CHAPTER 3
RESULTS

3.1 Toxicity analysis of fermented brown rice and rice bran (FBRA) in A/J mice

To examine whether FBRA affect the health of A/J mice, the female A/J mice were divided and received the FBRA treatment, according to Figure 23. At 21st weeks of age, all mice were sacrificed. The weight of body, liver, kidney, and lung were measured and the histology of liver and kidney was analyzed. During the 21 weeks of experimental period starting from week 6th, treatment with 5 and 10%FBRA (group 7 and 8, respectively) in A/J mice caused no significant differences in the final body weight and the weights of the liver, kidney or lung in comparison with control diet group (group 6) as shown in Table 4. In addition, there were no sign of toxicity in the liver and kidney of any mice in group 6 to 8 (which were received control diet, 5%FBRA and 10%FBRA, respectively) examined by the histological analysis.

3.2 Chemopreventive effect of FBRA against NNK-induced lung tumorigenesis

To further examine the chemopreventive effect of FBRA against NNK-induced lung tumorigenesis in *in vivo* model, the female A/J mice were divided into 8 groups, received the FBRA administration, and intra-peritoneal injected with 10μmol/L of nicotine-derived nitrosamine ketone (NNK), according to Figure 23. The incidences, multiplicities (number of tumor per mice) and size of lung lesions in both NNK-injected mice (group 1-5) and NNK-uninjected mice (group 6-8) were summarized in Table 5. NNK injection significantly induced the incidences of lung lesions (P<0.001) in female A/J mice. Whitish nodules in the lung were frequently detected in mice injected with NNK. Nevertheless, there was a mouse in the NNK-uninjected group (group 7) harbored a nodule in the lung, suggesting a spontaneous development of lung tumor in this mouse. However, there was no evidence of the development of lung tumors in NNK-uninjected mice (group 6-8). Histological analysis in NNK-injected mice (group 1-5) revealed that all lung lesions were

adenoma (Figure 29). In the pre-initiation of FBRA administration (mice in group 2 and 3), treatment with 10% FBRA (group 3) but not 5% FBRA (group 2) during NNK injection significantly reduced the multiplicity of lung lesions (P<0.01), when compared with the control NNK injection group (group 1). However, 10% FBRA treatment (group 3) had no significant effect on the incidence and tumor's size in comparison with control NNK injection group (group 1) as shown in Table 5. In the post-initiation FBRA administration (mice in group 4 and 5), treatment with 10% FBRA significantly decreased in both the multiplicity and tumor size (P<0.05) in comparison with control NNK injection group (group 1). However, it had no significant effect on the incidence of lung lesion as shown in Table 5.

3.3 The anti-proliferative effect of FBRA in lung lesions of NNK-injected mice

To investigate the effect of FBRA against NNK-induced lung tumorigenesis in the post-initiation treatment, immunohistochemical analysis for the expression of Ki67 protein, a marker for proliferative cell, was performed to examine the cell proliferative index in lung lesions of female A/J mice. Approximately 1,000 cells per microscopic field at 400x magnification were counted and Ki67 positive cells represent for proliferative cells. As results, administration of 10%FBRA in the diet to NNK-injected mice (group 5) significantly reduced (P<0.001) the cell proliferative index (Ki67 positive cells per total lung cells) in lung lesions, when compared with lung lesions in the NNK-injected mice fed with control diet. The cell proliferative index in lung lesion of NNK-injected mice fed with control diet were 114±25 cells per 1,000 total counted cells and 10% FBRA administration reduced the cell proliferative index in twice to 65±16 cells per 1,000 total counted cells as shown in Figure 30 and 31.

3.4 Effect of FBRA on the Cyp2a5 mRNA expression in the liver and lung tissues

To investigate the molecular mechanisms of the suppressing effect of FBRA against NNK-induced lung tumorigenesis in the pre-initiation treatment, female A/J mice were divided into 2 groups and received 10%FBRA treatment. After 2 weeks of FBRA treatment, all mice were sacrificed and their liver and lung tissues were collected. The mRNA expression levels from *Cyp2a5* gene in both liver and lung tissues were determined by quantitative real-time RT-PCR and the relative-mRNA expression of *Cyp2a5* gene were quantified and normalized with β–actin (arbitory

unit). Treatment of 10% FBRA significantly decreased the relative mRNA expression of *Cyp2a5* gene in the lung tissues from1.28±0.68 in the control diet group to 0.48±0.25 in 10% FBRA-treated group (P<0.05) as shown in Figure 32. However, 10%FBRA administration had no significantly effect on the relative mRNA expression of *Cyp2a5* gene in the liver tissues (1.51±0.74 in 10% FBRA-treated group vs. 1.27±0.33 in the control diet group).

Table 4 The weights of body, liver, kidney and lung of FBRA-treated A/J mice

Group	Treatment	No.[a]	Weight (grams)[b]			
			Body	Liver	Kidney	Lung
1	Control diet + NNK	**26**	**25.7±3.0**	**1.21±0.13**	**0.40±0.04**	**0.21±0.04**
2	5%FBRA (Pre-initiation) + NNK	**20**	**29.1±4.2**	**1.22±0.15**	**0.42±0.07**	**0.22±0.03**
3	10%FBRA (Pre-initiation) + NNK	**20**	**26.9±4.2**	**1.32±0.16**	**0.42±0.07**	**0.23±0.02**
4	5%FBRA (Post-initiation) + NNK	**20**	**28.0±2.8**	**1.23±0.17**	**0.43±0.08**	**0.21±0.04**
5	10%FBRA (Post-initiation) + NNK	**20**	**28.6±3.6**	**1.18±0.14**	**0.42±0.09**	**0.21±0.02**
6	Control diet alone	**5**	**28.0±4.2**	**1.27±0.14**	**0.44±0.07**	**0.23±0.02**
7	5%FBRA alone	**5**	**29.6±1.5**	**1.41±0.07**	**0.49±0.02**	**0.22±0.01**
8	10%FBRA alone	**5**	**26.1±1.8**	**1.30±0.08**	**0.42±0.04**	**0.23±0.01**

[a] Number of examined mice

[b] Representation in mean ± standard deviation.

Table 5 Incidences, multiplicities, and size of NNK-induced lung tumors in female A/J mice treated with FBRA

Group	Treatment	No.[a]	Incidence (%)[b]	Multiplicity[c]	Tumor size[d]
1	NNK alone	**26**	**26/26 (100)[e]**	**4.08±1.85**	**0.77±0.33**
2	5%FBRA (Pre-initiation) +NNK	**20**	**19/20 (95)**	**3.60±2.44**	**0.78±0.33**
3	10%FBRA (Pre-initiation) +NNK	**20**	**16/20 (80)**	**2.35±2.13[f]**	**0.78±0.32**
4	5%FBRA (Post-initiation) +NNK	**20**	**20/20 (100)**	**3.10±1.59**	**0.74±0.30**
5	10%FBRA (Post-initiation) +NNK	**20**	**20/20 (100)**	**3.00±1.52[g]**	**0.66±0.32[g]**
6	Control diet alone	**5**	**0/5 (0)**	**0.00±0.00**	**ND[h]**
7	5%FBRA alone	**5**	**1/5 (20)**	**0.20±0.45**	**ND**
8	10%FBRA alone	**5**	**0/5 (0)**	**0.00±0.00**	**ND**

[a] Number of examined mice

[b] Number of mice observed each lesion (%)

[c] Representation in mean ± standard deviation (Tumor per mouse)

[d] Representation in mean ± standard deviation (mm^3)

[e] Significantly difference from group 6 by student's t-test ($P<0.001$)

[f] Significantly difference from group 1 by student's *t*-test ($P<0.01$)

[g] Significantly difference from group 1 by student's t-test ($p<0.05$)

[h] Not determined

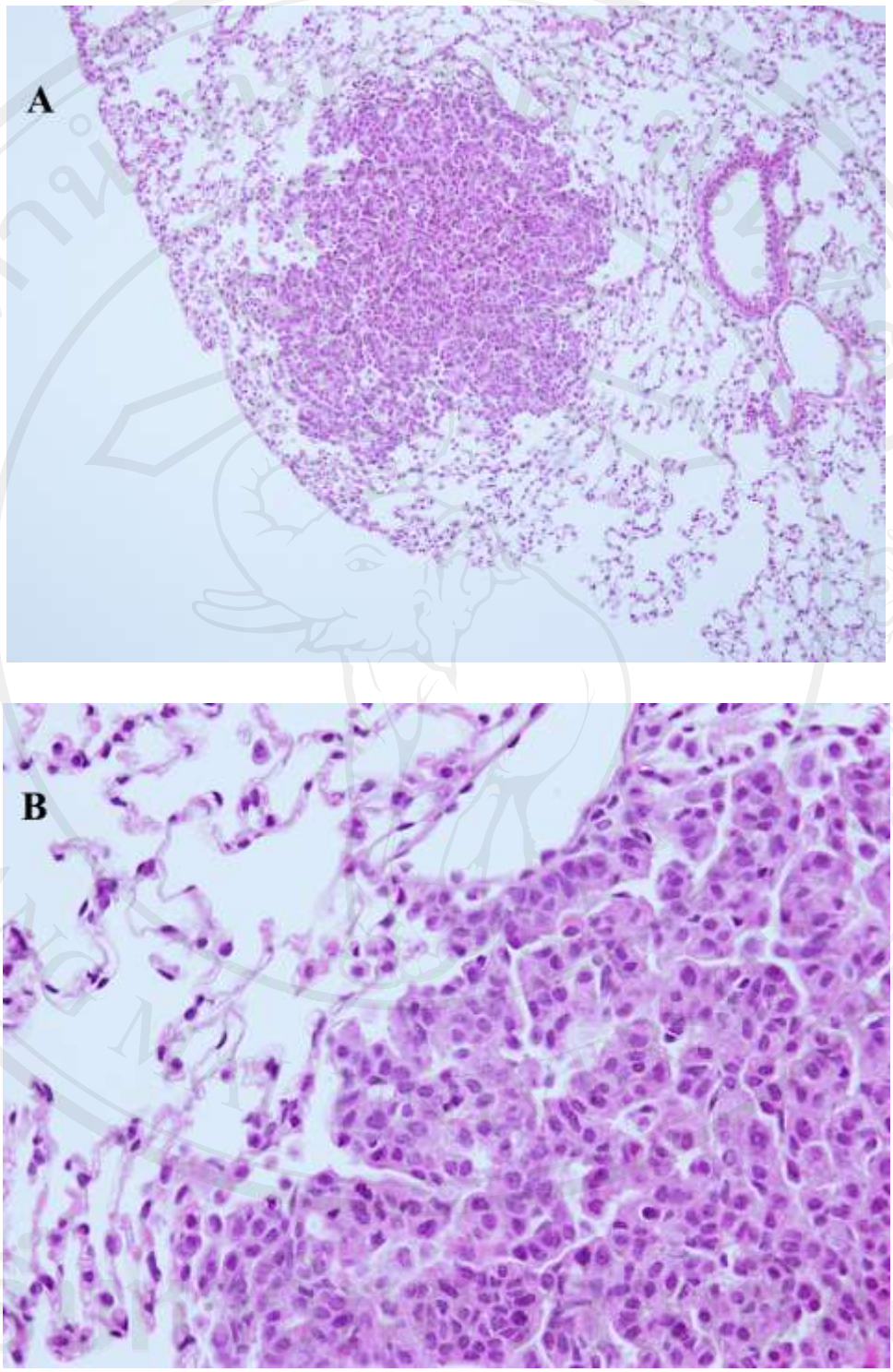

Figure 29 Histopathology of lung adenoma in NNK-treated mice. Lung tissue sections were provided and stained by H&E staining. Photomicrograph of mouse's lung adenoma was shown in 100x (A) and 400x (B) magnification.

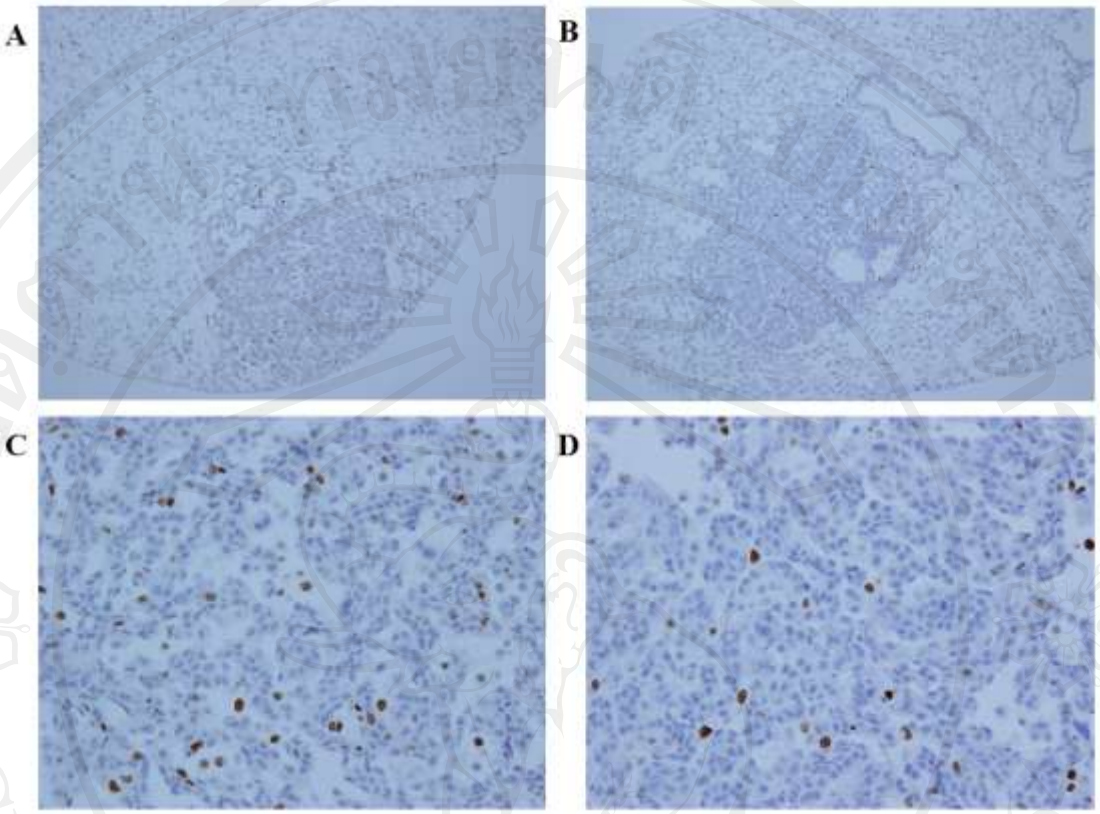

Figure 30 Effect of 10% FBRA treatment (B and D) in comparison with control diet (A and C) on the number of Ki67 positive cell in lung lesion. A and C displayed the Ki67-immunohistochemical staining of lung lesion from NNK-injected mice fed with control diet (group 1) in 100x and 400x magnification, respectively. B and D displayed the Ki67-immunohistochemical staining of lung lesion from NNK-injected mice fed with 10% FBRA (group 5) in 100x and 400x magnification, respectively.

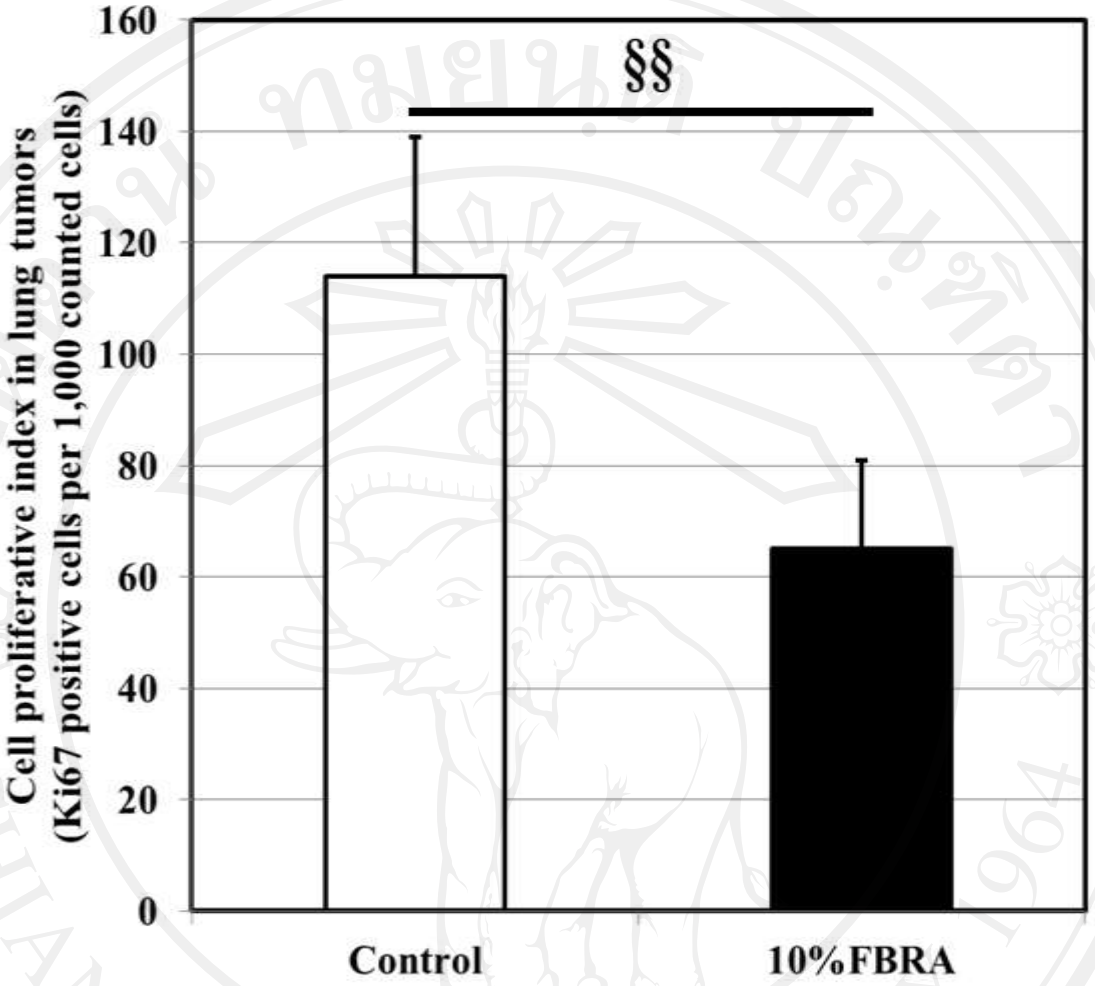

Figure 31 Effect of 10% FBRA treatment on the cell proliferative index of lung tumors in NNK-injected A/J mice. Lung tissue sections from female A/J mice in group 1 and 5 were stained with specific antibody for Ki67 protein and analyzed by immunohistochemistry. These results represented the mean of the ratio between Ki67 positive cells per 1,000 total counted cells in each microscopic field at 400x magnification. §§ $P<0.001$, vs. control group.

