ORIGINAL ARTICLE

The protective potential and possible mechanism of *Phyllanthus amarus* Schum. & Thonn. aqueous extract on paracetamol-induced hepatotoxicity in rats

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Abstract

Wongnawa, M., Thaina, P., Bumrungwong, N., Rattanapirun, P., Nitiruangjaras, A., Muso, A., and Prasartthong, V.
The protective potential and possible mechanism of *Phyllanthus amarus* Schum. & Thonn. aqueous extract on paracetamol-induced hepatotoxicity in rats
Songklanakarin J. Sci. Technol., 2006, 28(3) : 551-561

The hepatoprotective potential of *Phyllanthus amarus* Schum. & Thonn. was studied on paracetamol-induced hepatotoxicity in rats by measuring the levels of serum transaminase (SGOT and SGPT), alkaline phosphatase (ALP) and bilirubin, as well as by histopathological examination of the liver. Furthermore, the hepatoprotective mechanisms were investigated by determining the amount of paracetamol and its metabolites (glucuronide, sulfate, cysteine and mercapturic acid conjugates) in urine and pentobarbital-induced

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Received, 11 August 2005 Accepted, 21 October 2005

sleeping time to indicate the inhibition on cytochrome P450. The involvement of glutathione was evaluated by determining hepatic reduced glutathione. Its radical scavenging activity, iron chelating activity and total phenolic content were also determined. *P. amarus* aqueous extracts (0.8, 1.6 or 3.2 g/kg) were orally administered twice daily for 7 days prior, for 2 days after, or for 7 days prior and followed by 2 days after a single oral dose of paracetamol (3 g/kg). The results showed that the extract at the doses of 1.6 and 3.2 g/kg decreased the paracetamol-induced hepatotoxicity as indicated by the decrease in SGOT, SGPT, bilirubin and histopathological score while the ALP did not change. Moreover, it is suggested that the hepatoprotective mechanism of this plant was related neither to the inhibition on cytochrome P450, nor to the induction on sulfate and/or glucuronide conjugation pathways of paracetamol, but partly due to the protective effect on the depletion of hepatic reduced glutathione and also its antioxidant activity, especially the radical scavenging and iron chelating activity, which might be related to the high polyphenolic contents. These results support the value of *P. amarus*, which has been used in Thai folk medicine for the treatment of liver diseases.

Keywords : *Phyllanthus amarus*, paracetamol, cytochrome P450, glutathione, antioxidant activity

บทคัดย่อ

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ศักยภาพและกลไกการออกฤทธิ์ของน้ำต้มลูกใต้ใบในการป้องกันความเป็นพิษต่อตับของหนูขาวที่ได้รับการเหนี่ยวนำด้วยพาราเซทตะมอล

