyperglycemia and oppose the actions of hypoglycemic agents. The opposing effects of the two drugs are well known and the combination is seldom prescribed (Pleuvry, 2005).

Drug interactions occur not only with other medications, but also with food and herb.

Drug-drug interactions

Drug-drug interaction is a modification of the effect of a drug when administered with another drug. The effect may be an increase or a decrease in the action of either substance, or it may be an adverse effect that is not normally associated with either drug. Aronson, (2004) classifying of drug-drug interactions mechanistically gives major insight into how to predict, detect, and avoid them are pharmaceutical interactions, pharmacokinetic interactions and pharmacodynamic interactions.

Drug-food interactions

Drug-food interactions are the effect produced when some drugs and certain foods or beverages are taken at the same time. For example, grapefruit juice blocks the metabolism of some drugs in the GI tract, an action that can cause normal dosages of a drug to reach toxic levels in the plasma. Drug-food interactions can be a major source of patient inconvenience and nonadherence through disruptions in a patient daily schedule. Unless advised to the contrary, patients often take drugs with meals as a suitable adherence remainder and to lesson gastrointestinal side effects (Harris, 2001).

Drug-herbal interactions

Herbs have been used for medicinal purposes since the beginning of recorded time. About one third of drugs (including digitalis, morphine, atropine and several chemotherapeutic agents) were developed from plants. So, indeed, herbs can be potent products. Herbs can affect body functions; therefore, when herbs are taken concurrently with drugs, interactions are possible. The mechanism of action of many herbs has not been determined. Therefore, the exact mechanisms of drug-herb interaction are also unknown (Kuhn, 2002).

2.6 Herb-drug interactions

Herbal medicines are becoming popular worldwide, the use of herbal medicinal products among the general public is increasing over recent years since the World Health Organization urged its member countries to use folk healing practices and herbal medicines as part of the basic public health projects (Laddawan, 2001). This has also prompted Thailand to become interested in using herbal medicines and Thai traditional medicines. The Thai government began to support an attempt to develop herbal medicines and Thai herbal drugs systematically. Some items of herbal medicines were placed on the National list of Essential medicines in 1999 (The National Drug Committee, 2000) as part to promote the use of herbal medicine and provide diversity of alternatives for health care.

Clinical evidence for some important herb-drug interactions is presented, together with an explanation of pharmacodynamic interactions and pharmacokinetic effects involving CYP450 enzymes. However, the true extent of the incidence and significance of herb-drug interactions is still largely unknown, because unlicensed medicines are rarely prescribed by qualified clinicians, and it is only recently that schemes have been launched to encourage doctors or pharmacists to report adverse reactions to herbal medicines and herb-drug interactions (Williamson, 2006). It is important that potential herb-drug interactions should be identified in order to prevent adverse outcomes in patient concurrent consumption of herbal products and conventional drugs. Identification of the mechanism involved in any interaction is nessasary; it will offer the approaches to minimize their impact and can be design appropriate studies in humans.

Mechanism of herb-drug interactions

The overlapping substrate specificity in the biotransformational pathways of the physiologic systems is seen as the major reason for drug-drug, food-drug, and herb-drug interaction (Marchetti *et al*., 2007). The ability of different chemical moieties to interact with receptor sites and alter physiological environment can explain pharmacodynamic drug interactions while pharmacokinetic interactions arise from altered absorption, interference in distribution pattern as well as changes and competition in the metabolic and excretory pathways (Izzo, 2005). The major underlying mechanism of pharmacokinetic herb-drug interaction, like drug-drug interaction, is either the induction or inhibition of intestinal and hepatic metabolic enzymes particularly the CYP enzyme family.

The interaction of herbal products with hepatic enzymes can also result in induction and inhibition of metabolic enzymes. Induction is the increase in intestinal and hepatic enzyme activity as a result of increased mRNA transcription leading to protein levels higher than normal physiologic values. When this happens, there is a corresponding increase in the rate of drug metabolism affecting both the oral bioavailability and the systemic disposition. In the formulation and dosage design of oral medications, allowance is often made for pre-systemic metabolism in order to

achieve predictable systemic bioavailability. A disruption in this balance can result in significant changes in blood concentrations of the drugs. Herbal products have been shown to be capable of inducing CYP. Concomitant administration of enzyme-inducing herbal products and prescription drugs can therefore result in sub-therapeutic plasma levels of the latter with therapeutic failure as a possible clinical consequence.

Apart from enzyme induction, herbal products can also inhibit enzyme activities. The inhibition of CYP and other metabolic enzymes is usually competitive with instantaneous and inhibitor concentration-dependent effects (Zhang and Wong, 2005). Most inhibitors are also substrates of CYP (Zhou, 2008). This phenomenon alters pharmacokinetic profiles of xenobiotics significantly. As a result of the suppression of the anticipated pre-systemic intestinal and hepatic metabolism, unusually high plasma levels of xenobiotics are observed. Toxic manifestation could be the ultimate effect of this observation. An equally clinically important consequence of enzyme inhibition is drug accumulation due to subdued hepatic clearance. These effects will be of particular concerns in drugs with narrow therapeutic window or steep dose-response profiles. The induction and inhibition of CYPs by herbal products in the presence of a prescribed drug has led to adverse effects. Some of herbal medicines might be caused the adverse effect such as St John's wort (*Hypericum perforatum*), Gingko (*Ginkgo biloba*) and Grapefruit.

St John's wort (*Hypericum perforatum*) is one of the most widely used herbal antidepressants (Hoyland, 2011). It is a potent inducer of CYP3A4 and depending on the dose, duration and route of administration; it may induce or inhibit other CYP isozymes and P-glycoprotein (Madabushi *et al*., 2006). Studies from case reports indicate that, due to its inducing effects on CYP3A4, it significantly reduces the plasma levels of CYP3A4 substrates including cyclosporine, simvastatin, indinavir, warfarin, amitriptyline, tacrolimus, oxycodone, and nevirapine (Vlachojannis *et al*., 2011). It has also been reported that the alteration in the blood serum concentration of cyclosporine due to St John's wort has led to organ rejection in patients (Murakami *et al*., 2006). Reports of breakthrough bleeding and unplanned pregnancies due to interaction between St John's wort and oral contraceptives have also been documented (Hu *et al*., 2005). The group of drugs with the highest potential

for clinically significant pharmacokinetic drug interaction with St John's wort is the antidepressants as St John's wort itself is consumed by patients with depression. Its concomitant use with selective serotonin reuptake inhibitors like sertraline and paroxetine has been reported to result in symptoms of central serotonergic syndrome (Bonetto *et al*., 2007). It has also been said to increase the incidence of hypoglycemia in patients on tolbutamide without apparent alteration in the pharmacokinetic profile of tolbutamide (Mannel, 2004). It also inhibits the production of SN-38, an active metabolite of irinotecan, in cancer patients.

Amitriptyline is a substrate to both CYP3A4 and intestinal P-glycoprotein. The risk of therapeutic failure is thus high due to induction of CYP3A4-dependent metabolism activities resulting in poor oral bioavailability. In a study by Johne *et al*. (2002), a 21% decrease in the area under the plasma concentration-time curve of amitriptyline was observed in 12 depressed patients who were concomitantly administered with extracts of St John's wort and amitriptyline for 2 weeks.

Other CYP and P-glycoprotein substrates whose pharmacokinetic profile have been reportedly altered by St John's wort include anticoagulants like phenprocoumon and warfarin; antihistamines like fexofenadine; antiretroviral drugs including protease inhibitors and reverse transcriptase inhibitors; hypoglycemic agents such as tolbutamide; immunosuppressants like cyclosporine, tacrolimus, and mycophenolic acid; anticonvulsants such as carbamazepine; anti-cancer like irinotecan; bronchodilators like theophylline; antitussive like dextromethorphan; cardiovascular drugs like statins, digoxin, and dihydropyridine calcium channel blockers; oral contraceptives; opiates like methadone and loperamide; and benzodiazepines including alprazolam and midazolam (Hojo *et al*., 2011). Following a single dose administration of 300 mg standardized extracts of St John's wort containing 5% hyperforin in humans, a maximum plasma concentration of 0.17-0.5 μM hyperforin yielding a $[I]/Ki > 0.22$, *in vivo* extrapolation suggests a high possibility of *in vivo* pharmacokinetic drug interaction (Agrosi *et al*., 2000). Bray *et al*. (2002) confirmed through animal studies that St John's wort modulates various CYP enzymes. Dresser *et al*. (2007) demonstrated that St John's wort is capable of inducing CYP3A4 in healthy subjects through the observation of increased clearance of midazolam. Thus animal and human studies further confirm St John's wort as

containing both inhibitory and inducing constituents on various CYP isozymes. These effects may depend on dosage and duration of administration, and may also be species- and tissue-specific. While the individual phytochemical constituents of St John's wort have elicited varying effects on the metabolic activity of the CYP isozymes, whole extracts and major constituents especially hyperforin have been reported to inhibit the metabolic activities of CYP1A2, 2C9, 2C19, 2D6, and 3A4 via *in vitro* studies and *in vivo* studies (Hokkanen *et al*., 2011).

Gingko (*Ginkgo biloba*) have been reported to induce CYP 2C19-dependent omeprazole metabolism in healthy human subjects (Yin *et al*., 2004). Piscitelli *et al*. (2002) in a garlic–saquinavir interaction study reported 51% decrease in saquinavir oral bioavailability caused by the presence of garlic and attributable to garlic-induced CYP3A4 induction. Its effects on the warfarin pharmacokinetic have also been reported in animal models (Taki *et al*., 2012).

Grapefruit have been demonstrated to have a preventive influence on many chronic diseases, such as cancer and cardiovascular disease. Grapefruit juice, which has revealed herbal drug interaction potentials in flavonoid-containing herbal remedies (Alvarez *et al*., 2010). A related CYP inhibitor is rotenone. By interfering with the electron transfer of the heme iron, rotenone, a naturally occurring phytochemical found in several plants such as the jicama vine plant is known to inhibit CYP activity (Sanderson *et al*., 2004). Resveratrol, a natural polymer and tryptophan, an amino acid have been documented as potent CYP inhibitors (Rannug *et al*., 2006). The other studies have been overshadowed by the possible risk of interactions between drugs and grapefruit juice (Mertens-Talcott *et al*., 2006). The active ingredients of grapefruit are the flavonoid, naringin, furanocoumarin and 6', 7'-dihydroxybergamottin. The primary mechanism through which interactions are mediated is mechanism-based intestinal CYP3A4 inhibition by furanocoumarins resulting in increased bioavailability of administered medications that are substrates. Clinically relevant interactions seem likely for most felodipine, terfenadine, saquinavir, cyclosporine, midazolam, triazolam, verapamil, lovastatin, cisapride and astemizole. The importance of these interactions appears to be influenced by

individual patient susceptibility, type and amount of grapefruit juice and administration-related factors (Arayne *et al*. 2005; Seden *et al*., 2010).

Potential of *Phylanthus amarus* interactions on cytochrome P450s

Previous studies have reported that aqueous extract of *P. amarus* exert an inhibitory effect on several recombinant human CYP isoforms. Bhaskarannair *et al*. (2006) reported that an alcoholic extract of *P. amarus* inhibit CYP enzymes both *in vivo* as well as *in vitro*. This was studied using specific resorufin derivatives, as substrate for isoenzymes in the P450 super family. IC_{50} for 7-ethoxyresorufin-O-deethylase, (CYP1A1) was 4.6 mg/mL while those for 7-methoxyresorufin-O-demethylase (CYP1A2) was 7.725 µg/mL and 7-pentoxyresorufin-O-dependentylase (CYP2B1/2) was found to be 4.18 µg/mL indicating that the extract inhibited the P450 enzymes at very low concentration. Extract also inhibited the activity of aniline hydroxylase (an indicator of CYP 2E1 activity, IC_{50} = 50 µg/mL) and aminopyrine demethylase (an indicator of CYP 1A, 2A 2B, 2D and 3A activity, IC_{50}>1000 µg/mL). Oral administration of the extract was also found to reduce the elevated P450 enzyme activities produced by phenobarbitone by 50% at 250 mg/kg body weight. Appiah-Opong *et al*. (2008) also reported that *P. amarus* aqueous extracts inhibited CYP1A2 and CYP2C9 with IC_{50} values ranging between 28.3-134.3 µg/mL and between 63.4-425.9 µg/mL, respectively. Similarly, both CYP2D6 and CYP3A4 were inhibited by *P. amarus* whole plant, leaf, stem and root ,with IC_{50} values ranging between 45.8-182.0 µg/mL and between 79.2-158.8 µg/mL, respectively. In addition, Anannarukan *et al*. (2012) reported that *P. amarus* aqueous extract possessed inhibitory effects on CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 with IC50 of 74.30 ± 1.21, 34.80 ± 0.34, 180.40 ± 2.53, 49.41 ± 0.52 and 2.07 ± 0.03 µg/mL,respectively. The inhibitory effects of plant extracts on these CYPs indicate the possibilities of herb-drug interaction.

