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ผลของสารสกัดลูกใต้ใบต่อเภสัชจลนศาสตร์ของยามิดาโซแลมในกระต่าย

Effect of *Phyllanthus amarus* Schum. and Thonn. extract on the pharmacokinetics of midazolam in rabbits

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บทคัดย่อ

วัตถุประสงค์ของการศึกษานี้เพื่อประเมินผลของสารสกัดลูกใต้ใบที่ใช้รักษาโรคตับ ต่อเภสัชจลนศาสตร์ของยามิดาโซแลมในกระต่าย การศึกษาแบ่งเป็น 2 ช่วงห่างกัน 7 วัน ใช้กระต่ายจำนวน 4 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

จากการทดลองพบว่ากระต่ายที่ได้รับสารสกัดสมุนไพรลูกใต้ใบ มีค่าความเข้มข้นของยาในพลาสมาสูงสุด เวลาที่ระดับยาในพลาสมาสูงสุด พื้นที่ใต้กราฟระหว่างความเข้มข้นของกับเวลาที่ให้ยา ของยามิดาโซแลมเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (p<0.05) ส่วน ค่าครึ่งชีวิตเพิ่มขึ้นเล็กน้อย ในขณะที่อัตราการชำระยา มีค่าลดลงเมื่อเปรียบเทียบกับกระต่ายที่ได้รับยามิดาโซแลมเพียงอย่างเดียว แสดงว่าสารสกัดลูกใต้ใบอาจมีฤทธิ์ยับยั้งการทำงานของ CYP3A4 ซึ่งเป็นเอนไซม์ที่ใช้ในการเมตาบอลิซึมของยามิดาโซแลมในกระต่ายได้ ดังนั้นการรับประทานลูกใต้ใบร่วมกับมิดาโซแลมหรือยาแผนปัจจุบันบางชนิดที่เกิดเมตาบอลิซึมโดย CYP3A4 อาจทำให้ระดับยาในเลือดเพิ่มสูงขึ้นจนเกิดอาการข้างเคียงที่รุนแรงได้ จึงควรระวังการเกิดปฏิกิริยาระหว่างยากับสมุนไพรในผู้ป่วย

คำสำคัญ ลูกใต้ใบ ปฏิกิริยาระหว่างสมุนไพรกับยา ไซโตโครมพี 450 กระต่าย

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Abstract

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 crossover design with one week washout period. Four 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* medication.

The results showed that pretreatment with *P. amarus* increased the mean maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), time required to reduce the plasma concentration to one half its initial value (half-life; $T_{1/2}$), area under the drug concentration-time curves (AUC), whereas the total clearance (CL/F) was decreased compared with control group receiving a single oral dose of midazolam. It is suggested that *P. amarus* extract might inhibit CYP3A4 which is the enzyme responsible for midazolam metabolism in rabbits. Thus, coadministration of *P. amarus* and midazolam or other drugs which are CYP3A4 substrates may increase plasma drug concentration leading to serious side effects. Clinical relevance of herb-drug interaction between *P. amarus* and CYP3A4 substrates should be warranted.

Keywords: *P. amarus* Herb-drug interaction Cytochromes P450 rabbits

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Introduction

Phyllanthus amarus, belonging to the Euphorbiaceae family, is a medicinal plant that has been widely used in tropical and subtropical countries for treatment of several diseases such as liver disease, urinary tract infection, menstruation problem (Calixto et al., 1998). Their common activities include antioxidant (Kumaran et al., 2007), antimicrobial (Mazumder et al., 2006), hepatoprotective activity against paracetamol and ethanol (Wongnawa et al., 2006 and Pramyothin et al., 2007). *P. amarus* contains several lignans including phyllanthin and hypophyllanthin, which have been shown to have hepatoprotective and antioxidative properties (Krithika et al., 2009,). Pre-administration of *P. amarus* aqueous extract at the dose of 1,500 - 2,000 mg/kg have been reported to prevent DNA damage in hamster liver cells caused by dimethylnitrosamine (Sripanidkulchai et al., 2002) and significantly inhibited tumor growth (Rajeshkumar et al., 2002). Oral administration of an ethanolic extract of *P. amarus* (300 mg/kg) in rats showed hepatoprotective activity against aflatoxin B1-induced liver damage via its antioxidant activity (Naaz et al., 2007). In addition, more than 30% inhibition of HIV-replication was observed in HIV patients treated with 1,200 mg of *P. amarus* extract (Notka et al., 2004). Cytotoxicity to human adenocarcinoma cell line Caco-2 was also reported (Lawson-Evi et al., 2008).

