# CHAPTER IV
# ISOLATION OF ANTI-HEPATOTOXIC COMPOUND, PHYLLANTHIN, FROM THE WHOLE PLANT *OF PHYLLANTHUS AMARUS*

The whole plant of *Phyllanthus amarus* has been widely used in Thai traditional medicine to treat liver diseases. Hepatoprotective properties of the extract of *P. amarus* were demonstrated using animal models for paracetamol- (Wongnawa, *et al.* 2005), $CCl_4$- (Yadav, *et al.* 2008), and ethanol-induced liver toxicity (Pramyothin, *et al.* 2007). Numerous studies have reported the hepatoprotective effect of phyllanthin, which is a major *P. amarus* bioactive lignan. Previous reports have indicated that phyllanthin exerts a hepatoprotective effect, through antioxidant activity, against ethanol-induced liver disease in a primary rat hepatocyte culture (Chirdchupunseree and Pramyothin 2010). Phyllanthin showed significant protection against $CCl_4$-induced hepatic damage by enhancing antioxidant activity (Krithika, *et al.* 2011). Thus, the present study was initiated to ascertain the anti-hepatotoxic activity of phyllanthin against this well characterized hepatotoxin in human hepatoma HepG2 cell line.

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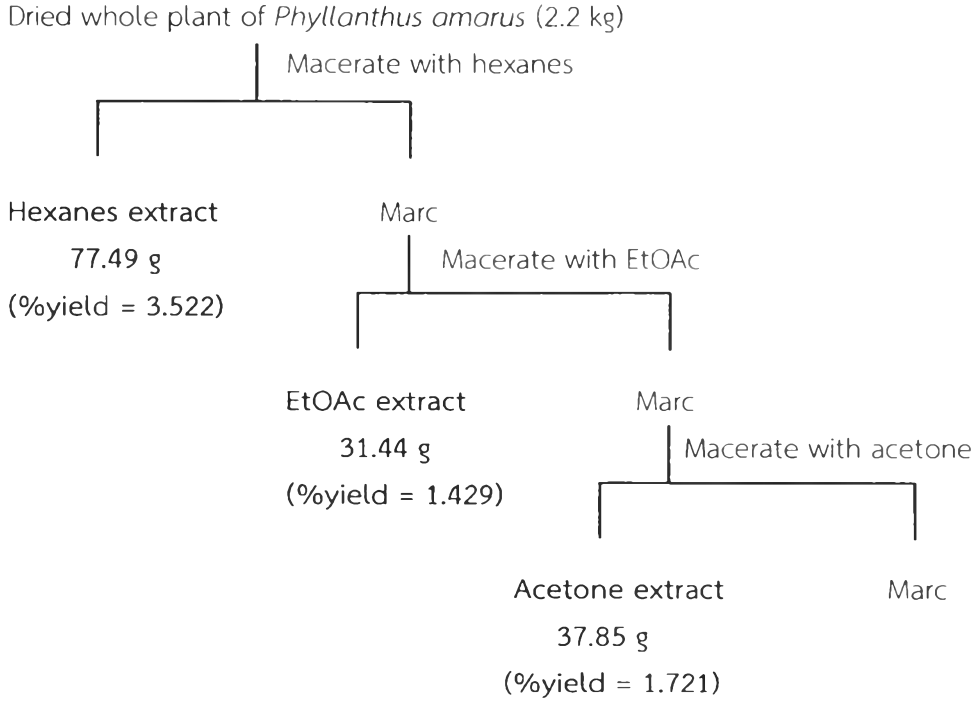
## 4.1 Materials and Methods

### 4.1.1 Plant materials

The whole plant of *P. amarus* used for the isolation were collected from Nakhon Sawan province of Thailand and authenticated by Assoc. Prof. Dr. Chaiyo Chaichantipyuth, the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. All organic solvents used throughout the investigation were commercial grade and were redistilled prior to use.

### 4.1.2 Sample preparation

The plant materials (2.2 kg) were shade dried, ground into powders, and extracted with hexanes, ethyl acetate (EtOAc), and acetone, respectively (3×15L). The crude extract was macerated at room temperature for 72 hr. Subsequently, the extract was filtered through Whatman No. 1 filter paper and evaporated to dryness by rotary evaporation under reduced pressure at 40°C, as presented in **Scheme 2**.