ว. สงขลานครินทร์ วทท. 2549 28(3) : 551-561

ได้ศึกษาฤทธิ์และกลไกการออกฤทธิ์ของน้ำต้มลูกใต้ใบในการป้องกันการเกิดพิษต่อตับของหนูขาวที่ได้รับพาราเซทตะมอล โดยวัดระดับเอนไซม์ transaminase (SGOT และ SGPT) alkaline phosphatase (ALP) และบิลิรูบินในซีรัม พร้อมทั้งศึกษาการเปลี่ยนแปลงทางจุลพยาธิวิทยาของตับ นอกจากนั้นยังได้ศึกษากลไกการออกฤทธิ์ในการป้องกันการเกิดพิษต่อตับ โดยวัดระดับพาราเซทตะมอลและสารแปรรูปของพาราเซทตะมอล (glucuronide, sulfate, cysteine และ mercapturic acid conjugates) ในปัสสาวะ และวัดระยะเวลาการนอนของหนู (pentobarbital-induced sleeping time) เพื่อศึกษาฤทธิ์ในการยับยั้งเอนไซม์ cytochrome P450 พร้อมทั้งวัดระดับรีดิวส์กลูตาไธโอนในตับของหนู ศึกษาฤทธิ์ในการจับกับอนุมูลอิสระ ฤทธิ์ในการจับกับเหล็ก และหาปริมาณสารฟีนอลในน้ำต้มลูกใต้ใบ การศึกษานี้ทำในหนูขาวใหญ่ที่ได้รับน้ำต้มลูกใต้ใบวันละ 2 ครั้งในขนาด 0.8, 1.6 และ 3.2 กรัม/กก. ที่เวลาต่าง ๆ กัน คือ 7 วันติดต่อกันก่อน 2 วันติดต่อกันหลัง หรือ 7 วันติดต่อกันก่อนร่วมกับ 2 วันติดต่อกันหลังได้รับพาราเซทตะมอลทางปากในขนาด 3 กรัม/กก. พบว่าน้ำต้มลูกใต้ใบในขนาด 1.6 และ 3.2 กรัม/กก. มีผลลดความเป็นพิษของพาราเซทตะมอลได้เมื่อพิจารณาจากค่า SGOT, SGPT, bilirubin และ histopathological score ที่ลดลงอย่างมีนัยสำคัญ ในขณะที่ ALP ไม่แตกต่างกัน นอกจากนั้นยังพบว่ากลไกการป้องกันการเกิดพิษต่อตับไม่เกี่ยวข้องกับการยับยั้งเอนไซม์ cytochrome P450 หรือการเหนี่ยวนำให้พาราเซทตามอลรวมตัวกับ sulfate หรือ glucuronide แต่น่าจะเนื่องจากฤทธิ์ในการป้องกันการขาดกลูตาไธโอนในตับ และฤทธิ์ต้านออกซิเดชั่นโดยเฉพาะฤทธิ์ในการจับกับอนุมูลอิสระและฤทธิ์ในการจับกับเหล็กซึ่งอาจเกี่ยวข้องกับการที่พบปริมาณสารฟีนอลสูง ผลการวิจัยครั้งนี้สนับสนุนสรรพคุณของลูกใต้ใบที่ใช้ในแพทย์แผนไทยเพื่อรักษาโรคตับ

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Phyllanthus amarus Schum. & Thonn. (F. Euphorbiaceae) is a small herb which has been used in Thai folk medicine for the treatment of fever, jaundice, ascites, hemorrhoid and diabetes (Pongboonrod, 1976). Apart from these medicinal uses, several reports showed anti- hepatitis B virus effect (Thyagarajan *et al*., 1988), hypoglycemic effect (Moshi *et al*., 1997), antinociceptive effect (Santos *et al*., 2000), the increase in life span of rats with hepatocellular carcinoma (Rajeshkumar and Kuttan, 2000), antitumour, antimutagenic and anticarcinogenic effect (Sripanidkulchai *et al*. and Rajeshkumar *et al*., 2002), anti-inflammatory effect (Kiemer *et al*., 2003) and chemoprotective effect (Kumar and Kuttan, 2005).

Paracetamol is widely used as analgesic and antipyretic agent. Its antipyretic and analgesic properties have been widely abused. Indiscriminate ingestion can lead to accidental poisoning and potentially lethal hepatotoxicity (Prescott *et al*., 1971).

Lignans from *P. amarus* (phyllanthin and hypophyllanthin) showed a protective effect on CCl_4-induced hepatotoxicity in isolated hepatocytes (Syamasundar *et al*., 1985). However, the protective effect of *P. amarus* on paracetamol-induced hepatotoxicity *in vivo* has not been evaluated. In this study, we investigated the protective potential of *P. amarus* aqueous extract on paracetamol-induced hepatotoxicity in rats by determining liver enzymes, studying histopathology of the liver, and also investigated its protective mechanism(s) by studying the effect on paracetamol metabolic pathway, pentobarbital- induced sleeping time and hepatic reduced glutathione. Moreover, radical scavenging activity, iron chelating activity and polyphenolic content of the extract were also evaluated.

Materials and Methods

1. Preparation of the plant extract

The whole plant of *P. amarus*, collected in Hat Yai, Songkhla, Thailand, during July-August, was identified. A voucher specimen (number 111555) was deposited in the PSU Herbarium. Dried plant (100 g) was minced and boiled with water (500 ml) for 15 min and then filtered. The filtrate was centrifuged, the supernatant was filtered and adjusted to 1 g/ml.

