CHAPTER III
ISOLATION OF ANTI-HEPATOTOXIC COMPOUND, ROSMARINIC ACID, FROM *THUNBERGIA LAURIFOLIA* LEAVES

Thunbergia laurifolia leaves are widely used as antipyretic and antidote agent in Thai traditional medicine. Recently, *T. laurifolia* leaves extracts showed potential hepatoprotective activity in ethanol-induced liver injury in male Wistar rats and in primary cultures of rat hepatocytes (Pramyothin, *et al.* 2005), decreased serum alanine aminotransferase (ALT), and decreased liver cell damage (Wonkchalee, *et al.* 2012). Previously studies have reported the hepatoprotective effect of rosmarinic acid, which is the major phenolic compound of *T. laurifolia* leaves. Rosmarinic acid showed protective potential against oxidative damage induced by *tert*-butylhydroperoxide (*t*-BHP) in HepG2 cells (Lima, *et al.* 2006). Furthermore, rosmarinic acid significantly decreased hepatic histopathological changes and ALT levels in CCl_4-intoxicated mice (Domitrovic, *et al.* 2013). Thus, the present study aims to investigate the anti-hepatotoxic substance, rosmarinic acid, from *T. laurifolia* on HepG2 cell line against ethanol induced toxicity.

3.1 Materials and Methods

3.1.1 Plant materials

The leaves of *T. laurifolia* used for the isolation were collected from Nakhon Sawan province of Thailand and were authenticated by Assoc. Prof. Dr. Chaiyo Chaichantipyuth from the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.1.2 Sample preparation

T. laurifolia plant materials (1 kg) were shade dried, ground into powders, and then macerated with ethanol at room temperature for 72 h. Subsequently, the ethanolic extract was filtered through Whatman No. 1 filter paper and evaporated to dryness by rotary evaporation under reduced pressure at 45°C.

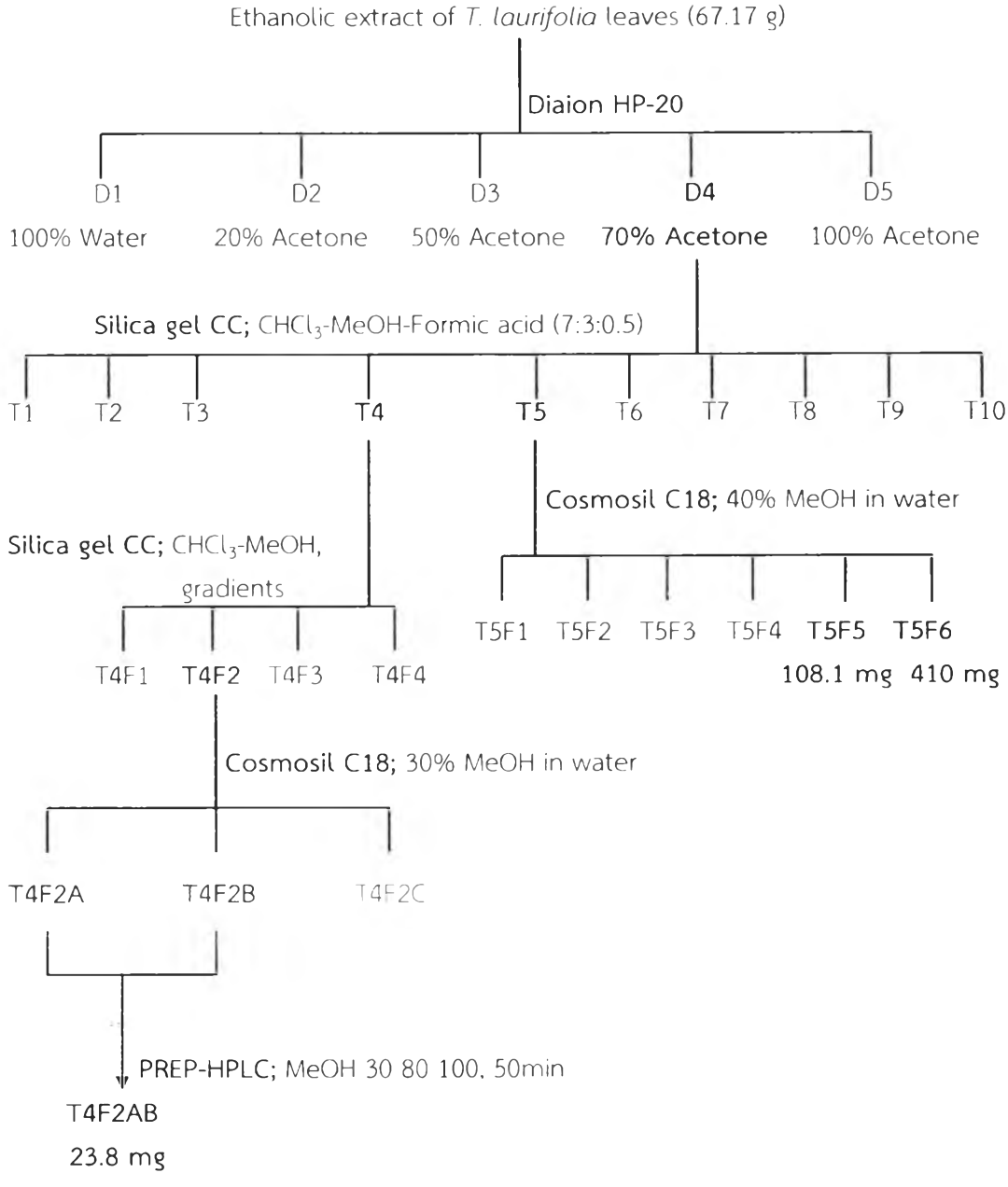
Throughout this work, all organic solvents used in the extraction and isolation procedure were of commercial grade and were redistilled prior to use. For HPLC method, all solvents were of HPLC grade.