Scheme 2 Extraction of the whole part from *P. amarus*

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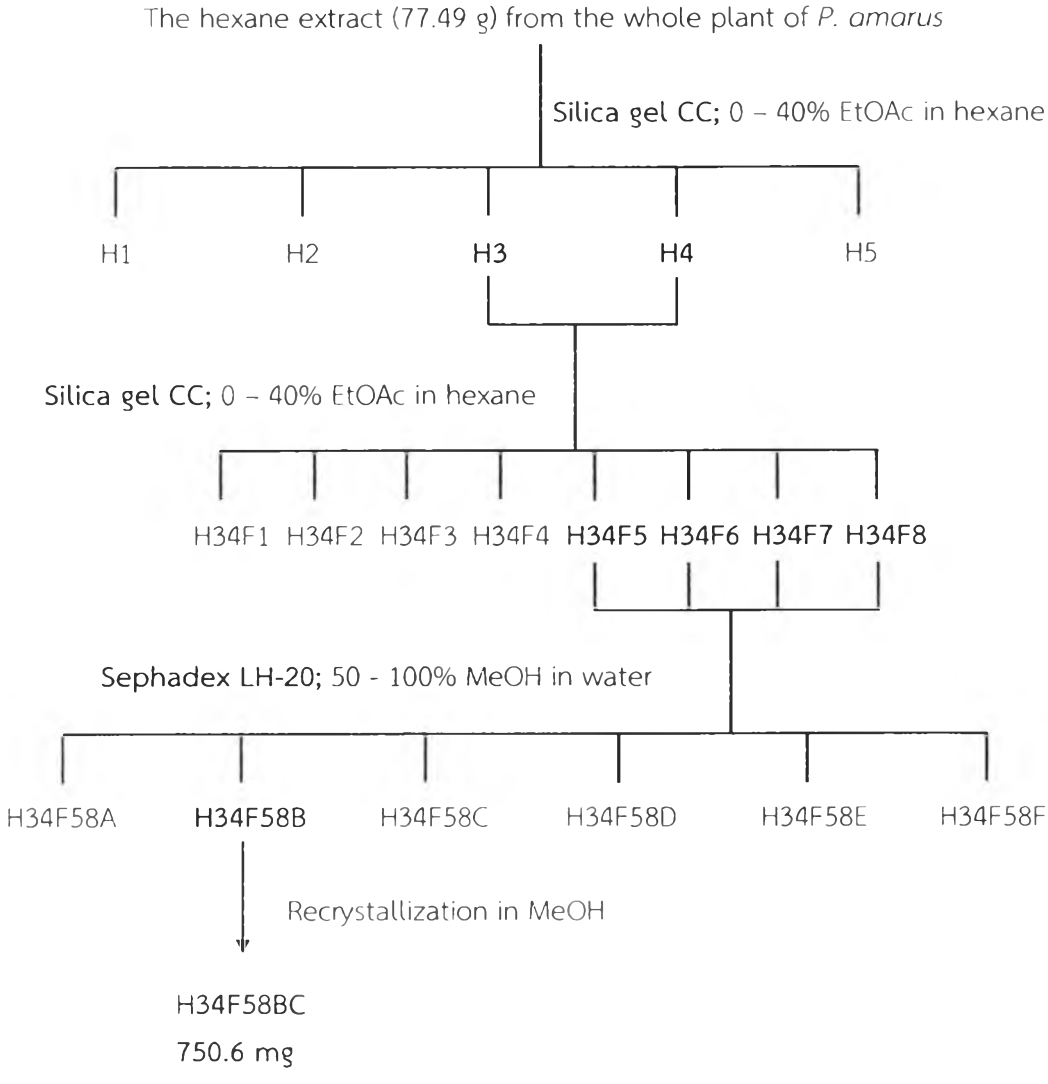
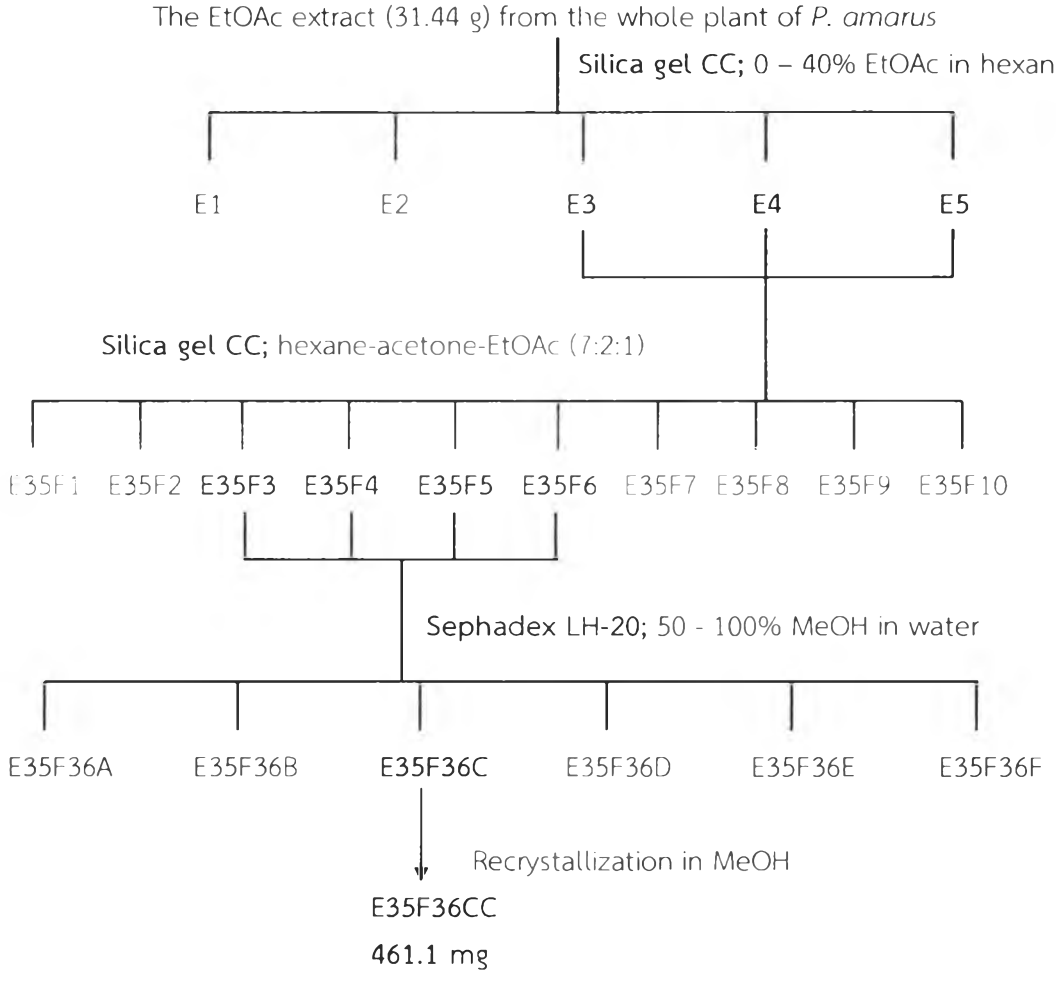
### 4.1.3 Extraction and isolation of phyllanthin