2. Test animals

Male Wistars rats (200-250 g) from the animal center, Faculty of Science, Prince of Songkla University, were housed at 22±3ºC with a 12 h. light-dark cycle and fed with pelleted diet and water *ad libitum*. The study protocol was approved by the Ethics Committee on Animal Experiment, Faculty of Science, Prince of Songkla University, Thailand.

3. Experimental procedures

3.1 Hepatoprotective study rats were divided into 13 groups. Group I was orally administered 50% sucrose (10 ml/kg) as normal control. Group II was given a single oral dose of paracetamol (3 g/kg) suspension (in 50% sucrose). Groups III-V were given *P. amarus* extracts (0.8, 1.6, 3.2 g/kg, respectively) orally twice daily (b.i.d.) for 7 days. Groups VI-VIII were given *P. amarus* extracts (0.8, 1.6, 3.2 g/kg, respectively) orally b.i.d. for 7 days prior to paracetamol dosing. Groups IX-XI were given *P. amarus* extracts (0.8, 1.6, 3.2 g/kg, respectively) orally b.i.d. for 2 days after paracetamol dosing. Groups XII-XIII were given *P. amarus* extracts (1.6, 3.2 g/kg, respectively) orally b.i.d. for 7 days prior to and for 2 consecutive days after paracetamol dosing.

3.2 Assay of serum GOT, GPT, ALP and bilirubin Forty-eight hours after paracetamol intoxication, the rats were sacrificed and blood was collected from the orbital plexus. The activity of serum glutamate-oxalate-transaminase (SGOT), serum glutamate-pyruvate-transaminase (SGPT), alkaline phosphatase (ALP) and total bilirubin were measured by Automated Analyzer (HITACHI 717).

3.3 Histopathological study of liver The hepatic tissue was fixed in 10% formalin and stained with hematoxylin and eosin dye. The histological sections were examined under a light microscope and the extent of necrosis was graded

as follows (Pramyothin *et al*., 1994): normal sections (0), minimal centrilobular necrosis (+1), extensive necrosis confined to centrilobular region (+2), necrosis extending from central zone to midzone or further to portal triad (+3) as shown in Figure 1.

3.4 Assay of paracetamol and its metabolites in urine Rats were divided into two groups. Group I was given a single oral dose of paracetamol (3 g/kg). Group II was orally given *P. amarus* extract (3.2 g/kg) b.i.d. for 7 days prior to paracetamol dosing. The urine was collected during 24 hr after paracetamol dosing. The concentration of paracetamol and its metabolites (glucuronide, sulfate, cysteine and mercapturic acid conjugates) were determined by the HPLC method as described by Miners *et al*. (1984).

3.5 Pentobarbital-induced sleeping time Rats were divided into 6 groups. Group I received distilled water. Group II was pretreated with phenobarbital sodium (60 mg/kg, i.p. daily) for 4 days. Group III was 30 min. pretreated with SKF-525A (15 mg/kg, i.p.). Group IV-VI were pretreated with *P. amarus* extracts (0.8, 1.6, 3.2 g/kg, respectively) b.i.d. orally for 7 days. Pentobarbital (35 mg/kg, i.p.) was injected and the sleeping time was recorded (Jayatilaka *et al*., 1990).

3.6 Assay of glutathione in liver Rats were divided into 4 groups. Group I was orally given 50% sucrose as control. Group II was given a single oral dose of paracetamol (3 g/kg) suspension. Group III was given *P. amarus* extract (3.2 g/kg) orally b.i.d. for 7 days. Group IV was given *P. amarus* extract (3.2 g/kg) orally b.i.d. for 7 days prior to paracetamol dosing. Six hours after paracetamol intoxication, the animals were sacrified. Hepatic reduced glutathione was determined as described by Mitchell *et al*. (1973).