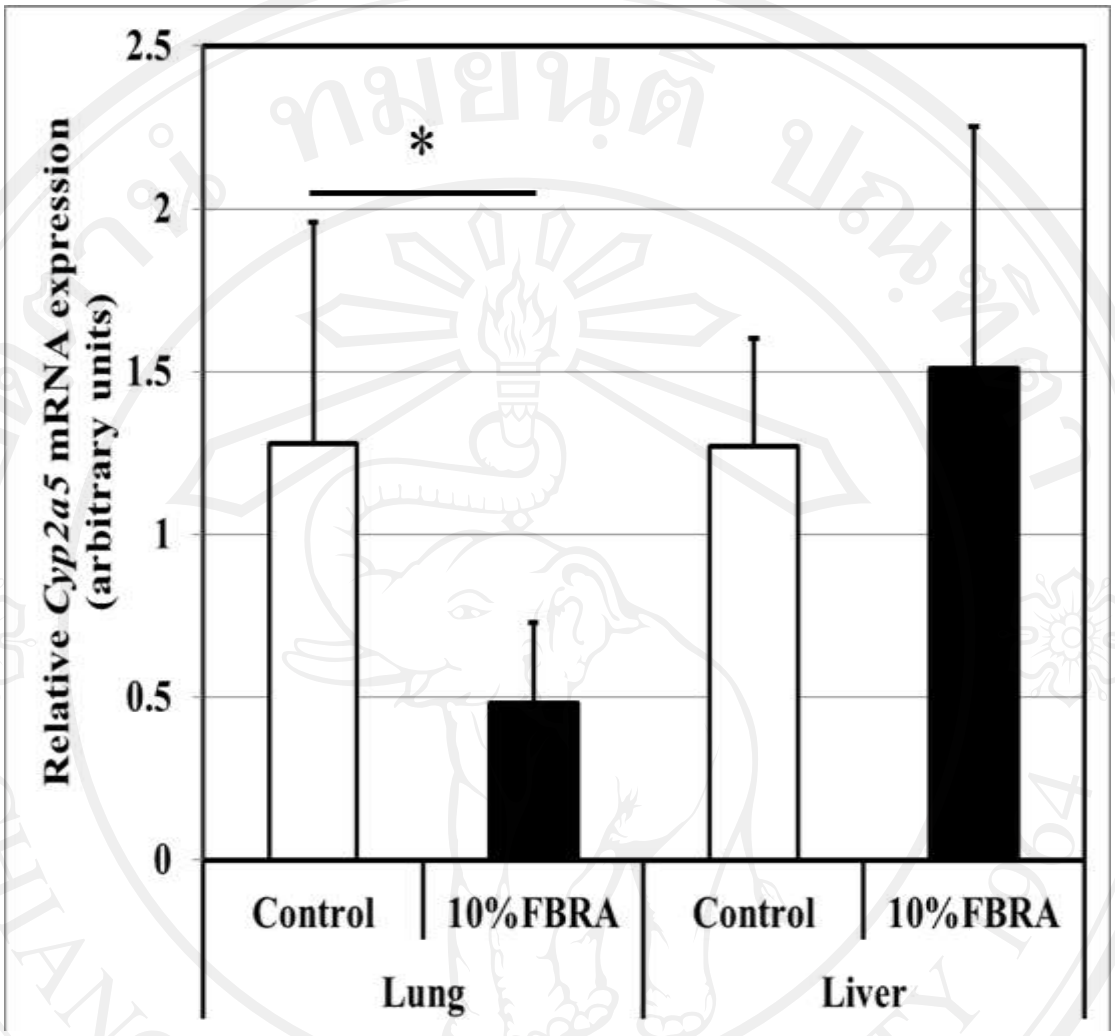

Figure 32 Effect of 10% FBRA treatment on the mRNA expression from *Cyp2a5* gene in both lung and liver tissues of NNK-injected A/J mice. After sacrifice, tissues of lung and liver from group 6 and 8 were collected. Quantitative real-time RT-PCR was used to monitor the mRNA expression from mouse *Cyp2a5* gene in both liver and lung tissue. These results were representative of mean from 3 independent experiments. The relative mRNA expression value of *Cyp2a5* gene was normalized with β-actin. * $P<0.05$, vs. control group.

3.5 Analysis for γ-tocotrienol concentration level in Thai rice

To explore the concentration level of γ-tocotrienol in Thai rice, six lines of Thai rice, including Khao Dawk Mali 105, Suphan Buri 1, Pathum Thani 1, RD31, RD41 and RD47, were collected and analysed the content of γ-tocotrienol by reverse phase high-performance liquid chromatography (HPLC) technique. HPLC profile of standard tocotrienols (α-, β-, γ-, and δ-tocotrienol) and tocopherols (α-, β-, γ-, and δ-tocopherol), which monitored at 292 nm, were shown in Figure 33. The quantitative data of γ-tocotrienol concentration level in Thai rice was shown in Table 6. All lines of Thai rice contained γ-tocotrienol with the range in between 15.32 to 54.81 mg/g. The content of γ-tocotrienol in rice was varied upon rice lines. Even the same rice lines, the content of γ-tocotrienol was still varied, depending on the cultivatation area as compared among the Khao Dawk Mali 105 from Maehongson, Sukhothai, and Suphanburi. Interstingly, the rice, which was cultivated in the northern region of Thailand, such as Maehongson and Sukhothai, contained higher content of γ-tocotrienol than the rice, which was cultivated in Suphanburi, the middle part of Thailand. In 2012, unpublished data from Asst. Prof. Dr. Chaiyavat Chaiyasut under NRCT grant showed that the concentation of γ-tocotrienol in Thai rice samples were changed after fermentation. After fermentation, the concentations of γ-tocotrienol in Khao Dawk Mali 105, Suphan Buri 1, Pathum Thani 1 and RD41 were increased 42, 101, 49 and 103%, respectively.

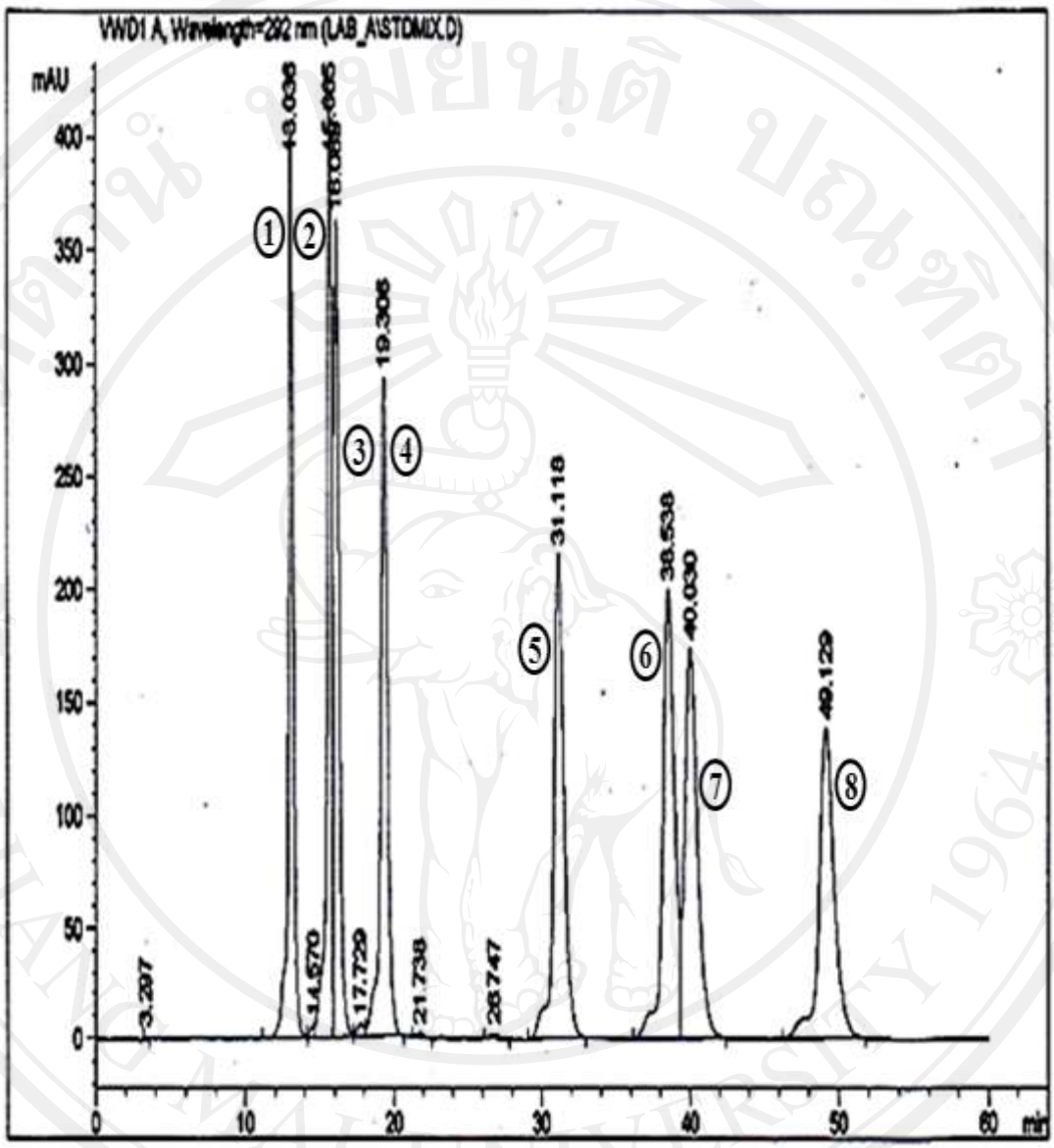

Figure 33 HPLC profile of standard tocotrienols and tocopherols monitored at 292 nm. Peak **1**: δ-Tocotrienol, **2**: γ-Tocotrienol, **3**: β-Tocotrienol, **4**: α-Tocotrienol, **5**: δ-Tocopherol, **6**: γ-Tocopherol, **7**: β-Tocopherol, **8**: α-Tocopherol

Table 6 The content of γ-tocotrienol in Thai rice

Lines	**Cultivated area in Thailand (Region/province)**	**Concentration of γ-tocotrienol (mg/100g of sample)**
Khao Dawk Mali 105	Northern/Maehongson	**36.82**
Khao Dawk Mali 105	Northern/Sukhothai	**34.92**
Khao Dawk Mali 105	Middle/Suphanburi	**18.99**
Suphan Buri 1	Middle/Suphanburi	**17.38**
Pathum Thani 1	Middle/Pathumthani	**37.68**
RD31	Middle/Suphanburi	**54.81**
RD31	Middle/Pathumthani	**15.32**
RD41	Middle/Suphanburi	**28.29**
RD47	Middle/Suphanburi	**31.28**

3.6 Prevention of the NNK-increased cell viability of lung cancer cell line A549 by γ-tocotrienol (γ-T_3) but not phytic acid (IP_6)

To investigate the carcinogenic effect of nicotine-derived nitrosamine ketone (NNK), which is classified as *carcinogenic to humans (Group 1)* by the International Agency for Research on Cancer (IARC), on the cell viability of human lung cells, A549 cells were treated with 0-10 μmol/L of NNK for 24, 48 and 72 hours. Finally, the cell viability was examined by MTT assay. As shown in Figure 34, treatment of NNK at concentrations of 10μmol/L significantly increased the cell viability of A549 cells in both 48 and 72 hours (P<0.05 and P<0.001, respectively) in comparison with NNK-untreated cells.

To further investigate the preventive effects of γ-tocotrienol and phytic acid against the NNK-increased cell viability of A549 cells, MTT assay was used to examine the cell viability of A549 cells. The A549 cells were pre-treated with 0-80 μmol/L of γ-tocotrienol or phytic acid for 12 hours, and then treated with 10μmol/L of NNK at 24 and 48 hours. As shown in Figure 35 and 36, γ-tocotrienol but not phytic acid pre-treatment significantly reduced the cell viability of NNK-treated A549 cells at both 24 and 48 hours (P<0.001) compared with NNK-treated A549 cells that were not pre-treatment with γ-tocotrienol or phytic acid. The preventive effect of γ-tocotrienol was both dose-response and time-dependent manner as shown in Table 7. The half maximal inhibitory concentration value (IC50) at 24 and 48 hours were 43.8±0.2 and 37.0±0.1 μmol/L, respectively. Furthermore, the 20%-inhibitory concentration value (IC20) at 24 and 48 hours were 22.7±3.3 and 21.5±0.5 μmol/L, respectively.

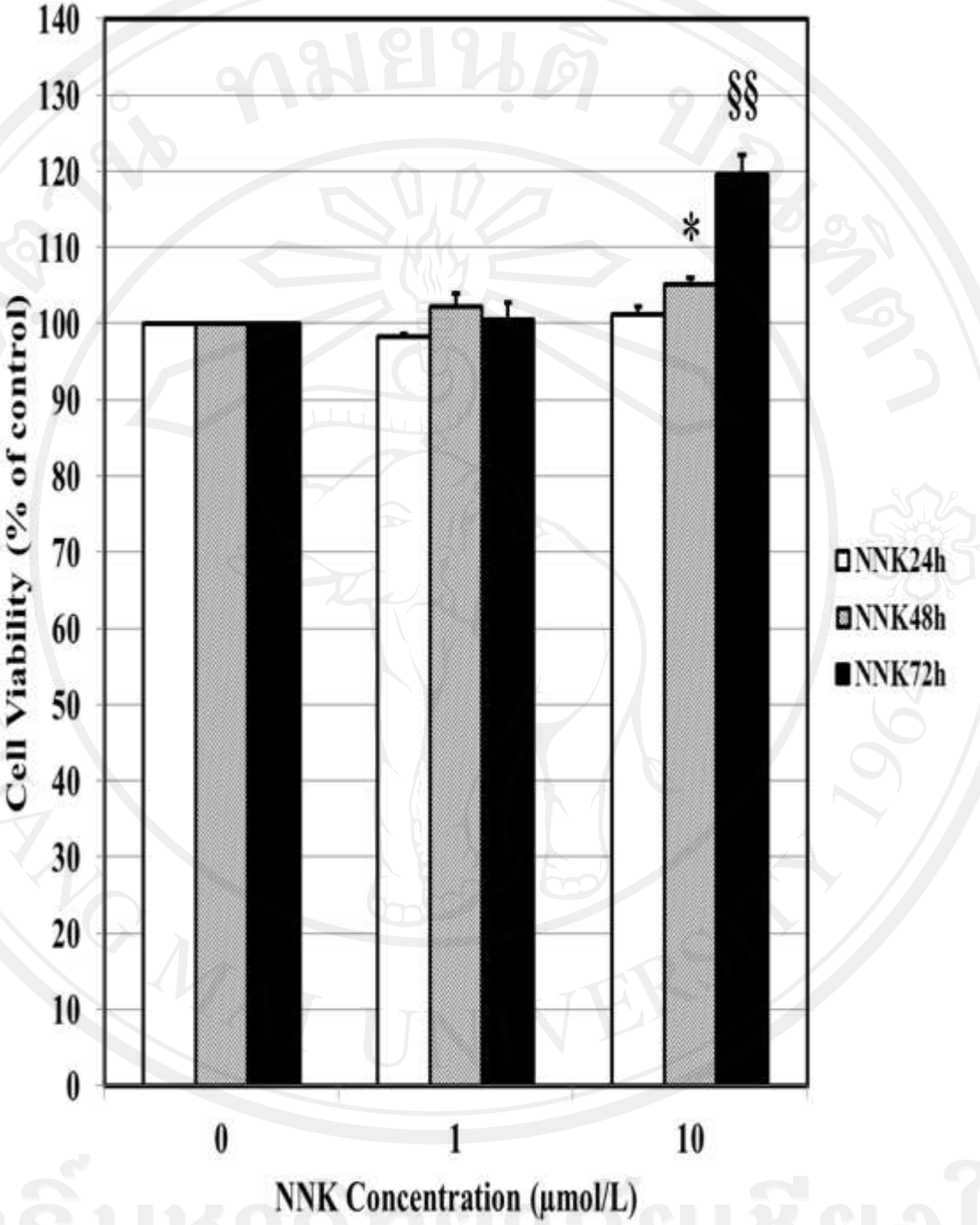

Figure 34 Effect of NNK on the cell viability of human lung cancer cell line A549. A549 cells were treated with NNK (0, 1, and 10µmol/L) for 24, 48 and 72 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and calculated relative to the negative control wells (0µmol/L of NNK). The results represent the mean of at least three independent experiments. * $P<0.05$, and §§ $P<0.001$, vs. control group.

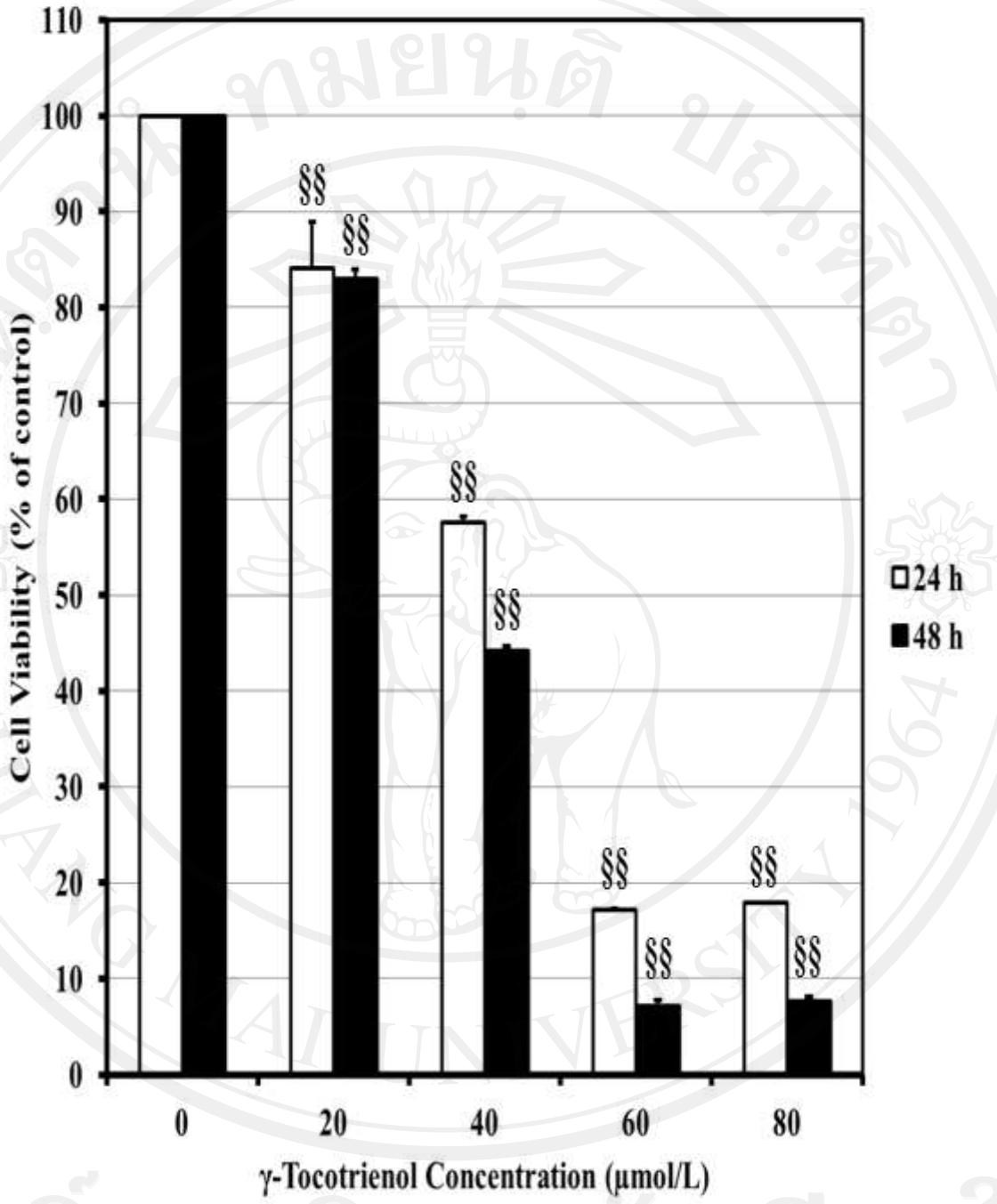

Figure 35 Preventive effect of γ-tocotrienol on the NNK-increased cell viability of A549 cells. A549 cells were pre-treated with 0-80 μmol/L of γ-tocotrienol for 12 hours, and then treated with NNK (10μmol/L) for 24 and 48 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and calculated relative to the negative control wells (0μmol/L of γ-tocotrienol). The results represent the mean of at least three independent experiments. §§ $P<0.001$, vs. control group.