Midazolam (MDZ) is a sedative drug with a rapid onset and short duration of action. MDZ is commonly used in clinical practice to induce anaesthesia, to treat status epilepticus and induce sedation in patients in intensive care units (Kumaran et al., 2007). Clearance of MDZ has been used as a marker for the activity of cytochrome P450 (CYP) 3A, the most abundant class of CYP enzymes (Luo et al., 2009). Midazolam is rapidly and extensively metabolized by CYP3A isoforms to 1-hydroxy and 4-hydroxy midazolam (Gorski et al., 1994; Moltke et al., 1995)

Since herbal medicines contain a number of phytochemicals, some chemicals are biologically active and capable of interacting with therapeutic drugs. Inhibition or induction of drug metabolizing enzymes, particularly CYP that are the major enzymes responsible for the metabolism of most of therapeutic drugs, are of major concern. The inhibitory effect of medicinal plants on these enzymes may result in increased plasma concentrations of several drugs leading to increased efficacy or toxicity of the drug. On the other hand, an induction of CYP may decrease plasma concentration of certain drugs, leading to reduced efficacy of the drug or treatment failure (Elvin-Lewis et al., 2001). Among these CYP enzymes, CYP3A4 is the most abundant in human liver and is involved in the metabolism of more than 50% of all prescribed drugs (Zhou, 2008). Taesotikul et al. (2011) reported that the inhibitory potency of the ethanolic and aqueous extract of *P. amarus* on CYP3A4 activity *in vitro* was about 2-3 orders of magnitude stronger than the known CYP3A4 inhibitors such as erythromycin and clarithromycin. Thus, concomitant administration of *P. amarus* with CYP3A4 may potentially lead to herb-drug interaction *in vivo*. Therefore, this study was aimed to investigate the effect of *P. amarus* on the pharmacokinetics of midazolam, a CYP3A4 substrate, in rabbits.

Materials and Methods

Animals

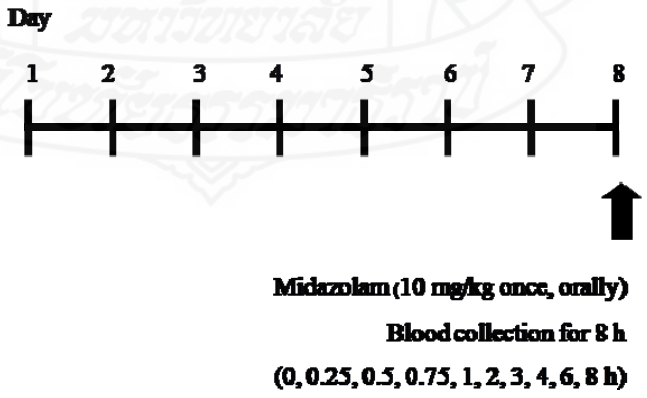
Four male New Zealand White rabbits obtained from The National Laboratory Animal Center, Mahidol University (NLAC-MU), Salaya, Nakornpathom, Thailand, were used in this study. The rabbits were at the age of 12-16 weeks old, weighing between 3.0 and 3.5 kg at the beginning of the study. The rabbits were 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 Faculty of Science, Prince of Songkla University, Thailand.

Study design

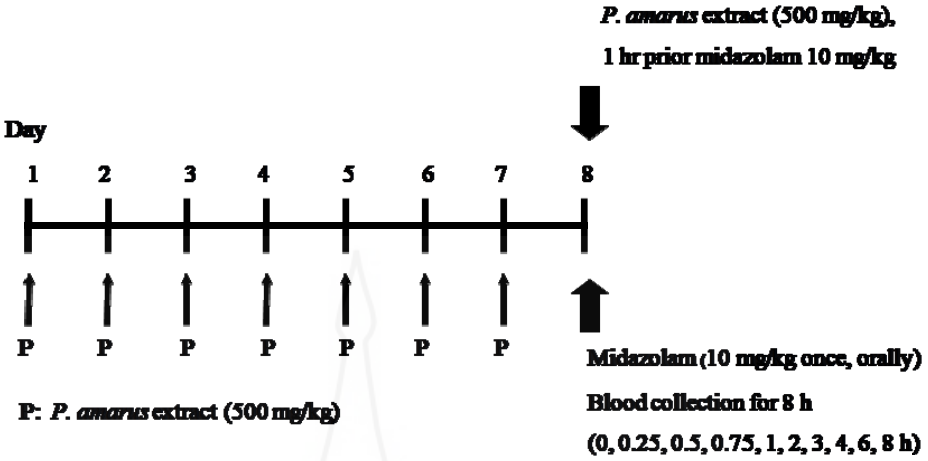
The study was an open-label, randomized, two-phase crossover design with one week washout period, of single-dose (midazolam) and multiple-dose (*P. amarus* extract). In the first phase, after 12 h fasted, the animal received a single oral dose of 10 mg/kg midazolam (Dormicum®, Hoffmann-La Roche Ltd., Basel, Switzerland) with 10 mL of water. Baseline pharmacokinetics of midazolam was evaluated. In the second phase, 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 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.

