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Original Article

Effect of *Phyllanthus amarus* extract on the pharmacokinetics of midazolam in rabbits

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Abstract

Phyllanthus amarus Schum. & Thonn. has been used for the treatment of various diseases. Previous *in vitro* study in human liver microsome 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. Midazolam plasma concentration time-profiles (0-8 h) after a single oral dose of 10 mg/kg midazolam were examined in rabbits receiving *P. amarus* extract compared to control. In treatment group, *P. amarus* extract (500 mg/kg) was orally administered for 7 days and on the experiment day prior to midazolam administration. The results showed that pretreatment with *P. amarus* significantly increased the mean maximum concentration (Cmax), time to reach maximum concentration (Tmax), area under curve (AUC0-8), and elimination half-life (T1/2) (2.9-, 1.6-, 2.8-, and 1.4-fold, respectively) compared with control group receiving a single oral dose of midazolam. The results suggest that *P. amarus* extract inhibits midazolam metabolism in rabbits probably by inhibition of CYP3A which is the enzyme responsible for midazolam metabolism. Therefore, coadministration of *P. amarus* and drugs which are CYP3A substrates may increase plasma drug concentration leading to serious side effects. Thus, concern over the clinical relevance of herb-drug interaction between *P. amarus* and CYP3A substrates should be warranted. However, further investigation is needed to be performed in human to reveal the clinically significant potential of these herb-drug interactions.

Keywords: *P. amarus*, midazolam, herb-drug interaction, cytochrome P450, rabbits

1. Introduction

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, hepatoprotective against ethanol-, paracetamol-, and carbon tetrachloride-toxicity, hypoglycemic, hypocholesterolemic, immunomodulatory, nephroprotective, radioprotective, and spasmolytic activities (Patel *et al.*, 2011, Pramyothin *et al.*, 2007, Wongnawa *et al.*, 2006). The secondary metabolites present in *P. amarus* are alkaloids (securinine, dihydrosecurinine, etc.), flavonoids (quercetin, astragalin, rutin,

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kaempferol, etc.), ellagitannins (gallic acid, ellagic acid, etc.), lignans (phyllanthin, hypophyllanthin, niranthin, etc.), triterpines (lupeol), sterols, and volatile oil (Patel *et al*., 2011). An alcoholic extract of *P. amarus* was found, *in vivo* as well as *in vitro*, to inhibit some cytochrome P450 isozymes (CYP 1A1, 1A2, 2B1/2, 2E1) which are responsible for activation of various procarcinogens, suggesting its inhibitory mechanism 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 that 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 more than 50% of all drugs. Various studies have demonstrated that inhibition of CYP3A4 increased plasma concentration of drugs which are CYP 3A4 substrate leading to serious side effects or toxicity (Norwack, 2008, Lilja *et al*., 2000, Westphal, 2000). Midazolam is a sedative drug with a rapid onset and short duration of action. After oral administration, maximum plasma levels are reached within 30 min and the drug is rapidly eliminated from plasma with the half-life of about 2 h. The oral bioavailability ranges from 31-72%. It is rapidly and extensively metabolized by CYP3A to 1-hydroxy and 4-hydroxy midazolam (Heizmann *et al*., 1983, Patki *et al*., 2003, Reves *et al*., 1985). 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*.

2. Materials and Methods

2.1 Chemicals and reagents

Midazolam tablets (Dormicum®, Hoffmann-La Roche Ltd., Basel, Switzerland) were purchased from the Food and Drug Administration, Ministry of Public Health, Bangkok, Thailand. Standard midazolam and diazepam were kindly provided by Dr Wirachai Samai, The Forensic Medicine and Toxicology Unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University. Standard phyllanthin and hypophyllanthin were kindly provided by Dr Wanida Sukketsiri, Department of Pharmacology, Faculty of Science, PSU. Methanol and acetonitrile (HPLC grade) were purchased from J.T. Baker. USA. All other chemicals and solvents were of analytical reagent grade. Water was purified by Milli Q Water Purification System, Millipore, USA.

2.2 Preparation of *P. amarus* extract

P. amarus powder was purchased from Lampang Herb Conservation, Lampang province, Thailand, and was identified by Assoc. Prof. Tanomjit Supavita, School of Pharmacy, Walailak University, Nakhon Si Thammarat. The powder was extracted twice in 70% ethanol (1:5) at room temperature for 7 days. The filtrate was evaporated at 60°C and then lyophillized. The yield of the extract was about 13% w/w. The extract was stored in a well-closed, light protected container at -20°C until used. A portion of the extract was freshly reconstituted in 10% of gum acacia at desired concentrations prior to the experiment.