Moreover, recently have report about the inhibitory effect of *P. amarus* and its major phytochemicals phyllanthin and hypophylanthin on CYP isoform. Both ethanolic and aqueous extract of *P. amarus* inhibited CYP1A2, CYP2D6, CYP2E1 and CYP3A4 in dose-dependent manner. However, the extracts

were compared to known CYP3A inhibitors, the IC_{50} value of both extracts on testosterone 6β-hydroxylation were higher than that of ketoconazole but lower than those of erythromycin and clarithromycin. Both extracts were weak inhibitors of CYP1A2, CYP2D6 and CYP2E1. Additionally, the ratios of K_{inact}/K_I for phyllanthin and hypophyllanthin were much higher when compared to several therapeutic drugs that act as mechanism-based inhibitors of CYP3A4 (Taesotikul *et al*., 2011). An *in vivo* study, the effect on CYP3A-mediated drug metabolism in rats after oral administration of *P. amarus* extract was investigated using midazolam (MDZ) as a probe substrate. Daily administrations of *P. amarus* extract (200 or 800 mg/kg/day) for 15 days in rats increased the activity and expression of CYP3A and CYP2B1/2. In contrast, the activities and expressions of CYP1A, CYP2C and CYP2E1 were not significantly changed. The dual effects of *P. amarus* extract on CYP enzymes were demonstrated. Single dose administration of the extract increased oral bioavailability of MDZ through inhibition of intestinal CYP3A whereas repeated administration of the extract slightly induced hepatic CYP3A and CYP2B1/2 in rats, which suggested that herb-drug interactions by *P. amarus* may potentially occur via CYP3A and 2B (Taesotikul *et al*., 2012).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Drugs

Midazolam tablets (Dormicum®, 15 mg/tablet, Batch No. B1772B01) were purchased from Ministry of Public Health, Food and Drug Administration, Bangkok, Thailand. Manufactured by Hoffmann-La Roche Ltd, Basel, Switzerland.

3.1.2 Plant materials

Phyllanthus amarus Schum. & Thonn. powder (4 kg, Lot no. 250554) were purchased from Lampang Herb Conservation, Thailand.

3.1.3 Chemicals and Reagents

Standard powder of midazolam (Alltech-Applied Science, USA), and diazepam were obtained from Department of Pathology, Faculty of Medicine, Prince of Songkla University, Thailand.

Standard powder of phyllanthin, hypophyllanthin were obtained from Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Methanol and acetonitrile (HPLC grade) were purchased from J.T. Baker NJ, USA.

Potassium dihydrogen phosphate (KH_2PO_4) and diethyl ether were purchased from Merck Darmstadt, Germany.

Water was purified by Milli Q Water Purification System, Milipore, Milford, MA, USA.

3.2 Equipments

3.2.1 High performance liquid chromatography (HPLC)

Agilent 1200 LC Series consisted of

a) Vacuum Degasser
b) Autosamplers
c) Thermostatted Column Compartment [G1316A/G1316B]
d) Variable Wavelength Detector (VWD) [G1314B/G1314C (SL)]
e) Quaternary pump

The signals from the detector were collected and analyzed using a computer equipped with Agilent Chemstation (Agilent Technologies INC., USA)

3.2.2 Instruments

a) Vortex mixer
b) Centrifuge machine
c) pH meter
d) Micropipette (100, 200, and 1,000 µL)
e) Pipette tip
f) Disposable needle (20-23G)
g) Heparin lock
h) Test tube with cap (10 mL)
i) Disposable syringe (3 mL, 5 mL, and 10 mL)
j) Eppendorf microcentrifuge tube (1.5 mL, 2 mL)
k) Nylon membrane disc, 4.7 mm×0.22 µm
l) Whatman filter paper no. 1

3.3 Methodology

3.3.1 Preparation of *P. amarus* extract and medication

P. amarus powder was extracted twice with 70% ethanol (1:5 w/v) at room temperature for 7 days. The filtrate was subsequently evaporated at 60 ºC and lyophilized by freeze-dryer. The final yield of the preparation was 13% w/w. The extract was stored at -20 ºC until used. The portion of the extract was freshly reconstituted in 10% of gum acacia at desired concentrations prior to the experiment.

Each animal received single dose of 500 mg/kg *P. amarus* extract orally for 7 days and once in the morning on day 8, 1 h. before midazolam administration**.**

3.3.2 Determination of phyllanthin and hypophyllanthin in *P. amarus* extract

Sample preparation

A sample solution of *P. amarus* extract was prepared by accurately weighing 1 g dried powder and dissolved in 1 mL of methanol. A sample solution was centrifuged at 1,300×g for 10 minutes. The supernatant was transferred into a microcentrifuge tube and diluted to 10 µg/mL, and 20 µL was injected into high performance liquid chromatography (HPLC) system for analysis.

Chromatographic conditions

The method was modified from Murugaiyah and Chan (2007) using the following condition:

Column	: Symmetry C_{18} column: reverse-phase column, 3.9 mm × 150 mm, particle size 5 µm, Waters Corporation, USA.
Guard column	: Zorbax reliance cartridge C_{18}, 4.6 mm × 12.5 mm
Mobile phase	: Mixture of acetonotrile: water (52:48 v/v)
Flow rate	: 1.0 mL/min
Injection volume	: 20 µL
Detector	: Variable-wavelength UV detector, 220 nm
Temperature	: 25 °C

Standard curve

The standard calibration curve of phyllanthin, hypophyllanthin were prepared by using concentration of standard phyllanthin, hypophyllanthin in methanol at 250, 500, 1000, 1500, 2000 and 2500 ng/mL. Standard calibration curves were constructed by the least-square linear regression of phyllanthin, hypophyllanthin concentration and peak area of phyllanthin, hypophyllanthin. Unknown concentrations of phyllanthin, hypophyllanthin in *P. amarus* extract were calculated from the standard curve by reverse prediction.

3.3.3 Determination of phyllanthin and hypophyllanthin in plasma

Sample preparation

The 50 µL of 3 µg/mL of internal standard (diazepam dissolved in methanol) was added to the 250 µL of plasma sample and 250 µL of acetonitrile for deproteinized. The mixture was vortex-mixed for 1 minute and centrifuged at 1,300×g for 10 minutes. The supernatant was transferred into a microcentrifuge tube and 50 µL was injected into high performance liquid chromatography (HPLC) system for analysis.

Chromatographic conditions

The method was modified from Murugaiyah and Chan (2007) using the following condition:

Column	: Symmetry C_{18} column: reverse-phase column, 3.9 mm × 150 mm, particle size 5 µm, Waters Corporation, USA.
Guard column	: Zorbax reliance cartridge C_{18}, 4.6 mm × 12.5 mm
Mobile phase	: Mixture of acetonotrile: water (52:48 v/v)
Flow rate	: 1.0 mL/min
Injection volume	: 50 µL
Detector	: Variable-wavelength UV detector, 220 nm
Temperature	: 25 °C

Standard curve

The working standard solution (10 µg/mL) was diluted to concentration of 250, 500, 1000, 1500, 2000 and 2500 ng/mL with drug-free plasma. Standard calibration curves were constructed by the least-square linear regression of phyllanthin, hypophyllanthin concentration and peak area ratio of phyllanthin, hypophyllanthin to diazepam (internal standard). Unknown concentrations of phyllanthin, hypophyllanthin in rabbit's plasma were calculated from the standard curve by reverse prediction.

Stock standard solution

The stock standard phyllanthin, hypophyllanthin and diazepam (internal standard) solutions at concentration of 1 mg/mL were prepared in methanol and stored at -20 °C under light protecting condition.

3.3.4 Determination of midazolam in plasma

Sample preparation

The 50 µL of 3 µg/mL of internal standard (diazepam dissolved in methanol) was added to the 250 µL of plasma sample and 1 mL of diethyl ether. The mixture was vortex-mixed for 5 minutes and centrifuged at 1,300×g for 10 minutes. The 800 µL of upper organic phase was separated and evaporated at 35 °C under air flow. The residue was reconstituted in 100 µL of the mobile phase and 50 µL was injected into high performance liquid chromatography (HPLC) system for analysis.

Chromatographic conditions

The method was modified from Megarbane *et al*. (2005) using the following condition:

Column	: Luna C_8 column: reverse-phase column C_8, 250 mm × 4.6 mm, particle size 5 µm, Phenomenex Inc., USA.
Guard column	: Security guard cartridges C_8, 4.0 mm×3.0 mm
Mobile phase	: Mixture of 0.025 M KH_2PO_4 (pH 4.6): Acetonitrile: Methanol (35:30:35, v/v/v)
Flow rate	: 1.2 mL/min
Injection volume	: 50 µL
Detector	: Variable wavelength UV detector, 210 nm
Temperature	: 25 °C

Standard curve

The working standard solution (10 µg/mL) of midazolam was diluted to concentration of 50, 200, 400, 600, 800 and 1000 ng/mL with drug-free plasma. Standard calibration curve was constructed by the least-square linear regression of midazolam concentration and peak area ratio of midazolam to diazepam (internal standard). Unknown concentrations of midazolam in rabbit's plasma were calculated from the standard curve by reverse prediction.

Stock standard solution

The stock standard midazolam and diazepam (internal standard) solutions at a concentration of 1 mg/mL were prepared in methanol and stored at -20 °C under light protecting condition. The working solution was prepared in methanol. The stock standard midazolam at a concentration of 1 mg/mL were stored at -20 °C and stable for at least 12 months (Mahjoub and Staub, 2000). The extracted samples of midazolam were stable at 4 °C in the dark for 10 days before analysis (Jurica *et al.*, 2007). Midazolam in whole blood stored on ice bath (0-4 °C) and at room temperature is stable at least 2 h. Samples stored in -20 °C remained stable for at least 3 months. Extracted sample were stable when kept at ambient temperature in mobile phase for 46 h prior to analysis (Zhang *et al*., 2010). Working standard solutions used to prepare a standard calibration curve were freshly prepared by diluting the solution with drug-free plasma.

3.4 Method validation

The method validation was carried out according to the guidance of the US FDA, 2001.

1. Lower limit of quantification (LLOQ)

The LLOQ was the lowest amount of analytical which can reliably to quantify under the condition. The response should be at least five times response compared to blank response. In addition, the analyze peak (response) in LLOQ sample should be measured with acceptable accuracy and precision, the percentage of coefficient of variation (%CV) should be less than 20 % and the %accuracy should be within the range of 80-120%.

2. Linearity/standard calibration curve

Linearity was evaluated using freshly prepared blank plasma spiked with 50, 200, 400, 600, 800, 1000 ng/mL of midazolam, 250, 500, 1000, 1500, 2000, 2500 ng/mL of phyllanthin, hypophyllanthin. Samples were quantified using peak area ratio of midazolam, phyllanthin, hypophyllanthin and diazepam (internal standard) as the assay parameter. Standard calibration curves were constructed by the least-square linear regression of peak area ratio of standard and concentration. The coefficient of determination (r^2) should be more than 0.99.

3. Precision and accuracy

To assay intraday precision and accuracy, drug free plasma was spiked with midazolam standard solution at 100, 500 and 900 ng/mL and with phyllanthin, hypophyllanthin standard solution at 375, 1250 and 2250 ng/mL. Five replications of each concentration were analyzed in one day.

To assay interday precision and accuracy, drug free plasma was spiked with midazolam standard solution at 100, 500 and 900 ng/mL and with phyllanthin, hypophyllanthin standard solution at 375, 1250 and 2250 ng/mL. Two replications of each concentration were analyzed for five days. The %CV of each concentration should be less than 15 % and the %accuracy of each concentration should be within the range of 85-115%. The percentage of coefficient of variation and accuracy were calculated as following:

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

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

4. Recovery

Recovery of plasma midazolam and phyllanthin, hypophyllanthin were assayed by comparing peak area of extracted drug in plasma with peak area of the drug in mobile phase. The percentage of recovery of an analyze should be consistent, precise and reproducible. The percentage of recovery was calculated as following:

$$\text{Recovery (\%)} = \frac{\text{Peak area ratio of standard in plasma}}{\text{Peak area ratio of standard in mobile phase}} \times 100$$

3.5 Animals

Nine male New Zealand White rabbits obtained from The National Laboratory Animal Center, Mahidol University (NLAC-MU), Salaya, Nakornpathom, Thailand, were used in this study. The rabbits were at the age of 12-16 weeks old, weighing between 3.0 and 3.5 kg at the beginning of the study. The rabbits were housed in The Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University, Thailand, under controlled environment (temperature 25±2 °C and relative humidity of approximately 60% with 12 h light/12 h dark cycle). They received standard pellet diet and water *ad libitum* and were acclimatized for 7 days, then fasted for 12 h before the experiment. Experimental protocol was ethically approved by the Ethics Committee of Prince of Songkla University, Thailand. (Appendix A).

3.5.1 Sample size calculation

This study determined the effect of *P. amarus* on the pharmacokinetics of midazolam in rabbits. However, there were no reports on the interaction of *P. amarus* and midazolam in humans. So, the study of Kupferschmidt *et al*. (1995) on the pharmacokinetic interaction between grapefruit juice and midazolam in human was therefore used to calculate the sample size. They found that the AUC of midazolam was significantly increased from 143 ± 26 to 217 ± 31 µg.h/mL when co-administered with grapefruit juice.

The AUC difference of midazolam (d) = 217-143 = 74 µg.h/mL.