3.1.3 Isolation of compounds from the ethanolic extract of *T. laurifolia*

The ethanolic extract (67.17 g) was dissolved in a small volume of water and then applied to an ion-exchange resin column using diaion HP-20. The column was eluted with a gradient mixture of water and acetone. Five fractions (D1 – D5, 500 mL each) were collected. Fraction D4 (70% acetone in water) was further separated on a silica gel column using $CHCl_3$-MeOH-formic acid (7:3:0.5 v/v/v) as the mobile phase to give ten fractions (T1 – T10). Fraction T4 (208.1 mg) was fractionated on a silica gel column eluted with $CHCl_3$ in MeOH gradient and then combined to yield 4 subfractions (T4F1 – T4F4). Subfraction T4F2 (45.4 mg) was purified on a cosmosil C18 (Nacalai Tesque Inc., Kyoto, Japan) column eluting with 30% MeOH in water. Sixty fractions (12 mL each) were collected and combined to give 3 subfractions (T4F2A – T4F2C). From HPLC chromatogram, subfraction T4F2A (34.4 mg) and T4F2B (11 mg) were combined and then were purified by PREP-HPLC PLC2020 (Gilson Inc., Middleton, USA); UV detection at an absorbance of 210 nm, Alantis®PrepT3; 5 µm 19×250 mm column (Waters company, USA), 5 mL injection volume, flow rate: 14 mL/min/tube, and gradient 0 – 100% MeOH from 0 – 50 min. Compound **T4F2AB** (23.8 mg) was obtained as white needles.

Fraction T5 (1.2225 g) was separated on a cosmosil C18 column using 40% MeOH in water as the mobile phase to give six combined fractions (T5F1 – T5F6). Subfraction **T5F5** (108.1 mg) and **T5F6** (410 mg) were obtained as white amorphous powder. Compound T5F6 spiked with standard rosmarinic acid was analyzed by HPLC (Agilent Technologies, USA); UV detection at an absorbance of 210 nm, Alantis®T3; 5 µm 6×250 mm column (Waters company, USA), 5 µL injection volume, flow rate: 1 mL/min, and gradient 0 – 100% MeOH from 0 – 50 min.

The isolation of compounds from the ethanolic extract of *T. laurifolia* is presented in **Scheme 1.**



Scheme 1 Isolation of compounds from *T. laurifolia* leaves

3.1.4 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. Silymarin was purchased from Sigma Chemical, Inc. (St. Louis, MO, USA). MTT was obtained from molecular probes® (life technologies™, Oregon, USA).

3.1.5 Cell viability

Cell viability was determined using MTT (3-[4, 5-di-methyl-thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) 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 (1×10^5 cells/mL) were cultured in 96-well plate with or without various concentrations of negative control; ethanol (1, 5, 10, 100, 500, and 1000 mM) and rosmarinic acid (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 the incomplete 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 (Corning Incorporated, NY, USA). The percentage of cell viability was calculated.

3.1.6 Anti-hepatotoxicity assay

3.1.6.1 Pretreatment

Rosmarinic acid 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 (1×10^5 cells/mL) for 24 h at 37°C and 5% CO_2. They were then treated with different agents for 24 h and then treated with ethanol (EtOH) 10 and 100 mM for 6, 12, and 24 h.

Group 1 – Control without treatment

Group 2 - 3 – EtOH 10 and 100 mM

Group 4 - 6 – Rosmarinic acid (RA) at 1, 5, and 10 μM + EtOH 10 mM

Group 7 - 9 – Rosmarinic acid (RA) 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

3.1.6.2 Post-treatment

For post-treatment, HepG2 cells were placed in a 96-well plate (1×10^5 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 and then received 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 + rosmarinic acid (RA) 1, 5, and 10 μM

Group 7 - 9 – EtOH 100 mM + rosmarinic acid (RA) 1, 5, and 10 μM

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

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

3.1.7 Statistical analysis

All data were expressed as 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.

3.2 Results and discussion

3.2.1 Anti-hepatotoxic of *T. laurifolia* crude extract

In the bioassay-guided for isolation and elucidation of anti-hepatotoxic compounds, *T. laurifolia* crude extracts were investigated by inducing ethanol toxicity and ascertained by MTT assay. Fraction D4 (70% acetone in water) increased %cell viability as compared to the effect of ethanol alone (**Table 3**). Thus, fraction D4 was chosen for isolation and elucidation of anti-hepatotoxic compounds.

Table 3 The anti-hepatotoxic activity against HepG2 cells of *T. laurifolia* crude extracts.

Treatment	MTT[a] (%)	MTT[a] (%)
Control	100	-
Ethanol (EtOH)	27.2±2.8*	100
D1 + EtOH	46.3±4.0*,**	207.7±18.0**
D2 + EtOH	35.9±1.2*	125.9±10.0
D3 + EtOH	52.2±2.4*,**	181.7±13.3**
D4 + EtOH	91.2±1.5*,**	316.5±13.5**
D5 + EtOH	42.9±3.2*,**	149.7±8.1**
D6 + EtOH	23.3±0.7*	107.7±7.3

[a]Values are mean±S.E.M., n = 8

*Significant difference from control (p<0.05)

**Significant difference from ethanol (p<0.05)

3.2.2 Extraction, isolation, purification, and identification of caffeic acid

The yield for the ethanolic extract of *T. laurifolia* was 6.717%w/w. Compound T4F2AB was purified by PREP-HPLC method (**Figure 19**) and obtained as white amorphous powder (23.8 mg, %yield = 0.238).

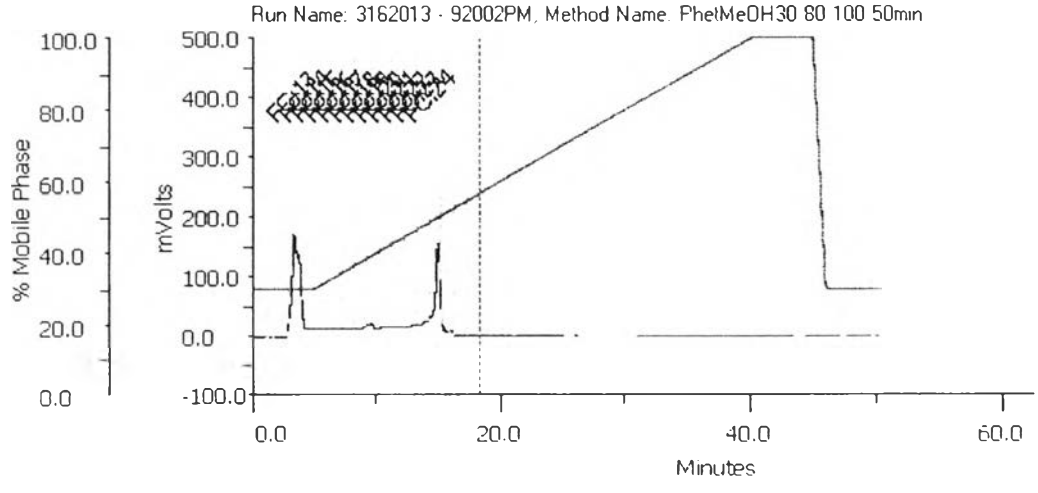
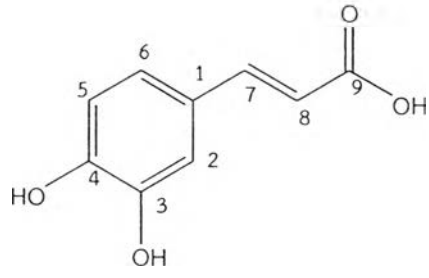