3.7 Assay of radical scavenging activity Radical scavenging activity was measured by a decrease in absorbance at 520 nm of a methanol sulution of coloured DPPH (1,1-diphenyl-2-picryl-hydrazyl) as described by Hatano *et al*. (1989). *P. amarus* aqueous extracts (100-300 µg/ml) were mixed (1:1) with DPPH solution (60 µM). The absorbances were measured after 30 min. EC_{50} was calculated from % inhibition. BHT (butylated hydroxytoluene) was used as a standard. All tests

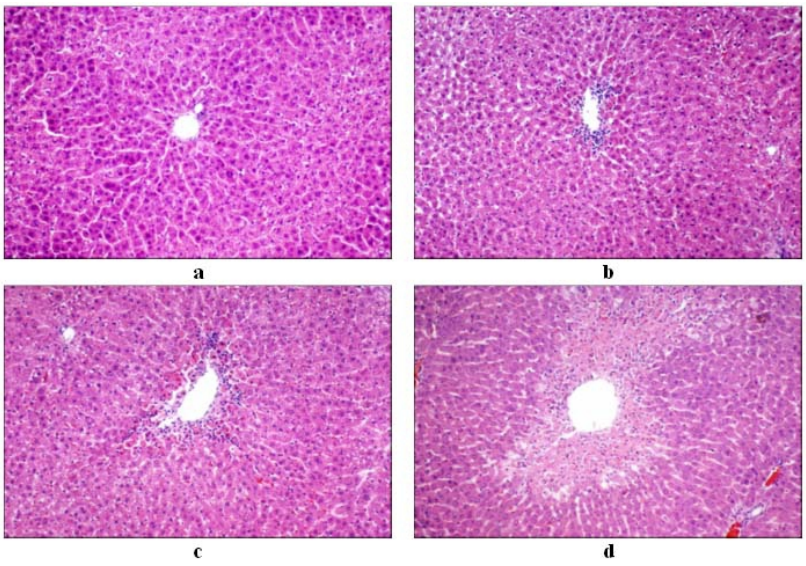

Figure 1. Histopathology of rat liver; a = Grade 0 (normal); b = Grade 1 (minimal centrilobular necrosis); c = Grade 2 (extensive necrosis confined to centrilobular region); d = Grade 3 (necrosis extending from central zone to midzone or further to portal triad).

were carried out in triplicate.

3.8 Iron chelating activity Interaction of of *P. amarus* aqueous extract and iron was studied by comparing the absorption spectra of $FeSO_4$ solution (1 mM in deionized water), *P. amarus* aqueous extract (0.4 mg/ml), mixed solution of $FeSO_4$ solution and *P. amarus* extract and followed by the addition of phenanthroline solution (5 μM) to the mixed solution, all at room temperature. The absorption spectra (200-800 nm) were recorded using UV-Vis spectrophotometer (SPECORD S100, Analytik Jena AG, Germany) as described by Tennesen and Greenhill (1992).

3.9 Total phenolic content determination Folin-Ciocalteu method (AOAC, 1995), with modification, was used to determine total phenolic content. To Folin-Ciocalteu reagent, *P. amarus* aqueous extract (0.1g/ml) or standard (0.5-4 mg/ml tannic acid) was added and mixed. After 5 min, sodium carbonate solution (7.5g %) was added and the mixture made up to 5 ml with distilled water and kept at room temperature for 30 min. The absorbance was measured at 760 nm on a Spectro 22 spectrophotometer (LaboMed. Inc, USA). All tests were carried out in tripicate.

4. Statistical analysis

Values are expressed as mean ± S.E.M. The biochemical parameters were statistically assessed by one-way analysis of variance (ANOVA). The difference between groups was evaluated by Student's *t*-test. Liver histopathological data were analyzed by Kruskal-Wallis test followed by Newman-Keuls test. $P<0.05$ was considered significant.