Type I error 5% ($\alpha = 0.05$), $Z_\alpha = 1.645$, and Type II error 10% ($\beta = 0.10$), $Z_\beta = 1.282$, Pooled variance (Sp^2) = 818.5

$$N = \frac{2(Z_\alpha + Z_\beta)^2 (Sp^2)}{d^2}$$

$$= \frac{2(1.645+1.282)^2(818.5)}{(74)^2}$$

$$= 2.56$$

$$\approx 3$$

A total sample size of 3 animals should be sufficient to detect a significant pharmacokinetic difference in AUC of midazolam. However, it is necessary to increase the sample size to offset the excluded or rejected from the study. Therefore, the sample size of animal used in this study was adjusted upward to 9 rabbits.

3.6 Protocol

Study design

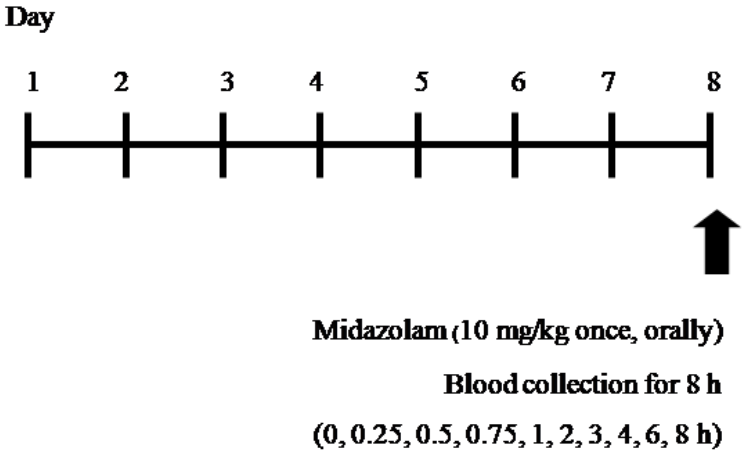
The study was an open-label, randomized, two-phase design with one week washout period, of midazolam, single-dose and *P. amarus* extract, multiple-dose.

Phase 1: After 12 h fasted, the animal received a single oral dose of 10 mg/kg midazolam (Dormicum®, Hoffmann-La Roche Ltd., Basel, Switzerland) with 10 mL of water. Blood samples were taken from marginal ear vein through a venous catheter before administration of midazolam (T 0) and at 0.25, 0.5, 0.75, 1, 2, 3, 4, 6 and 8 h post dose. The heparinized plasma was collected and stored at -20 ºC until analyzed.

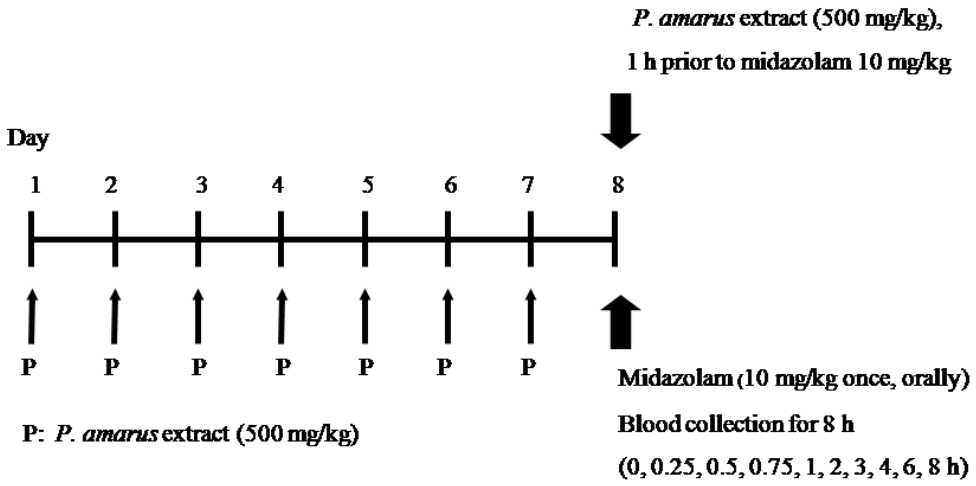
Phase 2: After one week washout period, a second midazolam pharmacokinetic characterization was performed on day 8 of *P. amarus* medication with the regimen presented below. Both phases of midazolam pharmacokinetics study were conducted identically. Blood samples were taken by the same manner as previously described in phase 1

Schematic plan of the study

Phase 1: A single oral dose of midazolam alone



Phase 2: *P. amarus* extract once daily, orally for 7 days and 1 h before a single oral dose of midazolam



Remarks: P = *P. amarus* extract (500 mg/kg, once daily, orally for 7 days)

3.7 Pharmacokinetics analysis

Plasma concentration of midazolam was plotted against time after the drug administration. The pharmacokinetic parameters of midazolam were determined using a non-compartmental method. The areas under the drug concentration-time (AUC) curves from 0 to 8 h (AUC_{0-8}) were calculated using the linear trapezoidal method from time 0 to 8 h after midazolam administration. The areas under the drug concentration time curves from zero to infinity ($AUC_{0-\infty}$) were calculated by the sum of AUC_{0-8} and ratio of the concentration at 8 h to the elimination rate constant (K_e). The maximum plasma concentrations (C_{max}) and the time to reach C_{max} (T_{max}) were obtained from the actual data. Clearance (CL/F) was calculated as dose/$AUC_{0-\infty}$. The elimination rate constant was obtained from the slope of the terminal log linear concentration-time value were determined using WinNonlin professional Software Version 1.1 (Pharsight, Mountain View, CA).

3.8 Statistical analysis

All pharmacokinetics parameters were expressed as the mean ± standard deviation ($\overline{X} \pm SD$). Paired *t*-test was applied for pair wise comparisons. The significance level was set at *p*-value less than 0.05. The software used was SPSS (SPSS for windows, Version 11.5, USA.).

CHAPTER 4

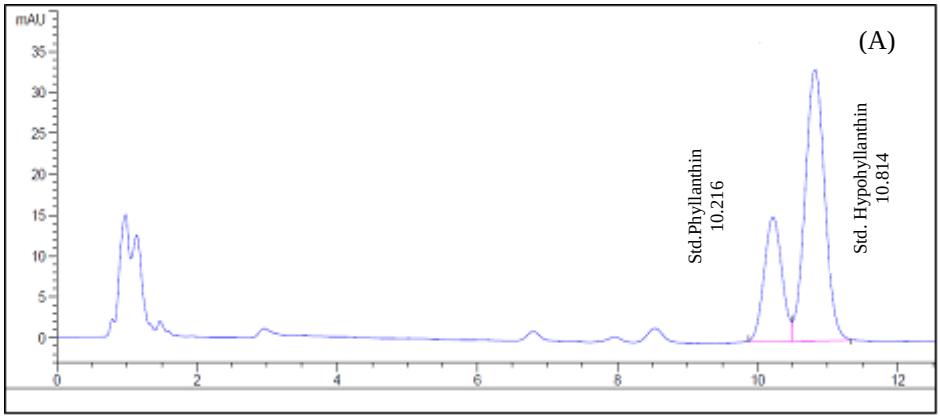
RESULTS

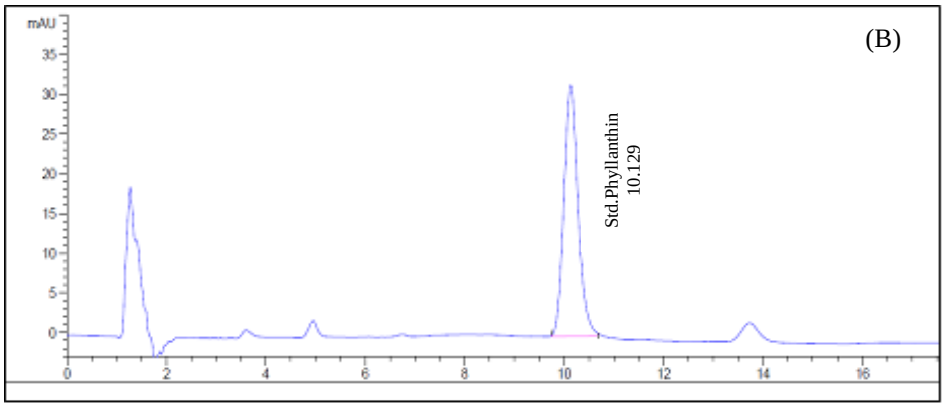
Chromatograms

4.1 Determination of phyllanthin and hypophyllanthin in *P. amarus* extract

4.1.1 Chromatograms

The chromatograms show that the peak of phyllanthin and hypophyllanthin in methanol were separated with no interference at around 10.2 and 10.8 min, respectively (Fig. 13A, 13B, 13C).





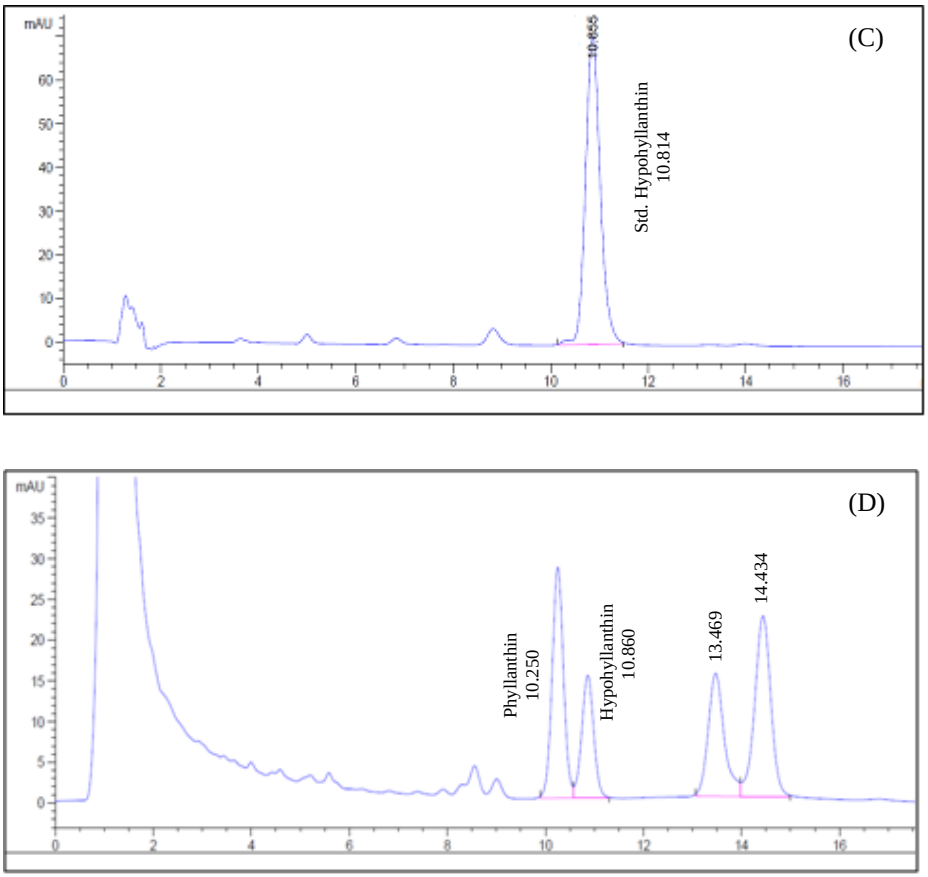


Fig. 13 Chromatograms of standard phyllanthin and hypophyllanthin (5 µg/mL) (A), phyllanthin (10 µg/mL) (B), and hypophyllanthin (10 µg/mL) (C) in methanol, and *P. amarus* ethanolic extract (D). Flow rate 1 mL/min.

4.1.2 Linearity of the standard calibration curve of phyllanthin and hypophyllanthin

The coefficient of determination (r^2) of the standard curves of phyllanthin and hypophyllanthin was 0.9993 and 0.9965, respectively. The linear regression equation of phyllanthin and hypophyllanthin was Y = 0.154X + 3.0391 and Y = 0.2827x - 15.03, respectively (Fig. 14A, 14B).

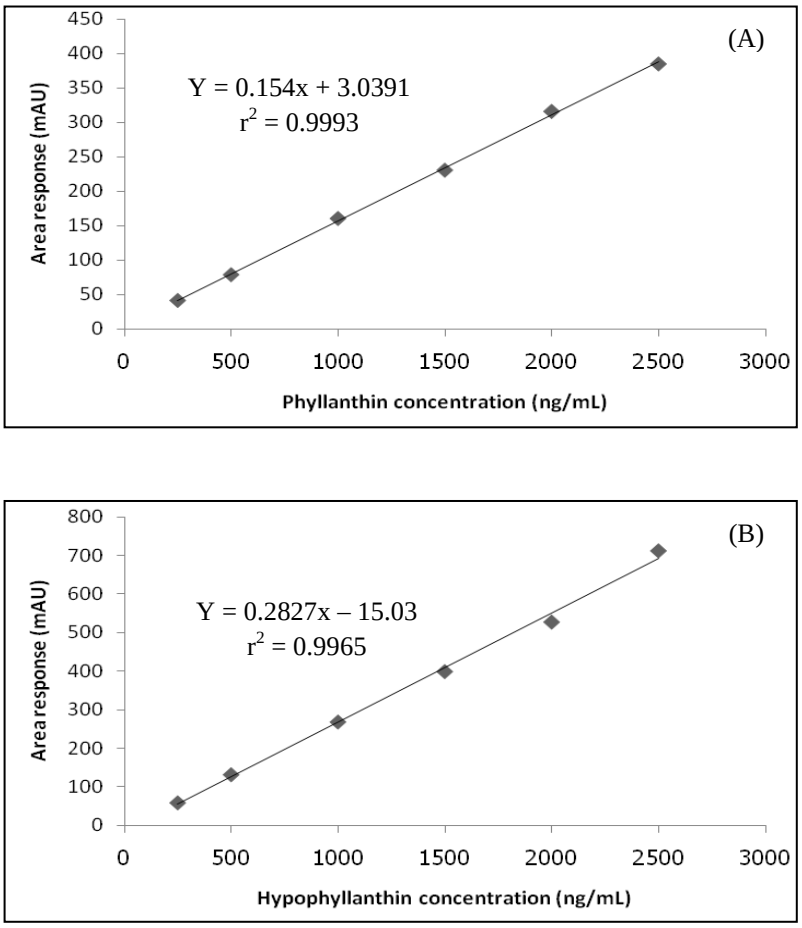


Fig. 14 Calibration curve of phyllanthin (A), hypophyllanthin (B) in methanol.