Figure 19 The preparative HPLC chromatogram and fraction collection of compound T4F2AB obtained using UV detection at 210 nm, Alantis®PrepT3; 5 µm 19×250 mm column (Waters company, USA). 5 mL injection volume, flow rate: 14 mL/min/tube, and gradient 0 – 100% MeOH from 0 – 50 min.

Its ^{13}C NMR spectrum (**Figure A1**) included one benzene ring at 149.4 (C-4), 146.8 (C-3), 127.8 (C-1), 122.8 (C-6), 116.5 (C-5), and 115.1 ppm (C-2), one carboxylic carbon at δ 171.5 ppm (C-9), and two olefinic carbons at δ 146.8 (C-7) and 115.1 ppm (C-8).

For NMR data (**Table 4**), the ^{1}H-NMR spectrum of this compound (**Figure A2**) showed a *trans*-double bond, 2 olefinic doublets, at δ 7.50 and 6.21 ppm (1H each, *d*, *J* = 15.9 Hz, H-7 and H-8), aromatic region 2 doublets at δ 7.02 (1H, *d*, *J* = 2 Hz), 6.77 ppm (1H, *d*, *J* = 8.2 Hz), and a double doublet at δ 6.92 ppm (1H, *dd*, *J* = 8.2, 2 Hz).

According to the [M-H] peak at *m/z* 179.0327 in the high resolution mass spectrum (**Figure A3**), its molecular formula could be identified as $C_9H_8O_4$. Comparison of these data with literature subsequently revealed compound T4F2AB to be the phenolic compound caffeic acid (Lee, *et al.* 2010).



Caffeic acid

Table 4 ^{1}H and ^{13}C-NMR assignments of compound T4F2AB and caffeic acid.

Position	T4F2AB		Caffeic acid*	
	δ_H	δ_C	δ_H	δ_C
1	-	127.8	-	127.8
2	7.02 (*d*, *J* = 2.0 Hz)	115.1	7.02 (*d*, *J* = 2.1 Hz)	115.6
3	-	146.7	-	146.8
4	-	149.4	-	149.5
5	6.77 (*d*, *J* = 8.2 Hz)	116.5	6.77 (*d*, *J* = 8.1 Hz)	116.5
6	6.92 (*dd*, *J* = 8.2, 2.0 Hz)	122.8	6.92 (*dd*, *J* = 8.4, 2.1 Hz)	122.8
7	7.50 (*d*, *J* = 15.9 Hz)	146.8	7.52 (*d*, *J* = 15.9 Hz)	147.0
8	6.21 (*d*, *J* = 15.9 Hz)	115.1	6.20 (*d*, *J* = 15.9 Hz)	115.1
9	-	171.5	-	171.1

Chemical shift values are reported as δ (ppm) at 300 MHz for ^{1}H and 75 MHz for ^{13}C in CD.OD. *(Lee, *et al.* 2010).

Caffeic acid, 3, 4-dihydroxycinnamic acid, is found in many plants as a key intermediate in the biosynthesis of lignins (Bhullar, *et al.* 2014). Caffeic acid has been reported to have a potent antiproliferative (Gomes, *et al.* 2003), antihypertensive agent (Li, *et al.* 2005), and inhibitor of angiotensin converting enzyme activity (Actis-Goretta, *et al.* 2006).

3.2.3 Extraction, isolation, purification, and identification of rosmarinic acid

Compound T5F5 and T5F6 (518.1 mg, %yield = 0.5181) were obtained as white amorphous powder. The chromatographic separation was carried out on reversed phase TLC (Merck, Darmstadt, Germany) using 40% MeOH in water as the mobile phase. From a TLC pattern, The R_f values of compound T5F5 and T5F6 were obtained near to the R_f value of standard rosmarinic acid (**Figure 20**).

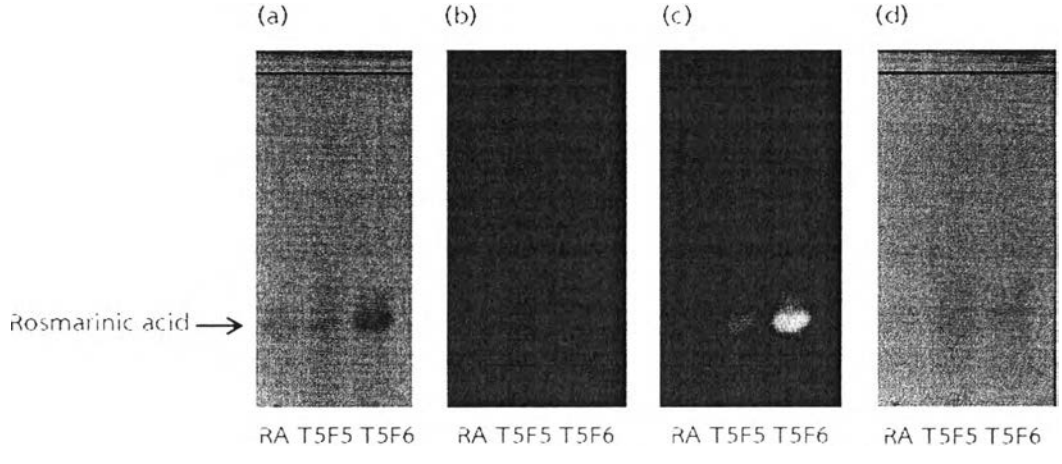


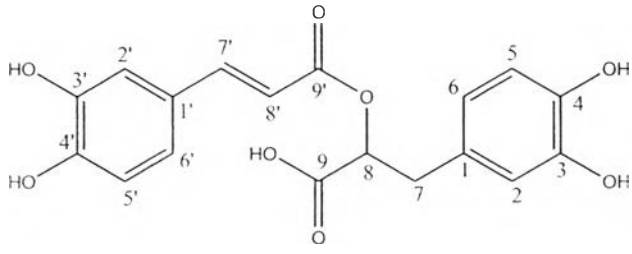
Figure 20 The TLC pattern of the standard rosmarinic acid, compound T5F5, and T5F6. The TLC plates were developed with 40% MeOH in water and then viewed under UV light at 254 nm (a), 366 nm (b), 366 nm after being sprayed with 20% H_2SO_4 solution (c), and under white light (d), respectively.