The hexane extract (77.49 g) was re-dissolved in a small volume of hexane and then applied to silica gel column chromatography. The column was eluted with 0 - 40% EtOAc in hexane. Fifty fractions were collected and combined according to their TLC patterns using hexane-EtOAc as mobile phases into 5 fractions (H1 – H5). Subfraction H3 – H4 were combined and re-chromatographed on a column of silica gel was eluted with 0 - 40% EtOAc in hexane. The forty fractions collected were combined to give 8 fractions (H34F1 – H34F8). Subfraction H34F5 – H5F8 were combined, separated on sephadex LH-20, and eluted with 50 - 100% MeOH in water (Murugaiyah and Chan 2007) to give thirty-five fractions. These fractions were combined into 6 fractions (H34F58A – H34F58F). Subfractions H34F58B was purified by recrystallization in MeOH to give compound H34F58BC (750.6 mg) as a white needle and was identified as phyllanthin as presented in **Scheme 3**.




**Scheme 3** Isolation of phyllanthin from the hexane extract.

The EtOAc extract (31.44 g) was re-dissolved in a small volume of EtOAc and then separated on silica gel and eluted with 0 – 40% EtOAc in hexane. Forty-seven fractions were collected. According to their chemical profile by TLC using EtOAc-hexane (3:2, v/v) as the mobile phase, these fractions were combined into 5 fractions (E1 – E5). Subfractions E3 – E5 were combined, purified on silica gel, and eluted with hexane-acetone-EtOAc (7:2:1, v/v/v). The fifty subfractions were combined into 10 fractions (E35F1 – E35F10). Subfractions E35F3 – E35F6 were combined, separated on Sephadex LH-20 with 50 - 100% MeOH in water, and then subfraction E35F36C was purified by recrystallization in MeOH. The compound E35F36CC (461.1 mg) was identified as **phyllanthin**, presented in **Scheme 4**.




**Scheme 4** Isolation of phyllanthin from the EtOAc extract.

### 4.1.4 Cells and Reagents

Human hepatoma (HepG2) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) containing 10% Fetal Bovine Serum (FBS, Hyclone, UK), 1% L-glutamine (Gibco®, Grand Island, NY, USA), and 1% antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin, Gibco®, Grand Island, NY, USA). HepG2 cells were incubated in a humidified atmosphere containing 5% $CO_2$ in air at 37 °C.

To prepare 1000 mL of Phosphate Buffer Saline (PBS), the ingredients of PBS solution including 8 g of NaCl, 0.2 g of KCl, 1.15 g of $Na_2HPO_4$, and 0.2 g of $KH_2PO_4$ were dissolved in ultrapure water and adjusted the pH 7.2 to 7.4 with NaOH. The solution was adjusted the volume to 1000 mL and then sterilized by autoclave.

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Silymarin, hepatoprotective drug, was purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). MTT (3-[4, 5-di-methyl-thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) was obtained from molecular probes® (life technologies™, Oregon, USA).

### 4.1.5 Cell viability

Cell viability was determined using MTT assay. MTT assay is based on the ability of viable cells to reduce MTT from yellow water-soluble dye to dark blue insoluble formazan crystal. In brief, HepG2 cells ($1\times10^5$ cells/mL) were cultured in 96-well plate with or without various concentrations of phyllanthin (1, 5, 10, 100, 1000, and 3000 µM) for 24 h. After 24 h incubation, medium was removed and 100 µL of MTT (0.4 mg/mL) in fresh medium was added to each well and incubated at 37°C for further 2 – 4 h. Subsequently 100 µL of DMSO (dimethylsulfoxide) was added to dissolve formazan crystals and the absorbance was measured at 570 nm using the microplate reader (Coming Incorporated, NY, USA). The percentage of cell viability was calculated.

### 4.1.6 Anti-hepatotoxic activity assay

#### 4.1.6.1 Pretreatment

In order to determine the hepatoprotective effect of phyllanthin, phyllanthin was dissolved in DMEM medium and serially diluted to achieve concentrations of 1, 5, and 10 µM. HepG2 cells were placed in a 96-well plate ($1\times10^5$ cells/mL) for 24 h at 37°C and 5% $CO_2$. They were then administered with different agents for 24 h and after that treated with ethanol (EtOH) 10 and 100 mM for 6, 12, and 24 h, respectively to induce hepatotoxicity.

**Group 1** – Control without treatment

**Group 2 - 3** –EtOH 10 and 100 mM

**Group 4 - 6** – Phyllanthin (PH) at 1, 5, and 10 µM + EtOH 10 mM

**Group 7 - 9** – Phyllanthin (PH) at 1, 5, and 10 µM + EtOH 100 mM

**Group 10 - 12** – Silymarin (SM) at 1, 5, and 10 µM + EtOH 10 mM

**Group 13 - 15** – Silymarin (SM) at 1, 5, and 10 µM + EtOH 100 mM

#### 4.1.6.2 Post-treatment

For recovery assay, HepG2 cells were placed in a 96-well plate ($1\times10^5$ cells/mL) for 24 h at 37°C and 5% $CO_2$. They were treated with EtOH 10 and 100 mM for 6, 12, and 24 h, respectively and then post-treatment with different agents for 24 h.

**Group 1** – Control without treatment

**Group 2 - 3** –EtOH 10 and 100 mM

**Group 4 - 6** –EtOH 10 mM + phyllanthin (PH) at 1, 5, and 10 µM

**Group 7 – 9** – EtOH 100 mM + phyllanthin (PH) at 1, 5, and 10 µM

**Group 10 - 12** – EtOH 10 mM + silymarin (SM) at 1, 5, and 10 µM

**Group 13 - 15** – EtOH 100 mM + silymarin (SM) at 1, 5, and 10 µM

### 4.1.7 Statistical analysis

All data were expressed as the mean±SEM. Results were statistically analyzed by the one-way analysis of variance (ANOVA) followed by Tukey test, using the Graph Pad/Instat program; values with $p<0.05$ were considered statistically significant.