2.3 Animals

Nine male New Zealand White rabbits, obtained from The National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand, weighing between 3.0 - 3.5 kg, were housed at The Southern Laboratory Animal Facility, Prince of Songkla University, Thailand, under controlled environment (temperature 25±2°C and relative humidity of approximately 60% with 12 h light/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. The experimental procedures were approved by the Committee on Animal Care (Ref. 03/54) and were in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Prince of Songkla University.

2.4 Study design

In phase 1, the baseline pharmacokinetics of midazolam were evaluated in the animals receiving a single oral dose of 10 mg/kg midazolam with 10 mL of water. In phase 2, after a 1-week washout period, the animals were orally administered with 500 mg/kg *P. amarus* extract once a day (o.d.) for 7 days and the last dose in the morning on day 8, one hour before midazolam administration. Blood samples (2 mL) were taken from marginal ear vein through a venous catheter before administration of midazolam (T0) 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°C until analysed within 1 month.

2.5 Midazolam analysis

Midazolam concentration in plasma was determined by HPLC method modified from Lehmann and Boulieu (1995). To the 250 μL of plasma sample, 100 μL of 0.1 N NaOH, 50 μL internal standard (diazepam in methanol, 3 μg/mL) and 1 mL of diethyl ether were added. The mixture was vortex-mixed for 5 minutes and centrifuged at 1,000 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 the HPLC system (Agilent Technologies) using C8 reversed-phase column (Phenomenex Inc, USA). The mobile phase consisted of 0.025 M KH_2PO_4 (pH 4.6):acetonitrile:methanol (35:30:35 v/v/v), at a flow rate of 1.2 mL/min. The peak was detected using a UV detector set at 210 nm. The standard midazolam and internal standard (diazepam) were eluted at 7 and 8 min,

respectively. The assay was validated according to the guideline (US. FDA, 2001) with the lower limit of quantification (LLOQ) of 50 ng/mL. The interday and intraday accuracies(104-110% and 95-100%, respectively), precision (% CV less than 10) and mean recovery (86-99%) were within the accepted ranges.

2.6 Phyllanthin and hypophyllanthin analysis

2.6.1 In *P. amarus* extract

The sample solution of *P. amarus* extract was prepared by accurately weighing 1 g dry powder extract and dissolving in 10 mL of methanol, then centrifuging at 1,300 g for 10 min. The supernatant was transferred into a microcentrifuge tube and diluted to 10 µg/mL and 20 µL was injected into the HPLC system (Agilent Technologies) using C18 reversed-phase column (Symmetry®, Waters, USA). The mobile phase consisted of acetonitrile:water (52:48 v/v), at a flow rate of 1.0 mL/min. The peak was detected using a UV detector set at 220 nm. The experiment was run in triplicate. Standard calibration curves were constructed by the least-square linear regression of phyllanthin and hypophyllanthin concentration and their peak areas. The concentrations of phyllanthin and hypophyllanthin in *P. amarus* extract were calculated from the standard curve by reverse prediction.

2.6.2 In plasma

Phyllanthin and hypophyllanthin concentration in plasma were determined by HPLC method modified from Murugaiyah and Chan (2007). To the 250 µL of plasma sample, 50 µL of internal standard (diazepam in methanol, 3 µg/mL) and 250 µL of acetonitride were added then vortex-mixed for 1 minute and centrifuged at 1,000 g for 10 minutes. The supernatant (50 µL) was injected into HPLC system (Agilent Technologies) using C18 reversed-phase column (Symmetry®, Waters, USA). The mobile phase consisted of acetonitrile:water (52:48 v/v), at a flow rate of 1.0 mL/min. The peak was detected using a UV detector set at 220 nm. The internal standard (diazepam), standard phyllanthin and hypophyllanthin were eluted at 5, 10.2 and 10.8 min, respectively. The assay was validated according to the guideline (US. FDA, 2001). The LLOQ were 211.18 ng/mL and 226.62 ng/mL for phyllanthin and hypophyllanthin, respectively. The intraday accuracy (85-106%), interday accuracy (91-108%), precision (% CV less than 10) and mean recovery (84 - 102%) were within the accepted ranges.