The ^{13}C NMR spectrum of this compound (**Figure A4**) displayed 18 carbons signals from one ester carbon at δ 171.4 ppm (C-9), one carboxylic carbon at δ 166.4 ppm (C-9'), two benzene rings at δ 148.9 (C-4'), 146.2 (C-4), 145.2 (C-3'), 144.3 (C-3), 127.8 (C-1), 125.8 (C-1'), 121.9 (C-6'), 120.4 (C-6), 117.0 (C-2), 116.1 (C-5'), 115.7 (C-2'), and 113.6 ppm (C-5), two olefinic carbons at δ 145.9 (C-7') and 116.1 ppm (C-8'), one methine carbon at δ 73.3 ppm (C-8), and one methylene carbon at δ 36.7 ppm (C-7).

The ^{1}H-NMR spectrum of this compound (**Figure A5**) showed a *trans*-double bond, 2 olefinic doublets, at δ 7.42 and 6.18 ppm (1H each, *d*, *J* = 15.0 Hz, H-7' and H-8'), two aromatic region, two singlets at δ 7.01 and 6.67 ppm (1H each, s, H-2' and H-2), four doublets at δ 6.88 (1H, *d*, *J* = 9.0 Hz, H-6'), 6.74 (1H, *d*, *J* = 9.0 Hz, H-5'), 6.63 (1H, *d*, *J* = 6.0 Hz, H-5), and 6.50 ppm (1H, *d*, *J* = 9.0 Hz, H-6), methylene proton (H-7) resonated at δ 3.10 (*d*, *J* = 14.0 Hz) and 2.93 ppm (*dd*, *J* = 14.0, 3.0 Hz). In addition, a methine double doublet at δ 5.01 ppm (*dd*, *J* = 6.0, 3.0 Hz, H-8).

The mass spectrum (**Figure A6**) showed $[M+Na]^+$ peak at *m/z* 383.0793, suggesting the molecular formula $C_{18}H_{16}O_8$. Comparison of the data of compound T5F5 and rosmarinic acid isolated from the fruits of *Helicteres isora* L. (Satake, *et al.* 1999) is shown in **Table 5**.

From the results, compound T5F5 and T5F6 were identified as rosmarinic acid by TLC and NMR methods. Rosmarinic acid was first reported as a major constituent from *T. laurifolia* leaves (Suwanchaikasem 2011). The presence of rosmarinic acid in *T. laurifolia* may be related to the hepatoprotective activity (Pramyothin, *et al.* 2005, Wonkchalee, *et al.* 2012) as previously reported for *T. laurifolia*. Therefore, should be investigated in the relevance of anti-hepatotoxic activity.



Rosmarinic acid

Table 5 ^{1}H- and ^{13}C-NMR assignments of compound T5F5 and rosmarinic acid.

Position	T5F5		Rosmarinic acid*	
	δ_H	δ_C	δ_H	δ_C
1	-	127.8	-	128.7
2	6.67 (*s*)	117.0	6.68 (*d*, *J* = 2.0 Hz)	116.9
3	-	144.3	-	144.2
4	-	146.2	-	144.0
5	6.63 (*d*, *J* = 6.0 Hz)	113.6	6.64 (*d*, *J* = 8.0 Hz)	113.6
6	6.50 (*d*, *J* = 6.0 Hz)	120.4	6.52 (*dd*, *J* = 8.0, 2.0 Hz)	120.0
7	2.93 (*dd*, *J* = 14.0, 3.0 Hz) 3.10 (*d*, *J* = 14.0 Hz)	36.7	2.89 (*dd*, *J* = 14.3, 8.6 Hz) 2.99 (*dd*, *J* = 14.3, 4.1 Hz)	36.9
8	5.01 (*dd*, *J* = 6.0, 3.0 Hz)	73.3	5.02 (*dd*, *J* = 8.6, 4.1 Hz)	75.5
9	-	171.4	-	172.1
1'	-	125.8	-	125.7
2'	7.01 (*s*)	115.7	7.06 (*d*, *J* = 2.0 Hz)	115.6
3'	-	145.2	-	145.2
4'	-	148.9	-	148.8
5'	6.74 (*d*, *J* = 9.0 Hz)	116.1	6.77 (*d*, *J* = 8.2 Hz)	116.1
6'	6.88 (*d*, *J* = 9.0 Hz)	121.9	7.01 (*dd*, *J* = 8.2, 2.0 Hz)	121.5
7'	7.42 (*d*, *J* = 15.0 Hz)	145.9	7.46 (*d*, *J* = 15.9 Hz)	145.4
8'	6.18 (*d*, *J* = 15.0 Hz)	116.1	6.24 (*d*, *J* = 15.9 Hz)	116.1
9'	-	166.4	-	166.3

Chemical shift values are reported as δ (ppm) at 300 MHz for ^{1}H and 75 MHz for ^{13}C in DMSO-d_6. *(Satake, *et al.* 1999).

3.2.4 Preliminary concentration-dependent experiment on cell viability

The viability of HepG2 cells exposed to ethanol (EtOH) at the concentrations of 1, 5, 10, 100, 500, and 1000 mM, respectively for 24 h were determined by MTT method. Ethanol reduced the cell viability in a dose-dependent manner with significant effects (**Figure. 21**). The concentrations of ethanol at 10 and 100 mM were chosen for subsequent interaction experiments.