4.1.3 Content of phyllanthin and hypophyllanthin in *P. amarus* ethanolic extract

The average content of phyllanthin and hypophyllanthin in *P. amarus* extract were 296.69±8.04 and 96.56±4.36 mg/g, respectively (Table 2 and 3).

Table 2. The content of phyllanthin in *P. amarus* extract

N	**Content of phyllanthin (mg/g)**
1	295.00
2	307.27
3	287.79
$\overline{X}$ **±SD**	296.69±8.04

Table 3. The content of hypophyllanthin in *P. amarus* extract

N	**Content of hypophyllanthin (mg/g)**
1	96.54
2	101.92
3	91.23
$\overline{X}$ **±SD**	96.56±4.36

4.2 Assay validation of phyllanthin and hypophyllanthin in plasma

4.2.1 Chromatograms

The chromatograms showed that peaks of phyllanthin, hypophyllanthin and its internal standard (diazepam) were well separated from the other peaks in plasma as shown in Fig. 15. The internal standard, standard phyllanthin and hypophyllanthin were eluted at around 5, 10.2 and 10.8 min, respectively.

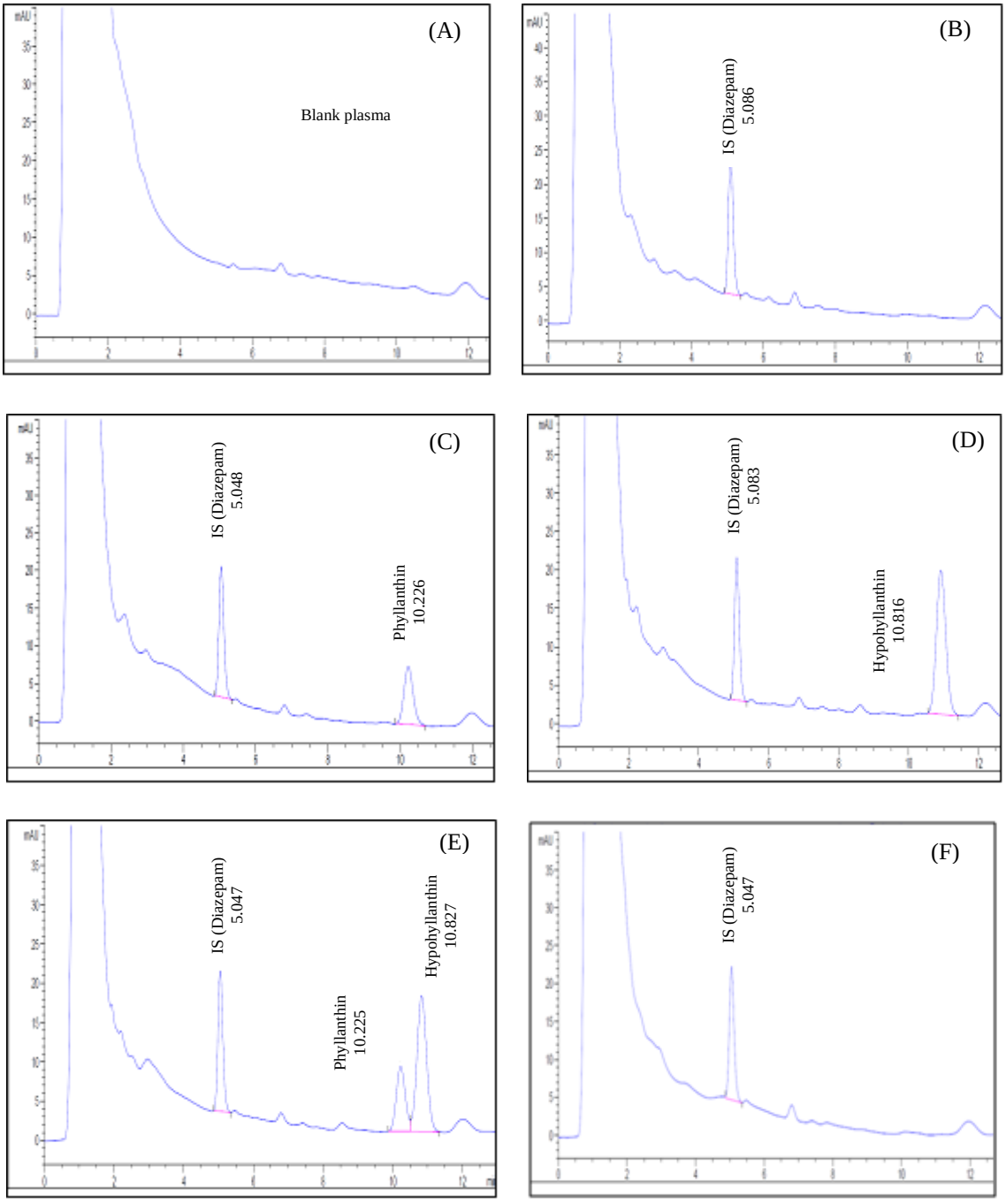


Fig. 15 Chromatograms of blank plasma (A), blank plasma spiked with diazepam; internal standard (5 µg/mL) (B) and phyllanthin (C), hypophyllanthin (D), standard phyllanthin and hypophyllanthin (1,500 ng/mL) (E) and plasma obtained from a rabbit after oral administration of the last dose of 500 mg/kg *P. amarus* extract (F). Flow rate 1 mL/min.

4.2.2 Linearity of the standard calibration curve of phyllanthin and hypophyllanthin in plasma

The coefficient of determination (r^2) of the standard curves of phyllanthin and hypophyllanthin were 0.9951 and 0.9977, respectively. The linear regression equation of phyllanthin and hypophyllanthin were Y = 0.0005X + 0.0671 and Y = 0.0013x - 0.0215, respectively (Fig. 16A, 16B).

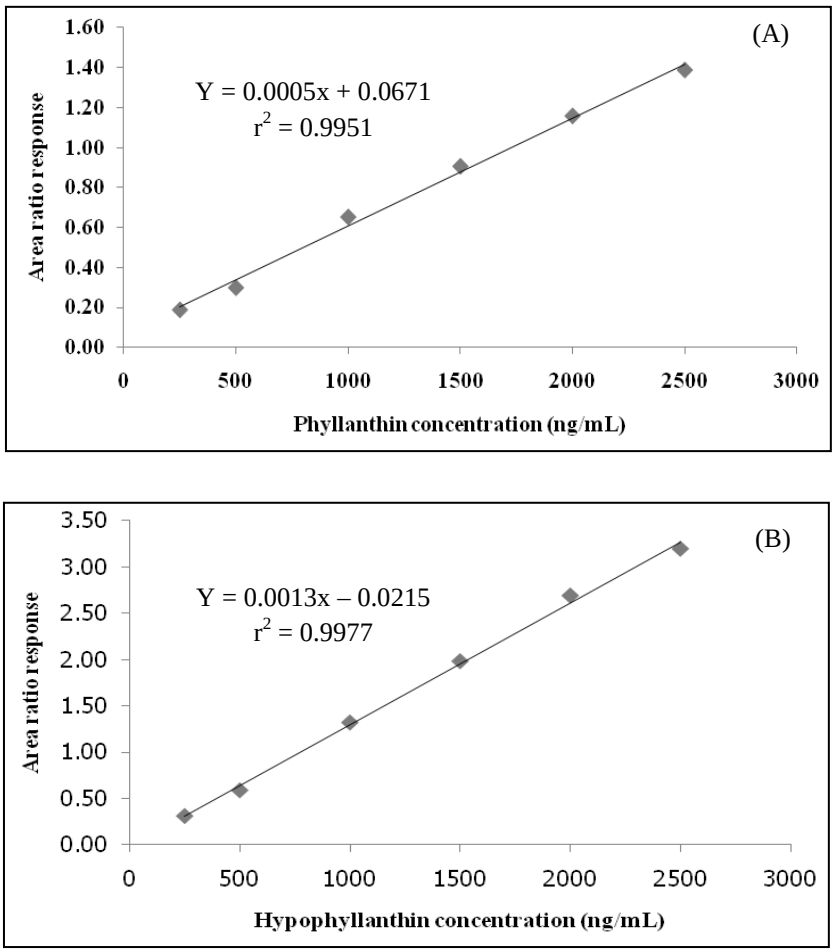


Fig. 16 Calibration curve of phyllanthin (A), hypophyllanthin (B) in plasma

4.2.3 Lower limit of quantification (LLOQ)

The LLOQ of phyllanthin and hypophyllanthin in plasma were 211.18 ng/mL and 226.62 ng/mL, respectively (Table 4, 5) with acceptable precision of (% CV) of 4.14% and 4.19%, accuracy of 86.07% and 92.24, respectively (Table 6, 7).

Table 4. Lower limit of quantitation (LLOQ) of phyllanthin in plasma

N	Peak area ratio	Concentration (ng/mL)
1	0.18	200.60
2	0.18	218.33
3	0.19	210.56
4	0.19	204.99
5	0.17	221.44
	$\overline{X}$ ±SD	**211.18±8.76**
	%CV	**4.14**
	%Accuracy	**86.07**

Table 5. Lower limit of quantitation (LLOQ) of hypophyllanthin in plasma

N	Peak area ratio	Concentration (ng/mL)
1	0.30	219.55
2	0.30	222.78
3	0.31	240.30
4	0.31	217.97
5	0.30	232.54
	$\overline{X}$ ±SD	**226.62±9.50**
	%CV	**4.19**
	%Accuracy	**92.24**

4.2.4 Precision

Precision of the assay procedure was assessed from %CV of peak area ratio of phyllanthin, hypophyllanthin and their concentrations from intraday and interday assay, as shown in Table 6 and 7. The %CV of intraday and interday of phyllanthin ranged between 1.17-8.97%, 0.83-9.50%, respectively and those of hypophyllanthin ranged between 1.36-3.17%, 1.57-3.56%, respectively.

4.2.5 Accuracy

Accuracy of the assay of phyllanthin, hypophyllanthin in plasma were assessed by determining three phyllanthin, hypophyllanthin concentrations (375, 1250 and 2250 ng/mL) in five replicates for intraday and interday. The intraday and interday accuracy of phyllanthin range between 85.71-106.45%, 92.98-106.32%, respectively and hypophyllanthin range between 98.57-102.14%, 91.01-105.63%, respectively (Table 6, 7).

Table 6. The intraday and interday precision of the analytical method of phyllanthin

Concentration (ng/mL)	Mean area ratio ±SD	Mean concentration ±SD (ng/mL)	CV (%)	Accuracy (%)
Intraday (n=5)				
375	0.252±3.99	310.16±3.51	8.97	85.71
1250	0.781±1.92	1191.83±3.68	1.17	95.34
2250	1.503±4.65	2395.01±3.09	1.50	106.45
Interday (n=5)				
375	0.254±2.91	348.70±9.99	3.75	92.98
1250	0.757±3.01	1353.00±6.06	0.83	108.28
2250	1.276±3.54	2392.41±6.25	9.50	106.32

Table 7. The intraday and interday precision of the analytical method of hypophyllanthin

Concentration (ng/mL)	Mean area ratio ±SD	Mean concentration ±SD (ng/mL)	CV (%)	Accuracy (%)
Intraday (n=5)				
375	0.503±0.026	380.69±26.18	3.17	98.57
1250	1.725±0.261	1291.57±17.45	1.36	102.14
2250	3.102±0.302	2321.40±24.95	1.68	100.30
Interday (n=5)				
375	0.495±1.91	369.64±3.19	3.04	91.01
1250	1.765±1.31	1276.78±6.06	3.56	105.63
2250	3.137±3.33	2256.78±4.25	1.57	99.82

4.2.6 Recovery

Efficacy of liquid-liquid extraction procedure was assessed from the percentage recovery as shown in Table 8 and 9. The mean recoveries of phyllanthin, hypophyllanthin at the concentration of 375, 1250, and 2250 ng/mL were 102.40%, 96.32% and 84.36%, respectively for phyllanthin, and 87.06%, 81.95% and 79.12%, respectively for hypophyllanthin.

Table 8. The recovery of phyllanthin in plasma (n=5)

Concentration (ng/mL)	Mean recovery±SD (%)	CV (%)
375	102.40±2.57	4.62
1250	96.32±4.33	1.07
2250	84.36±1.20	1.36

Table 9. The recovery of hypophyllanthin in plasma (n=5)

Concentration (ng/mL)	Mean recovery±SD (%)	CV (%)
375	87.06±4.89	3.32
1250	81.95±2.17	1.38
2250	79.12±2.61	1.33

4.2.7 The plasma concentration of phyllanthin and hypophyllanthin

Phyllanthin and hypophyllanthin could not be detected in plasma samples at one hour after oral administration of the last dose 500 mg/kg *P. amarus* extract from all rabbits with the LLOQ of phyllanthin, hypophyllanthin in plasma were 211.18 ng/mL and 226.62 ng/mL respectively.