## 4.2 Results and discussion

### 4.2.1 Extraction, isolation, purification, and identification of phyllanthin

The yield of the hexane, EtOAc, and acetone extracts from the whole plant of *P. amarus* were 3.522%, 1.429%, and 1.721% w/w, respectively. Compound H34F58BC and compound E35F36CC (1.2117 g, %yield = 1.1124) were isolated from the hexane and EtOAc extracts as white needles, appeared as an orange spot on TLC upon spraying with Dragendorff's reagent, and heated. This false-positive reaction with Dragendorff's spray reagent has been reported for a variety of non-nitrogenous oxygenated compounds possessing proteins, some coumarins, lignans, ketone or lactone functions (Finkelstein 1978, Habib 1980).

The $^{13}$C-NMR spectrum (**Figure A7**) exhibited the signal of a lignan, including two benzene ring at δ 148.7 ppm (C-3, C-3'), 147.1 ppm (C-4, C-4'), 133.7 ppm (C-1, C-1'), 121.1 ppm (C-6, C-6'), 112.2 ppm (C-2, C-2'), 111.0 ppm (C-5, C-5') ,six methoxyls at δ 58.6 ppm (9 (9')-$OCH_3$), 55.9 ppm (3 (3')-$OCH_3$), 55.8 ppm (4 (4')-$OCH_3$), four methylenes at δ 72.6 ppm (C-9, C-9'), 35.0 ppm (C-7, C-7'), and two olefinic methines at δ 40.8 ppm (C-8, C-8').

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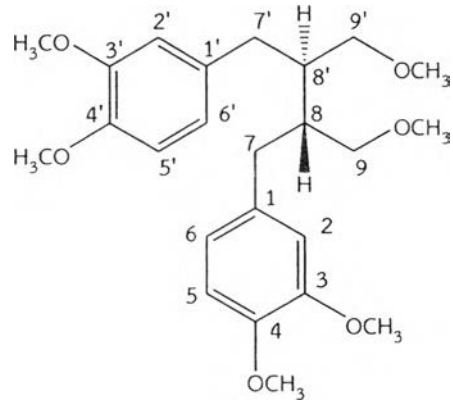
According to the $^{1}$H-NMR spectrum of this compound (**Figure A8**) showed 3 methoxyl singlets at δ 3.28 ppm (9 (9')-$OCH_3$), 3.80 ppm (3 (3')-$OCH_3$), and 3.85 ppm (4 (4')-$OCH_3$), the aromatic region displayed 2 doublets at δ 6.65 ppm (2H, $J$ = 1.8 Hz, H-2, H-2'), 6.75 ppm (2H, $J$ = 8.1 Hz, H-5, H-5'), and 1 doublet of doublet at δ 6.62 ppm (2H, $J$ = 8.1, 1.8 Hz, H-6, and H-6'), showed 2 methylene multiplet at δ 3.29 ppm, 3.27 ppm (4H, H-9, H-9'), 2.65 ppm, and 2.60 ppm (4H, H-7, H-7'), and methine displayed 1 multiplet at δ 2.01 ppm (2H, H-8, H-8').

From GC-MS data, its molecular formula was determined as $C_{24}H_{34}O_6$ from its molecular ion peak at m/z 418.4 in the mass spectrum (**Figure A9**). Consequently, comparison of the $^{1}$H and $^{13}$C-NMR data of compound H34F58BC with previous reported (Somanabandhu, *et al.* 1993) (**Table 6**) led to the identification of this compound as phyllanthin, which is one of the most common lignans. This compound is widely distributed in members of the genus *Phyllanthus* such as *P. urinaria* (Fang, *et al.* 2008), *P. niruri* (Murugaiyah and Chan 2007), and *P. simplex* (Chouhan and Singh 2011). It was also found as the major lignans constituent of *P. amarus*.



Phyllanthin

Table 6 $^{1}H$ and $^{13}C$-NMR assignments of compound H34F58BC and phyllanthin.

| Position | H34F58BC | | Phyllanthin* | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 (1') | - | 133.7 | - | 133.6 |
| 2 (2') | 6.65 (*d*, *J* = 1.8 Hz) | 112.2 | 6.59 (*d*, *J* = 1.8 Hz) | 112.2 |
| 3 (3') | - | 148.7 | - | 148.7 |
| 4 (4') | - | 147.1 | - | 147.1 |
| 5 (5') | 6.75 (*d*, *J* = 8.1 Hz) | 111.0 | 6.73 (*d*, *J* = 8.1 Hz) | 111.0 |
| 6 (6') | 6.62 (*dd*, *J* = 8.1, 1.8 Hz) | 121.1 | 6.61 (*dd*, *J* = 8.1, 1.8 Hz) | 121.0 |
| 7 (7') | 2.60 (*m*)<br>2.65 (*m*) | 35.0 | 2.59 (*dd*, *J* = 13.8, 7.3 Hz)<br>2.66 (*dd*, *J* = 13.8, 7.5 Hz) | 34.9 |
| 8 (8') | 2.01 (*m*) | 40.8 | 2.01 (*m*) | 40.7 |
| 9 (9') | 3.26 (*dd*, *J* = 13.8, 7.8 Hz)<br>3.29 (*dd*, *J* = 13.8, 5.2 Hz) | 72.6 | 3.25 (*dd*, *J* = 13.8, 7.8 Hz)<br>3.28 (*dd*, *J* = 13.8, 5.4 Hz) | 72.8 |
| 3 (3')-$OCH_3$ | 3.80 (*s*) | 55.9 | 3.78 (*s*) | 55.9 |
| 4 (4')-$OCH_3$ | 3.85 (*s*) | 55.8 | 3.82 (*s*) | 55.7 |
| 9 (9')-$OCH_3$ | 3.27 (*s*) | 58.6 | 3.27 (*s*) | 58.7 |

Chemical shift values are reported as δ (ppm) at 300 MHz for $^{1}H$ and 75 MHz for $^{13}C$ in $CDCl_3$. *(Somanabandhu, *et al.* 1993)

### 4.2.2 Preliminary concentration – dependent experiment on cell viability

The viability of HepG2 cells exposed to phyllanthin at the concentrations of 1, 5, 10, 50, 100, 1000, and 3000 μM, respectively for 24 h was examined using a MTT assay. Phyllanthin produced toxicity in HepG2 cells in a dose-dependent manner (Figure 29). The concentrations of phyllanthin at 1, 5, and 10 μM respectively were chosen non – cytotoxic for subsequent interaction experiments.