Results

1. Hepatoprotective effect on paracetamol-induced hepatotoxicity

Table 1 shows that a single oral dose of paracetamol (3 g/kg) induced a significant increase in SGOT, SGPT, ALP, bilirubin and histopathological score in comparison with the normal controls. *P. amarus* aqueous extract itself at the dose of 0.8-3.2 g/kg administered for 7 days had no effect on the enzymes level and the histology of liver was normal. Treatment of rats with *P. amarus* aqueous extracts at the dose of 1.6, 3.2 g/kg for 7 days before paracetamol administration resulted in a significant reduction of the biochemical parameters. The SGOT and SGPT levels were reduced by about 50% , while serum bilirubin was decreased by about 20%. Post-treatment with *P. amarus* showed less effect. *P. amarus* aqueous extracts treatment for 7 days before and 2 days after paracetamol dosing markedly reduced SGOT, SGPT and bilirubin by about 70%, 60% and 40%, respectively. The histopathological score correlated well with the changes of serum markers, while the ALP was unchanged.

2. Effect on paracetamol metabolism

Figure 2 shows that the proportions of paracetamol and its metabolites: glucuronide, sulfate, cysteine and mercapturic acid conjugates excreted in urine in *P. amarus* pretreated group (21.36±2.7, 43.9±2.7, 23.18±3.4, 1.27±0.4 and 10.68±1.0 %, respectively) were not significantly different from the control group (18.2±2.6, 41.84±3.2, 26.37±4.0, 1.57±0.4 and 11.46±0.8 %, respectively).

3. Effect on pentobarbital-induced sleeping time

Figure 3 shows that pentobarbital-induced sleeping time in the rats pretreated with phenobarbital was shortened (5.3±0.7 min.) and that of rats pretreated with SKF-525A was prolonged (98.5±7.7 min.), whereas pretreatment with *P. amarus* extracts (0.8, 1.6, 3.2 g/kg) did not prolong the sleeping time (48.6±2.5, 43.0±3.3 and 50.6±4.0 min., respectively), as compared with the control group (46.2±4.9 min.).

4. Effect on hepatic reduced glutathione

Table 2 shows that paracetamol (3 g/kg) caused a marked decrease in hepatic GSH content (1.91±0.2 μmol/g) at 6 hour post-dosing when compared with the control group (7.2±0.3 μmol/g). *P. amarus* extract (3.2 g/kg b.i.d. orally for 7 days) slightly but significantly increased GSH (7.9±0.3 μmol/g). The depletion of GSH in the

Table 1. Effects of paracetamol and *P. amarus* aqueous extract on serum GOT, GPT, ALP, bilirubin and histopathological change in rats (mean±S.E.M., n = 7-19).

Treatment	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Bilirubin (mg%)	Histopatho-logical score
Control	106±5	53±2	282±19	0.04±0.01	0
Paracetamol 3 g/kg	3569±615*	2622±460*	353±27*	0.24±0.04*	2.9±0.1*
P. amarus extract					
0.8 g/kg	124±4	45±3	257±26	0.04±0.01	0
1.6 g/kg	107±4	56±3	288±36	0.03±0.01	0
3.2 g/kg	111±5	54±5	273±29	0.03±0.01	0
b.i.d. for 7 days					
P. amarus extract					
0.8 g/kg	3116±757	1796±333	380±31	0.22±0.02	2.4±0.2
1.6 g/kg	1660±489[a]	1239±351[a]	351±31	0.18±0.03	2.3±0.2[a]
3.2 g/kg	2111±810	1106±350[a]	328±21	0.19±0.05	2.5±0.2
b.i.d. for 7 days before paracetamol					
P. amarus extract					
0.8 g/kg	3092±929	2205±753	379±45	0.22±0.04	2.7±0.2
1.6 g/kg	2162±781	1390±383	443±42	0.25±0.06	2.2±0.4[a]
3.2 g/kg	2416±649	1546±493	349±16	0.23±0.05	2.4±0.2
b.i.d. for 2 days after paracetamol					
P. amarus extract					
1.6 g/kg	921±265[a]	994±334[a]	293±26	0.14±0.02[a]	2.3±0.3[a]
3.2 g/kg	1210±391[a]	1538±536	303±40	0.18±0.05	2.0±0.4[a]
b.i.d. for 7 days before and 2 days after paracetamol					

***significantly different from control group ($P < 0.05$)**
[a] significantly different from paracetamol group ($P < 0.05$)

Table 2. Effects of *P. amarus* aqueous extract and paracetamol on hepatic reduced glutathione in rats (mean±S.E.M., n = 6-9).