4.3 Assay validation of midazolam in plasma

4.3.1 Chromatograms

The chromatograms showed that peak of midazolam and its internal standard (diazepam) were well separated from the other peaks in plasma as shown in Fig. 17 and Fig. 18. The standard midazolam and internal standard were eluted at around 7 and 8 min, respectively.

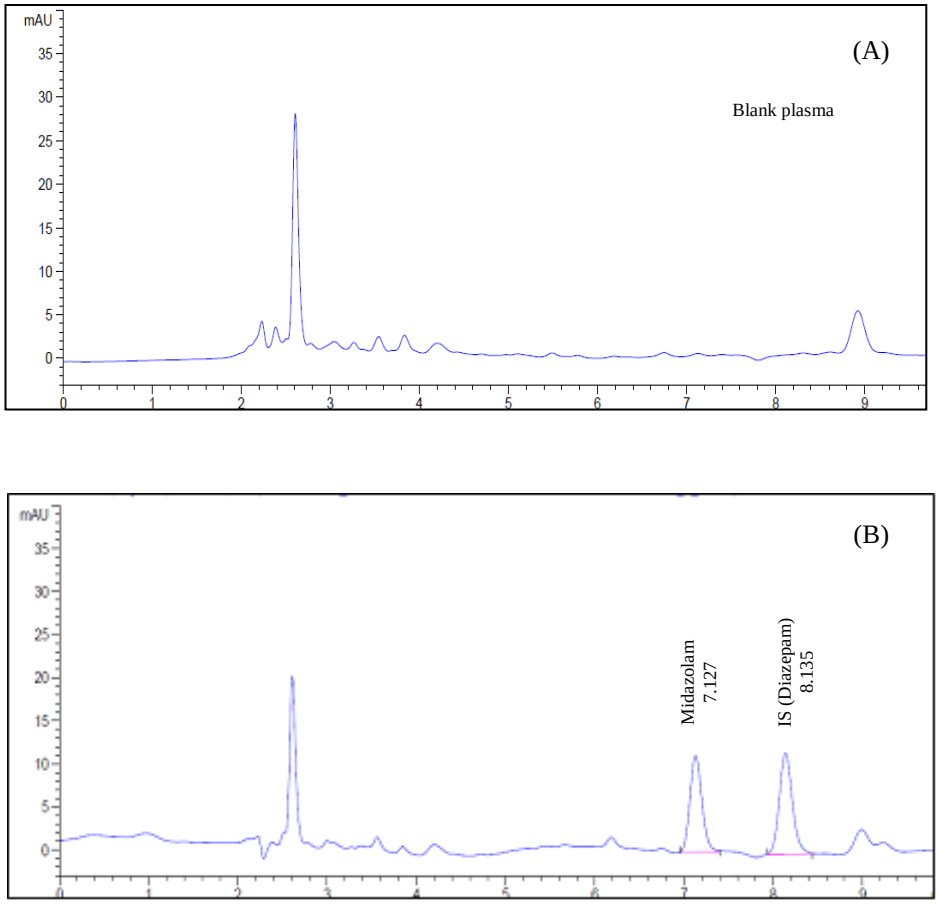


Fig. 17 Chromatograms of blank plasma (A), and blank plasma spiked with standard midazolam (500 ng/mL) and internal standard; diazepam (3 µg/mL) (B). Flow rate was 1.2 mL/min.

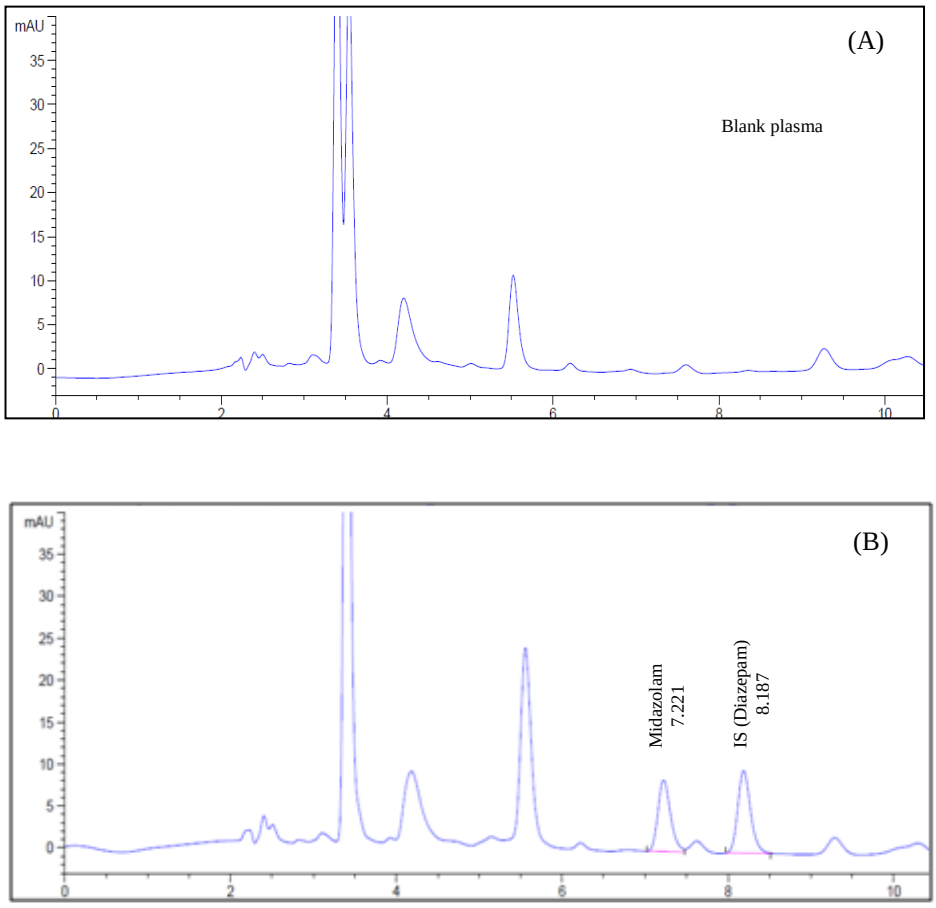


Fig. 18 Chromatograms of plasma obtained from a rabbit before treatment with midazolam (A), and after 1 h receiving a single oral dose of 10 mg/kg midazolam after oral administration at 1 h (B). Flow rate was 1.2 mL/min.

4.3.2 Linearity of the standard calibration curve

The coefficient of determination (r^2) of the standard curves of midazolam was 0.9996. The linear regression equation of midazolam was Y = 0.0026X - 0.0031 (Fig. 19).

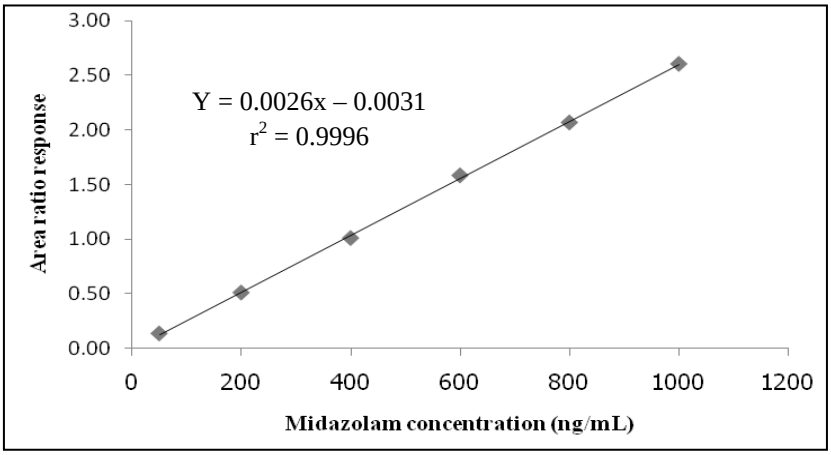


Fig. 19 Calibration curve of midazolam in plasma

4.3.3 Lower limit of quantitation (LLOQ)

The LLOQ of midazolam was 46.76 ng/mL with acceptable precision (%CV) 1.59% and accuracy of 102.17% (Table 10).

Table 10. Lower limit of quantitation (LLOQ) of midazolam in plasma

N	Peak area ratio	Concentration (ng/mL)
1	0.10	47.40
2	0.12	45.78
3	0.11	48.54
4	0.14	44.77
5	0.15	47.35
	$\overline{X}$ ±SD	**46.76±1.48**
	%CV	**1.59**
	%Accuracy	**93.52**

4.3.4 Precision

Precision of the assay procedure was assessed from %CV of area ratio response of midazolam at each concentration from intraday and interday assay, which are shown in Table 11. The %CV of intraday and interday of midazolam ranged between 0.58-3.43% and 0.02-0.67%, respectively.

4.3.5 Accuracy

Accuracy of the assay of midazolam in plasma was assessed by determining three midazolam concentrations (100, 500 and 900 ng/mL) in five replicates for intraday and interday. The intraday and interday accuracy of midazolam ranged between 104.58%-110.82% and 95.25-100.05%, respectively (Table 11).

Table 11. The intraday and interday precision of the analytical method of midazolam

Concentration (ng/mL)	Mean area ratio ± SD	Mean concentration ±SD (ng/mL)	CV (%)	Accuracy (%)
Intraday (n=5)				
100	0.201±0.09	110.85±3.81	3.43	110.82
500	1.147±0.04	539.16±3.66	0.67	107.83
900	1.792±0.05	941.42±3.49	0.58	104.58
Interday (n=5)				
100	0.217±0.01	95.64±9.99	10.44	95.25
500	1.046±0.08	486.61±6.06	1.24	97.32
900	1.907±0.09	900.45±6.25	0.67	100.05

4.3.6 Recovery

Efficacy of liquid-liquid extraction procedure was assessed from the percentage recovery as shown in Table 12. The mean recoveries of midazolam at the concentration of 100, 500, and 900 ng/mL were 86.24%, 95.10%, and 99.31%, respectively.

Table 12. The recovery of midazolam in plasma (n=5)

Concentration (ng/mL)	Mean recovery±SD (%)	CV (%)
100	86.24±2.87	3.07
500	95.10±4.47	3.41
900	99.31±0.20	1.11

4.4 Midazolam pharmacokinetics study

Phase 1: Pharmacokinetics of a single oral dose of 10 mg/kg midazolam alone

After a single oral dose of 10 mg/kg midazolam alone in nine rabbits, the mean values of C_{max}, AUC_{0-8}, $AUC_{0-\infty}$, $AUMC_{0-8}$, $AUMC_{0-\infty}$, MRT_{0-8}, $MRT_{0-\infty}$, λ_z, $T_{1/2}$, T_{max}, V_z/F, and CL/F of midazolam were 164.87±140.15 ng/mL, 362.39±281.88 ng.h/mL, 439.76±360.02 ng.h/mL, 867.89±813.87 $ng.h^2/mL$, 1819.89±2135.38 $ng.h^2/mL$, 1.98±0.80 h, 3.24±1.56 h, 0.39±0.19 h^{-1}, 2.19±1.12 h, 0.44±0.27 h, 0.18±0.21 L/kg, and 0.08±0.10 L/h/kg, respectively.

Phase 2: Pharmacokinetics of a single oral dose of 10 mg/kg midazolam after *P. amarus*

The mean values of C_{max}, AUC_{0-8}, $AUC_{0-\infty}$, $AUMC_{0-8}$, $AUMC_{0-\infty}$, MRT_{0-8}, $MRT_{0-\infty}$, λ_z, $T_{1/2}$, T_{max}, V_z/F, and CL/F of a single oral dose of 10 mg/kg midazolam after *P. amarus* in nine rabbits were 474.15±322.46 ng/mL, 1024.78±1094.74 ng.h/mL, 1424.27±1749.62 ng.h/mL, 2936.18±3993.38 $ng.h^2/mL$, 8709.61±13726.84 $ng.h^2/mL$, 2.30±0.68 h, 4.25±2.15 h, 0.26±0.18 h^{-1}, 3.18±1.49 h, 0.72±0.29 h, 0.07±0.05 L/kg, and 0.02±0.02 L/h, respectively.

Mean plasma concentration-time profiles of midazolam before and after pretreatment with *P. amarus* are shown in Figure 20 and the mean pharmacokinetic parameters are shown in Table 13. In this study, when a single oral dose of 10 mg/kg midazolam were administered after *P. amarus* the mean $C_{max,}$ T_{max} AUC_{0-8} and $T_{1/2}$ of midazolam was significantly ($p < 0.05$) increased when compared with midazolam alone by 187.59% (474.15±322.46 from 164.87±140.15 ng/mL), 63.63% (0.72±0.29 from 0.44±0.27 h), 182.78% (1024.78±1094.74 from 362.39±281.88 ng.h/mL), 45.20% (3.18±1.49 from 2.19±1.12 h) and the mean $AUC_{0-\infty}$, $AUMC_{0-8,}$ $AUMC_{0-\infty}$, MRT_{0-8} and $MRT_{0-\infty}$ of midazolam were insignificantly increased when compared with midazolam alone by 223.95% (1424.27±1749.62 from 439.76±360.02 ng.h/mL), 238.31% (2936.18±3993.38 from 867.89±813.87 $ng.h^2/mL$), 378.58% (8709.61±13726.84 from 1819.89±2135.38 $ng.h^2/mL$), 16.16%

(2.30±0.68 from 1.98±0.80 h), 31.17% (4.25±2.15 from 3.24±1.56 h), respectively. Furthermore, the mean values for the λ_z, V_z/F and CL/F of midazolam were insignificantly decreased when compared with midazolam alone.