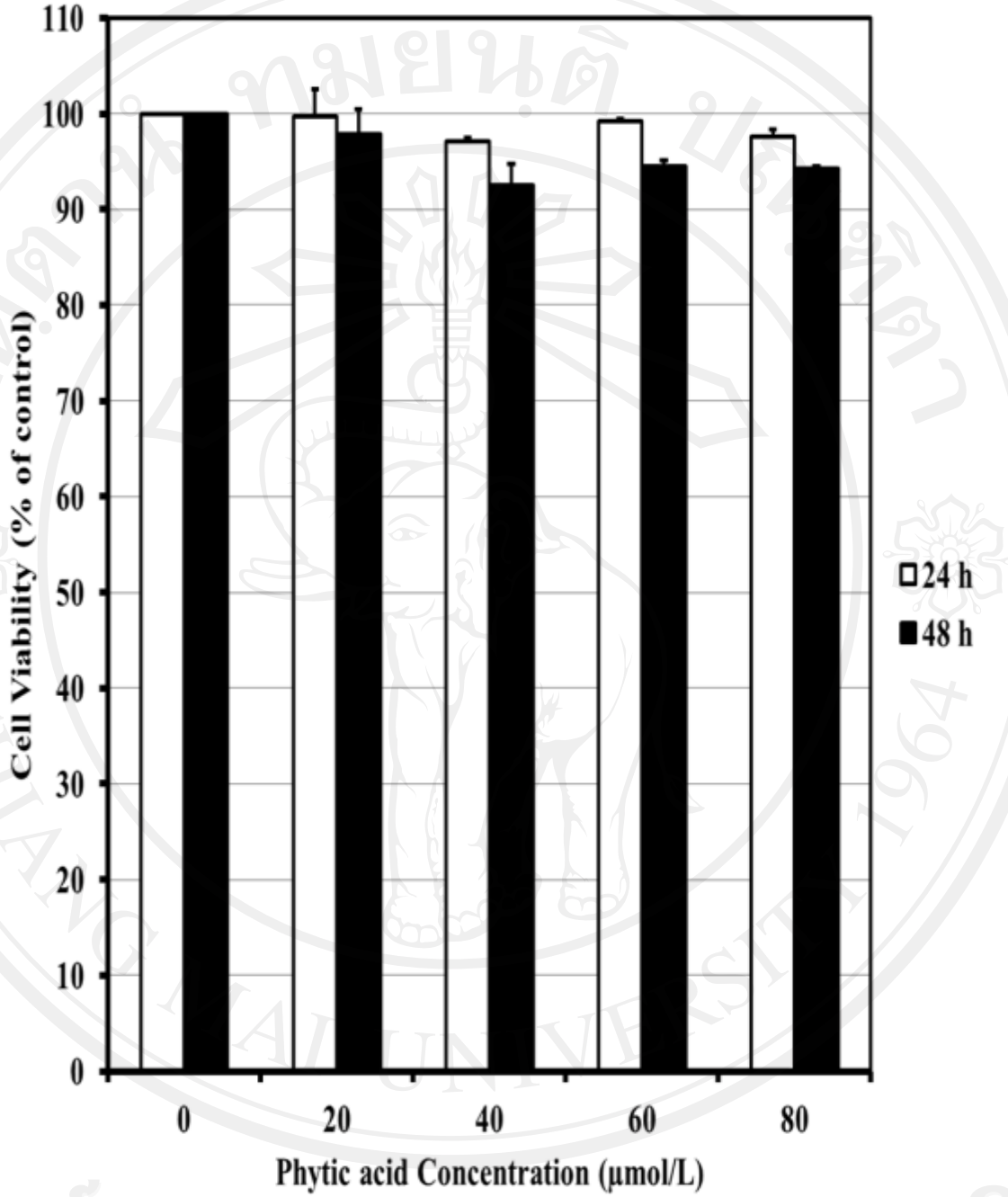

Figure 36 Effect of phytic acid on the NNK-increased cell viability of A549 cells. A549 cells were pre-treated with 0-80 µmol/L of phytic acid for 12 hours, and then treated with NNK (10µmol/L) for 24 and 48 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and calculated relative to the negative control wells (0µmol/L of phytic acid). The results represent the mean of at least three independent experiments.

Table 7 The inhibitory concentrations (IC) of γ-tocotrienol on the cell viability of NNK-treated A549 cells

The inhibitory concentration (IC)	γ-Tocotrienol Concentration (μmol/L)[a]	
	At 24 hours	At 48 hours
20	22.7±3.3	21.5±0.5
50	43.8±0.2	37.0±0.1
80	58.6±0.1	53.0±0.1

[a] Representation in mean ± standard deviation

3.7 Prevention of the NNK-increased cell proliferation of human lung cell line A549 by γ-tocotrienol but not phytic acid

To investigate the carcinogenic effect of NNK on the cell proliferation of human lung cells, the cell proliferation of A549 cells were measured by BrdU cell proliferation assay after 0-10 μmol/L of NNK treatment for 24 hours. As shown in Figure 37, treatment of NNK at concentrations of 10μmol/L significantly increased the cell proliferation in A549 cells at 24 hours (P<0.05) compared with NNK-untreated A549 cells.

To further investigate the preventive effect of γ-tocotrienol and phytic acid against the NNK-increased cell proliferation of A549 cells, the A549 cells were pre-treated with 0-80 μmol/L of γ-tocotrienol or phytic acid for 12 hours, and then treated with 10μmol/L of NNK for 24 hours. In comparison with NNK-treated A549 cells that were not pre-treatment with γ-tocotrienol or phytic acid, γ-tocotrienol but not phytic acid pre-treatment significantly inhibited the cell proliferation of NNK-treated A549 cells at 24 hours (P<0.001) as shown in Figure 38 and 39. The preventive effect of γ-tocotrienol was dose-response manner as shown in Table 8. The 20%- and 50%-inhibitory concentration values (IC20 and IC50, respectively) at 24 hours were 35.0±0.2 and 43.2±0.2 μmol/L, respectively.

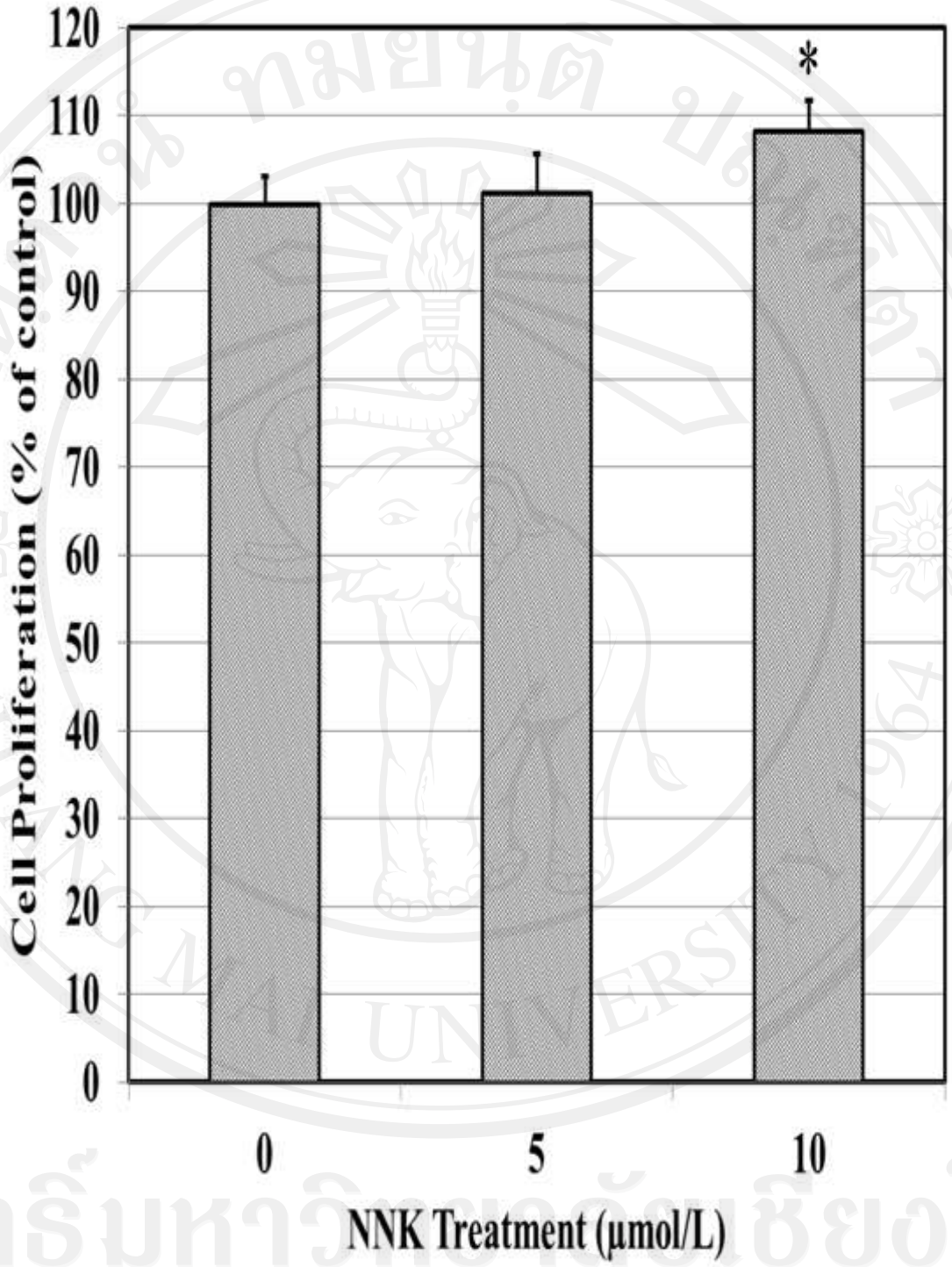

Figure 37 Effect of NNK on the cell proliferation of A549 cells. A549 cells were treated with 0-10 μmol/L of NNK for 24 hours. Each concentration was repeated in three wells. The cell proliferation was determined by BrdU cell proliferation assay and the values were calculated relative to the negative control wells (0μmol/L of NNK). * $P<0.05$, vs. control group.

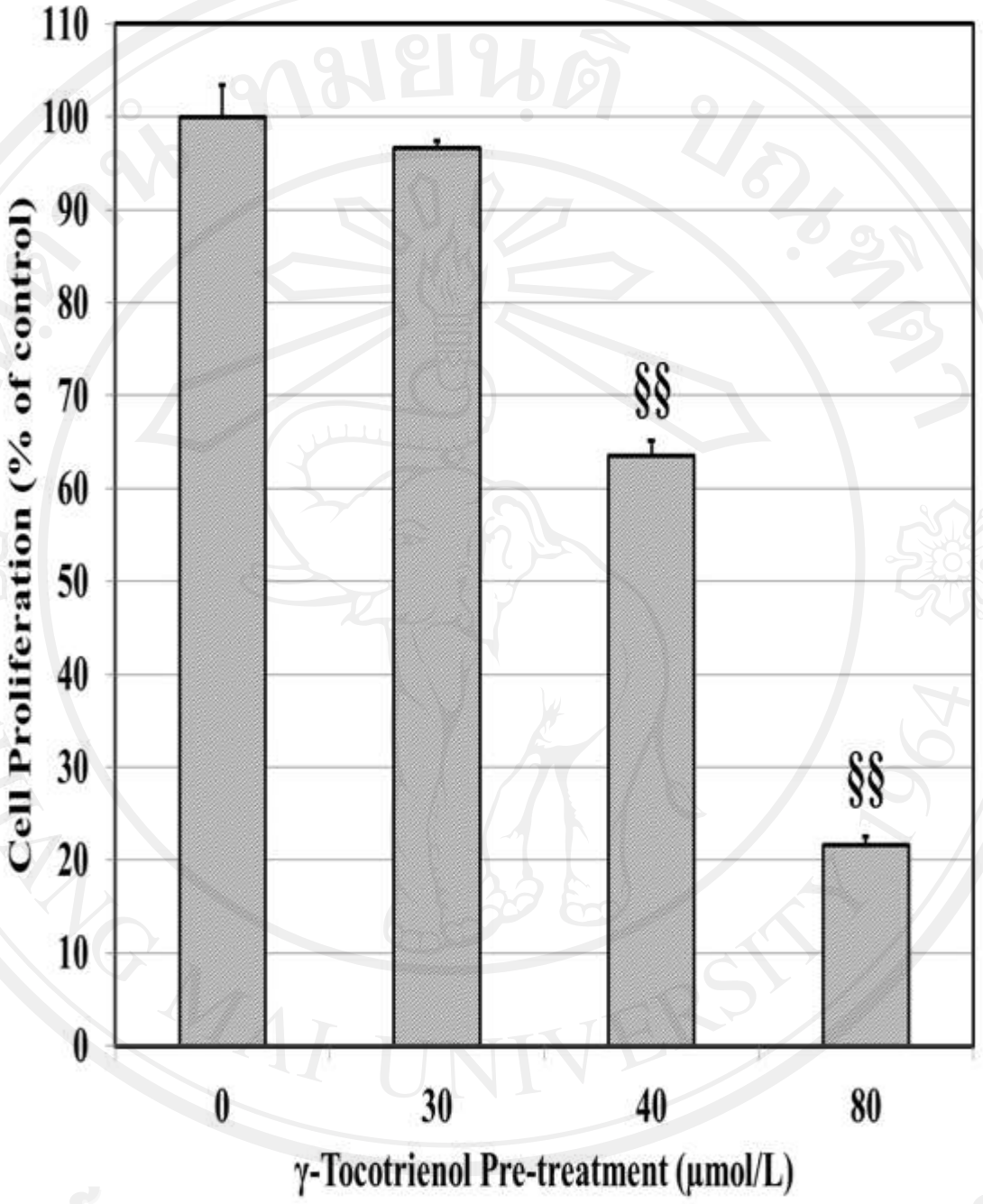

Figure 38 Inhibitory effect of γ-tocotrienol on the NNK-induced cell proliferation of A549 cells. A549 cells were pre-treated with 0-80 μmol/L of γ-tocotrienol for 12 hours, and then treated with NNK (10μmol/L) for 24 hours. Each concentration was repeated in three wells. The cell proliferation was determined by BrdU cell proliferation assay and the values were calculated relative to the negative control wells (0μmol/L of γ-tocotrienol). §§ $P<0.001$, vs. control group.

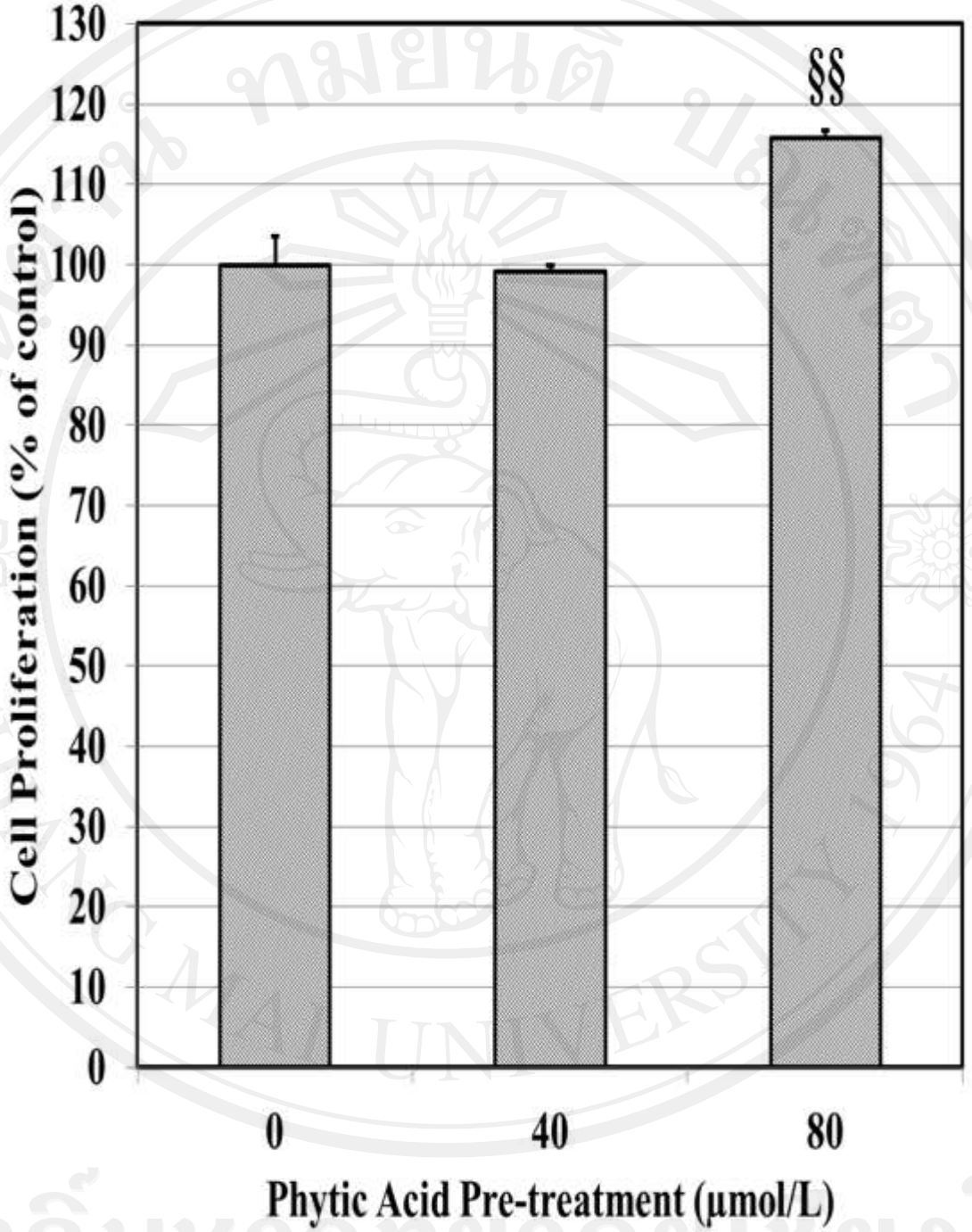

Figure 39 Effect of phytic acid on the NNK-induced cell proliferation of A549 cells. A549 cells were pre-treated with 0-80 µmol/L of phytic acid for 12 hours, and then treated with NNK (10µmol/L) for 24 hours. Each concentration was repeated in three wells. The cell proliferation was determined by BrdU cell proliferation assay and the values were calculated relative to the negative control wells (0µmol/L of phytic acid). §§ $P<0.001$, vs. control group.

Table 8 The inhibitory concentrations (IC) of γ-tocotrienol on the cell proliferation of NNK-treated A549

The inhibitory concentration (IC)	Concentration of γ-tocotrienol[a] At 24 hours
20	**35.0±0.2**
50	**43.2±0.2**
80	**> 80**

[a] Representation in mean ± standard deviation (μmol/L)

3.8 The cell cycle arrest activity of γ-tocotrienol in NNK-treated A549 cells

To investigate the effect of γ-tocotrienol on cell cycle distribution of NNK-treated A549 cells, the cell cycle distribution was analyzed by flow cytometry. A549 cells were pre-treated with 0-60 μmol/L of γ-tocotrienol for 12 hours, and then treated with 10μmol/L of NNK for 24 hours. In compared with NNK-treated A549 cells that were not pre-treatment with γ-tocotrienol, pre-treatment of γ-tocotrienol at concentrations of 30μmol/L significantly increased the G0/G1 cell cycle population in NNK-treated A549 cells ($P<0.05$) as shown in Figure 40. These results suggested that γ-tocotrienol induced cell cycle arrest at G0/G1 phase in NNK-treated A549 cells. Furthermore, pre-treatment of high dose of γ-tocotrienol, at concentrations of 40 and 60μmol/L significantly increased the subG1 cell cycle population in NNK-treated A549 cells. These results suggested that high dose of γ-tocotrienol induced cell death in NNK-treated A549 cells. Therefore, these results indicated that γ-tocotrienol induced cell death through G0/G1 cell cycle arrest.

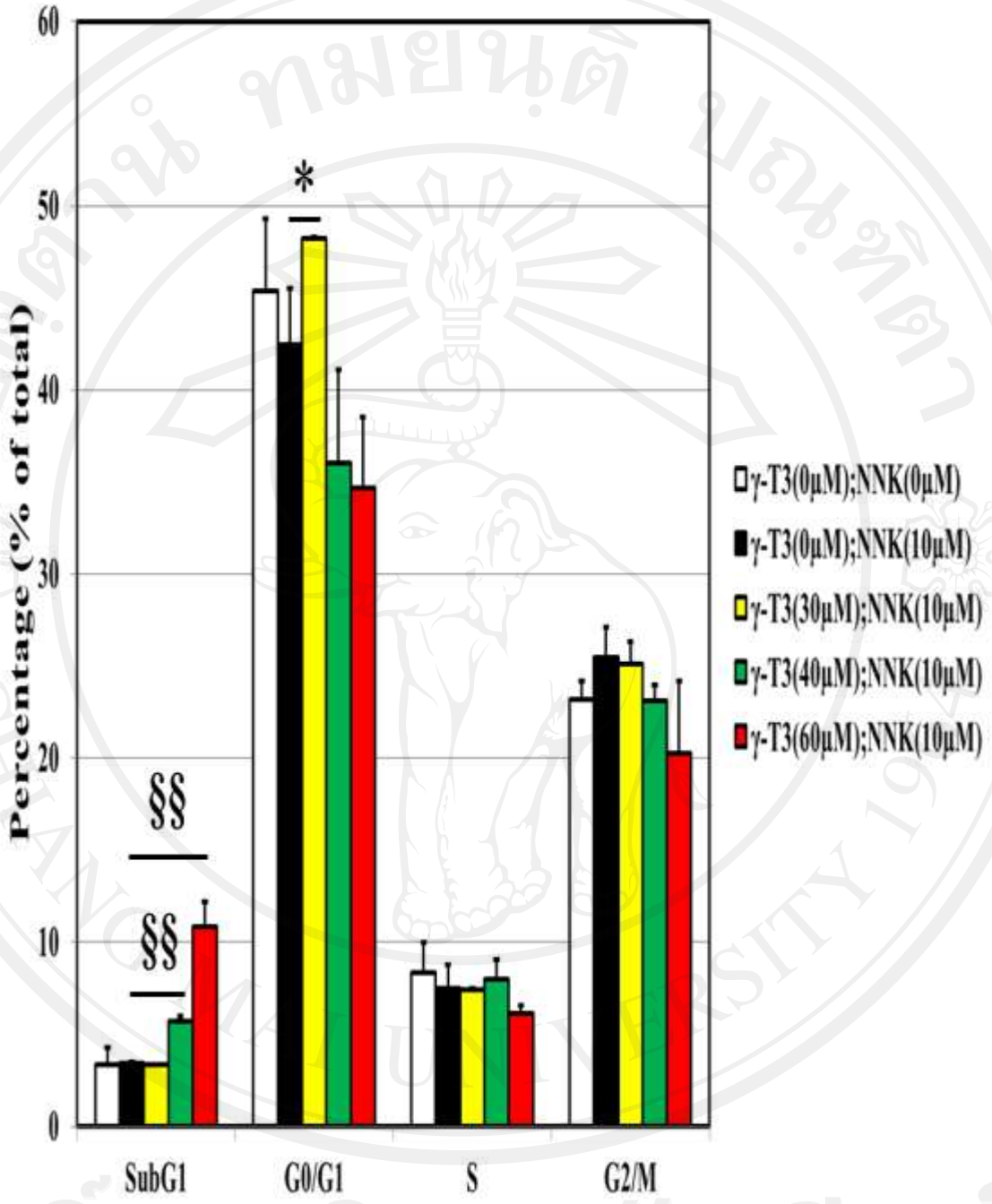

Figure 40 Effect of γ-tocotrienol on cell cycle distribution of NNK-treated A549 cells. A549 cells were pre-treated with 0-60 μmol/L of γ-tocotrienol for 12 hours, and then treated with 10μmol/L of NNK for 24 hours. Finally, the cells were harvested and the cell cycle distribution was analyzed by flow cytometry. The results represent the mean of at least three-independent experiments. * $P< 0.05$, and §§ $P<0.001$, vs. control group (γ-T3 at 0 μM and NNK at 10 μM).