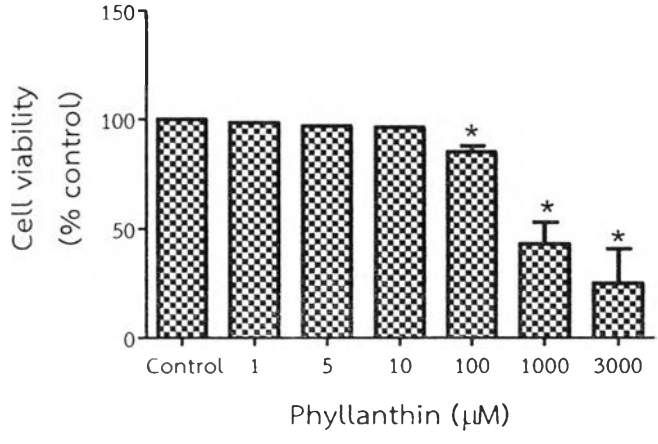



Figure 29 Phyllanthin induced cytotoxicity in HepG2 cells as measured by MTT assay. HepG2 cells were treated with different concentrations of phyllanthin for 24 h. Results are expressed as percentage of control and are mean±SD, (n = 3).

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### 4.2.3 Antihepatotoxic activity of phyllanthin in HepG2 cells

Antihepatotoxic activity of phyllanthin in HepG2 cell line was demonstrated by inducing ethanol toxicity and ascertained by MTT (3-[4, 5-di-methyl-thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay. The present study was designed to ascertain the antihepatotoxic activity of phyllanthin, which was isolated from *P. amarus*, against this well characterized hepatotoxin in human hepatoma (HepG2) cell line. Silymarin, a hepatoprotective drug, was used as the positive control.

#### 4.2.3.1 Phyllanthin protect HepG2 cells from ethanol-induced toxicity

In order to investigate the hepatoprotective effect of phyllanthin on ethanol-induced toxicity in HepG2 cells, were pretreated with various concentrations of phyllanthin (1, 5, and 10 μM) or silymarin for 24 h. After 24 h, HepG2 cells were treated with 10 mM and 100 mM of ethanol for various time periods (6, 12, and 24 h). After finish time periods, cell viability was analyzed by MTT assay.

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From the results, both 10 and 100 mM of ethanol significantly decreased cell viability on HepG2 cells in time-dependent. Phyllanthin at 1, 5, and 10 μM were able to prevent the loss of cell viability induced by ethanol. In time period experiments, the protective effect of phyllanthin was observed only when the ethanol was applied to cells for 12 h (**Figure 30a – 30b**).

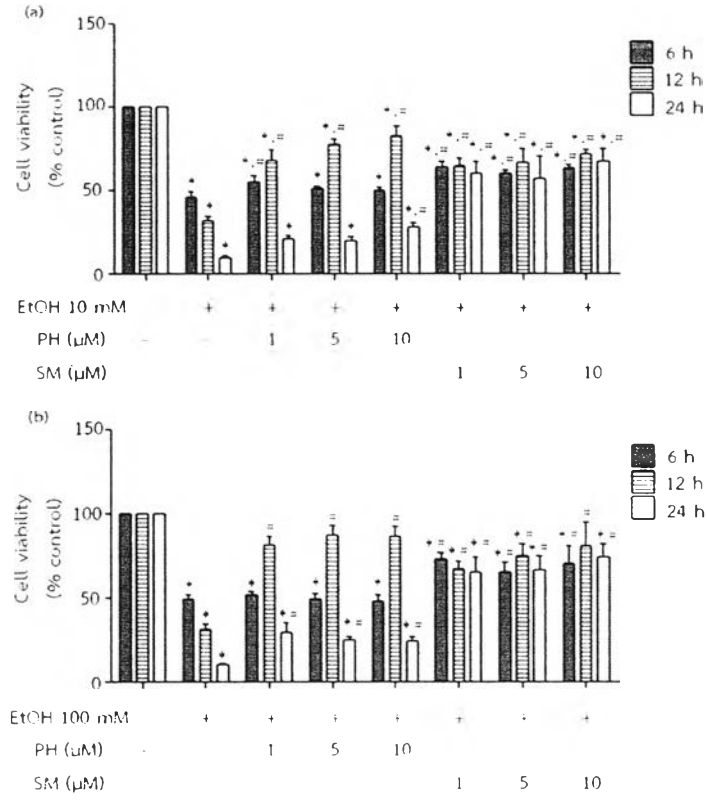


**Figure 30** Hepatoprotective effect of pretreatment with phyllanthin (PH) on HepG2 cells induced by ethanol (EtOH) 10 mM (a) and 100 (b) after 6, 12, and 24 hours, respectively. The experiments were carried out at least three times in triplicate. Each column represents the mean ± SEM. *Significant difference with control ($p<0.05$) and #Significant difference with ethanol, respectively, as determined by ANOVA.