Schematic plan of 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



Preparation of *P. amarus* extract and medication

P. amarus powder was purchased from Lampang Herb Conservation, Lampang province, Thailand. The powder was extracted twice in 70% ethanol (1:5) at room temperature for 7 days. The filtrate was subsequently evaporated at 60 ºC and lyophilized by freeze-dryer to dryness. 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.

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 of 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 ºC 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 were eluted at 7 and 8 min, respectively. The assay was validated according to the guideline (US. FDA, 2001) with the limit of quantification of 50 ng/mL. The within-day and between-day accuracies for the analysis were 99-109% and 96-97% with precision value below 6.7%. Mean recoveries were between 89 and 99%.

Pharmacokinetics and statistical analysis

Plasma concentration of midazolam was plotted against time after the drug administration. Pharmacokinetic parameters [maximum plasma concentrations (C_{max}), time to reach C_{max} (T_{max}), area under the drug concentration-time curves, (AUC), total clearance (CL/F), time required to reduce the plasma concentration to one half its initial value (half-life; $T_{1/2}$)] of midazolam were determined by a non-compartment model was performed by WinNonlin professional Software Version 1.1 (Pharsight, Mountain View, CA).

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

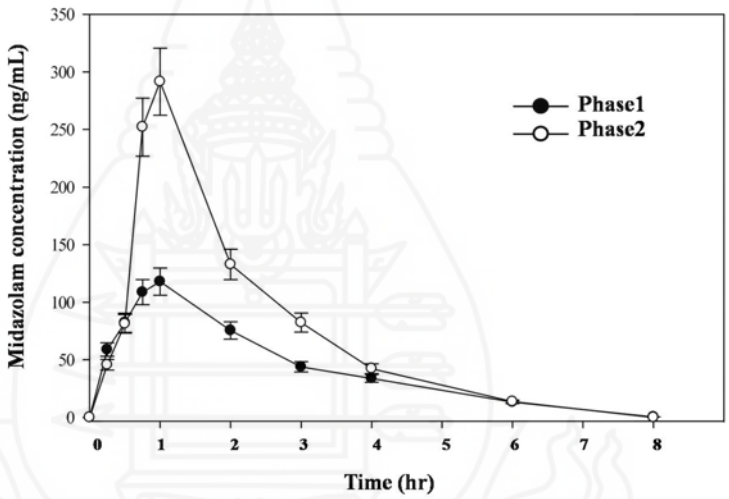


Figure 1. Mean plasma concentration time-profiles of midazolam in 4 rabbits after a single oral dose of 10 mg/kg midazolam (Phase 1) and after pretreatment with 500 mg/kg *P. amarus* o.d. orally for 7 days and once before midazolam administration (Phase 2)

Table 1. Pharmacokinetic parameters (X ± SD) of midazolam in 4 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
	Phase1	Phase 2	
C_{max} (ng/L)	116.62±47.91	356.87±47.52	0.008*
T_{max} (h)	0.50±0.102	1.00±0.00	0.016*
AUC_{0-8}(ng.h/L)	208.65±77.40	583.37±228.30	0.031*
$AUC_{0-\infty}$(ng.h/L)	296.80±161.45	663.63±189.82	0.023*
$T_{1/2}$ (h)	1.95±1.11	3.39±1.31	0.079
CL/F (L/h/kg)	0.045±0.02	0.016±0.01	0.061

*Significantly different between phase 1 and phase 2 ($p < 0.05$)

Results

Effects of *P. amarus* on midazolam pharmacokinetics

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) were shown in Figure 1. The mean C_{max}, T_{max}, AUC_{0-8} and $AUC_{0-\infty}$ were increased (356.87±47.52 VS 116.62±47.91, 1.00±0.00 VS 0.50±0.102, 583.37±228.30 VS 208.65±77.40, 663.63±189.82 VS 296.80±161.45, respectively), whereas the CL/F was decreased (0.016±0.01 VS 0.045±0.02) significantly after *P. amarus* pretreatment. $T_{1/2}$ was slightly increase (3.39±1.31 VS 1.95±1.11) but it is not significantly different, as shown in Table 1.