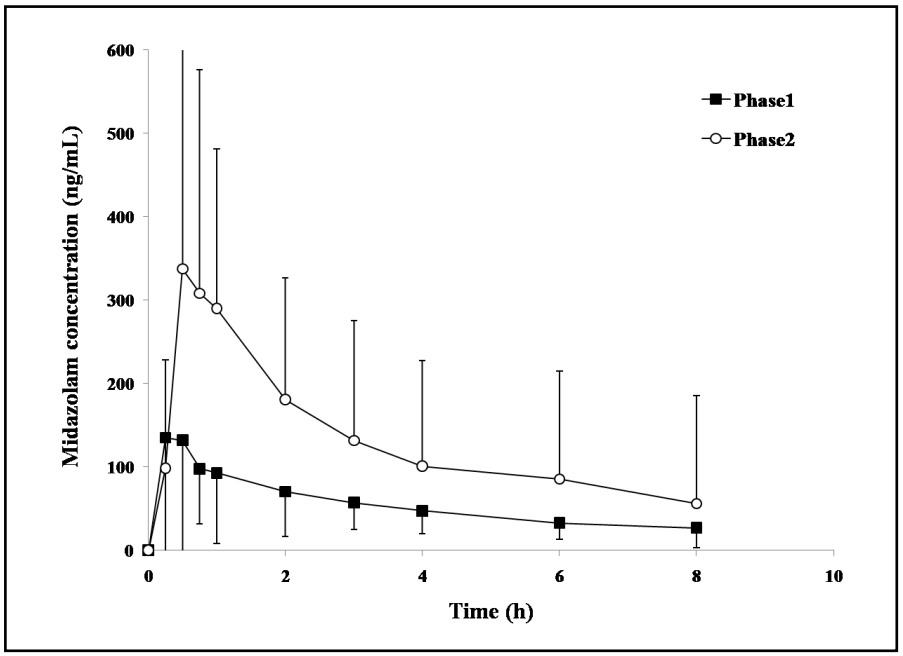


Fig. 20. Plasma concentration time-profiles of midazolam ($\overline{X}$ ±SD) in 9 rabbits after a single oral dose of 10 mg/kg midazolam (Phase 1) and after pretreatment with 500 mg/kg *P. amarus*, once a day for 7 days and once before midazolam administration (Phase 2)

Table 13. Pharmacokinetics parameters ($\overline{X}$ ±SD) and % change of midazolam of the nine rabbits receiving a single oral dose of 10 mg/kg midazolam (Phase 1) compared with after pretreatment with *P. amarus* extract for 7 days (Phase 2)

Parameters	Phase 1	Phase 2	%Change	*p*-value
C_{max} (ng/mL)	164.87±140.15	474.15±322.46	187.59	0.004
AUC_{0-8} (ng.h/mL)	362.39±281.88	1024.78±1094.74	182.78	0.048
$AUC_{0-\infty}$ (ng.h/mL)	439.76±360.02	1424.27±1749.62	223.95	0.050
$AUMC_{0-8}$ ($ng.h^2/mL$)	867.89±813.87	2936.18±3993.38	238.31	0.100
$AUMC_{0-\infty}$ ($ng.h^2/mL$)	1819.89±2135.38	8709.61±13726.84	378.58	0.130
MRT_{0-8} (h)	1.98±0.80	2.30±0.68	16.16	0.150
$MRT_{0-\infty}$ (h)	3.24±1.56	4.25±2.15	31.17	0.105
λ_z (h^{-1})	0.39±0.19	0.26±0.18	-33.34	0.170
$T_{1/2}$ (h)	2.19±1.12	3.18±1.49	45.20	0.038
T_{max} (h)	0.44±0.27	0.72±0.29	63.63	0.030
V_Z/F (L/kg)	0.18±0.21	0.07±0.05	-61.11	0.145
CL/F (L/h/kg)	0.08±0.10	0.02±0.02	-75.00	0.085

CHAPTER 5

DISCUSSION AND CONCLUSION

Phyllanthus amarus, is well known for its medicinal properties and has long been used in folk medicine worldwide. It is easily found in all regions of Thailand. *P. amarus* contains various phytochemicals, which are biologically active and may capable of interacting with therapeutic drugs. Inhibition or induction of drug metabolizing enzymes, particularly cytochromes P450 (CYP) that are the major enzymes responsible for the metabolism of most of therapeutic drugs, are of major concern. The inhibitory effect of herbs on these CYP enzymes may result in increased plasma concentrations of several drugs thus leading to increased efficacy or toxicity of the drug. On the other hand, an induction effect on CYP enzymes may decrease plasma concentration of certain drugs leading to reduced efficacy of the drug or treatment failure (Elvin-Lewis 2001).

CYP3A plays a substantial role in the metabolism of a vast array of pharmaceutical products, of particular concern are compounds that are predominantly or exclusively metabolized by CYP3A4 (Scott and Halpert, 2005). CYP3A4, the most abundant isozyme in human liver as well as in intestine, involves in the metabolism of more than 50% of all prescribed drugs (Zhou, 2008). Induction or inhibition of CYP3A4 by various herbs has been reported to be clinically important. For example, coadministration of St. John's Wort (*Hypericum perforatum*, a CYP 3A4 inducer) extract in patients receiving tacrolimus, an immunosuppressant, reduced tacrolimus blood concentration and increasing the risk of organ rejection (Mai *et al*, 2003). Coadministration of grapefruit juice (a CYP 3A4 inhibitor) with simvastatin, an anti-lipidemic drug, increased the mean peak serum concentration of simvastatin 12-fold and increased the risk for myopathy (Lilja *et al*, 2000).

Most induction and inhibition of drug metabolizing enzymes by herbal component have been documented in several *in vitro* studies. These studies determined the potential of herbal component to alter the pharmacokinetics of a drug. Affinity and CYP specificity for an inhibitor or inducer can be also studied *in vitro*. However, this does not necessarily mean that the compound would cause clinically significant herb-drug interactions. For example, milk thistle showed *in vitro* inhibitory activity on various CYP enzymes in human liver microsome and hepatocytes but had no significant effect on these enzymes on long term treatment in human (Venkataramanan *et al*., 2006). For such interactions to take place *in vivo*, the concentration of the substrate, inducer and inhibitor, should be high enough to affect the clinical outcome (Pelkonen *et al.*, 2008).

Previous *in vitro* studies have reported that the extract of *P. amarus* exerted an inhibitory effect on several CYP isoforms. Bhaskarannair *et al*. (2006) reported that an alcoholic extract of *P. amarus* inhibited CYP1A1, 1A2, 2B1/2 and 2E1 in rat liver microsome with the IC_{50} between 4.6-50 µg/mL and oral administration of the extract was also found to reduce the elevated P450 enzyme activities produced by phenobarbitone by 50% at the dose of 250 mg/kg in rats. According to Appiah-Opong *et al*. (2008), *P. amarus* aqueous extracts of whole plant, leaf, stem and root showed inhibitory potential towards recombinant human CYP heterologously expressed in *Escherichia coli* with the IC_{50} values ranging between 28.3-134.3 µg/mL on CYP1A2 and CYP2C9. Similarly, both CYP2D6 and CYP3A4 were inhibited with the IC_{50} ranging between 45.8-182.0 µg/mL. In addition, Anannarukan *et al*. (2012) reported that *P. amarus* aqueous extract possessed inhibitory effects on CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 with the IC_{50} ranging from 2.07 to 180.40 µg/mL. The potent inhibitory effects of this plant extract on these CYPs suggested the possibilities of herb-drug interaction *in vivo*.

In the present study, the effect of *P. amarus* ethanolic extract on midazolam pharmacokinetics was examined in rabbits in order to determine the inhibitory effect of *P. amarus* on CYP3A *in vivo* using midazolam as a substrate probe. Midazolam is a sedative drug with a rapid onset and short duration of action widely used for hypnosis, sedation, pre-operative, to treat status epilepticus, induction

and maintenance of anesthesia and sedation of patients in intensive care units (Reves *et al*. 1985). It is rapidly and extensively metabolized by CYP3A to 1-hydroxy and 4-hydroxy midazolam (Patki *et al*., 2003). It is one of the best *in vivo* probe drugs for the study of CYP3A4 activity (Zhou *et al*., 2005) not only because it is a substrate of CYP 3A, but also it is unaffected by P-glycoprotein or other known transporters and presents negligible adverse effects at the dose used for probe studies (Bjornsson *et al*., 2003). Midazolam can provide a measure of human CYP3A4 and 3A5 activity related to both intestinal and hepatic metabolism, respectively (Lin *et al*., 2002) Moreover, Elbarbry *et al*. (2009) has reported that midazolam hydroxylase activity could be determined by quantification of 1-OH and 4-OH midazolam formation in rabbit as well as in human hepatic microsomes suggesting that the data obtained by using rabbit as an animal model can be applied to determine CYP3A activity in human, although the isoform found in rabbit is CYP3A6, while those found in human are CYP3A4/3A5 (Patki *et al*., 2003, Vossen *et al*., 2007).

It was found from this study that oral pretreatment of *P. amarus* ethanolic extract (500 mg/kg, daily for 7 days and once 1 h before midazolam) increased C_{max}, T_{max}, AUC_{0-8} and $T_{1/2}$ (2.9, 1.6, 2.8 and 1.4-fold, respectively) while decreased CL/F (4-fold) of midazolam indicating that *P. amarus* inhibit the activity of CYP3A, the enzyme responsible for midazolam metabolism. According to the US-FDA drug interaction guideline (FDA 2006) which classified CYP3A inhibitors based on their *in vivo* fold-change in the plasma AUC of substrate, *P. amarus* at the dose of 500 mg/kg can be classified as moderate CYP inhibitor because the increase in the AUC of oral midazolam is in the range of 2-5 folds. The results were supported by previous *in vitro* data showing that ethanolic extract of *P. amarus* and its major lignans, phyllanthin and hypophyllanthin, were potent mechanism-based inhibitors of CYP 3A4 activity in human liver microsome (Taesotikul *et al*., 2011). Moreover, *in vivo* study in rats also showed that single dose administration of *P. amarus* ethanolic extract (800 mg/kg/day) increased oral bioavailability of midazolam through inhibition of intestinal CYP 3A since it was affected by oral but not intravenous administration (Taesotikul *et al*., 2012). In the present study, it was also revealed that the inhibitory activity of CYP3A by *P. amarus* extract occurred inspite of the very low concentration of phyllanthin and hypophyllanthin in plasma. Since the content of

phyllanthin and hypophyllanthin, which are major compounds found in the *P. amarus* extract at the dose of 500 mg/kg, was found to be about 148.34 and 48.28 mg/kg, respectively, but it could not be detected at the LLOQ of 211.18 and 226.62 ng/mL, respectively whereas the IC_{50} in *in vitro* study were more than 1000 ng/mL which suggested that the effect might take place in the intestine more than in the liver. Although Sukhaphirom *et al.* (2012) reported the competitive inhibitory effects of phyllanthin and hypophyllanthin on P-glycoprotein activity in *in vitro* model of Caco-2 cells, it might not be related to the increase in midazolam bioavailability when coadministered with *P. amarus* because midazolam is not a P-glycoprotein substrate. In general, the CYP inhibitors that have been shown to be mechanism-based inactivators form quasi-irreversible complexes with the heme iron-atom of the enzyme. These compound generally require catalytic activation by the enzyme to transient intermediates that then coordinate very tightly to the prosthetic heme in the CYP active site leading to inhibition (Hollenberg, 2002). This might be one of the possible mechanisms of *P. amarus* to inhibit CYP activity since *P. amarus* has been shown to possess iron chelating activity (Wongnawa *et al*., 2006; Kumaran and Karunakaran 2007). Moreover, deferasirox, a well known iron chelator, as well as some phytochemicals with iron chelating activity such as curcumin, quercetin and catechin also inhibit CYP activity (Cho *et al*., 2012; Jiao *et al*., 2006; Hatcher *et al*., 2008; Skerjanec *et al*., 2010; Galanello *et al*., 2012; Leopoldini *et al*., 2008; Choi *et al*., 2011; Chow *et al*., 2006; Morel *et al*., 1993).