The normal HepG2 cells were disturbed in the control group without treatment. In the ethanol groups, 10 mM (**Figure 31**) and 100 mM (**Figure 32**), showed cell damage as a results of toxicity on HepG2 cells. Meanwhile, pretreatment with phyllanthin at 1, 5, and 10 μM for 24 h increased cell viability when treated with ethanol at 12 h. It can be compared with that of silymarin group, the hepatoprotective drug.

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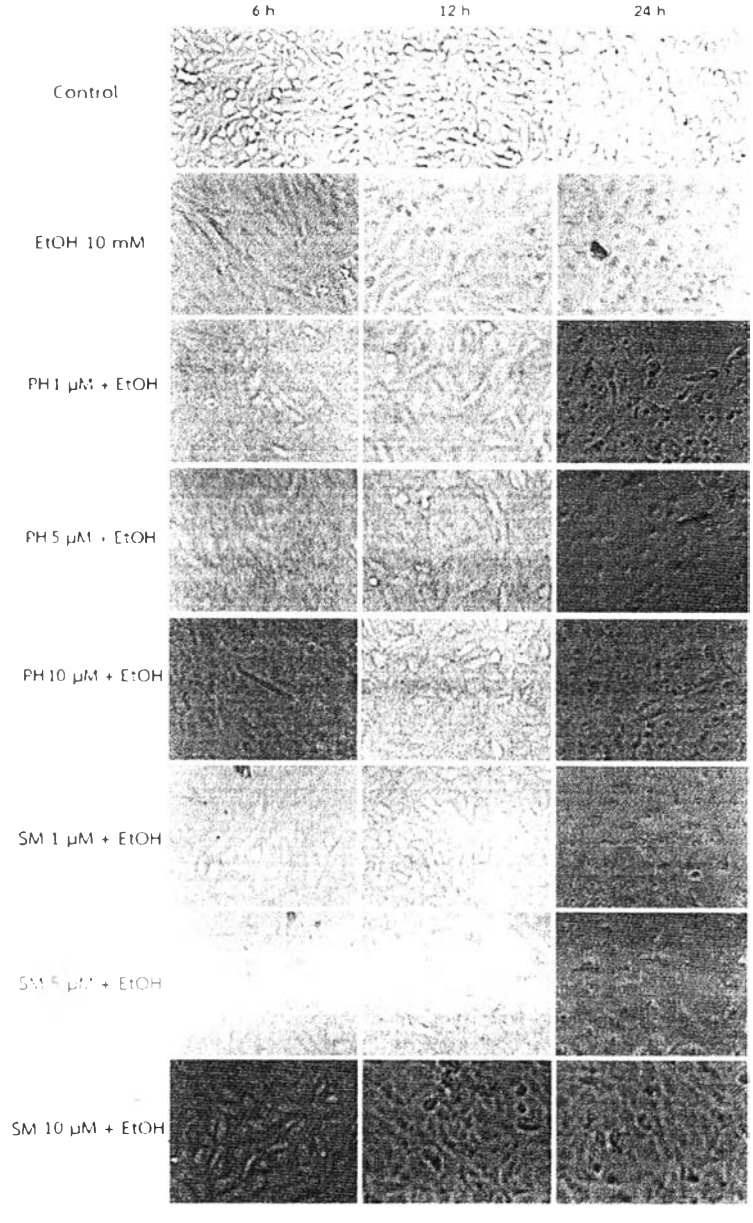


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**Figure 31** Hepatoprotective effect of phyllanthin (PH) at various concentrations (1, 5, and 10 µM) pretreatment for 24 h before induced liver damage by ethanol (EtOH) 10 mM at 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