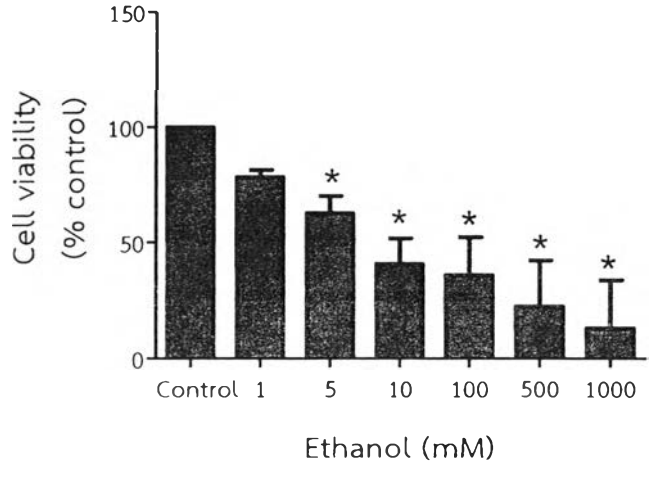


Figure 21 Ethanol induced cytotoxicity in HepG2 cells as measured by MTT assay. HepG2 cells were treated with different concentrations of ethanol for 24 h. Results are expressed as percentage of control and are mean±SD, (n = 3). *Significant difference with control ($p<0.05$)

For the viability of HepG2 cells exposed to rosmarinic acid, the concentrations of rosmarinic acid at 1, 5, 10, 100, 1000, and 3000 μM, respectively for 24 h was examined using a MTT assay. Rosmarinic acid produced toxicity in HepG2 cells in a dose-dependent manner (**Figure 22**). Non – cytotoxic concentrations of rosmarinic acid at 1, 5, and 10 μM, respectively were chosen for subsequent interaction experiments.

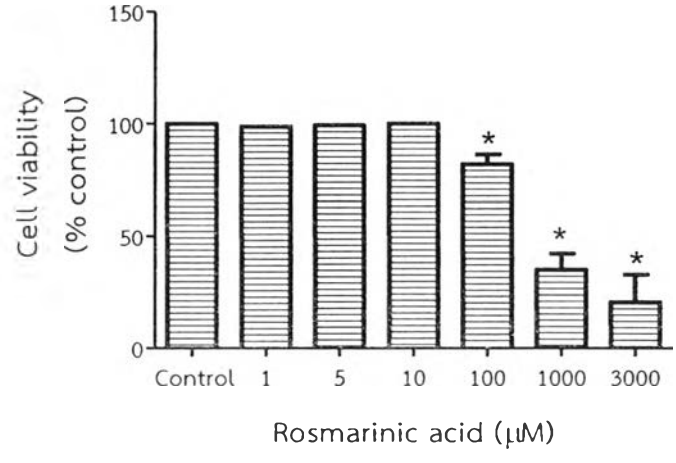


Figure 22 Rosmarinic acid induced toxicity in HepG2 cells as measured by MTT assay. HepG2 cells were treated with different concentrations of rosmarinic acid for 24 h. Results are expressed as percentage of control and are mean±SD, (n = 3). *Significant difference with control ($p<0.05$)

3.2.5 Anti-hepatotoxic activity of rosmarinic acid in HepG2 cells

Anti-hepatotoxic activity of rosmarinic acid 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 anti-hepatotoxic activity of rosmarinic acid, which was isolated from *T. laurifolia*, against this well characterized hepatotoxin in human hepatoma (HepG2) cell line. The immortalized hepatoma cell lines from humans are widely used to investigate the antihepatotoxic activity of plant crude extracts and their compounds. HepG2 cell lines are preferred to primary cultures from animals, as these are cells from humans which can minimize any species related differences that can occur during the extrapolation of the results (Thabrew, *et al.* 1997). Silymarin, which is a hepatoprotective drug, was used as the positive control.

3.2.5.1 Rosmarinic acid protect HepG2 cells from ethanol-induced toxicity

In order to investigate the hepatoprotective effect of rosmarinic acid on ethanol-induced toxicity in HepG2 cells were clarified. HepG2 cells were pretreated with various concentrations of rosmarinic acid (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. From the results, rosmarinic acid 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 rosmarinic acid was observed only when the rosmarinic acid pretreatment was applied to cells for at least induced with ethanol at 12 h.

Pretreatment of the HepG2 cells with the concentrations of rosmarinic acid at 1, 5, and 10 μM could prevent hepatotoxic induced by ethanol at 6 and 12 h (**Figure 23a – 23b**). These results illustrated the influence of concentration and pretreatment time on the hepatoprotective effect of rosmarinic acid against ethanol-induced toxicity in HepG2 cells.

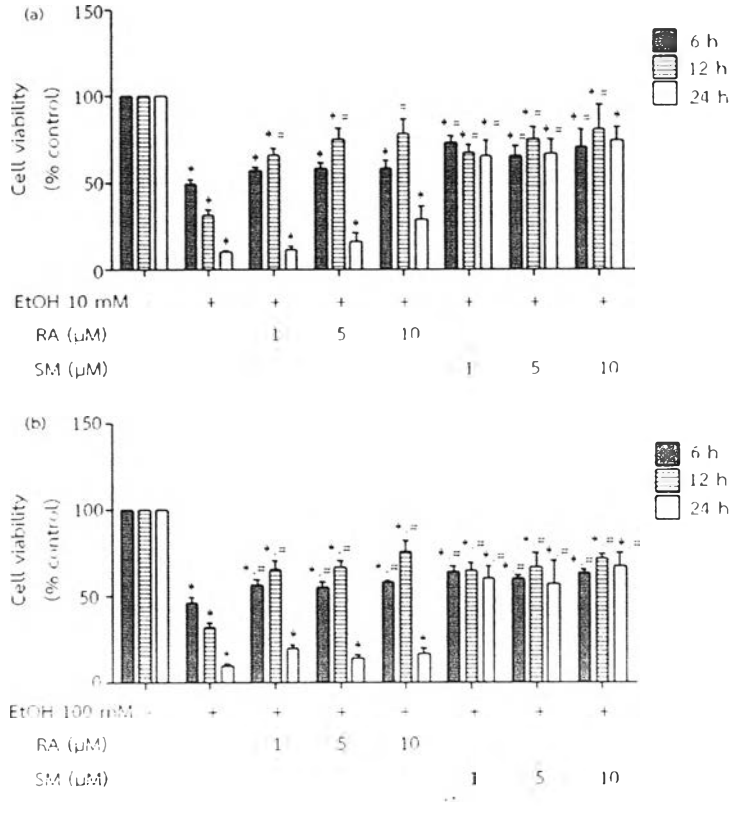


Figure 23 Hepatoprotective effect of pretreatment with rosmarinic acid (RA) and silymarin (SM) on HepG2 cells for 24 h after that induced by ethanol (EtOH) 10 mM (a) and 100 mM (b) for 6, 12, and 24 hours, respectively. The experiments were carried out at least three times in triplicate. Each column represents the mean±SEM.*Significant difference with control ($p<0.05$) and ¤Significant difference with ethanol, respectively, as determined by ANOVA.