After orally administered *P. amarus* extract at 500 mg/kg/day for 7 days, there were no significant differences in the mean body weight (data not shown).

Discussion and conclusion

P. amarus, a tropical plant in the family Euphorbiaceae, has long been used in folk medicine in several countries around the world and easily found in all regions of Thailand. It has been demonstrated that *P. amarus* extract exhibited several pharmacological effects including antitumor, anti-inflammatory and antiviral effects as well as anti-nephrotoxicity and a hepatoprotective effect (Faremi et al., 2008). In addition, phyllanthin and hypophyllanthin, the major lignans of this plant, have hepatoprotective effect on carbontetrachloride-induced hepatotoxicity as well as antihyperuricemic activity in rat (Chirdchupunseree and Pramyothin, 2010). Previous reports have shown that aqueous extract of *P. amarus* exerted an inhitory effect on several recombinant human CYP isoforms such as CYP1A2, CYP3A4 and CYP2B6 (Appiah-Opong et al., 2008). Moreover, oral administration of the methanolic extract of this plant to phenobarbitone (a CYP1A and CYP2B inducer) pretreated rats could inhibit CYP1A and CYP2B activities (Hari Kumar and Kuttan, 2006) which suggested that the potential herb-drug interactions may occur when *P. amarus* extract was co-administered with therapeutic drugs metabolized by CYP.

In the present study, the effect of *P. amarus* ethanolic extract on midazolam pharmacokinetics were examined in rabbits in order to determine the inhibitory effect of *P. amarus* on CYP3A4 *in vivo* using midazolam as a substrate probe. It was found 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 and $T_{1/2}$ (about 3-fold, 2-fold, 2.8-fold and 1.7-fold, respectively) while decreased CL/F (about 2.7- fold) of midazolam indicating that *P. amarus* inhibit the activity of CYP3A4, the enzyme responsible for midazolam metabolism. Since midazolam is mainly metabolized via CYP3A4 while it is not a substrate of P-glycoprotein, thus it is considered as one of the best *in vivo* probe drugs for the study of CYP3A4 activity (Zhou et al., 2005). Moreover, Elbarbry *et al*. (2009) has been 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. Since CYP 3A4 is an enzyme responsible for metabolism of various drugs, thus co-administration of *P. amarus* may increase plasma level of those drugs leading to increased serious side effects. However, clinical investigation should be performed to reveal the significant potential of these herb-drug interactions in human.

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References

Appiah-Opong, R., Commandeur, J.N., Axson, C., & Vermeulan, N.P. (2008). Interactions between cytochromes P450, glutathione S-transferases and Ghanaian medicinal plants. Food and Chemical Toxicology, 46, 3598-603.

Chirdchupunseree, H., & Pramyothin, P. (2010). Protective activity of phyllanthin in ethanol treated primary culture of rat hepatocytes. Journal of Ethnopharmacology, 128, 172-176.

Calixto, J.B., Santos, A.R., Cechinel-Filho, V., & Yunes, R.A. (1998). A review of the plants of the genus Phyllanthus: their chemistry, pharmacology, and therapeutic potential. Medicinal Research Reviews, 18, 225-258.

Elbarbry, F., Attia, A., & Shoker, A. (2009). Validation of a new HPLC method for determination of midazolam and its metabolites: Application to determine its pharmacokinetics in human and measure hepatic CYP3A activity in rabbits. Journal of Pharmaceutical and Biomedical Analysis, 50, 987-993.

Elvin-Lewis M. (2001). Should we be concerned about herbal remedies. Journal of Ethnopharmacology, 75, 141-164.

Faremi, T.Y., Suru, S.M., Fafunso, M.A., & Obioha, U.E. (2008). Hepatoprotective potentials of *Phyllanthus amarus* against ethanol-induced oxidative stress in rats. Food and Chemical Toxicology, 46, 2658-2664.

Gorski, J.C., Hall, S.D., Jones, D.R, Van den Branden, M. & Wrighton, S.A. (1994). Regioselective biotransformation of midazolam by members of the human cytochrome P450 3A (CYP3A) subfamily.Biochemical Pharmacology, 47, 1643-1653.

Hari Kumar, K.B., & Kuttan, R. (2006). Inhibition of drug metabolizing enzymes (cytochrome P450) *in vitro* as well as *in vivo* by *Phyllanthus amarus* SCHUM & THONN. Biological and Pharmaceutical Bulletin, 29, 1310-1313.

Krithika, R., Mohankumar, R., Verma, R.J., Shrivastav, P.S., Mohamad, I.L., Gunasekaran, P., & Narasimhan, S. (2009). Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line. Chemico-Biological Interactions, 181, 351-358.