3.9 Effect of NNK on the expression of cell cycle regulatory proteins and MAPK signal-transducing proteins in A549 cells

To investigate the carcinogenic effect of NNK on the expression of cell cycle regulatory proteins in A549 cells, the protein expression levels of proliferating cell nuclear antigen (PCNA), cyclinE and cyclinD1 were determined by western blotting. NNK treatment at concentrations of 10μmol/L induced the protein expression levels of PCNA, cyclinE and cyclinD1 in time-dependent manner as shown in Figure 41A. In addition, NNK treatment induced the protein expression levels of PCNA, cyclinE and cyclinD1 in dose-response manner as shown in Figure 41B and 41C.

To further investigate the effect of NNK on the mitogen-activated protein kinase (MAPK) pathway, which triggered the cell proliferation through increasing cyclinD1 protein, the protein expression levels of phosphorylated MEK1/2 (p-MEK1/2) and Kirsten rat sarcoma viral oncogene homolog (K-ras) were determined by western blotting. As shown in Figure 42A and 42B, NNK treatment at concentrations of 10μmol/L induced the expression levels of both p-MEK1/2 and K-ras proteins in time-dependent manner. These results suggested that NNK induced the expression of K-ras protein (during 15 minutes) prior to inducing expression of p-MEK1/2 protein (during 2 hours). In conclusion, these results indicated that NNK induced cell cycle progression through MAPK signaling.

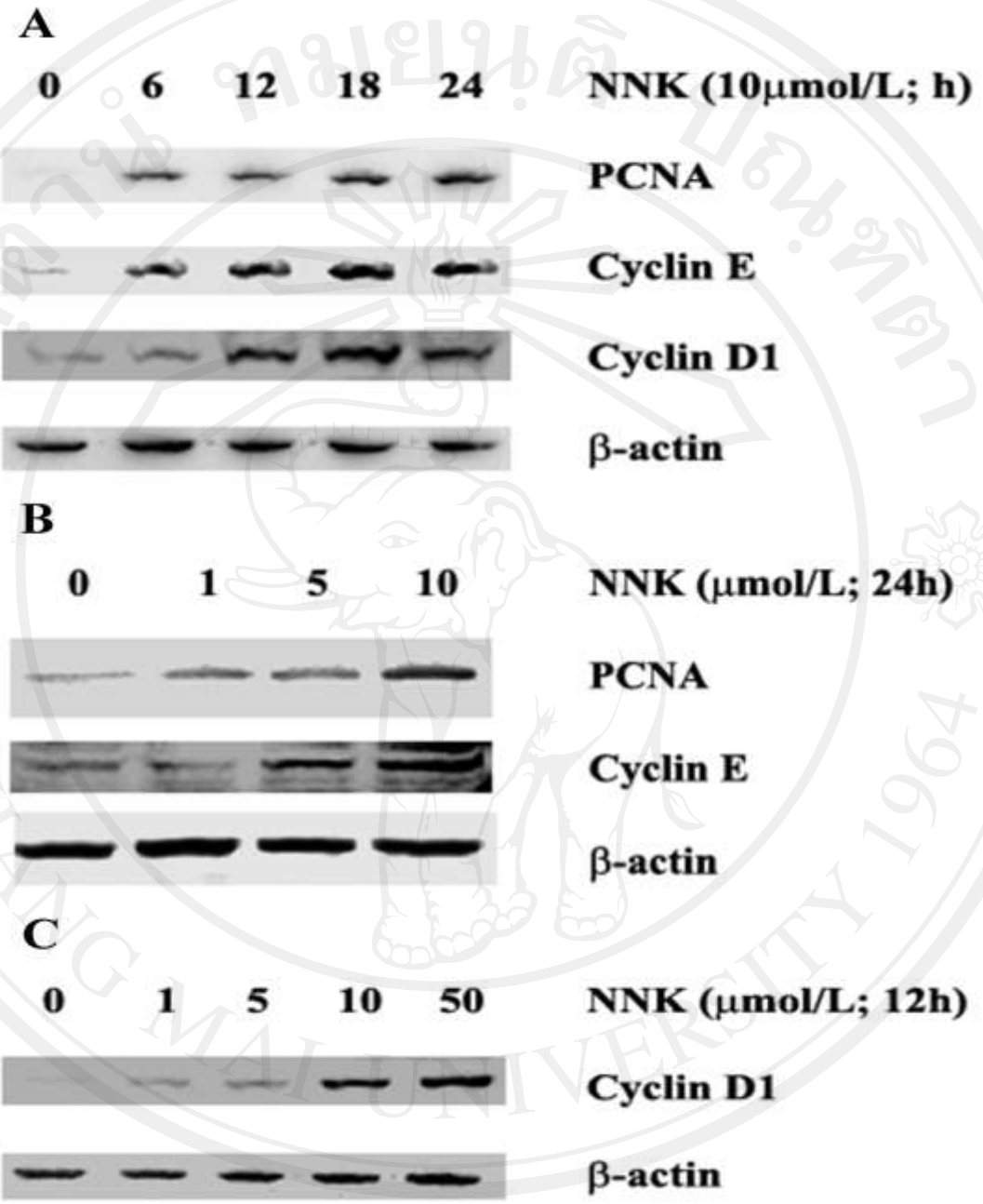

Figure 41 The carcinogenic effect of NNK on the expression of cell cycle regulatory proteins in A549 cells, demonstration in both time-dependent (A) and dose-response (B and C) manner. A549 cells were treated with indicated doses of NNK for indicated times, and then total cell lysates were harvested. The expression of cell cycle regulatory proteins such as PCNA, cyclinE and cyclinD1 was analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

A; The expression of cell cycle regulatory proteins such as PCNA, cyclinE and cyclinD1 after 10μmol/L of NNK treatment for 0-24 hours

B; The expression of cell cycle regulatory proteins such as PCNA and cyclinE after 0-10μmol/L of NNK treatment for 24 hours

C; The expression of cell cycle regulatory protein such as cyclinD1 after 0-50 μmol/L of NNK treatment for 12 hours

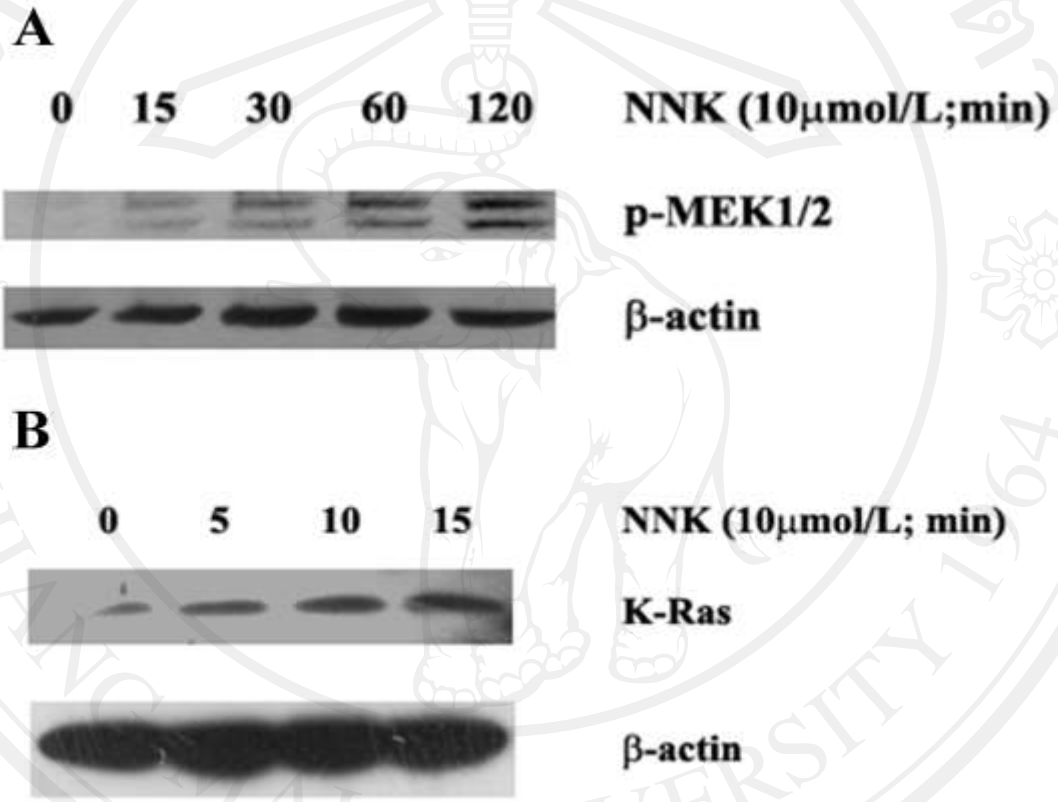

Figure 42 The effect of NNK on the expression of p-MEK1/2 (A) and K-ras (B) proteins in A549 cells, demonstration in time-dependent manner. A549 cells were treated with 10 μmol/L of NNK for indicated times, and then total cell lysates were harvested. The expression of the p-MEK1/2 and K-ras proteins was analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

A; The expression of p-MEK1/2 protein after 10 μmol/L of NNK treatment for 0-120 minutes

B; The expression of K-ras protein after 10 μmol/L of NNK treatment for 0-15 minutes

3.10 Preventive effect of γ-tocotrienol on the NNK-induced overexpression of cell cycle regulatory proteins and MAPK signal-transducing proteins in A549 cells

To investigate the chemopreventive effect of γ-tocotrienol on the NNK-induced overexpression of cell cycle regulatory proteins in A549 cells, the expression of PCNA, cyclinE and cyclinD1 proteins was determined by western blotting. Pre-treatment of γ-tocotrienol at concentrations of 30μmol/L for 12 hours, before 10μmol/L of NNK treatment, prevented the NNK-induced overexpression of PCNA, cyclinE and cyclinD1 proteins in time-dependent manner as shown in Figure 43A. Furthermore, γ-tocotrienol pre-treatment for 12 hours before 10μmol/L of NNK treatment also decreased the NNK-induced overexpression of PCNA, cyclinE and cyclinD1 proteins in dose-response manner as shown in Figure 43B and 43C.

To further investigate the effect of γ-tocotrienol on the NNK-induced overexpression of MAPK signal-transducing proteins in A549 cells, the expression of p-MEK1/2 and K-ras proteins was determined by western blotting. As shown in Figure 44A and 44B, γ-tocotrienol pre-treatment for 12 hours before 10μmol/L of NNK treatment only suppressed the NNK-induced overexpression of p-MEK1/2 protein but not K-ras protein in dose-response manner. These results suggested that the chemopreventive effect of γ-tocotrienol against the NNK-induced cell cycle progression was involved in suppressing the NNK-induced MAPK signal transduction due to down-regulation of p-MEK1/2 protein.

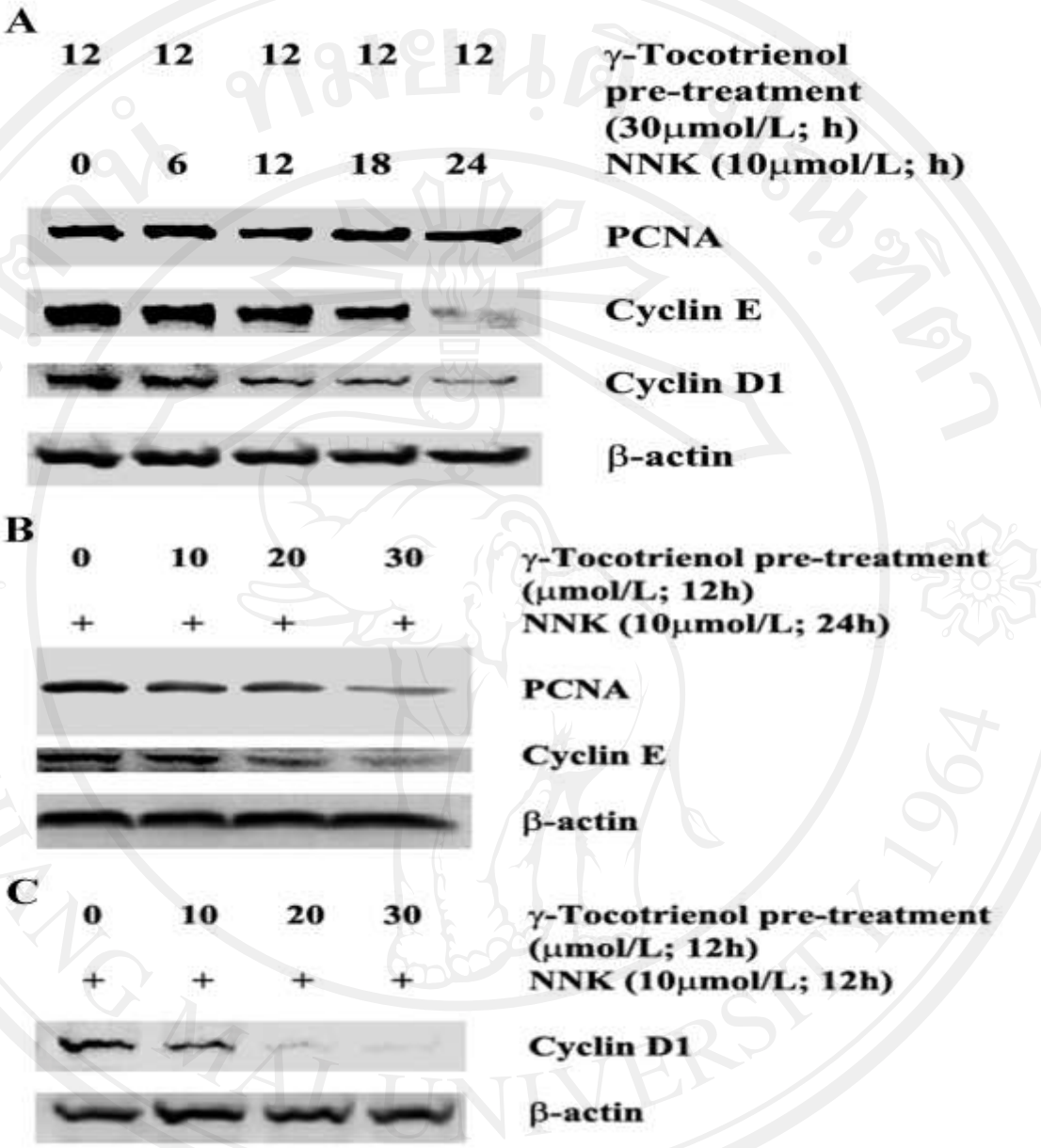

Figure 43 The preventive effect of γ-tocotrienol on the NNK-induced overexpression of cell cycle regulatory proteins in A549 cells, demonstration in both time-dependent (A) and dose-response (B and C) manner. A549 cells were pre-treated with indicated doses of γ-tocotrienol for 12 hours, and then treated with 10μmol/L of NNK for indicated times. Total cell lysates were harvested and analyzed the expression of the cell cycle regulatory proteins, such as PCNA, cyclinE and cyclinD1 by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

A; The expression of the cell cycle regulatory proteins, such as PCNA, cyclinE and cyclinD1 in A549 cells pre-treated with 30μmol/L of γ-tocotrienol for 12 hours before treatment of NNK at concentrations of 10μmol/L for 0-24 hours

B; The expression of the cell cycle regulatory proteins, such as PCNA and cyclinE in A549 cells pre-treated with 0-30μmol/L of γ-tocotrienol for 12 hours before treatment of NNK at concentrations of 10μmol/L for 24 hours

C; The expression of the cell cycle regulatory proteins, such as cyclinD1 in A549 cells pre-treated with 0-30μmol/L of γ-tocotrienol for 12 hours before treatment of NNK at concentrations of 10μmol/L for 12 hours

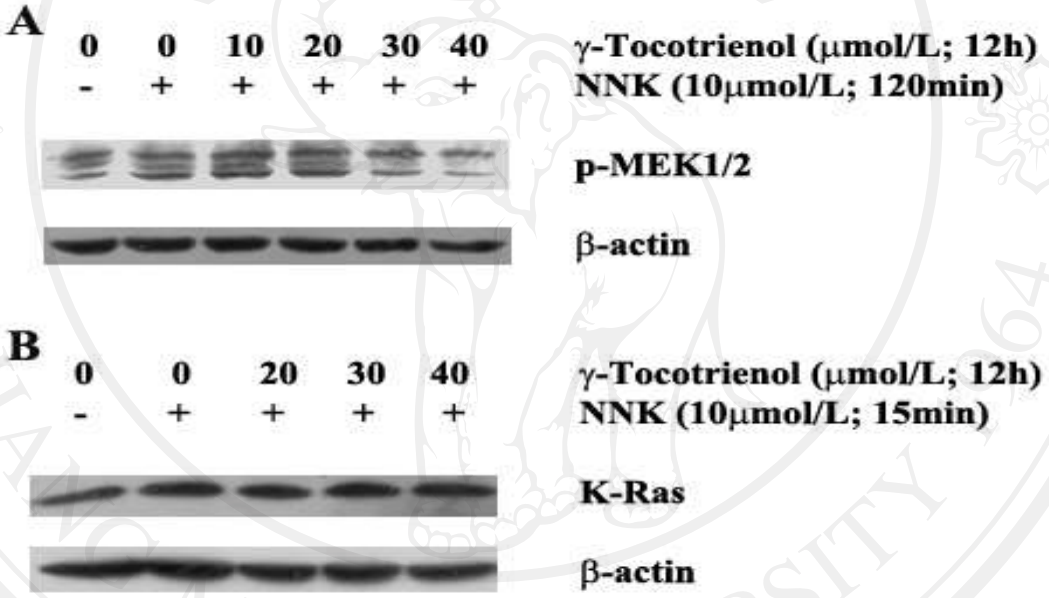

Figure 44 The preventive effect of γ-tocotrienol on the NNK-induced over-expression of p-MEK1/2 (A) and K-ras (B) proteins in A549 cells, demonstration in dose-response manner. A549 cells were pre-treated with indicated doses of γ-tocotrienol for 12 hours, and then treated with 10μmol/L of NNK for indicated times. Total cell lysates were harvested and analyzed the expression of p-MEK1/2 and K-ras proteins by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

A; The expression of p-MEK1/2 protein in A549 cells pre-treated with 0-40μmol/L of γ-tocotrienol for 12 hours before 10μmol/L of NNK treatment for 120 minutes

B; The expression of K-ras protein in A549 cells pre-treated with 0-40μmol/L of γ-tocotrienol for 12 hours before of 10 μmol/L NNK treatment for 15 minutes

3.11 Effect of γ-tocotrienol and phytic acid on the NNK-induced DNA damage in A549 cells

NNK has been shown to induce DNA damage and leads to gene mutation, which contributed to lung tumorigenesis (311). To examine the chemopreventive effect of γ-tocotrienol and phyic acid on the NNK-induced DNA damage in A549 cells, comet assay was used to monitor the DNA damage in NNK-treated A549 cells. The A549 cells were pre-treated with 30 μmol/L of γ-tocotrienol or 80 μmol/L of phytic acid for 12 hours, and then treated with 10μmol/L of NNK for 3 hours. Treatment of cells with 10μmol/L of NNK for 3 hours clearly induces DNA damage as determined by the Comet Assay (Figure 45B) in compared with NNK-untreated cells (Figure 45A). However, pre-treatment with both γ-tocotrienol (30 μmol/L) or phytic acid (80 μmol/L) for 12 hours in NNK-treated A549 cells had no effected on NNK-induced DNA damage as shown in Figure 45C and 45D. These results suggested that the chemopreventive effect of γ-tocotrienol against NNK-induced lung cell proliferation was not involved in the prevention of NNK-induced DNA damage.