The perfectly normal HepG2 cells were disturbed in the control group. In the ethanol groups, 10 mM (**Figure 24**) and 100 mM (**Figure 25**), showed cell damage as a results of toxicity on HepG2 cells. Meanwhile, pretreatment with rosmarinic acid at

1, 5, and 10 μM for 24 h reduced cell infarctions after treated with ethanol at 6 and 12 h. It can be compared with that of silymarin group, the hepatoprotective drug.

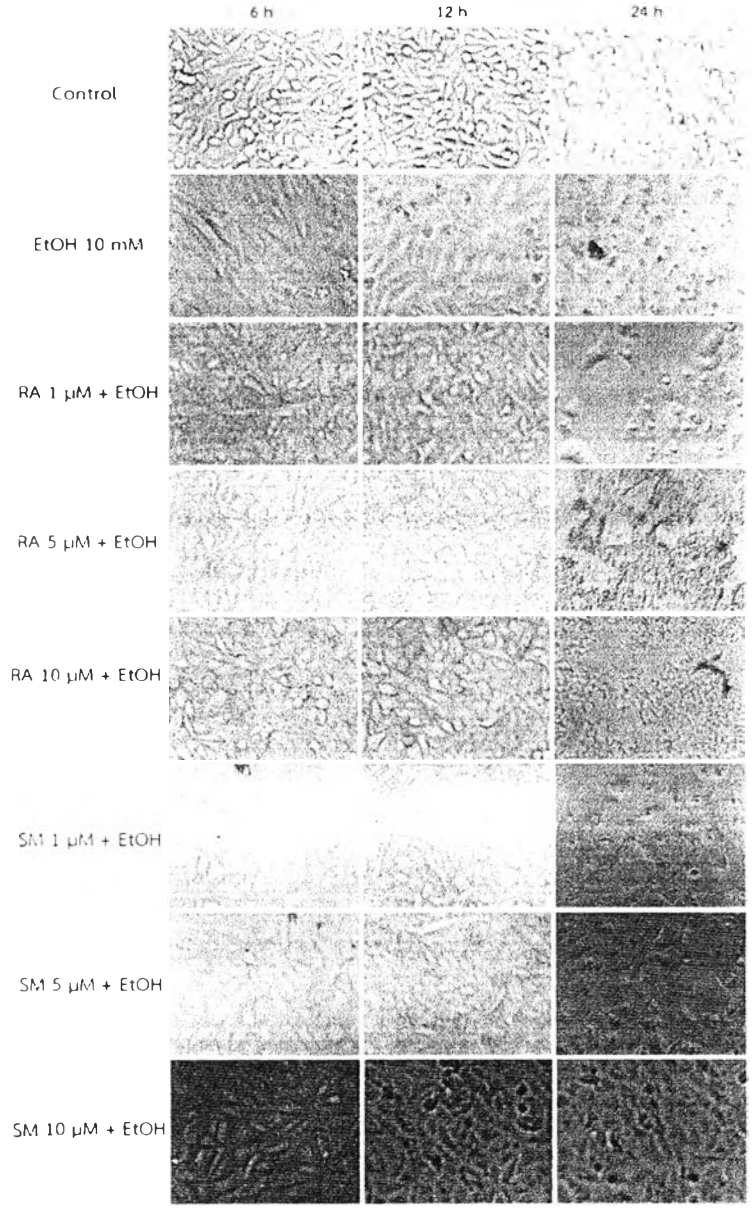


Figure 24 Effect of rosmarinic acid (RA) at various concentrations (1, 5, and 10 μM) pretreatment for 24 h before induced liver damage by ethanol (EtOH) 10 mM at 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope,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