2.7 Pharmacokinetics and statistical analysis

Plasma concentrations of midazolam were plotted against time after the drug administration. Pharmacokinetic parameters of midazolam were determined by a noncompartment method with the use of WinNonlin Professional Software Version 1.1 (Pharsight, Mountain View, CA). 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 (Ke). 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. Half life ($T_{1/2}$), the time for concentration to decrease by half, was calculated by ratio of 0.693/Ke. All pharmacokinetic parameters were expressed as the mean ± standard deviation (X±SD). Statistical analysis was performed using Student's paired t-test. Differences were considered as statistically significant when the *p*-value was less than 0.05. The software used was the SPSS (Version 11.5, SPSS Inc, Chicago, IL, USA).

3. Results

The contents of phyllanthin and hypophyllanthin in *P. amarus* extract were 296.69±8.04 mg/g and 96.56±4.36 mg/g, respectively. Mean plasma concentration time-profiles of midazolam 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) are shown in Figure 1. The mean C_{max}(164.87± 140.15 VS 474.15±322.46 ng.mL), T_{max}(0.44±0.27 VS 0.72±0.29 h), AUC_{0-8}(362.39±281.88 VS 1024.78±1094.74 ng.h/mL) and $T_{1/2}$(2.19±1.12 VS 3.18±1.49 h) of midazolam were significantly increased after pretreatment with *P. amarus*, whereas the $AUC_{0-\infty}$(439.76±360.02 VS 1424.27±1749.62 ng.h/mL) was non-significantly increased, Ke(0.39±0.19 VS 0.26±0.18 h^{-1}) and CL/F(0.08±0.10 VS 0.02±0.02 L/h/kg) were non-significantly decreased (Table 1). Phyllanthin and hypophyllanthin

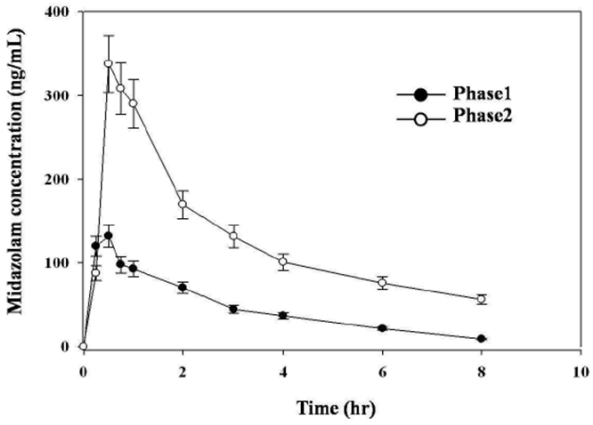

Figure 1. Mean plasma concentration-time profiles of midazolam in rabbit (N=9) after a single oral dose of 10 mg/kg midazolam (Phase 1) and after pretreatment with 500 mg/kg *P. amarus* o.d. orally for 7 days and once before midazolam administration (Phase 2).

Table 1. Pharmacokinetic parameters (X ± SD) of midazolam in 9 rabbits receiving a single oral dose of 10 mg/kg midazolam (Phase 1) compared with after pretreatment with 500 mg/kg *P. amarus* o.d. orally for 7 days and once before midazolam administration (Phase 2).

Parameters	Midazolam		p-value
	Phase 1	Phase 2	
T_{max} (h)	0.44±0.27	0.72±0.29*	0.030
C_{max} (ng/L)	164.87±140.15	474.15±322.46*	0.004
AUC_{0-8} (ng.h/L)	362.92±281.88	1024.78±1094.74*	0.048
$AUC_{0-\infty}$ (ng.h/L)	439.76±360.02	1424.27±1749.62	0.050
$T_{1/2}$ (h)	2.19±1.12	3.18±1.49*	0.038
Ke (h^{-1})	0.39±0.19	0.26±0.18	0.141
CL/F (L/h/kg)	0.08±0.10	0.02±0.02	0.085

* significantly different compared with phase 1

were undetectable in all plasma samples at one hour after oral administration of the last dose of *P. amarus*.