Treatment	Liver GSH	
	μmol/g	%
Control (50% sucrose)	7.20±0.25	100
Paracetamol (3g/kg) single oral dose	1.91±0.15*	26.49
P.amarus extract (3.2 g/kg) b.i.d.orally for 7 days	7.95±0.25*	110.26
P.amarus extract (3.2 g/kg) b.i.d.orally for 7 days before paracetamol (3 g/kg)	2.53±0.24[a]	35.09

***significantly different from control group ($P < 0.05$)**
[a] significantly different from paracetamol group ($P < 0.05$)

rats treated with paracetamol was partially prevented (2.53±0.3 μmol/g) by *P. amarus* pre-treatment.

5. DPPH radical scavenging activity

The aqueous extract of *P. amarus* exhibited a maximum DPPH radical scavenging activity of

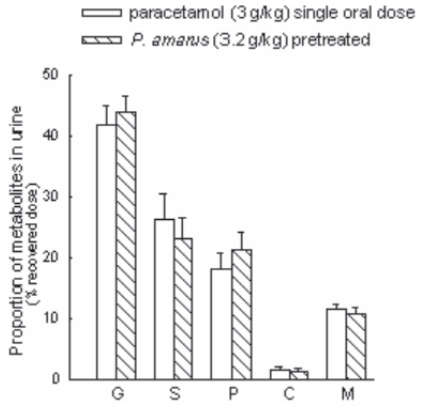

Figure 2. Effect of *P. amarus* extract on urinary paracetamol (P) and its metabolites (G = glucuronide, S = sulfate, C = cysteine, M = mercapturic acid conjugates) after a 3 g/kg dose of paracetamol in rats (mean±S.E.M., n =10).

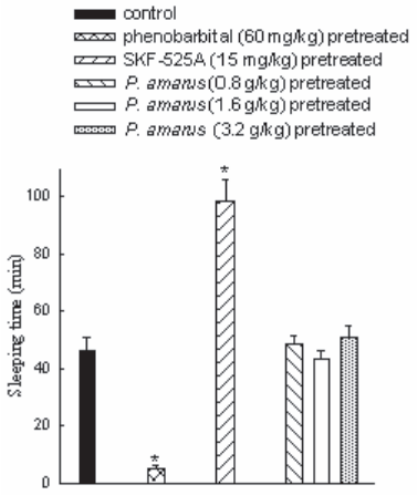

Figure 3. Effects of *P. amarus* extract, phenobarbital and SKF-525A pretreatment on pentobarbital-induced sleeping time in rats. (mean±S.E.M., n = 9-12)
***significantly different from control**

88% at the concentration of 150 µg/ml with the EC_{50} value of 45 µg/ml, which is approximately two times more than that of the standard BHT (EC_{50} = 20 µg/ml).

6. Iron chelating activity

Figure 4 shows that *P. amarus* aqueous extract has an absorption peak at 280 nm while $FeSO_4$ solution shows no peak. The interaction between *P. amarus* and Fe (II) was observed by an increase in the absorbances at 240, 310, 390 and 590 nm and the absence of the peak at 280 nm. When phenanthroline (an iron chelating agent) was added to the sample, the spectrum shifted back and was similar to that of *P. amarus* aqueous extract measured in the absence of iron.

7. Total phenolic compounds

The level of total phenolic compounds determined by the Folin-Ciocalteau reagent was 35.6±0.3 mg expressed as tannic acid equivalents per gram of *P. amarus* dried weight.