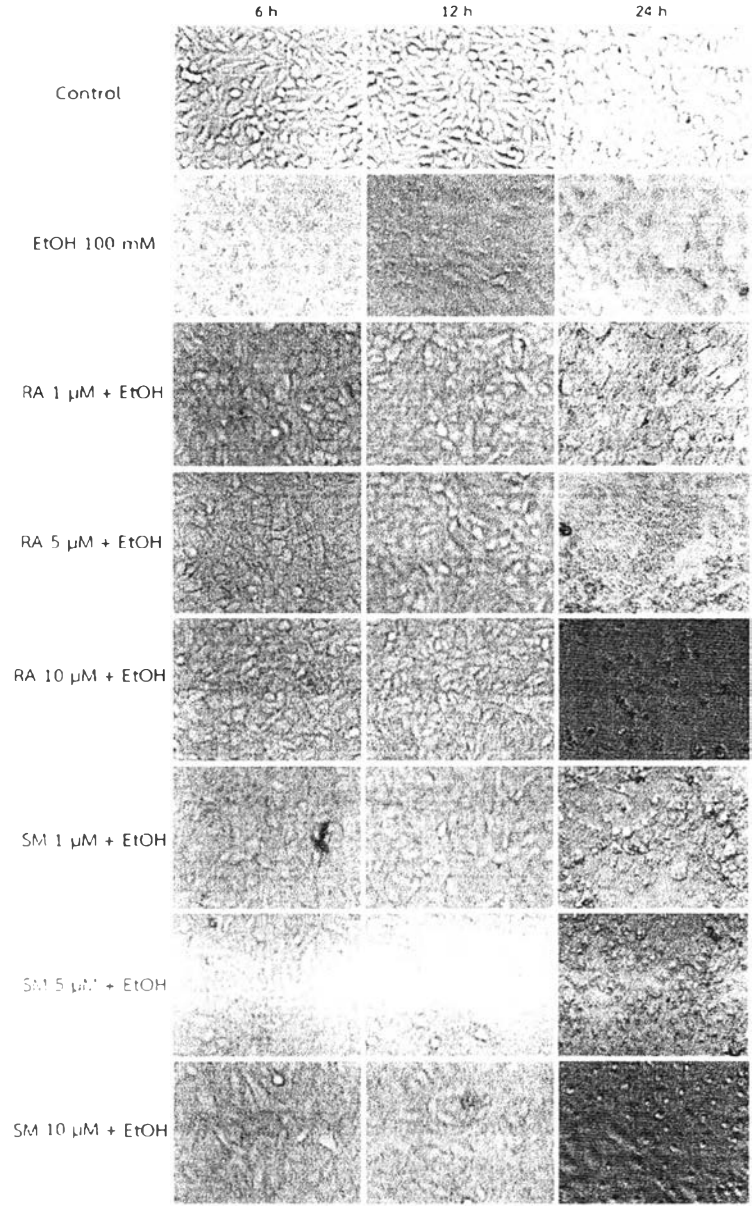


Figure 25 Effect of rosmarinic acid (RA) at various concentrations (1, 5, and 10 µM) pretreatment for 24 h before induced liver damage by ethanol (EtOH) 100 mM at 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

In pretreatment study demonstrated that rosmarinic acid, a purified phenolic compound from leaves of *T. laurifolia*, showed potential protection against ethanol-induced toxicity in HepG2 cells.

3.2.5.2 Effect of rosmarinic acid post-treatment in HepG2 cells

In order to investigate the effect of rosmarinic acid post-treatment on ethanol-induced toxicity in HepG2 cells were clarified. HepG2 cells were treated with 10 and 100 mM of ethanol for various time periods (6, 12, and 24 h) and received with various concentrations of rosmarinic acid or silymarin (1, 5, and 10 μM) for 24 h. After 24 h, cell viability was analyzed by MTT assay. From the results, the received rosmarinic acid at 1, 5, and 10 μM groups after being treated with ethanol 10 mM and 100 mM for 12 h showed significant increased %cell viability higher than treated with ethanol 10 mM and 100 mM for 6 and 24 h. For 24 h, HepG2 cells were treated with rosmarinic acid group and ethanol groups, it is possible to increase damage to the cells (**Figure 26a – 26b**).

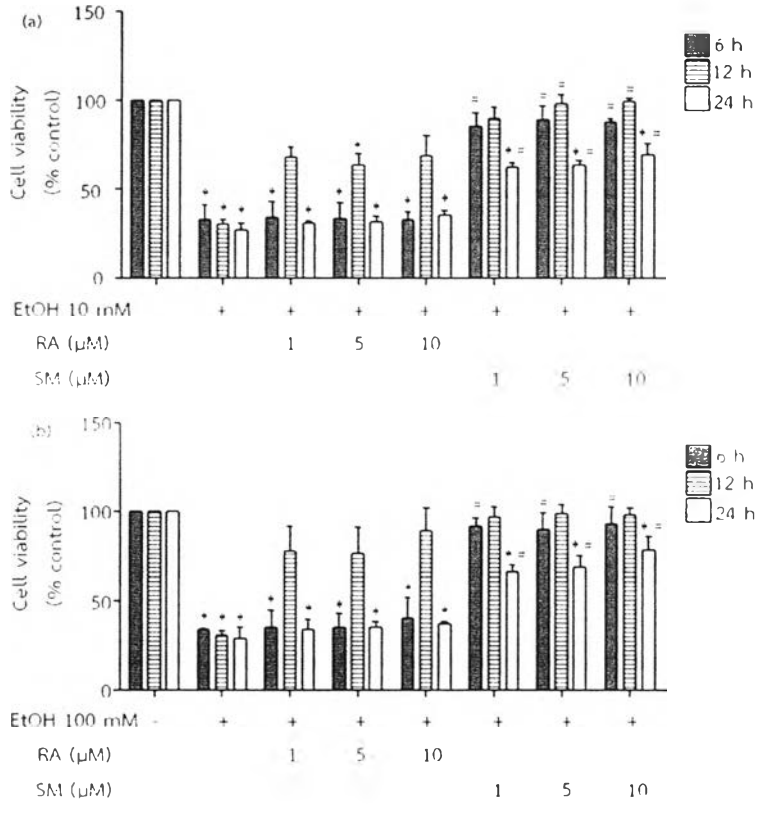


Figure 26 Effect of post-treatment with rosmarinic acid (RA) and silymarin (SM) on HepG2 cells induced by ethanol (EtOH) 10 mM (a) and 100 mM (b) at 6, 12, and 24 hours, respectively. The experiments were carried out at least three times in triplicate. Each column represents the mean±SEM. *Significant 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. In the ethanol groups, 10 mM **(Figure 27)** and 100 mM **(Figure 28)**, showed cell damage as a results of toxicity on HepG2 cells. Meanwhile, post-treatment with rosmarinic acid at 1, 5, and 10 μM after induced toxicity by ethanol for 12 h increased cell viability.