4. Discussion and Conclusions

Some phytochemicals contained in medicinal plants are biologically active and capable of interacting with therapeutic drugs. Inhibition or induction of drug metabolizing enzymes, particularly CYPs that are the major enzymes responsible for the metabolism of most therapeutic drugs, is of major concern. The induction of CYP may decrease plasma concentration of certain drugs, leading to reduced efficacy of the drug or treatment failure. On the other hand, an inhibitory effect of medicinal plants on CYP may cause increased plasma concentrations of several drugs leading to increased efficacy, side effect or toxicity of the drug (Elvin-Lewis, 2001). Among these CYP enzymes, CYP3A4 is the most abundant in human liver, as well as in intestine, and is involved in the metabolism of more than 50% of all prescribed drugs (Zhou *et al*., 2008). Induction or inhibition of CYP3A4 by various herbs has been reported to be clinically important. For example, administration of St. John's Wort (*Hypericum perforatum*, a CYP 3A4 inducer) extract to patients receiving tacrolimus, an immunosuppressive drug, markedly reduced tacrolimus blood concentration with the risk of organ rejection (Mai *et al.*, 2003) and coadministration of simvastatin (a lipid lowering drug) with grapefruit juice (a CYP 3A4 inhibitor) increased the mean peak serum concentration of simvastatin 12.0-fold with increase risk for myopathy (Lilja *et al.*, 2000). 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 (Reves *et al*.,1985). It is rapidly and extensively metabolized by CYP3A to 1-hydroxy and 4-hydroxy midazolam (Patki *et al*., 2003). Midazolam is one of the best *in vivo* probe drugs for the study of CYP3A4 activity not only because it is a substrate of CYP 3A, but 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; Galetin *et al*., 2005; Zhou *et al*., 2008). It can provide a measure of human CYP3A4 and 3A5 activity related to both intestinal and hepatic metabolism, respectively (Lin *et al*., 2002). Moreover, midazolam hydroxylase activity could be determined in rabbit as well as in human hepatic microsomes (Elbarbry *et al*., 2009) 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 (Daujat *et al*., 1991; Patki *et al*., 2003), it has been demonstrated that CYP 3A6 is strongly similar to CYP3A4 both in intestine and liver (McKinnon *et al*., 1993, Franklin, 1995).

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}, AUC_{0-8} and $T_{1/2}$ (2.9, 2.8 and 1.4-fold, respectively) of midazolam indicating the inhibitory activity of *P. amarus* on CYP3A, the enzyme responsible for midazolam metabolism. Although a large variation was noticed, it is consistent with previous *in vitro* and *in vivo* reports showing large interindividual variability in genetic expression for CYP3A leading to variability (20-60 fold) of oral bioavailability and metabolism of CYP3A substrates (Thummel *et al.*, 1994; Thummel *et al.,* 1996; Wilkinson, 1996). According to the drug interaction guideline (US FDA, 2006), *P. amarus* in this study was classified as a moderate CYP 3A inhibitor because the increase in the area under curve of oral midazolam was in the range of 2-5 fold. This inhibitory effect on CYP3A was consistent with previous *in vitro* data showing that ethanolic extract of *P. amarus* and its major lignans, phyllanthin and hypo-

phyllanthin, were potent mechanism-based inhibitors of CYP 3A4 activity in human liver microsome (Taesotikul *et al*., 2011). In the present study, *P. amarus* ethanolic extract containing phyllanthin and hypophyllanthin 297 mg/g and 96 mg/g, respectively, showed the inhibitory activity on CYP3A although the plasma concentration of phyllanthin and hypophyllanthin could not be detected at the LLOQ of 211.18 and 226.62 ng/mL, respectively, whereas the IC50 in *in vitro* study was 800 ng/mL (Taesotikul *et al*., 2011), and the clearance of midazolam was not significantly decreased which suggested that the inhibitory effect might take place mainly in the intestine rather than in the liver. 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). Concerning the mechanism of inhibition, CYP inhibitors that have been shown to be mechanism-based inactivators require catalytic activation by the enzyme to transient intermediates that then form quasi-irreversible complexes with the heme iron atom of the enzyme 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 (Morel *et al*., 1993; Chow *et al*., 2006; Jiao *et al*., 2006; Leopoldini *et al*., 2006; Hatcher *et al*., 2008; Skerjanec *et al*., 2010; Choi *et al*., 2011; Cho *et al*., 2012; Galanello *et al*., 2012). It is noted that Tmax was increased after pretreatment with *P. amarus*, which is inconsistant with the inhibitory effect on intestinal CYP3A. This effect might result from the delayed gastric emptying effect which is attributed by the stringent effect of tannin, a major component in *P. amarus* (Chaudhari and Mengi, 2006; Umoh *et al.*, 2013).

In conclusion, the present study has demonstrated that pretreatment with *P. amarus* ethanolic extract (500 mg/kg) for a short period (7 days) increased the plasma level of midazolam probably by inhibition of CYP3A. Since CYP 3A is an enzyme responsible for the metabolism of various drugs, co-administration of *P. amarus* may increase plasma level of these drugs leading to increased efficacy or serious side effects. Thus, clinical investigation needs to be performed to reveal the significant potential of these herb-drug interactions in human.

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