3.12 Effect of γ-tocotrienol and phytic acid on the NNK-induced DNA methyl transferase 1 (DNMT1) nuclear accumulation in A549 cells

Hypermethylation at multiple promoters of tumor suppressor genes is founded in various types of cancer. NNK has been shown to induce DNMT1 nuclear accumulation and subsequent hypermethylation on the promoter of many tumor suppressor genes in lung cancer (98). To investigate that NNK induced DNMT1 accumulation in the nucleus of A549 cells, the cells were treated with 10μmol/L of NNK for 0-180 minutes, and then the nuclear accumulation of DNMT1 was analyzed by western blotting. After 10μmol/L of NNK treatment, the nuclear accumulation of DNMT1 was induced with increasing time of NNK exposure as shown in Figure 46.

To investigate the chemopreventive effect of γ-tocotrienol and phyic acid against the NNK-induced DNMT1 nuclear accumulation in A549 cells, the cells were pre-treated with 30μmol/L of γ-tocotrienol or 80μmol/L of phytic acid for 12 hours before treatment with 10μmol/L of NNK for 0-180 minutes. Pre-treatment with γ-tocotrienol (Figure 47) but not phytic acid (Figure 48) prevented the accumulation of DNMT1 in the nucleus of NNK-treated A549 cells.

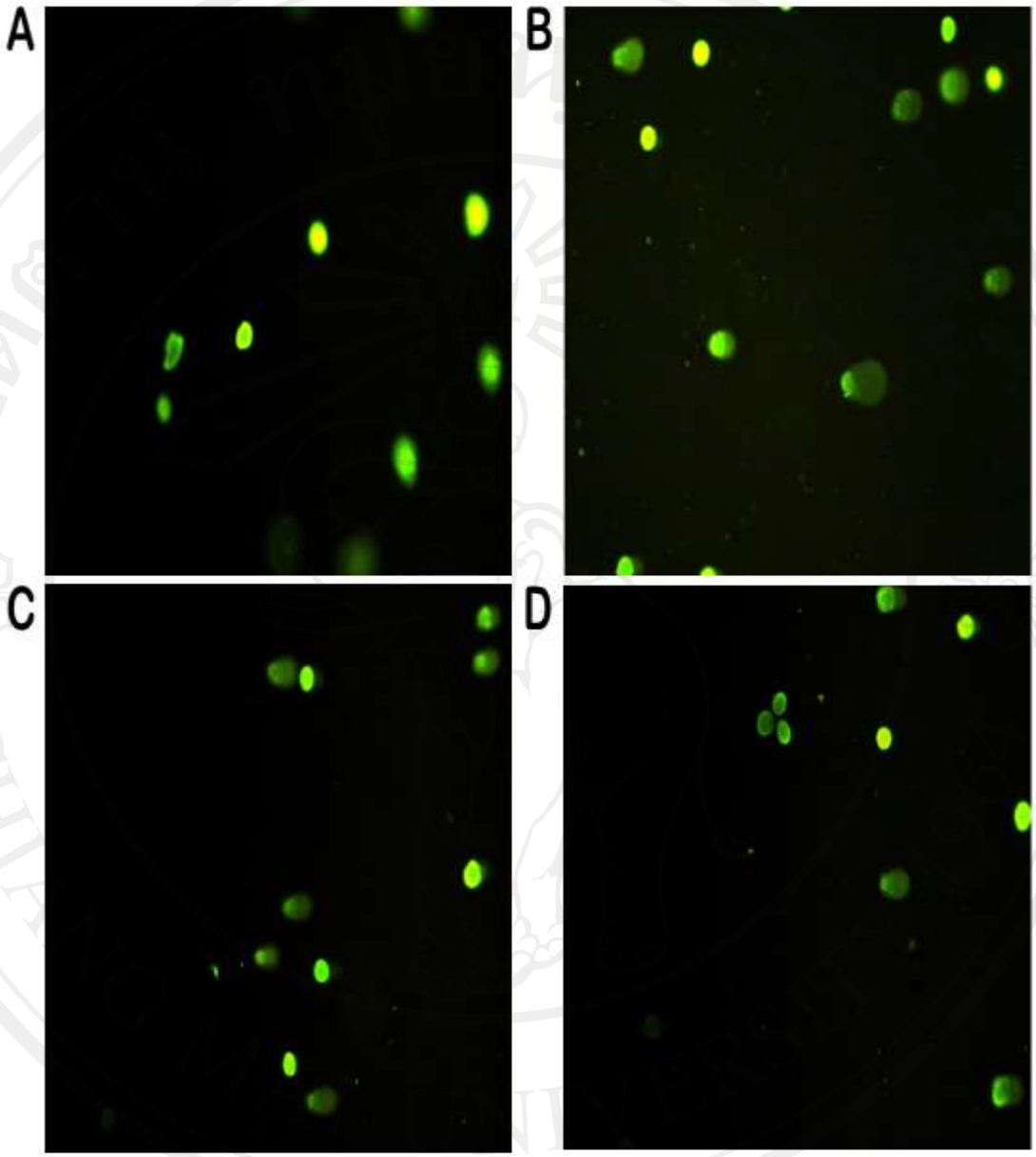

Figure 45 Effects of γ-tocotrienol or phytic acid pre-treatment on the NNK-induced DNA damage in A549 cells. DNA damage was analyzed by the Comet Assay.

A; Control NNK-untreated A549 cells

B; A549 cells were treated 10μmol/L of NNK for 3 hours

C; A549 cells were pre-treated with 30μmol/L of γ-tocotrienol for 12 hours, and then treated with 10μmol/L of NNK for 3 hours

D; A549 cells were pre-treated with 80μmol/L of phytic acid for 12 hours, and then treated with 10μmol/L of NNK for 3 hours

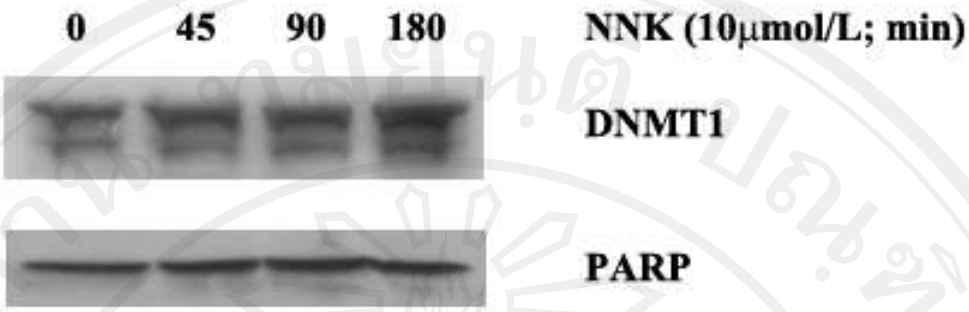

Figure 46 Effect of NNK treatment on the DNA methyl transferase 1 (DNMT1) nuclear accumulation in A549 cells. A549 cells were treated with 10μmol/L of NNK for 0-180 minutes. The nuclear lysates were collected and analysed the levels of DNMT1 protein in the nucleus by western blotting. These results were representative of 3 independent experiments with similar results and PARP was used as a loading control.

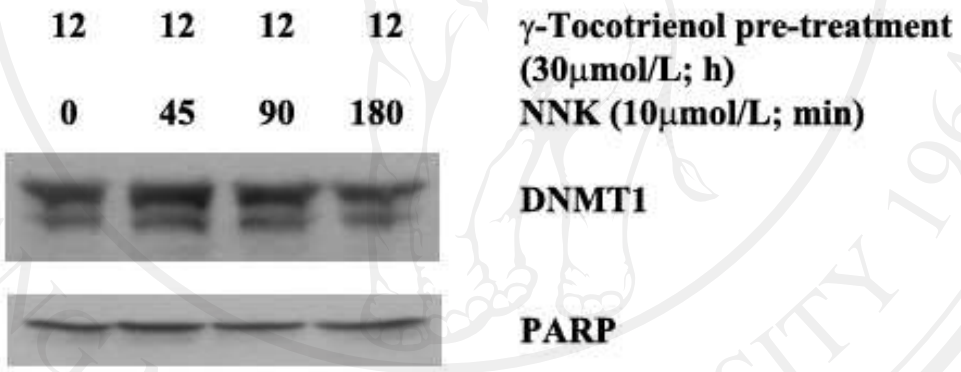

Figure 47 Preventive effect of γ-tocotrienol pre-treatment on the NNK-induced DNMT1 nuclear accumulation in A549 cells. A549 cells were pre-treated with 30μmol/L of γ-tocotrienol for 12 hours before treatment with 10μmol/L of NNK for 0-180 minutes. The nuclear lysates were collected and analysed the levels of DNMT1 protein in the nucleus by western blotting. These results were representative of 3 independent experiments with similar results and PARP was used as a loading control.

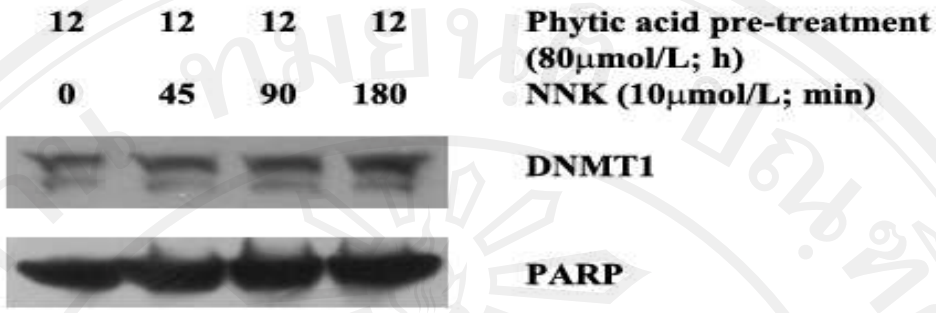

Figure 48 Effect of phytic acid pre-treatment on the NNK-induced DNMT1 nuclear accumulation in A549 cells. A549 cells were pre-treated with 80μmol/L of phytic acid for 12 hours before treatment with 10μmol/L of NNK for 0-180 minutes. The nuclear lysates were collected and analysed the levels of DNMT1 protein in the nucleus by western blotting. These results were representative of 3 independent experiments with similar results and PARP was used as a loading control.

3.13 Effect of γ-tocotrienol and phytic acid on the NNK-induced cytochrome P450 isotype 2A6 (CYP2A6) and isotype 2A13 (CYP2A13) overexpression in A549 cells

Cytochrome P450 or CYP plays a central role in the enzymatic activation of many environmental chemicals to generate reactive intermediates. CYP2A6 and CYP2A13 enzymes have been shown to play a major role in NNK metabolism and activation in human (17, 19, 87). To examine the effect of NNK on the expression of cytochrome P450 isotypes 2A6 (CYP2A6) and 2A13 (CYP2A13) in A549 cells, western blotting was used to monitor the expression of CYP2A6 and CYP2A13 proteins in NNK-treated A549 cells. Treatment of A549 cells with increasing concentrations of NNK induced the overexpression of both cytochrome P450 isotype 2A6 (CYP2A6) and isotype 2A13 (CYP2A13) as shown in Figure 49.

To further investigate the effect of γ-tocotrienol and phytic acid on the NNK-induced overexpression of CYP2A6 and CYP2A13 proteins in A549 cells, the cells were pre-treated with 0-30 μmol/L of γ-tocotrienol or 0-80 μmol/L of phytic acid for 12 hours before treatment with 10μmol/L of NNK for 12 hours. Pre-treatment with γ-tocotrienol but not phytic acid decreased the overexpression of CYP2A6 and CYP2A13 proteins in NNK-treated A549 cells as shown in Figure 50 and 51. These decreasing activities of γ-tocotrienol were dose-response manner.

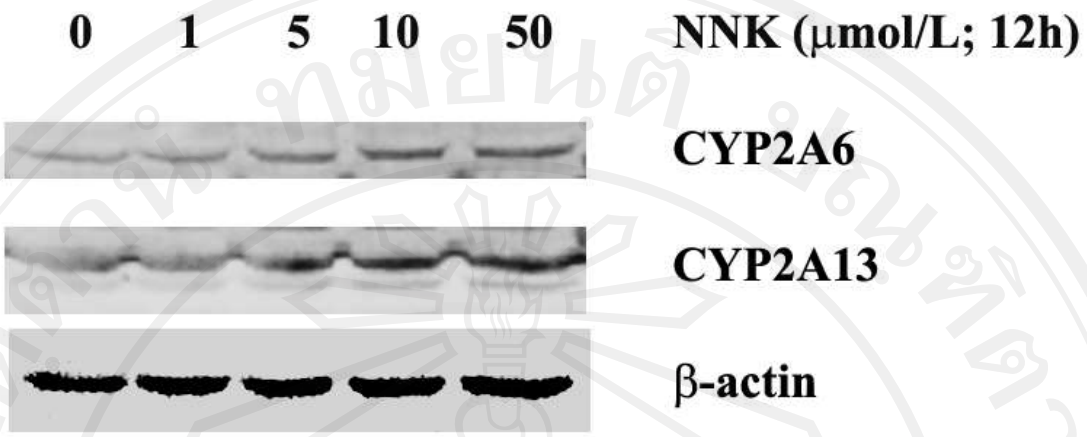

Figure 49 Effect of NNK on the expression of cytochrome P450 isotypes 2A6 (CYP2A6) and 2A13 (CYP2A13) proteins in A549 cells. A549 cells were treated with 0-50 μmol/L of NNK for 12 hours. The expression of CYP2A6 and CYP2A13 proteins was analysed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

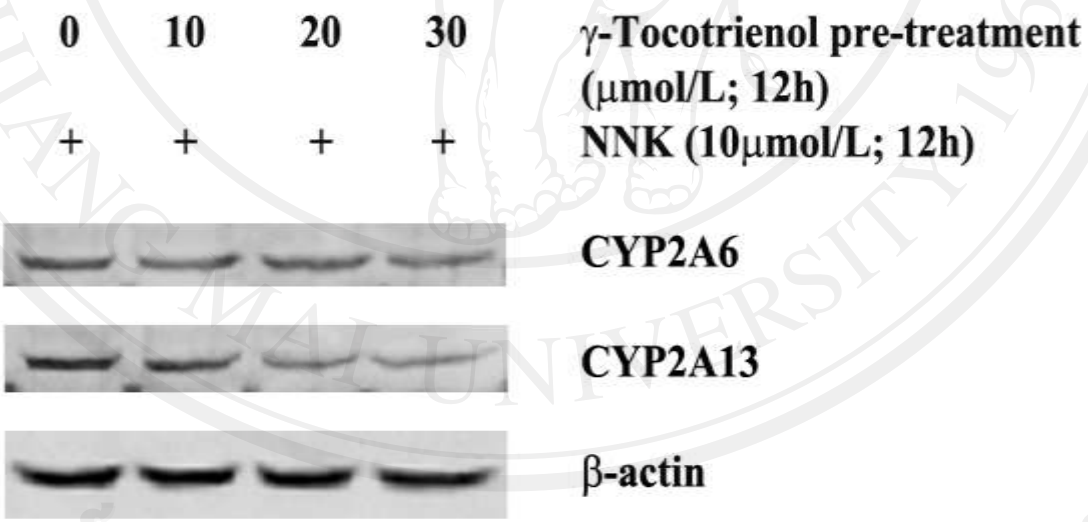

Figure 50 Suppressive effect of γ-tocotrienol pre-treatment on the NNK-induced overexpression of CYP2A6 and CYP2A13 proteins in A549 cells. A549 cells were pre-treated with 0-30 μmol/L of γ-tocotrienol for 12 hours before treatment with 10μmol/L of NNK for 12 hours. Total cell lysates were collected and the expression of CYP2A6 and CYP2A13 proteins was analysed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

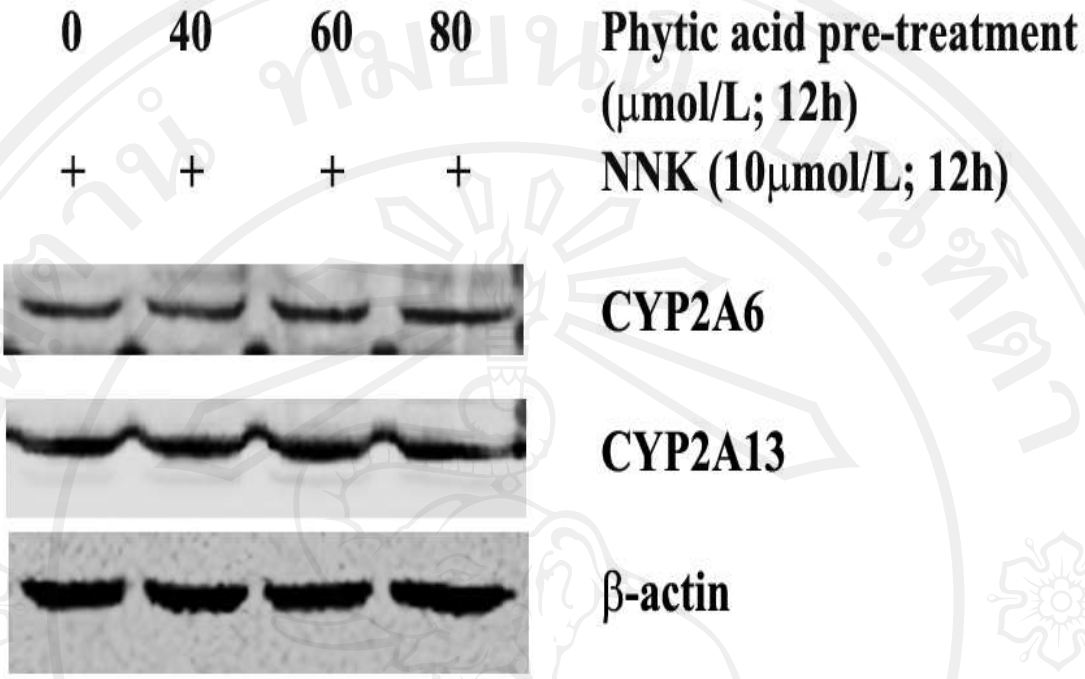

Figure 51 Effect of phytic acid pre-treatment on the NNK-induced overexpression of CYP2A6 and CYP2A13 proteins in A549 cells. A549 cells were pre-treated with 0-80 μmol/L of phytic acid for 12 hours before treatment with 10μmol/L of NNK for 12 hours. The expression of CYP2A6 and CYP2A13 proteins was analysed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

3.14 Effect of NNK on the mRNA expression from *CYP2A6* and *CYP2A13* genes in A549 cells

To further investigate the effect of NNK on the mRNA expression from *CYP2A6* and *CYP2A13* genes in A549 cells, quantitative real-time RT-PCR was used to monitor the mRNA expression from *CYP2A6* and *CYP2A13* genes in NNK-treated A549 cells. With increasing times of treatment from 0-12 hours, treatment with 10μmol/L of NNK had no significantly effect on the mRNA expression from both *cytochrome P450 isotype 2A6 (CYP2A6)* and *cytochrome P450 isotype 2A13 (CYP2A13)* genes in A549 cells as shown in Figure 52.