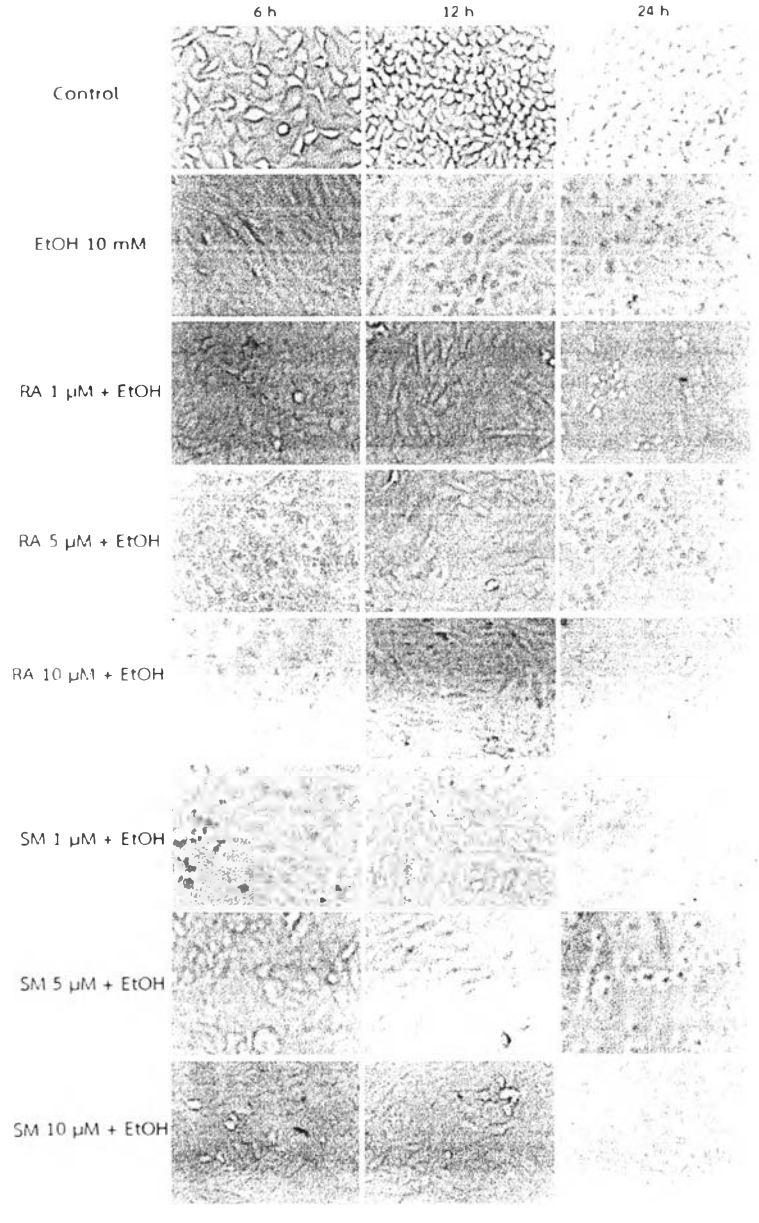


Figure 27 Effect of rosmarinic acid (RA) at various concentrations (1, 5, and 10 μM) post-treatment after ethanol (EtOH) 10 mM induced liver damage for 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

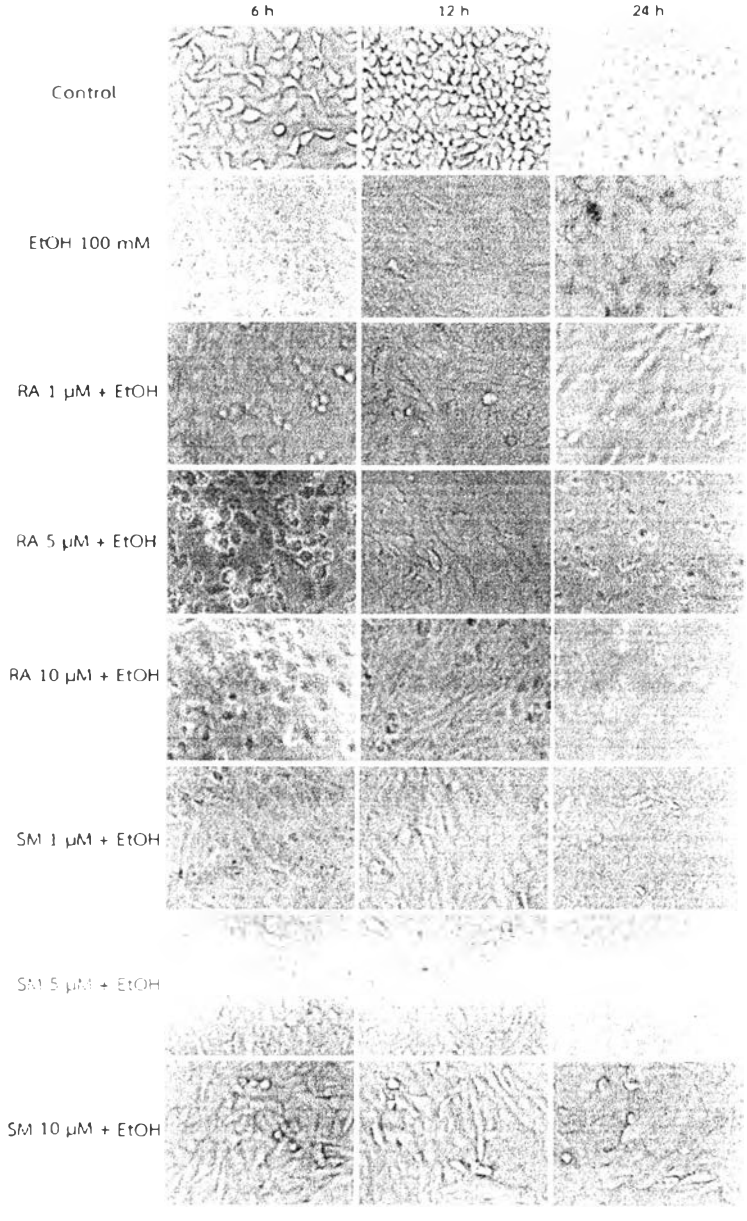


Figure 28 Effect of rosmarinic acid (RA) at various concentrations (1, 5, and 10 µM) post-treatment after ethanol (EtOH) 100 mM induced liver damage for 6, 12, and 24 h. Control; HepG2 cells without treatment and SM; treated with silymarin. HepG2 cells were captured by phase contrast microscope, magnification ×100.

In post-treatment study demonstrated that rosmarinic acid showed potential cell viability against ethanol-induced toxicity.

3.3 Conclusion

Ethanol is one major factor led to liver damage. Our study attempted to isolate and identify the anti-hepatotoxic compound from *T. laurifolia* leaves. As a result, a potent anti-hepatotoxic compound, rosmarinic acid, was isolated from this plant. This research study confirmed the anti-hepatotoxic effect of rosmarinic acid against ethanol-induced in human hepatoma HepG2 cells. The most important result in this research study is that rosmarinic acid could significantly increase the cell viability of HepG2 cells treated with ethanol. In summary, this study herein showed for the first time that rosmarinic acid exhibited a post-treatment against ethanol-induced human hepatoma HepG2 cells damage. The bioactivity of rosmarinic acid could be beneficial for treatment the liver disease with the leaves of *T. laurifolia* in traditional medicine.