Kumaran, A., & Karunnakaran, R.J. (2007). *In vitro* antioxidant activities of methanol extracts of five *Phyllanthus* species from India, LWT-Swiss Society of Food Science and Technology, 40, 344-352.

Lawson-Evi, P., Eku-Gadegbeku, K., Agbonon, A., Aklikokou, K., Mokha, S., Creppy, E.E., & Gbessor, M. (2008). Toxicological assessment on extracts of *Phyllanthus amarus* Schum. and Thonn. Scientific Research And Essays, 3, 410-415.

Lehmann, B., & Boulieu, R. (1995). Determination of midazolam and its unconjugated 1-hydroxy metabolite in human plasma by high-performance liquid chromatography. Journal of Chromatography B: Biomedical Sciences and Applications. 674, 138-142.

Luo, W., Zhao, M., Yang, B., Shen, G., & Rao, G. (2009). Identification of bioactive compounds in *Phyllanthus emblica* L. fruit and their free radical scavenging activities, Food Chemistry, 114, 499-504.

Mazumder, A., Mahato, A., & Mazumder, R. (2006). Antimicrobial potentiality of *Phyllanthus amarus* against drug resistant pathogens. Natural Product Research, 20(4), 323-326.

Moltke, L.L., Greenblatt, D.J., Schmider, J., Harmatz, J.S., & Shader, R.I. (1995).Metabolism of drugs by cytochrome P450 3A isoform. Clinical Pharmacokinetics, 29(Suppl. 1), 33-43.

Naaz, F., Javed, S., & Abdin, M.Z. (2007). Hepatoprotective effect of ethanolic extract of *Phyllanthus amarus* Schum. et Thonn. on aflatoxin B_1-induced liver damage in mice. Journal of Ethnopharmacology, 113, 503-509.

Notka, F., Meier, G., & Wagner, R. (2004). Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication *in vitro* and *ex vivo*. Antiviral Research, 64, 93-102.

Pramyothin, P., Ngamtin, C., Poungshompoo, S., & Chaichantipyuth, C. (2007). Hepatoprotective activity of *Phyllanthus amarus* Schum. et. Thonn.extract in ethanol treated rats: *In vitro* and *in vivo* studies. Journal of Ethnopharmacology, 114, 169-173.

Rajeshkumar, N.V., Joy, K.L., Kuttan, G., Ramsewak, R.S., Nair, M.G., & Kuttan, R. (2002). Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. Journal of Ethnopharmacology, 81, 17-22.

Seeka, P., Niwattisaiwong, N., Warisnoicharoen, W.,Winitthana, T., Tantisira, M.H., & Lawanprasert, S. (2012). Effects of the standardized extract of *Centella asiatica* ECa 233on human cytochrome P450. Thai Journal of Pharmacology Science, 36, 34-37.

Sripanidkulchai, B., Tattawasart, U., Laupatarakasem, P., Vinitketkumneun, U., Sripanidkulchai, K., Furihata, C., & Matsushima, T. (2002). Antimutagenic and anticarcinogenic effects of *Phyllanthus amarus*. Phytomedicine, 9, 26-32.

Taesotikul, T., Dumrongsakulchai, W., Wattanachai, N., Navinpipat, V., Somanabandhu, A., Tassaneeyakul, W. & Tassaneeyakul, W. (2011). Inhibitory effects of *Phyllanthus amarus* and its major lignans on human microsomal cytochrome P450 activities: evidence for CYP3A4 mechanism-based inhibition. Drug Metabolism and Pharmacokinetics, 26, 154-161.

U.S. Food and Drug Administration. (2001). Guidance for industry-bioanalytical method validation. http://www. fda.gov/CDER/GUIDANCE/index.htm. [February 5, 2010]

Wongnawa, M., Thaina, P., Bumrungwong, N., Rattanapirun, P., Nitiruangjarat, A., Muso, A., & Prasartthong, V. (2006). The protective potential and possible mechanism of *Phyllanthus amarus* Schum. & Thonn. Aqueous extract on paracetamol-induced hepatotoxicity in rats. Songklanakarin Journal of Science and Technology, 28(3), 551-561.

Zhou, S., Yung Chan, S., Cher Goh, B., Chan, E., Duan, W., Huang, M., & McLeod, H.L. (2005). Mechanism based inhibition of cytochrome P450 3A4 by therapeutic drugs. Clinical Pharmacokinetics, 44, 279-304.

Zhou, S.F. (2008). Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. Current Drug Metabolism, 9, 310-322.