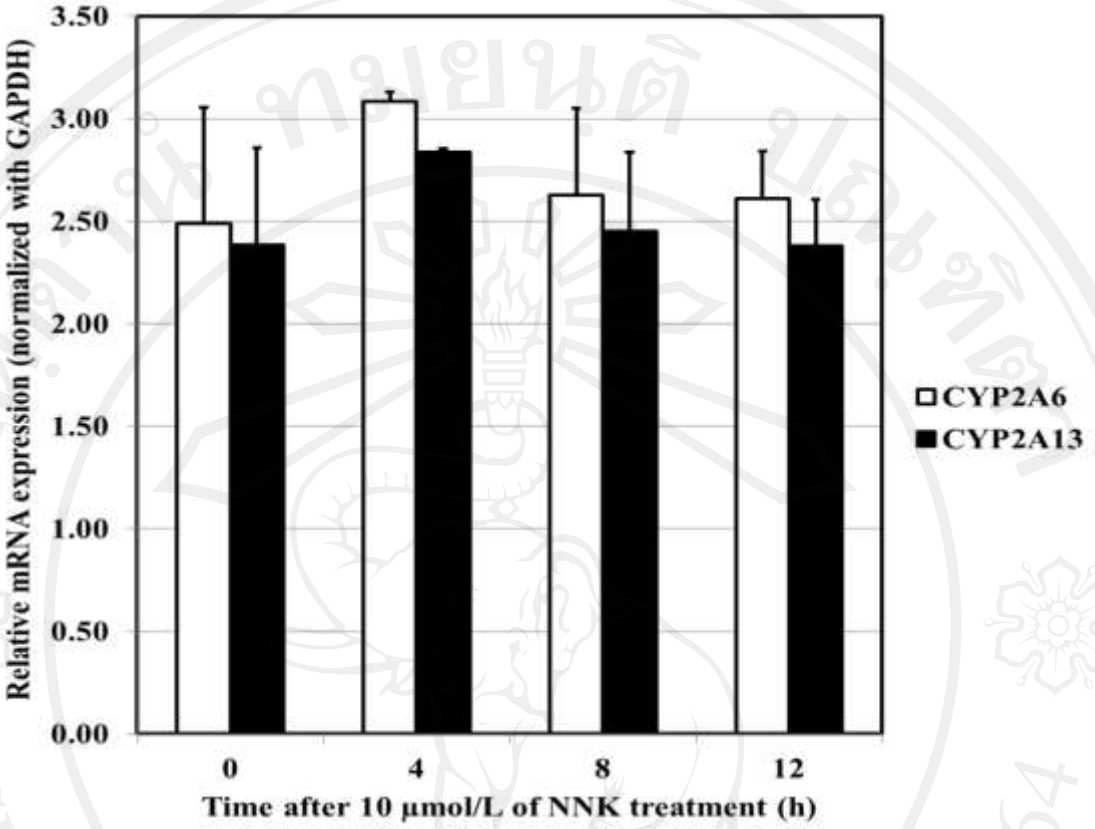

Figure 52 Effect of NNK on the mRNA expression from *CYP2A6* and *CYP2A13* genes in A549 cells. A549 cells were treated with 10µmol/L of NNK for 0-12 hours. CYP2A6 and CYP2A13 mRNA were isolated and analyzed by quantitative real-time RT-PCR. These results were representative of mean ± SD from 3 independent experiments. The relative mRNA expression value of *CYP2A6* and *CYP2A13* genes was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.15 Decreasing cell viability of A549 cells by γ-tocotrienol but not phytic acid

To investigate the effect of γ-tocotrienol (γ-T_3) and phytic acid (IP_6) on the cell viability of A549 cells, MTT assay was used to measure the cell viability of A549 cells. The A549 cells were treated with 0-90 µmol/L of γ-tocotrienol or 0-200 µmol/L of phytic acid for 12, 24, 36 and 48 hours. As shown in Figure 53 and 54, γ-tocotrienol but not phytic acid significantly decreased the cell viability of A549 cells at 12, 24, 36 and 48 hours. The suppressive effect of γ-tocotrienol was both dose-response and time-dependent manner as shown in Table 9. The half maximal inhibitory concentration value or IC50 at 12, 24, 36 and 48 hours were 48.4±1.0, 39.5±0.9, 34.8±0.7 and 33.3±0.5, respectively.

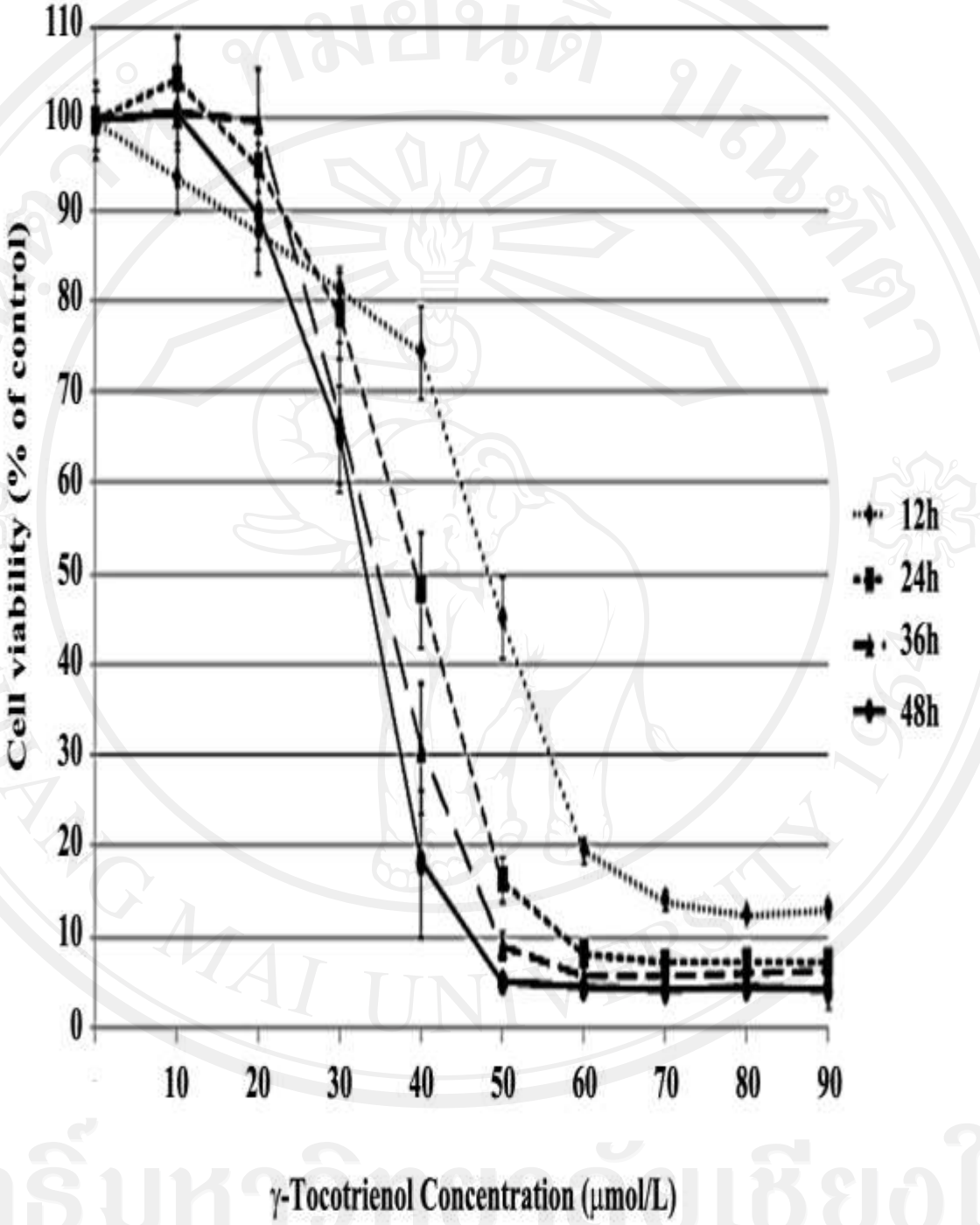

Figure 53 Suppressive effect of γ-tocotrienol on the cell viability of A549 cells. A549 cells were treated with 0-90 μmol/L of γ-tocotrienol for 12, 24, 36 and 48 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and calculated relative to the negative control wells (0μmol/L of γ-tocotrienol). The results represent the mean of at least three independent experiments.

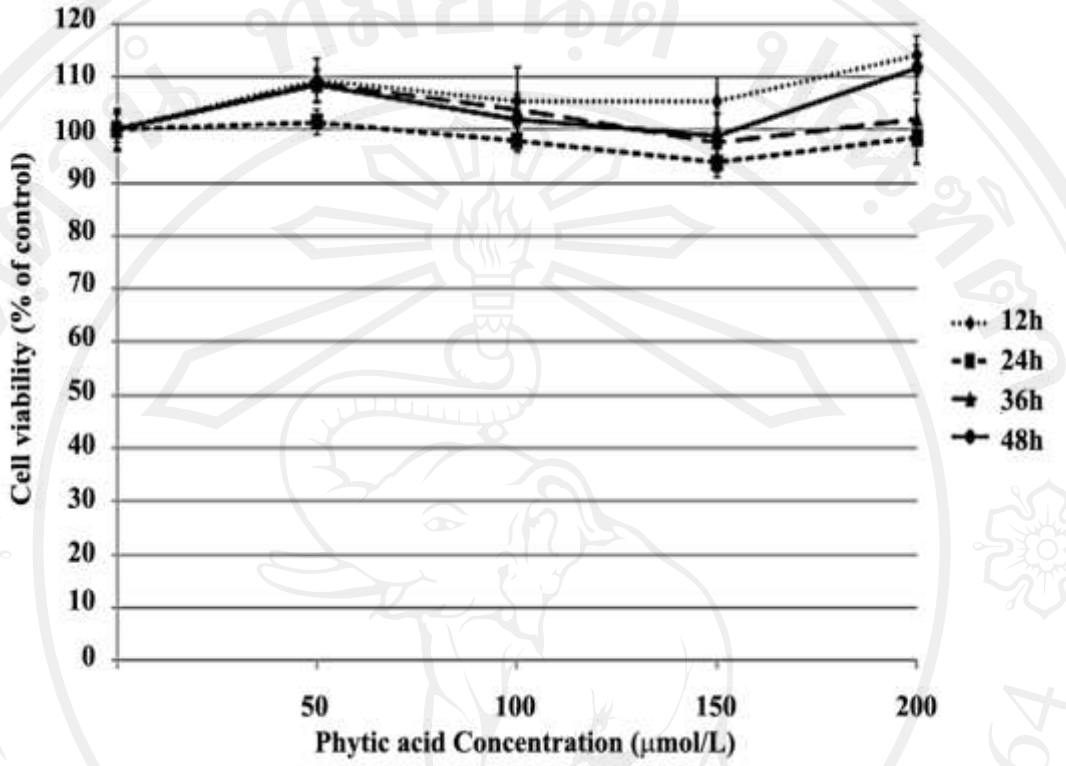

Figure 54 Effect of phytic acid on the cell viability of A549 cells. A549 cells were treated with 0-200 μmol/L of phytic acid for 12, 24, 36 and 48 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and calculated relative to the negative control wells (0μmol/L of phytic acid). The results represent the mean of at least three independent experiments.

Table 9 The inhibitory concentrations (IC) of γ-tocotrienol in A549 cells

The inhibitory concentration (IC)	γ-Tocotrienol Concentration (μmol/L)[a]			
	12 hours	24 hours	36 hours	48 hours
20	32.1±1.2	29.2±1.0	26.1±0.8	24.0±0.8
50	48.4±1.0	39.5±0.9	34.8±0.7	33.3±0.5
80	59.9±0.2	48.9±0.1	45.1±0.1	39.6±0.1

[a] Representation in mean ± standard deviation

3.16 Decreasing cell proliferation of A549 cells by γ-tocotrienol

To investigate the effect of γ-tocotrienol on the cell proliferation of A549 cells, A549 cells were treated with 0-100 μmol/L of γ-tocotrienol for 24 hours before analyzed for cell proliferation by BrdU cell proliferation assay. After treatment with γ-tocotrienol for 24 hours, the cell proliferation of A549 cells were significantly decreased in dose-response manner as shown in Figure 55. The half maximal inhibitory concentration or IC50 of γ-tocotrienol for the inhibition of A549 cell proliferation at 24 hours was 55.63±0.56 μmol/L.

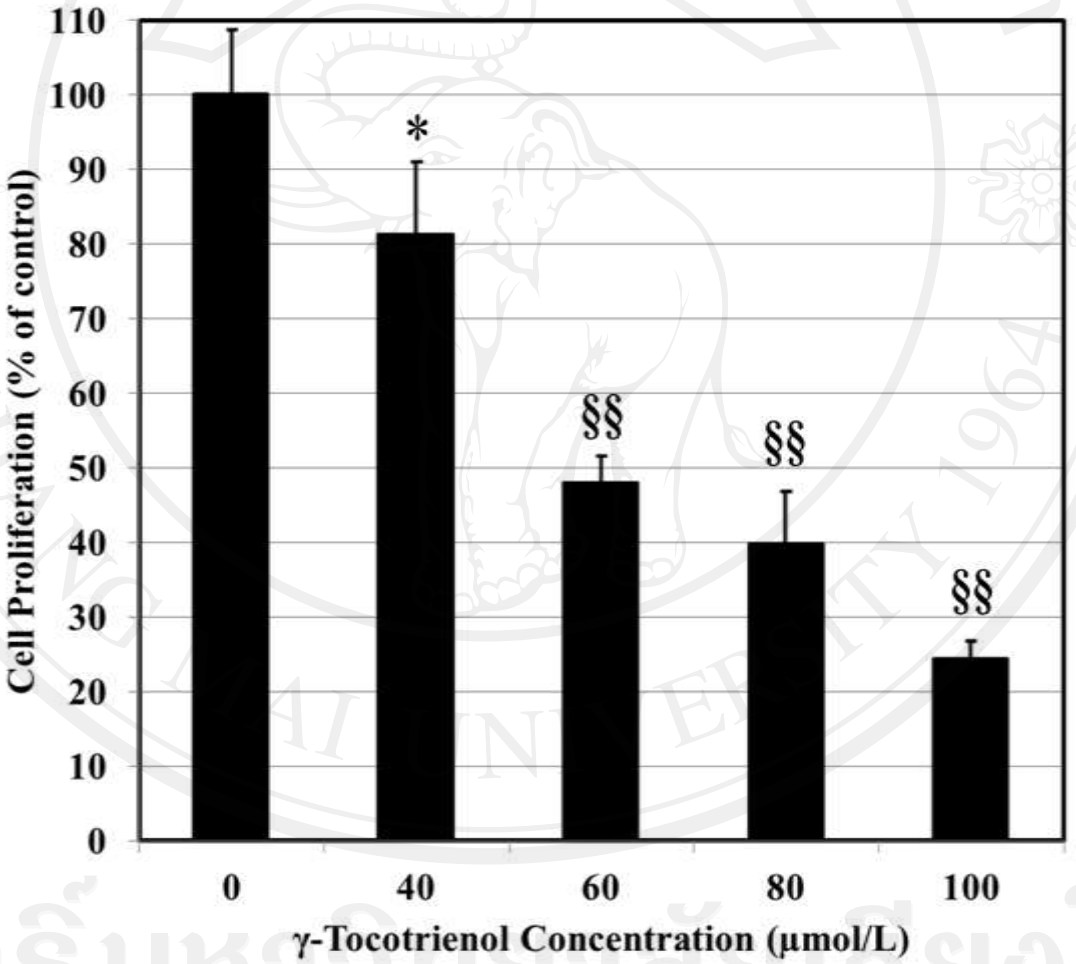

Figure 55 Effect of γ-tocotrienol on the cell proliferation of A549 cells. A549 cells were treated with 0-100 μmol/L of γ-tocotrienol for 24 hours. Each concentration was repeated in three wells. The cell proliferation was determined by BrdU cell proliferation assay and the values were calculated relative to the negative control group. * $P<0.05$, and §§ $P<0.001$, vs. control group (0μmol/L of γ-tocotrienol)

3.17 Induction of G0/G1 cell cycle arrest in A549 cells by γ-tocotrienol

To determine whether the inhibitory effect γ-tocotrienol on the cell proliferation of A549 cells was due to the cell cycle arrest activity, the cell cycle distribution of γ-tocotrienol-treated A549 cells was analyzed by flow cytometry. After γ-tocotrienol treatment for 24 hours, the cell cycle distribution of A549 cells was significantly changed as shown in Figure 56. Treatment of γ-tocotrienol at concentrations of 60μmol/L significantly increased the G0/G1 phase population and decreased the G2/M (mitosis) phase population, while treatment of γ-tocotrienol at concentrations of 40μmol/L had no significantly effect on cell cycle distribution. These results suggested that γ-tocotrienol treatment induced G0/G1 cell cycle arrest in A549 cells.

The cyclinD1/cyclin-dependent kinase (Cdk) complex has been identified as the G1 phase cell cycle regulatory proteins. Moreover, the downregulation of cyclinD1 protein expression leads to G0/G1 cell cycle arrest and decreases the proliferation rate (108). To further investigated the effect of γ-tocotrienol on cyclinD1 protein expression in A549 cells, western blotting was used to monitor the expression of cyclinD1 protein in γ-tocotrienol-treated A549 cells. The treatment of γ-tocotrienol for 24 hours decreased the expression of cyclinD1 protein in a dose-response manner as shown in Figure 57. These results indicated that γ-tocotrienol induced G0/G1 cell cycle arrest in A549 cells through the downregulation of cyclinD1 protein expression.

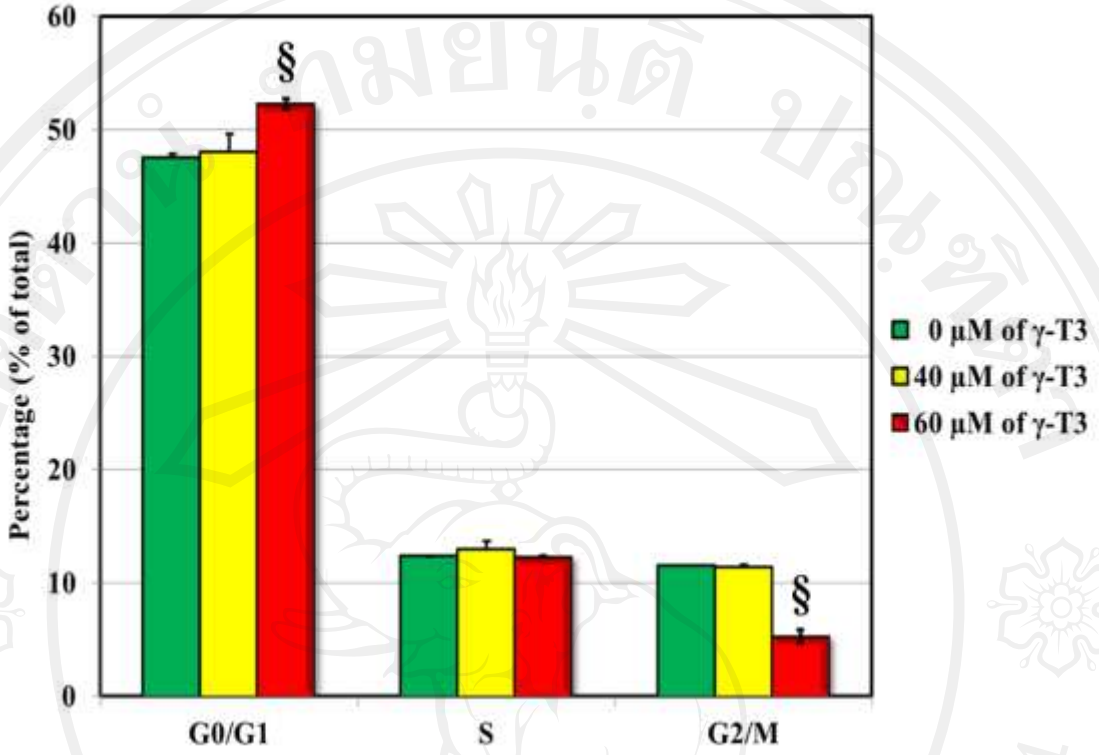

Figure 56 Effect of γ-tocotrienol on the cell cycle distribution of A549 cells. A549 cells were treated with 0, 40, and 60µmol/L of γ-tocotrienol for 24 hours and the cell cycle distribution was analyzed by flow cytometry. The results represent the mean of at least three-independent experiments. § P<0.005, vs. control group (0µM of γ-T_3)

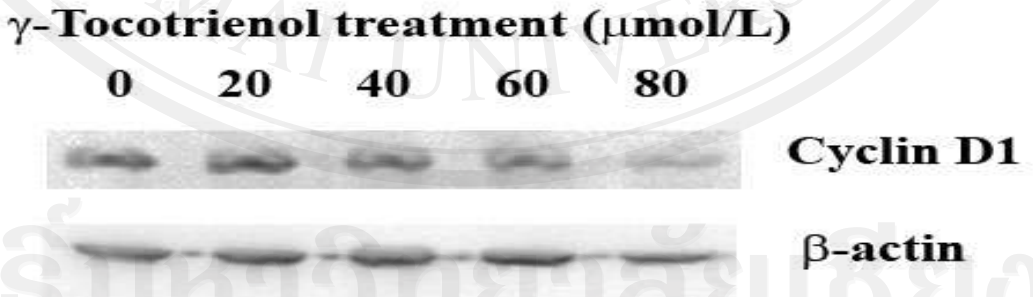

Figure 57 Effect of γ-tocotrienol on the expression of cyclin D1 protein. A549 cells were treated with 0-80 µmol/L of γ-tocotrienol for 24 hours. The total cell lysates were collected and analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

3.18 Induction of apoptosis in A549 cells by γ-tocotrienol

To determine whether the inhibitory effect γ-tocotrienol on cell viability of A549 cells was due to the induction of apoptosis, the morphological changes in γ-tocotrienol-treated A549 cells were observed by the scanning electron microscopy (SEM) technique. As shown in Figure 58, increasing times of γ-tocotrienol treatment (60μmol/L) clearly changed the morphology of A549 cells. The condensation of the nucleus and the loss of microvilli were appeared after γ-tocotrienol treatment for 12 hours. After 24 hours, the cell became spherical as its cytoskeleton, which holds the cell shape, was digested. Moreover, the nucleus condensed completely and segregated into several fragments. Finally, the cell broke into several vesicles (apoptotic bodies) and these blebs or vesicles were formed on its surface after 36 hours of γ-tocotrienol treatment.