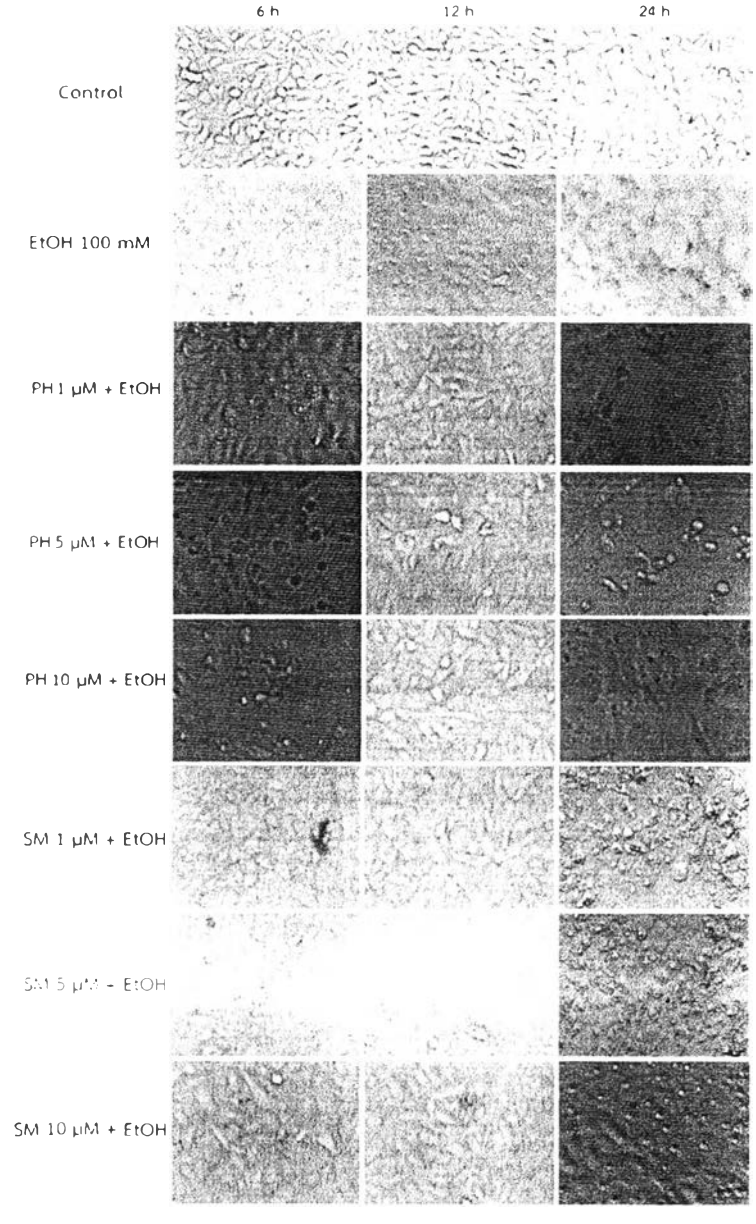


**Figure 32** Hepatoprotective effect of phyllanthin (PH) at various concentrations (1, 5, and 10 µM) pretreatment for 24 h before induced liver damage by ethanol (EtOH) 100 mM at 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

In hepatoprotective study demonstrated that phyllanthin, a purified lignan compound from the whole plants of *P. amarus*, showed potential protection against ethanol-induced toxicity in HepG2 cells.

### 4.2.3.2 Effect of phyllanthin post-treatment in HepG2 cells

In order to investigate the effect of phyllanthin post-treatment on ethanol-induced toxicity in HepG2 cells, were treated with 10 mM and 100 mM of ethanol for various time periods (6, 12, and 24 h) and received with various concentrations of phyllanthin or silymarin (1, 5, and 10 μM) for 24 h. After 24 h, cell viability was analyzed by MTT assay. Ethanol 10 mM and 100 mM were decreased %cell viability on HepG2 cells. From the results, the received phyllanthin at 1, 5, and 10 μM groups after treated with ethanol 10 mM and 100 mM for 12 h showed significant increase %cell viability higher than treated with ethanol for 6 and 24 h. For 24 h, HepG2 cells were treated with phyllanthin and ethanol groups, it is possible to increase damage to the cells (**Figure 33a – 33b**).

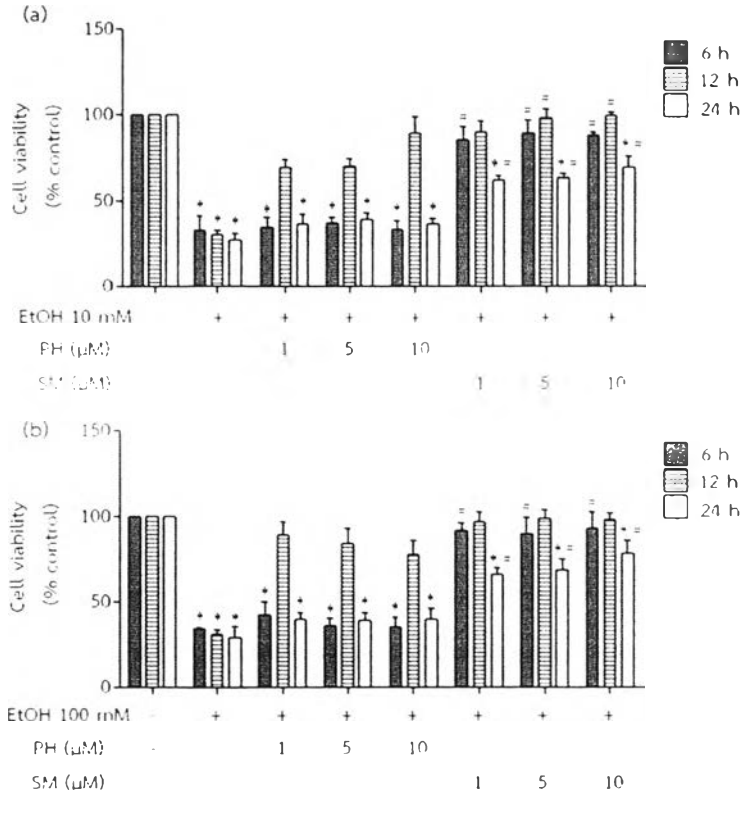


**Figure 33** Effect of post-treatment with phyllanthin (PH) for 24 h on HepG2 cell lines induced by ethanol (EtOH) 10 mM (a) and 100 mM (b) at 6, 12, and 24 hours. The experiments were carried out at least three times in triplicate. Each column represents the mean ± SEM. *Significant difference with control ($p<0.05$) and #Significant difference with ethanol, respectively, as determined by ANOVA.

The normal HepG2 cells were disturbed in the control group. In the ethanol groups, 10 mM (**Figure 34**) and 100 mM (**Figure 35**), were showed cell damage as a results of toxicity on HepG2 cells. Meanwhile, post-treatment with phyllanthin at 1, 5, and 10 μM after induced toxicity by ethanol for 12 h that were increased cell viability.