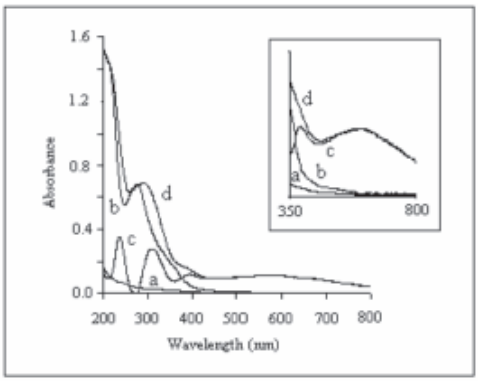

Figure 4. Absorption spectra of (a) $FeSO_4$ (1 mM) in deionized water (b) *P. amarus* aqueous extract (0.4 mg/ml) (c) *P. amarus* aqueous extract (0.4 mg/ml) after addition of $FeSO_4$ (1 mM) (d) *P. amarus* aqueous extract (0.4 mg/ml) after addition of $FeSO_4$ (1 mM) and phenanthroline (5 μM). (Inset shows details in the region 350-800 nm.)

Discussion

Liver injury induced by paracetamol is commonly used as a model for the screening of hepatoprotective drugs (Mitchell *et al*., 1973). Raised serum enzyme (SGOT, SGPT, ALP) levels in intoxicated rats can be attributed to the damaged structural integrity of the liver, because these enzymes are located in cytoplasm and are released into circulation after cellular damage (Sallie *et al*., 1991). The present study showed that *P. amarus* aqueous extract itself had no effect on the liver, whereas *P. amarus* aqueous extract pretreatment showed the potential to protect against paracetamol-induced hepatotoxicity in rats as judged from the decrease in SGOT, SGPT and serum bilirubin with a significant effect at the dose of 1.6 g/kg. The result was confirmed by histopathological study (Table 1). However, at the dose of 3.2 g/kg these parameters tended to be decreased although some were not significantly different from those of the paracetamol group, but the extent of decrease was not significantly different from those of 1.6 g/kg group. This might be because of the slight difference in standard deviation. Moreover, the activity was not dose-related which is unexplainable by this study. Further experiment with higher doses should be performed to reveal whether the response is dose-related.

The mechanism of cell damage appears to be mediated by the metabolic activation of paracetamol via cytochrome P450 (CYP) activity, especially CYP 2E1, to a highly reactive toxic metabolite (N-acetyl-*p*-benzoquinoneimine, NAPQI), which is normally conjugated with hepatocellular reduced glutathione (GSH) leading to depletion in GSH. Then NAPQI covalently binds with cellular proteins, including the Ca^{2+}-ATPase of the endoplasmic reticulum which is the Ca^{2+}-export system of the plasma membrane and mitochondrial proteins. Rises in intracellular free Ca^{2+} result from this damage and contribute to hepatocyte death (Halliwell and Gutteridge,1999). GSH, the predominant intracellular nonprotein sulfhydryl present in the cytosol, is a strong nucleophile able to react with electrophiles such as NAPQI and it is a reducing agent that contributes to the protection of cells against oxidative stress. The concentration of intracellular GSH, therefore, is a key determinant of the extent of paracetamol-induced hepatic injury. When GSH stores are below a critical level (about 30% of normal), they are no longer adequate to sustain detoxification of reactive metabolite. At this point, the disruption of cellular structure and function occurs (Dahm and Jones, 1996). After conjugation with GSH, the glutathione-derived conjugates (cysteine and mercapturic acid conjugates) are then excreted in urine in proportion to

the toxic metabolite formed. However, sulfation and glucuronidation are the major pathways of paracetamol metabolism. (Miners *et al*., 1984).

Our study on paracetamol metabolism showed that the urine proportions of cysteine and mercapturic acid conjugates in rats pretreated for 7 days with *P. amarus* aqueous extract were not different from those of the control group. These findings suggest that *P. amarus* extract did not inhibit CYP activity, which is responsible for paracetamol metabolism to produce toxic metabolite (Halliwell and Gutteridge,1999). Moreover, *P. amarus* extract did not induce paracetamol sulfation or glucuronide conjugation since the sulfate and glucuronide conjugates were present to the same extent in both control and treated group (Figure 2). This result was confirmed by the finding that *P. amarus* extract did not prolong pentobarbital-sleeping time, while phenobarbital (a CYP inducer) shortened and SKF-525A (a CYP inhibitor) prolonged the sleeping time (Figure 3). Since pentobarbital is metabolized by CYP, any drugs with an inhibitory effect on CYP are expected to prolong pentobarbital sleeping time (Fugimoto *et al*., 1960). Therefore, it is revealed that *P. amarus* aqueous extract is devoid of any inhibitory effect on CYP. These results suggest that the hepatoprotective effect of *P. amarus* aqueous extract was not related to the effect on the metabolic pathway of paracetamol, but mediated perhaps through other mechanism(s).