To further determine the other signature event of apoptosis in γ-tocotrienol-treated A549 cells, flow cytometry was used to analyze the translocation of phosphatidylserine (PS) from inner to the outer membrane surface, which is a signature event of early apoptosis. Treatment of cells with 60 μmol/L of γ-tocotrienol significantly increased the percentage of cells in early apoptosis phase and significantly decreased the percentage of viable cells in time-dependent manner (24 and 48 hours) compared with the untreated cells (0 hour) at $P<0.01$ as shown in Figure 59. Moreover, treatment with 60μmol/L of γ-tocotrienol also significantly increased the percentage of cells in late apoptosis phase in time-dependent manner at $P<0.01$.

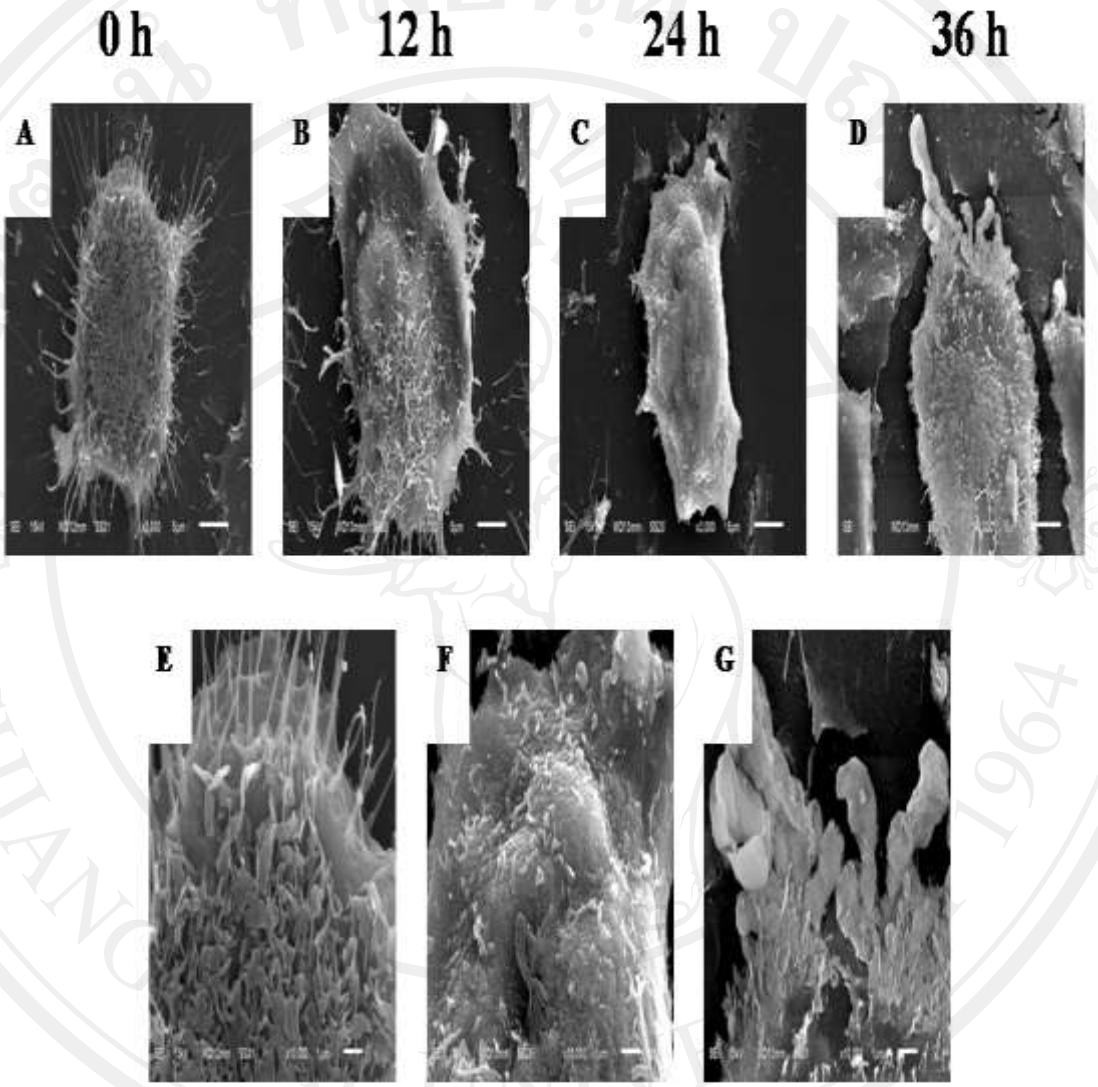

Figure 58 The morphological changes in γ-tocotrienol-treated A549 cells. The A549 cells were treated with γ-tocotrienol at concentrations of 60μmol/L for the indicated periods of time. Samples were preceded for scanning electron microscopy and analyzed by JEOL JSM-6610LV. *A-D*, the morphology of γ-tocotrienol-treated cells for 0, 12, 24 and 36 hours, respectively at 3,000 × magnification; bar indicates 5 μm. *E-G*, the morphology changes in γ-tocotrienol-treated cells at 10,000 × magnification. E, normal A549 cell (untreated cell). F, nuclear condensation and segregation. G, cell blebing.

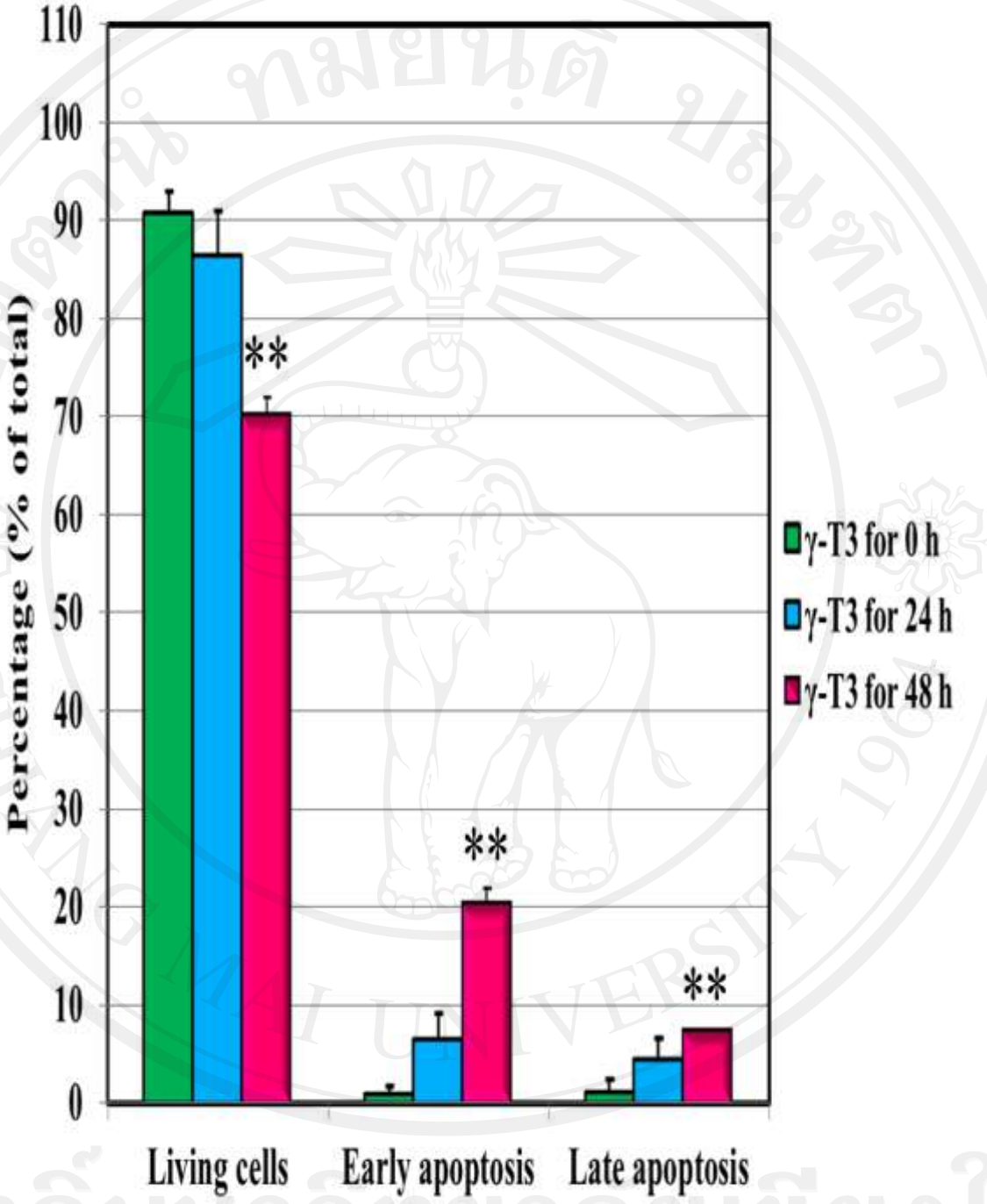

Figure 59 Effect of γ-tocotrienol on the induction of apoptosis in A549 cells, characterized by the translocation of phosphatidylserine (PS). A549 cells were treated with 60μmol/L of γ-tocotrienol for 0-48 hours. The translocation of phosphatidylserine (PS) from inner to outer membrane was analysed by flow cytometry. The results represent the mean of at least three-independent experiments. ** P<0.01, vs. control group (γ-T_3 for 0 h).

3.19 Induction of apoptosis via both intrinsic and extrinsic pathways in A549 cells by γ-tocotrienol

To confirm that γ-tocotrienol induced apoptosis in A549 cells, western blotting was used to monitor the expression of apoptosis-related proteins, including cleaved poly(ADP-ribose) polymerase (PARP) and procaspase-3 in γ-tocotrienol-treated A549 cells. As shown in Figure 60, γ-tocotrienol treatment increased the expression of cleaved-PARP protein in dose-response manner, suggested that γ-tocotrienol induced the cleavage of PARP. Moreover, treatment of γ-tocotrienol decreased the expression of pro-caspase-3 protein, suggested that γ-tocotrienol induced the activation of caspase-3. These results indicated that γ-tocotrienol induced apoptosis in A549 cells.

There are two well-known pathways that lead to apoptosis, intrinsic and extrinsic pathways. The extrinsic pathway initiated by extracellular signals and involved with the activation of caspase-8. The intrinsic pathway begins when an injury occurs within the cell. The intrinsic pathway involved with activation of caspase-9 and mitochondrial instability (increasing ratio of pro-apoptotic proteins per anti-apoptotic proteins) (187). To test which pathway that involved with γ-tocotrienol-induced apoptosis in A549 cells, the protein expression of pro-caspase-8, pro-caspase-9, pro-apoptotic protein bax, and anti-apoptotic protein bcl-xL were determined by western blotting. As shown in Figure 60, γ-tocotrienol treatment decreased the expression of pro-caspase-8 protein in dose-response manner, suggested that γ-tocotrienol induced apoptosis via extrinsic pathway. However, treatment of γ-tocotrienol also decreased the expression of pro-caspase-9 and bcl-xL protein, but increased the expression of bax protein in the dose-response manner, which were the patterns of the apoptosis via intrinsic pathway (activation of caspase9 and increasing ratio of bax/bcl-xL proteins). These results indicated that γ-tocotrienol induced apoptosis in A549 cells via both intrinsic and extrinsic pathways.

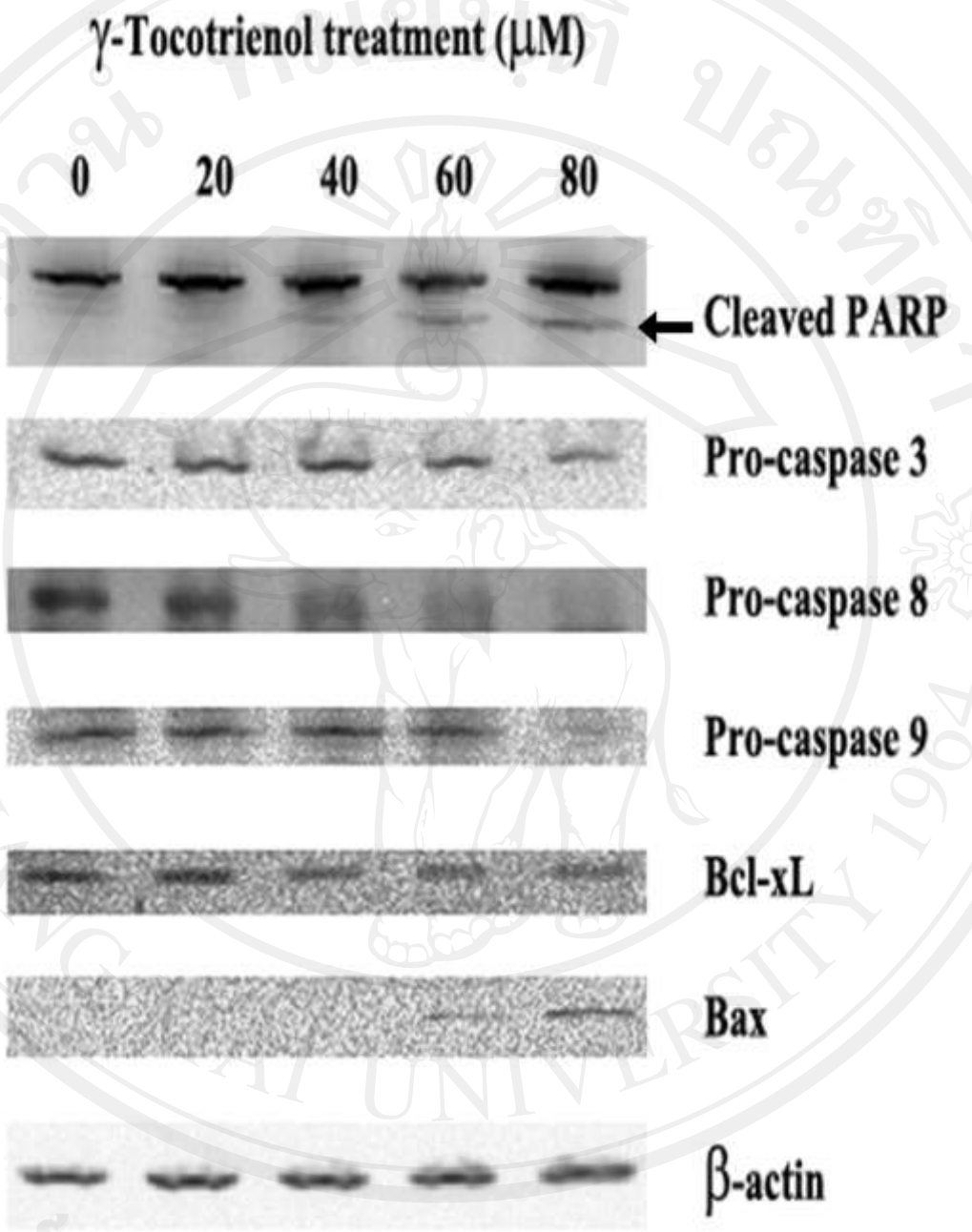

Figure 60 Effect of γ-tocotrienol on the induction of apoptosis in A549 cells. A549 cells were treated with 0-80 μmol/L of γ-tocotrienol for 24 hours. The apoptosis-related proteins, including cleaved PARP, procaspase-3, procaspase-8, procaspase-9, bcl-XL, and bax, were analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

3.20 Toxicity analysis of fermented brown rice and rice bran (FBRA) in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

To examine whether FBRA affect the health of *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice, a mouse model for study about human colorectal tumorigenesis, all mice were divided and received the FBRA administration, according to Figure 24. At 20th weeks of age, all mice were sacrificed, and their livers and kidneys were collected. The weights of body, liver, and kidney were measured, and the histology of liver and kidney were analyzed. During the 20 weeks of experimental period starting from week 5th, although there were significant different in the final body weight between the *Apc*$^{Min/+}$ mice received control diet and 5% FBRA (group 1 vs. group 2; $P<0.005$) and the weight of liver between the *Apc*$^{Min/+}$ mice received control diet and 10% FBRA (group 1 vs. group 3; $P<0.01$), but the histological analysis of liver and lung tissues confirmed that no evidence of toxicity was appeared as shown in Table 10.

3.21 Chemopreventive effect of FBRA against the colorectal tumorigenesis in *Apc*$^{Min/+}$ mice

To examine the chemopreventive effects of FBRA administration on the development of colorectal tumors in *Apc*$^{Min/+}$ mice, a model for study about human colorectal tumorigenesis, all mice were divided into 6 groups and received the FBRA administration since 5 weeks of age, according to Figure 24. At 20th weeks of age, all mice were sacrificed, and their intestine was collected. The incidences, multiplicities (number of tumor per mice), and size of colonic tumors in both *Apc*$^{Min/+}$ mice (group 1-3) and *Apc*$^{+/+}$ mice (group 4-6) were summarized in Table 11. As expected, only the *Apc*$^{Min/+}$ mice developed colorectal cancer due to their mutation of *Apc* gene. However, there were no significant differences in the incidences, multiplicities and size of tumors in colon among the control diet-, 5%FBRA- and 10%FBRA-received *Apc*$^{Min/+}$ mice (mice in group 1, 2, and 3, respectively).

Table 10 The weights of body, liver, and kidney of FBRA-treated *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

Group	Phenotype	Treatment	No.[a]	Weight (g)[b]		
				Body	Liver	Kidney
1	*Apc*$^{Min/+}$	Control diet	21	25.2±3.6	1.41±0.31	0.30±0.08
2	*Apc*$^{Min/+}$	5%FBRA	24	22.6±2.2[c]	1.36±0.30	0.30±0.05
3	*Apc*$^{Min/+}$	10%FBRA	21	26.7±3.0	1.67±0.31[d]	0.33±0.08
4	*Apc*$^{+/+}$	Control diet	18	27.3±6.1	1.21±0.34	0.33±0.12
5	*Apc*$^{+/+}$	5%FBRA	11	27.8±4.4	1.23±0.19	0.33±0.06
6	*Apc*$^{+/+}$	10%FBRA	18	28.8±3.9	1.30±0.26	0.34±0.13

[a] Number of examined mice

[b] Representation in mean ± standard deviation

[c] Significantly difference from group 1 by student's *t*-test ($p<0.005$)

[d] Significantly difference from group 1 by student's *t*-test ($p<0.01$)

Table 11 Incidences, multiplicities, and size of colonic tumors in colon in FBRA-treated *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

Group	Phenotype	Treatment	No.[a]	Incidence (%)[b]	Multiplicity[c]	Tumor size[d]
1	*Apc*$^{Min/+}$	Control diet	21	19/21 (90)	2.9±1.8	18.1±20.9
2	*Apc*$^{Min/+}$	5%FBRA	24	20/24 (83)	2.3±1.9	17.3±18.8
3	*Apc*$^{Min/+}$	10%FBRA	21	20/21 (95)	2.5±1.7	21.6±23.9
4	*Apc*$^{+/+}$	Control diet	18	0/18 (0)	0.0±0.0	ND
5	*Apc*$^{+/+}$	5%FBRA	11	0/11 (0)	0.0±0.0	ND
6	*Apc*$^{+/+}$	10%FBRA	18	0/18 (0)	0.0±0.0	ND

[a] Number of examined mice

[b] Number of mice observed each lesion (%)

[c] Number of total tumor per mouse (Mean ± standard deviation)

[d] Mean ± standard deviation of the sizes of colonic tumor (mm^3)

[e] ND, Not determined

3.22 Toxicity analysis of FBRA in dextran sodium sulfate (DSS)-treated *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

To examine whether FBRA affect the health of DSS-treated *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice, each type of mice was divided into 3 groups and received the FBRA administration since 4 weeks of age, according to Figure 25. All groups of mice were given 2% (w/v) DSS in drinking water for 1 week at 5 weeks of age. Dextran sodium sulfate or DSS is a chemical that irritated and generated the inflammation in gastrointestinal tract. At week 12th, all mice were sacrificed, and their liver and kidney were collected. The weights of body, liver, and kidney were measured and the histology of liver and kidney were analyzed. During the 12 weeks of experimental period, there was no significant difference in the weights of body, liver, and kidney among the groups of *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice as shown in Table 12. Additionally, the histological analysis of liver and lung tissues confirmed that no evidence of toxicity was appeared.