However, repeated administration of *P. amarus* extract (200 mg/kg/day and 800 mg/kg/day for 15 days) in rats slightly induced the activity and expression of hepatic CYP 3A and CYP 2B1/2 (Taesotikul *et al*., 2012). The opposing effects of herbs on CYP were often observed depending on the dose, duration of administration and may also be species- and tissue-specific. For example, acute administration of hyperforin, the primary constituent of St. John's Wort, inhibited CYP 3A4 activity, where as upon chronic exposure, the inductive effect predominated (Komoroski *et al*., 2004). In addition, the activities of various CYP were inhibited by kavalactones, the active principle of Kava, through a competitive mechanism, whereas these lactones were responsible for the induction of CYP3A23 gene expression mediated via PXR activation (Negi *et al*., 2008). It was suggested that an inducing effect of *P. amarus*

extract might be resulted from other phytochemical such as quercetin since previous report have been shown that short-term exposure of quercetin can strongly inhibit cortisol metabolism mediated by CYP3A4 and also long-term exposure caused an increase in CYP3A mRNA expression *in vitro*. It is therefore possible that other compounds in the extract such as quercetin may play some role in induction of CYP3A after multiple dose administration of the extract (Pal and Mitra 2006; Taesotikul *et al*., 2012).

In conclusion, the present study has demonstrated that pretreatment of *P. amarus* for a short period (7 days) increased the oral bioavailability of midazolam suggesting that this effect was due to inhibition of CYP3A. However, clinical investigation should be performed to reveal the significant potential of the herb-drug interactions in human.

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APPENDIX

APPENDIX A

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Ref. 03/2011

April 5 , 2011

This is to certify that the research project entitled "Effects of *Phyllanthus amarus* Schum.& Thonn. on the Pharmacokinetics of Midazolam in Rabbits" which was under conducted by Assoc.Prof.Malinee Wongnawa, Faculty of Sciences, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

APPENDIX B

Chemicals preparation

Preparation of phosphate buffer solution

Aqueous phosphate buffer (0.025 M, pH 4.6) was prepared by dissolving 3.4022 g of potassium di-hydrogen phosphate in 1,000 mL of ultrapure water and adjusting pH with ortho-phosphoric acid or sodium hydroxide. The buffer solution was filtered through a 0.22 µm nylon membrane filter and degassed in the ultrasonic bath for 30 minutes before use.

APPENDIX C

Plasma concentration time-profiles of midazolam at each time of blood drawn from individual rabbit after a single oral dose of 10 mg/kg midazolam (Phase 1) and after pretreatment with 500 mg/kg *P. amarus* o.d. orally for 7 days and once before midazolam administration (Phase 2)