The administration of paracetamol to rats caused a severe depletion of hepatic GSH which can be attributed to GSH consumption by glutathione transferase for metabolite conjugation and to GSH oxidation for the defence against the produced oxidative stress (Halliwell and Gutteridge,1999). Our results (Table 2) showed that *P. amarus* extract itself caused a slight but significant increase in GSH. As well as in rats pretreated with *P. amarus*, the GSH level in paracetamol intoxication at 6 hour post-dosing was raised to above the critical level (35.09% of normal), while those of the paracetamol alone group was below the critical level (26.49% of normal). This activity might be one of the hepatoprotective mechanisms of *P. amarus* aqueous extract.

Apart from the primary mechanism of paracetamol toxicity, it appears that toxic oxygen species (i.e. superoxide anion radical, hydrogen peroxide, hydroxyl radical) may play a role in the hepatocellular toxicity caused by paracetamol activation to NAPQI (Dahm and Jones,1996). Our study showed that *P. amarus* aqueous extract exhibited DPPH radical scavenging activity with the EC_{50} of 45 µg/ml, which is about two times less than standard BHT (EC_{50} = 20 µg/ml). While ascorbic acid, which has been reported to possess hepatoprotective effect probably by scavenging the reactive intermediates generated by the microsomal mixed-function oxidase enzymes, showed the DPPH radical scavenging activity with the EC_{50} of 59 µg/ml (Yuan *et al*., 2005; Lake *et al*., 1981). In addition, our spectroscopic studies revealed that the yellowish *P. amarus* aqueous extract interacts with colorless $FeSO_4$ solution resulting in a blue-black solution with a change in the UV spectrum (Figure 4), and the shifting of the spectrum after addition of phenanthroline which is an iron chelator suggested that *P. amarus* aqueous extract possesses iron chelating activity. This activity might be responsible for prevention of catalytic metal ion (such as iron, copper) involved in the Fenton-type reaction which can generate highly reactive hydroxyl radicals. Moreover, a high total phenolic content (35.6 mg/g) of *P. amarus* was also exhibited in this study. Several active compounds in *P. amarus* have been identified, such as lignans (phyllanthin and hypophyllanthin), flavonoids (quercetin and astragarin), ellagitannins (amarinic acid) and hydrolysable tannins (phyllanthisiin D and amarin) (Rajeshkumar and Kuttan, 2000). These compounds which contain polyphenol groups might be responsible for the antioxidant activity of *P. amarus* aqueous extract.

Conclusion

Based on the above data, it is concluded that *P. amarus* aqueous extract exhibited the hepatoprotective activity against paracetamol in-

toxication. The hepatoprotective mechanism was related to neither the inhibition of cytochrome P450 nor the induction of sulfate and/or glucuronide conjugation, but partly due to the protective effect on GSH depletion accompanying with its antioxidant activity, especially the radical scavenging and iron chelating activity which were related to the high polyphenolic contents. However, other possible mechanisms involved in hepatoprotective effect such as induction of glutathione linked detoxification system (glutathione-s-transferase, glutathione peroxidase, glutathione reductase) and other antioxidant enzymes (superoxide dismutase, catalase) should be further investigated. These results support the value of *P. amarus* which has been used in Thai folk medicine for the treatment of liver disease.

Acknowledgements

This research was supported by grants from The Ministry of Public Health and Prince of Songkla University, Thailand. We are grateful to Assistant Professor Choathip Purintavoragul, Department of Biology, Faculty of Science, Prince of Songkla University for the identification of the plant.

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