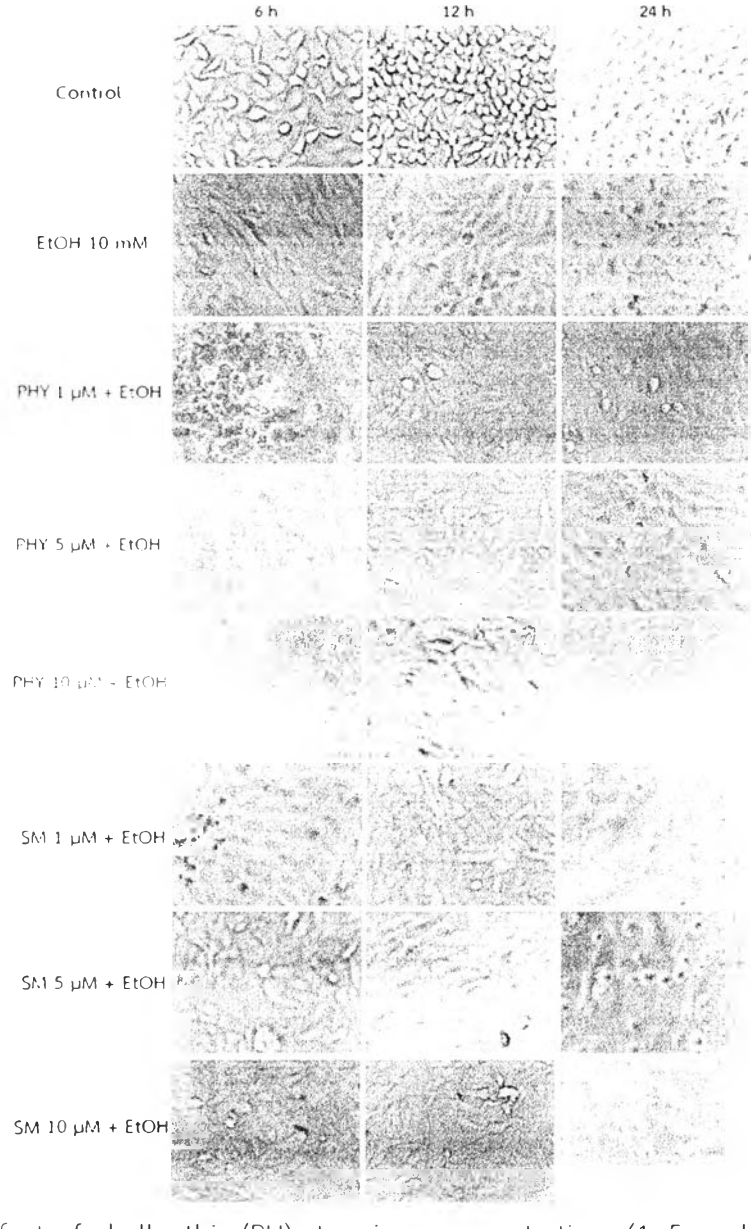


**Figure 34** Effect of phyllanthin (PH) at various concentrations (1, 5, and 10 μM) post-treatment after ethanol (EtOH) 10 mM induced liver damage for 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

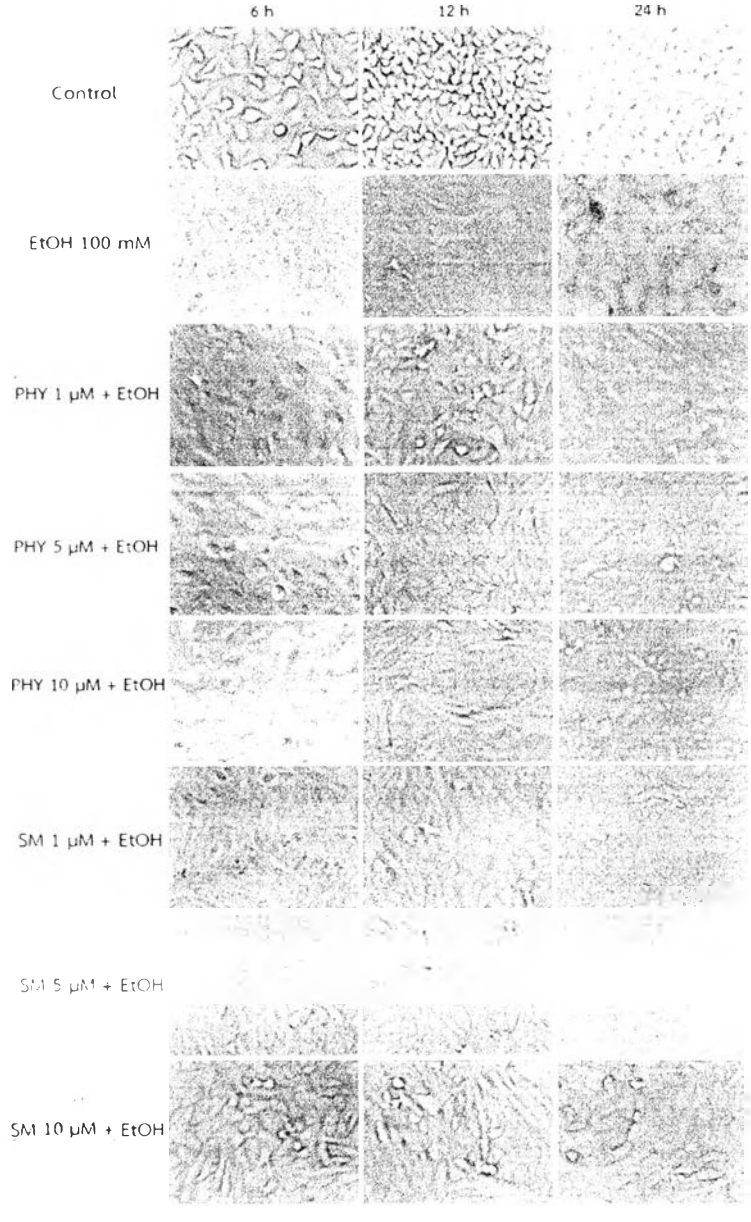


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**Figure 35** Effect of phyllanthin (PH) at various concentrations (1, 5, and 10 µM) post-treatment after ethanol (EtOH) 100 mM induced liver damage for 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

## 4.3 Conclusion

Phyllanthin was a major lignan constituent of *P. amarus*. The whole plant of *P. amarus* was used for the treatment of many liver diseases in folk medicine. From *in vitro* experiments, these results suggested the anti-hepatotoxic activity of *P. amarus* by hepatoprotective effect (pretreatment) and post-treatment against ethanol-induced toxicity causing human hepatoma HepG2 cells damage. This important information of the pharmacological activity of *P. amarus* for treatment and/or prevent the toxic effect of ethanol on liver.