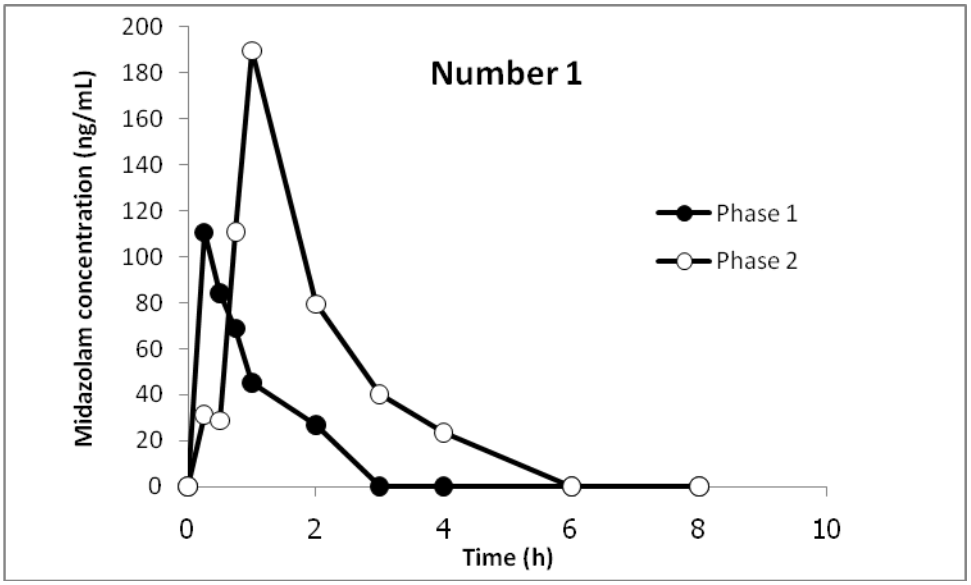

Fig. 21 Plasma midazolam concentration-time profiles of rabbit No. 1

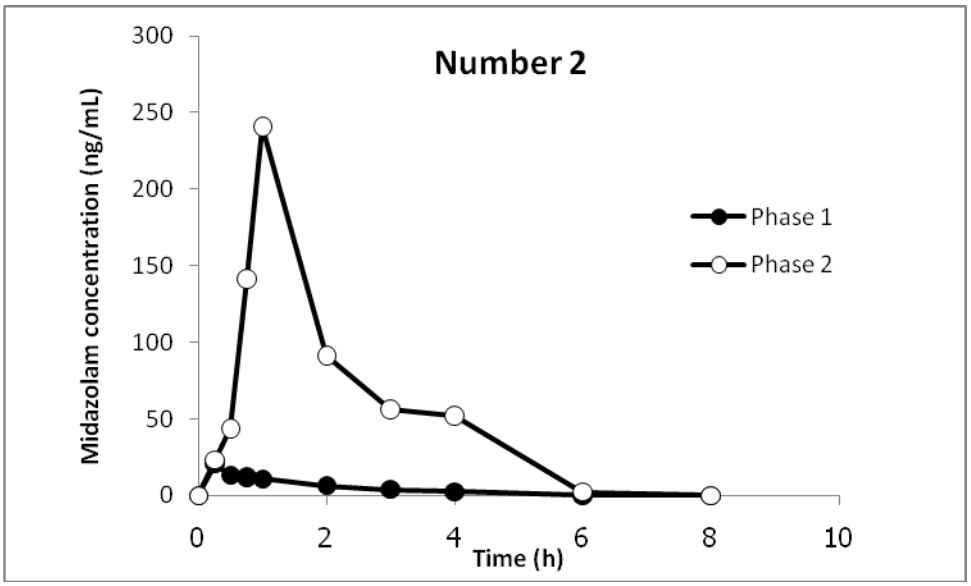

Fig. 22 Plasma midazolam concentration-time profiles of rabbit No. 2

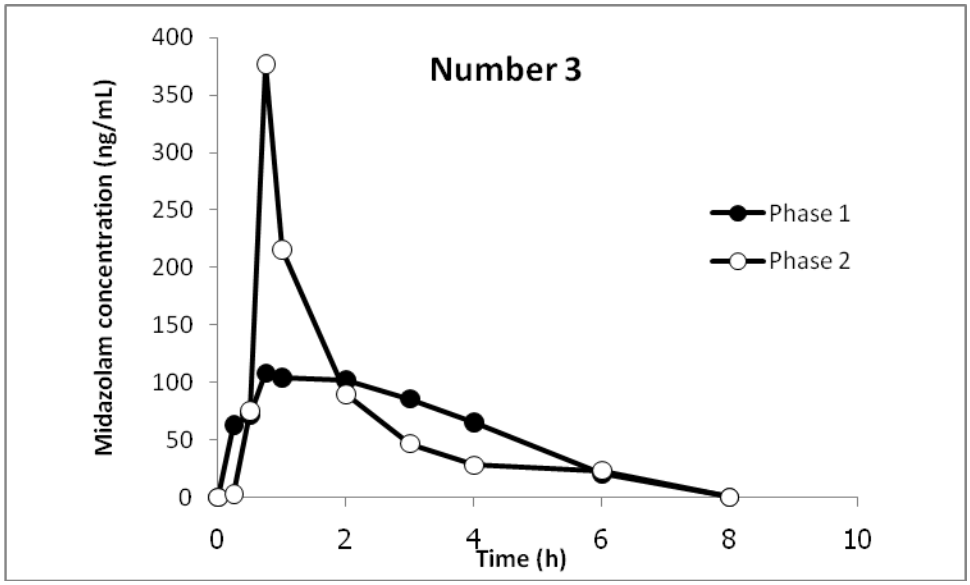

Fig. 23 Plasma midazolam concentration-time profiles of rabbit No. 3

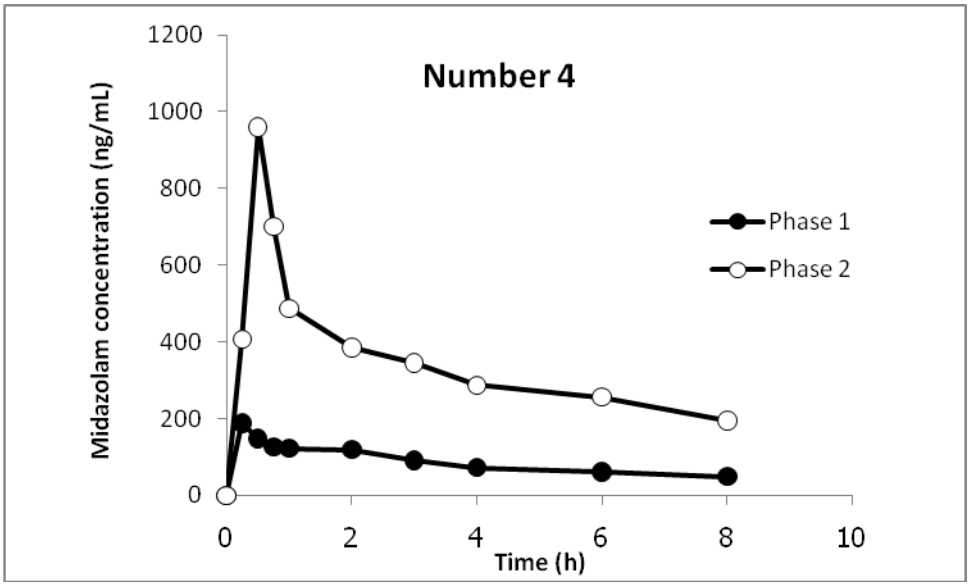

Fig. 24 Plasma midazolam concentration-time profiles of rabbit No. 4

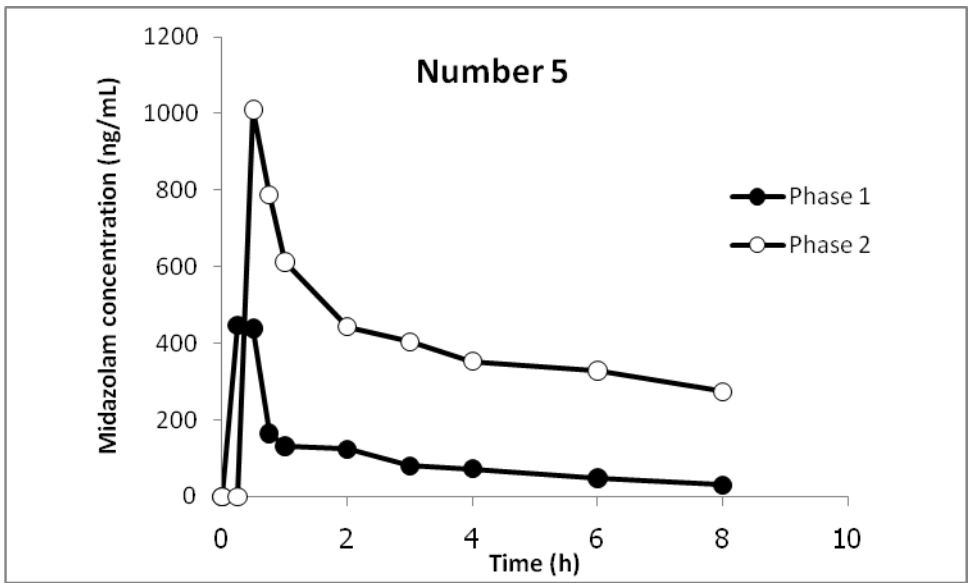

Fig. 25 Plasma midazolam concentration-time profiles of rabbit No. 5

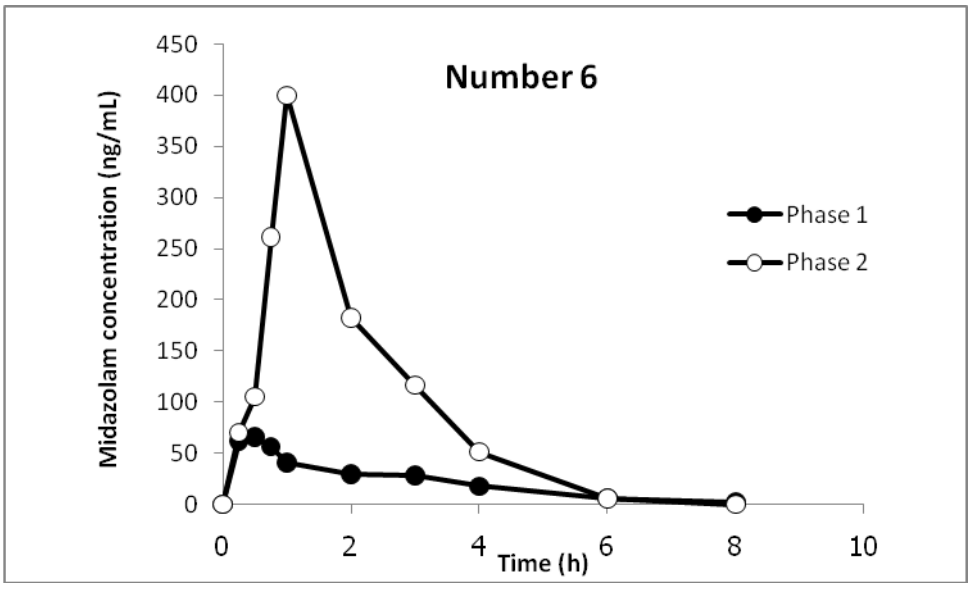

Fig. 26 Plasma midazolam concentration-time profiles of rabbit No. 6

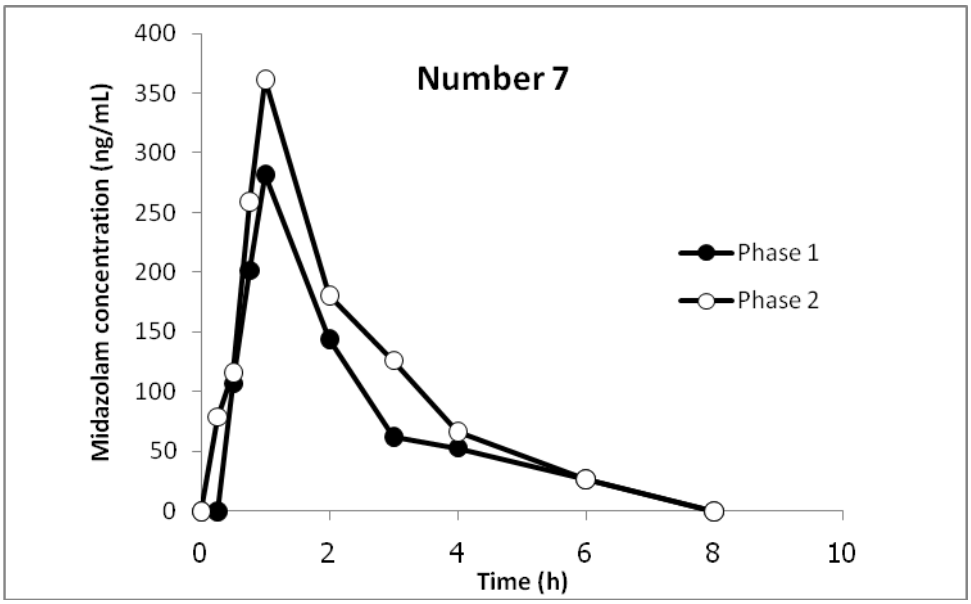

Fig. 27 Plasma midazolam concentration-time profiles of rabbit No. 7

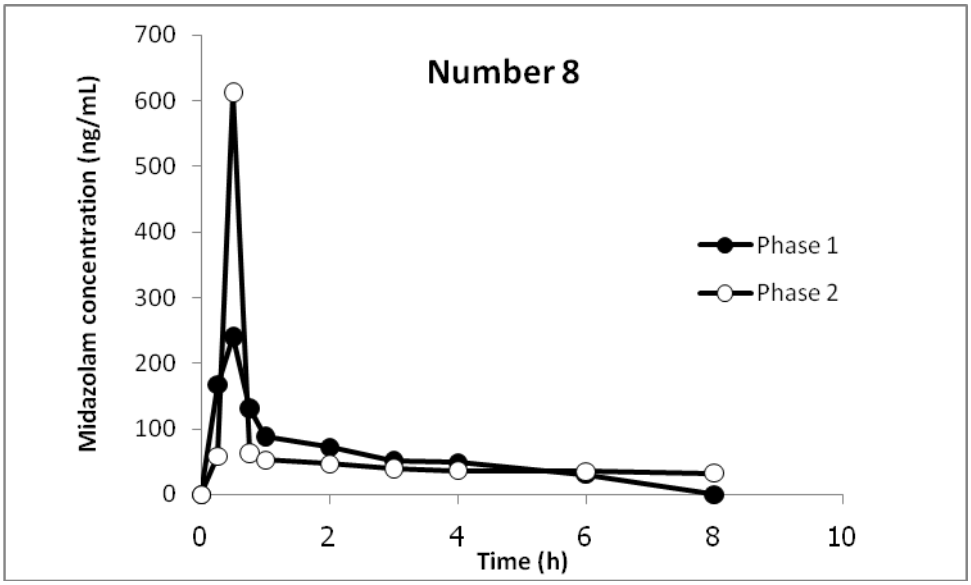

Fig. 28 Plasma midazolam concentration-time profiles of rabbit No. 8

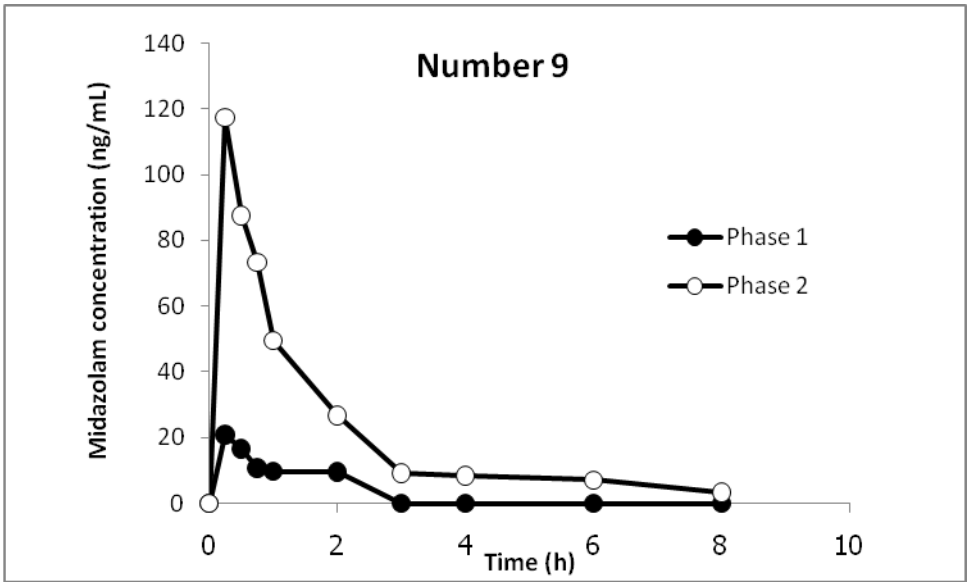

Fig. 29 Plasma midazolam concentration-time profiles of rabbit No. 9

APPENDIX D

Table 14. Plasma concentration of midazolam of each rabbit receiving a single oral dose of 10 mg/kg midazolam alone (Phase 1)

Rabbit No.	Plasma midazolam concentration (ng/mL) at each time of blood drawn (h)									
	0	**0.25**	**0.5**	**0.75**	**1**	**2**	**3**	**4**	**6**	**8**
1	0.00	110.66	84.10	68.81	45.19	26.67	ND	ND	ND	ND
2	0.00	20.74	13.30	11.86	10.89	6.53	3.99	2.43	ND	ND
3	0.00	62.83	71.94	108.00	103.87	101.83	85.64	65.05	20.37	ND
4	0.00	187.92	148.76	126.57	122.59	119.07	90.74	72.87	61.52	49.28
5	0.00	447.47	439.47	164.33	131.34	123.35	79.66	72.29	47.61	29.60
6	0.00	62.13	66.17	56.34	40.97	29.66	28.22	17.69	6.14	1.84
7	0.00	ND	106.97	201.59	281.68	143.90	61.91	52.90	26.85	ND
8	0.00	167.09	240.30	131.64	88.41	71.90	50.81	48.61	30.50	ND
9	0.00	20.92	16.69	10.86	9.75	9.62	ND	ND	ND	ND
$\overline{X}$	0.00	134.97	131.97	97.78	92.74	70.28	57.28	47.41	32.71	26.91
SD	0.00	140.74	134.53	65.93	84.22	53.51	32.07	27.44	19.74	23.83

LLOQ = 50 ng/mL
ND = Not detectable

Table 15. Plasma concentration of midazolam of each rabbit receiving a single oral dose of 10 mg/kg midazolam after pretreatment with 500 mg/kg *P. amarus* extract once a day for 7 days (Phase 2)

Rabbit No.	Plasma midazolam concentration (ng/mL) at each time of blood drawn (h)									
	0	**0.25**	**0.5**	**0.75**	**1**	**2**	**3**	**4**	**6**	**8**
1	0.00	31.12	28.75	110.99	189.70	79.52	40.33	23.43	ND	ND
2	0.00	23.13	43.62	141.65	240.90	91.33	56.45	52.13	2.20	ND
3	0.00	2.80	75.33	376.90	215.31	89.55	46.62	27.84	23.22	ND
4	0.00	406.52	959.87	700.36	487.45	386.22	345.93	287.33	256.18	194.65
5	0.00	ND	1009.24	787.23	613.36	443.14	403.99	353.07	327.83	273.74
6	0.00	70.36	105.32	261.56	399.83	182.30	116.35	51.30	5.51	ND
7	0.00	78.70	115.70	259.36	361.07	180.27	126.11	66.71	26.58	ND
8	0.00	58.16	612.55	62.84	52.67	46.54	39.09	36.59	35.09	32.54
9	0.00	117.31	87.56	73.38	49.49	26.82	9.21	8.49	7.06	3.46
$\overline{X}$	0.00	98.51	337.55	308.25	289.98	180.63	131.56	100.77	85.46	56.04
SD	0.00	129.52	407.77	267.98	191.34	146.24	143.67	126.67	129.41	103.46

LLOQ = 50 ng/mL
ND = Not detectable

Table 16. Pharmacokinetic parameters of midazolam of each rabbit receiving a single oral dose of 10 mg/kg midazolam alone (Phase 1) compared with after pretreatment with 500 mg/kg *P. amarus* extract once a day for 7 days (phase 2)

Rabbit No.	C_{max} (ng/mL)		AUC_{0-8} (ng.h/mL)		$AUC_{0-\infty}$ (ng.h/mL)		$AUMC_{0-8}$ ($ng.h^2/mL$)		$AUMC_{0-\infty}$ ($ng.h^2/mL$)		MRT_{0-8} (h)	
	Phase 1	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**
1	110.66	189.70	120.81	316.24	140.51	420.99	85.25	471.76	192.22	1685.77	0.79	1.61
2	20.74	240.90	32.44	433.04	34.65	435.51	41.88	862.01	69.23	899.93	1.40	2.00
3	108.01	376.89	451.39	472.70	533.65	619.75	1071.18	840.16	2203.98	3110.29	2.49	1.87
4	187.92	959.87	638.86	2691.49	999.69	3941.76	2210.70	8746.89	6761.59	26779.86	3.23	3.25
5	447.47	1009.24	781.17	3138.61	877.10	4970.70	1923.32	10997.69	3001.63	37916.44	2.46	3.50
6	66.17	399.83	170.31	745.82	174.51	751.04	386.91	1371.01	430.12	1456.22	2.27	1.85
7	281.68	361.07	592.10	798.72	638.55	862.78	1179.41	1592.30	1819.27	2445.32	2.09	2.06
8	240.30	612.55	446.62	459.54	512.51	641.30	891.25	1236.16	1774.24	3705.56	2.14	2.69
9	20.92	117.31	27.83	166.85	46.69	174.61	21.14	307.61	126.72	387.05	0.92	1.84
$\overline{X}$	164.87	474.15	362.39	1024.78	439.76	1424.27	867.89	2936.18	1819.89	8709.61	1.98	2.30
SD	140.15	322.46	281.88	1094.74	360.02	1749.62	813.87	3993.38	2135.38	13726.84	0.80	0.68

Table 17. Pharmacokinetic parameters of midazolam of each rabbit receiving a single oral dose of 10 mg/kg midazolam alone (Phase 1) compared with after pretreatment with 500 mg/kg *P. amarus* extract once a day for 7 days (phase 2) (continued)

Rabbit No.	$MRT_{0-\infty}$ (h)		λ_z (h^{-1})		$T_{1/2}$ (h)		T_{max} (h)		V_z/F (L/kg)		CL/F (L/h/kg)	
	Phase 1	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**	**Phase 1**	**Phase 2**
1	1.37	4.00	0.81	0.02	0.86	3.79	0.25	1.00	0.09	0.13	0.07	0.02
2	2.00	2.07	0.52	0.47	1.32	1.47	0.25	1.00	0.55	0.05	0.29	0.02
3	4.13	5.02	0.20	0.14	3.49	5.08	0.75	0.75	0.09	0.12	0.02	0.02
4	6.76	6.79	0.16	0.16	4.44	4.45	0.25	0.50	0.06	0.02	0.01	0.00
5	3.42	7.63	0.31	0.15	2.25	4.64	0.25	0.50	0.04	0.01	0.01	0.00
6	2.46	1.94	0.44	0.51	1.58	1.35	0.50	1.00	0.13	0.03	0.06	0.01
7	2.85	2.83	0.37	0.29	1.89	2.36	1.00	1.00	0.04	0.04	0.02	0.01
8	3.46	5.78	0.32	0.18	2.19	3.87	0.50	0.50	0.06	0.09	0.02	0.02
9	2.71	2.22	0.41	0.45	1.71	1.56	0.25	0.25	0.53	0.13	0.21	0.06
$\overline{X}$	3.24	4.25	0.39	0.26	2.19	3.18	0.44	0.72	0.18	0.07	0.08	0.02
SD	1.56	2.15	0.19	0.18	1.12	1.49	0.27	0.29	0.21	0.05	0.10	0.02

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Kaewmeesri, P., Wongnawa, M., Mahatthanatrakul, W., Ridtitid, W. and Sriwiriyajan, S. Effect of *Phyllanthus amarus* Schum. and Thonn. extract on the pharmacokinetics of midazolam in rabbits. The 3rd STOU Graduate Research Conference, September 3th, 4th, 2013, Nonthaburi, Thailand.