3.23 Chemopreventive effect of FBRA against the inflammation-related colorectal tumorigenesis in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

To examine the chemopreventive effects of FBRA administration against the inflammation-related colorectal tumorigenesis in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice, each type of mice was divided into 3 groups and received the FBRA administration since 4 weeks of age, according to Figure 25. All groups of mice were given 2% (w/v) DSS in drinking water for 1 week at 5 weeks of age. Dextran sodium sulfate or DSS is a chemical that irritated and generated the inflammation in gastrointestinal tract. All mice were sacrificed at 12 weeks of age and about one third of total mice, which are exposed to DSS, in *Apc*$^{Min/+}$ groups (group 1-3) were died because their severe colitis due to DSS treatment. However, there was no significant difference in the surviving rate among these groups. After sacrification, intestine of mice was collected. The incidences, multiplicities (number of tumor per mice), and size of tumors in colon of DSS *Apc*$^{Min/+}$ mice (group 1-3) and *Apc*$^{+/+}$ mice (group 4-6) were summarized in Table 13. As expected, all *Apc*$^{Min/+}$ mice developed the tumors in the colon, but no colonic tumor was found in *Apc*$^{+/+}$ mice. Administration with both 5%FBRA (group 2) and 10%FBRA (group 3) to *Apc*$^{Min/+}$ mice significantly suppressed the multiplicities of total tumors in the colon of mice in comparison with control diet group (11.3±5.6 in

group 2, and 9.7±4.8 in group 3 vs. 20.5±6.9 in group 1; $P<0.02$ and $P< 0.005$, respectively). However, there was no significant difference in the size of colonic tumors between FBRA-treated mice (mice in group 2 and 3) and control diet-fed mice (mice in group 1) as shown in Table 13.

Table 12 The weights of body, liver, and kidney of FBRA-administered and DSS-treated *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

Group	Phenotype	Treatment	No.[a]	Weight (g)[b]		
				Body	Liver	Kidney
1	*Apc*$^{Min/+}$	Control diet +DSS	10	21.6±2.7	1.17±0.16	0.30±0.03
2	*Apc*$^{Min/+}$	5%FBRA +DSS	7	21.1±3.3	1.13±0.23	0.31±0.06
3	*Apc*$^{Min/+}$	10%FBRA +DSS	6	20.2±3.8	1.04±0.21	0.29±0.05
4	*Apc*$^{+/+}$	Control diet +DSS	11	24.6±4.8	1.19±0.29	0.35±0.06
5	*Apc*$^{+/+}$	5%FBRA +DSS	11	25.5±3.1	1.38±0.24	0.38±0.07
6	*Apc*$^{+/+}$	10%FBRA +DSS	8	26.2±3.6	1.35±0.37	0.40±0.03

[a] Number of examined mice

[b] Representation in means ± standard deviation (grams)

Table 13. Incidences, multiplicities, and size of colonic tumors in colon in FBRA-treated *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

Group	Phenotype	Treatment	No.[a]	Incidence (%)[b]	Multiplicity[c]	Tumor size[d]
1	*Apc*$^{Min/+}$	Control diet+DSS	10	10/10 (100)	20.5±6.9	5.1±7.5
2	*Apc*$^{Min/+}$	5%FBRA+DSS	7	7/7 (100)	11.3±5.6[e]	5.1±5.9
3	*Apc*$^{Min/+}$	10%FBRA+DSS	6	6/6 (100)	9.7±4.8[f]	3.3±3.6
4	*Apc*$^{+/+}$	Control diet+DSS	11	0/11 (0)	0.0±0.0	ND[g]
5	*Apc*$^{+/+}$	5%FBRA+DSS	11	0/11 (0)	0.0±0.0	ND
6	*Apc*$^{+/+}$	10%FBRA+DSS	8	0/8 (0)	0.0±0.0	ND

[a] Number of examined mice

[b] Number of mice observed each lesion (%)

[c] Number of total tumor per mouse (mean ± standard deviation)

[d] Mean ± standard deviation of the sizes of colonic tumor (mm^3)

[e] Significantly difference from group 1 by student's t-test (P<0.02)

[f] Significantly difference from group 1 by student's t-test (P<0.005)

[g] ND, Not determined

3.24 Effect of FBRA on the mRNA expression of the inflammation-related genes

To investigate the molecular mechanisms involved in suppressing effect of FBRA against the inflammation-related colorectal tumorigenesis, *Apc*$^{+/+}$ mice were divided into 4 groups, received the FBRA administration since 4 weeks of age, and were given 1.5% (w/v) DSS in drinking water for 1 week at 5 weeks of age, according to Figure 26. All mice were sacrificed at 7 weeks of age and their colonic tissues were collected. Quantitative real-time RT-PCR technique was used to analyze the mRNA expression of inflammation-related genes, including *Cox2*, *iNos* and *Tnfα*, in colon tissues of DSS-treated and DSS-untreated *Apc*$^{+/+}$ mice. The relative mRNA expression of *Cox2*, *iNos* and *Tnfα* genes were quantified and normalized with β-actin (arbitrary unit). Treatment with DSS significantly increased the mRNA expression of *Cox2*, *iNos*, and *Tnfα* genes in colonic mucosa (P<0.03) as shown in Table 14. Importantly, 10% FBRA administration in DSS-treated mice significantly suppressed the mRNA expression of *Cox2* gene (0.38±0.37 unit in 10%FBRA group vs. 1.84±1.09 unit in control diet group; P<0.04) and *iNos* gene (0.03±0.05 unit in 10%FBRA group vs. 0.58±0.44 unit in control diet group; P<0.02) in comparison with control diet-fed DSS-treated mice. In contrast, no significant difference was detected in the mRNA expression of *Tnfα* gene (0.52±0.32 unit in 10%FBRA group vs. 0.95±0.36 unit in control diet group) in comparison with control diet-fed DSS-treated mice as shown in Table 14.

3.25 The anti-proliferative effect of FBRA in colonic crypts of DSS-treated mice

To further examine the effect of FBRA on the cell proliferative index of colonic crypts in the DSS-treated and DSS-untreated *Apc*$^{+/+}$ mice, the *Apc*$^{+/+}$ mice were divided into 4 groups, received the FBRA administration since 4 weeks of age, and were given 1.5% (w/v) DSS in drinking water for 1 week at 5 weeks of age, according to Figure 26. All mice were sacrificed at 7 weeks of age and their colonic tissues were collected. Immunohistochemical analysis for the expression of Ki67, a marker for proliferative cell, was performed to examine the cell proliferative index in colonic crypts of DSS-treated and DSS-untreated *Apc*$^{+/+}$ mice. Administration of 10%FBRA in DSS-treated *Apc*$^{+/+}$ mice significantly decreased the number of Ki67-positive cells in comparison with the control diet-fed DSS-treated *Apc*$^{+/+}$ mice (138±76

cells per 1,000 counted cells in 10%FBRA group vs. 475±126 cells per 1,000 counted cells in control diet group; $P<0.001$) as shown in Figure 61 and 62. These results suggested that 10% FBRA decreased the cell proliferative index in the colonic crypt cells of DSS-treated mice. On the contrary, 10% FBRA administration had no significantly effect on the cell proliferative index in the colonic crypt cells of DSS-untreated mice when compared with the control diet-fed group (138±56 cells per 1,000 counted cells in 10%FBRA group vs. 153±51 cells per 1,000 counted cells in control diet group).

Table 14 The effect of FBRA on the mRNA expression levels of *Cox2*, *iNos*, and *Tnfα* genes in the colonic mucosa

Phenotype	Treatment	No.[a]	Ratio of targeted mRNA/β-actin mRNA[b]		
			Cox2	*iNos*	*Tnfα*
Apc$^{+/+}$	Control diet+DSS	6	1.84±1.09	0.58±0.44	0.95±0.36
Apc$^{+/+}$	10%FBRA+DSS	4	0.38±0.37[c]	0.03±0.05[d]	0.52±0.32
Apc$^{+/+}$	Control diet	4	0.13±0.17[e]	3.45×10^{-6} $\pm1.88\times10^{-6}$[e]	2.83×10^{-8} $\pm5.18\times10^{-8}$[e]
Apc$^{+/+}$	10%FBRA	4	0.01±0.01[f]	3.41×10^{-6} $\pm5.91\times10^{-7}$[f]	7.88×10^{-9} $\pm7.35\times10^{-9}$[f]

[a] Number of examined mice

[b] Representation in means ± standard deviation

[c] Significantly difference from control diet+DSS group by student's *t*-test ($P<0.04$)

[d] Significantly difference from control diet+DSS group by student's *t*-test ($P<0.02$)

[e] Significantly difference from control diet+DSS group by student's *t*-test ($P<0.03$)

[f] Significantly difference from 10%FBRA+DSS group by student's *t*-test ($P<0.03$)

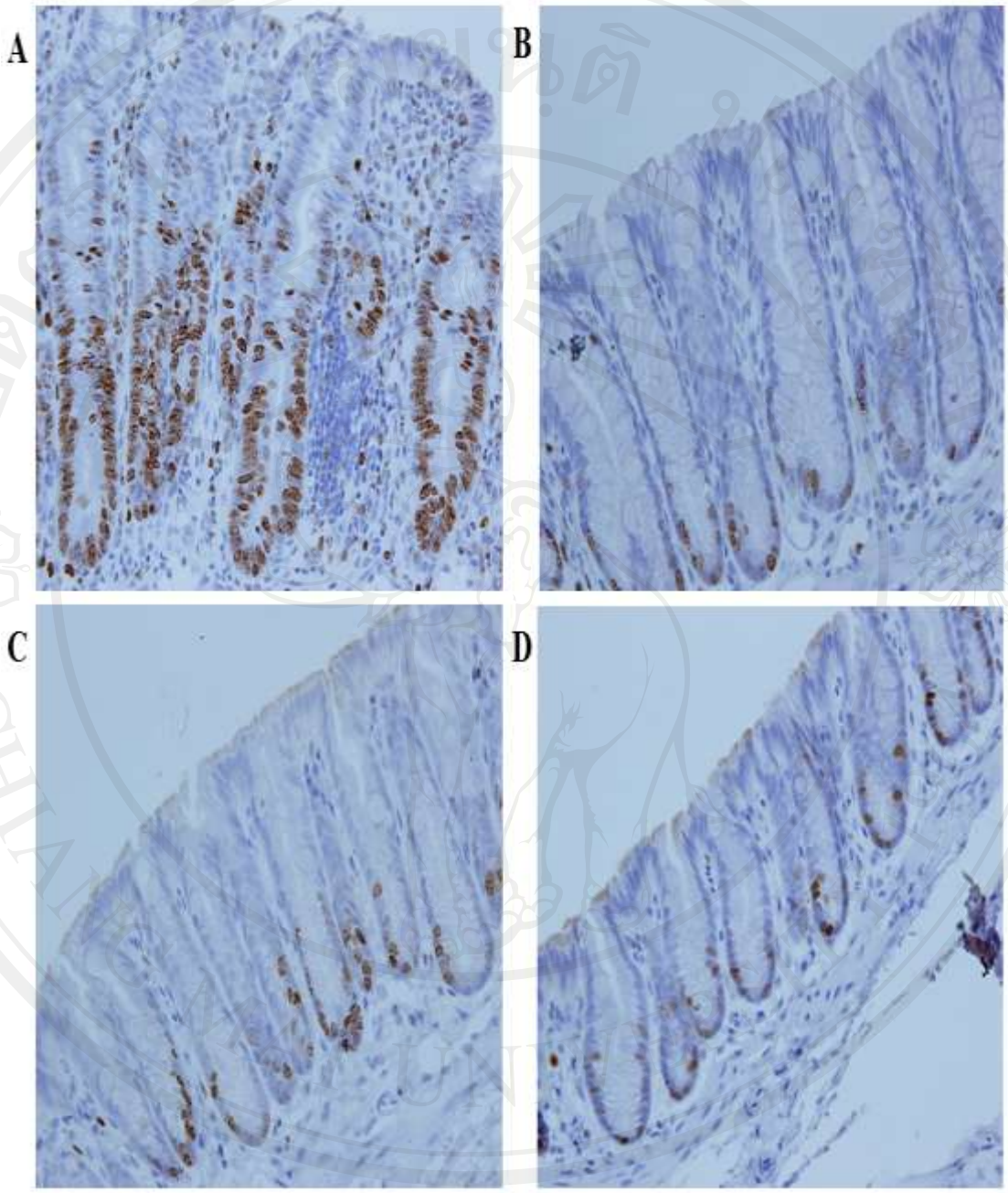

Figure 61 Effect of 10% FBRA treatment (B and D) in comparison with control diet (A and C) on the number of Ki67 positive cell in colonic crypts. A and C displayed the Ki67-immunohistochemical staining of colon tissue sections from DSS-treated *Apc*$^{+/+}$ mice fed with control diet (group 1) and DSS-untreated *Apc*$^{+/+}$ mice fed with control diet (group 3), respectively. B and D displayed the Ki67-immunohistochemical staining of colon tissue sections from DSS-treated *Apc*$^{+/+}$ mice fed with 10% FBRA (group 2) and DSS-untreated *Apc*$^{+/+}$ mice fed with 10% FBRA (group 4), respectively.

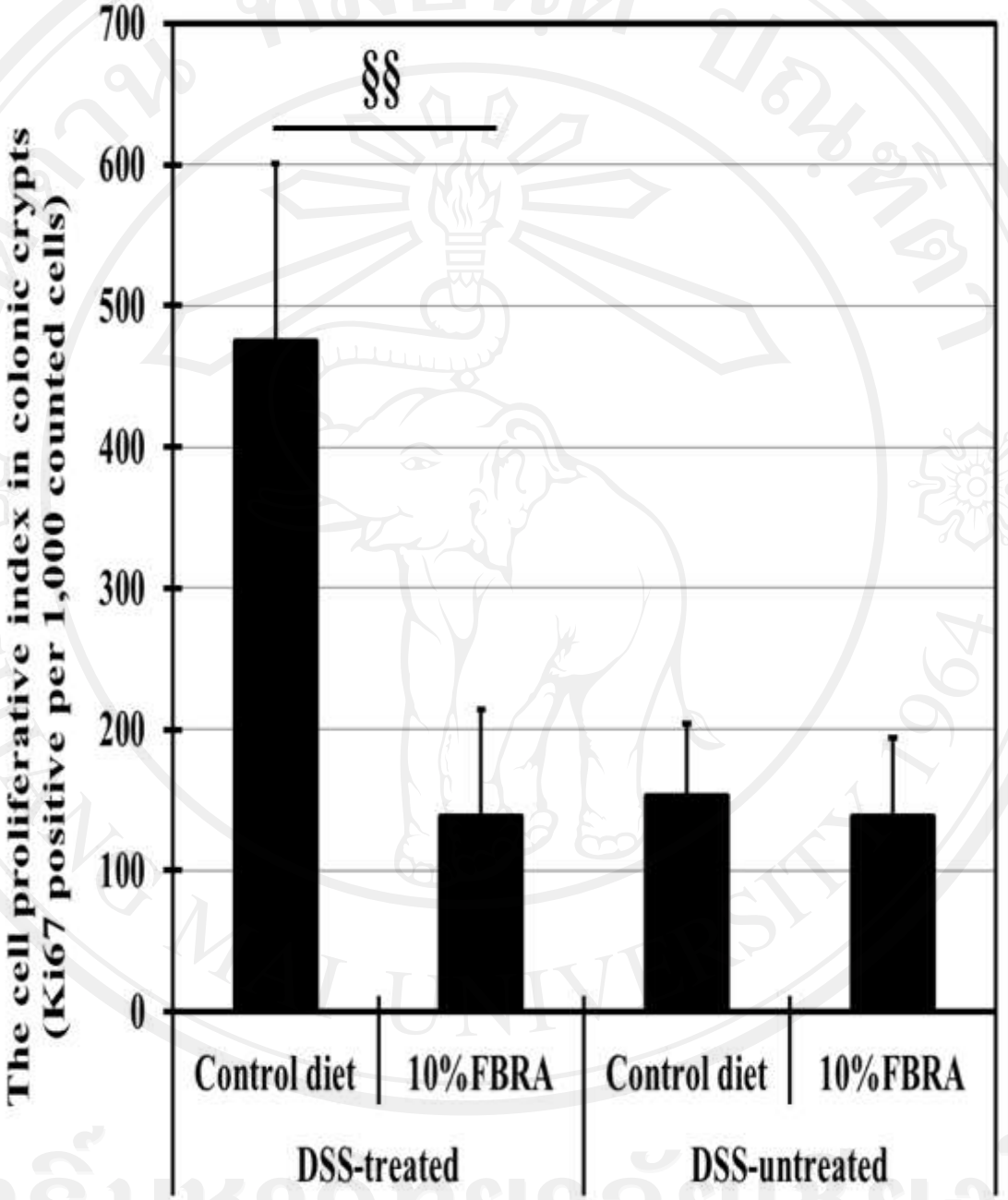

Figure 62 Effect of 10%FBRA on the cell proliferative index in colonic crypts of DSS-treated *Apc*$^{+/+}$ mice. Colonic sections from DSS-treated and DSS-untreated *Apc*$^{+/+}$ mice were performed, stained with specific antibody for Ki67, and analyzed by immunohistochemistry. These results represented the mean of ratio between Ki67 positive cells per 1,000 counted crypt cells in each microscopic field at 400x magnification. §§ P<0.001, vs. control diet fed DSS-treated group.

3.26 Effect of γ-tocotrienol and phytic acid on the cell viability of SW480 cells

To investigate the effect of γ-tocotrienol and phytic acid on the cell viability of human colorectal cells, SW480 cells were treated with 0-60 μmol/L of γ-tocotrienol or 0-80 μmol/L of phytic acid for 12 and 24 hours. Finally, the cell viability was examined by MTT assay. As shown in Figure 63, γ-tocotrienol treatment decreased the cell viability of SW480 cells at both 12 and 24 hours. This suppressive effect of γ-tocotrienol was both dose-response and time-dependent manner as shown in Table 15. However, phytic acid treatment at concentrations of 0-80 μmol/L had no suppressive effect on the cell viability of SW480 cells at both 12 and 24 hours as shown in Figure 64. These results indicated that phytic acid at concentration of 0-80 μmol/L had no toxicity effect on human colorectal cell line SW480.

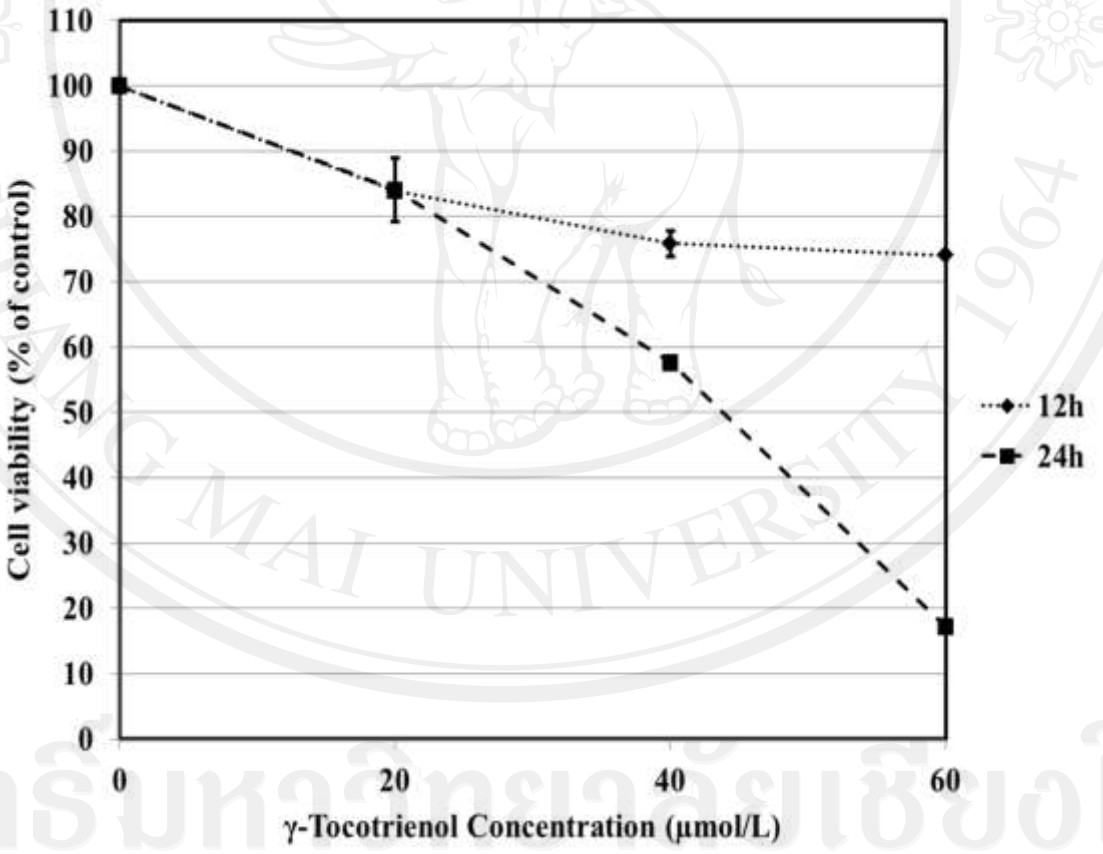

Figure 63 Effect of γ-tocotrienol on the cell viability of SW480 cells. SW480 cells were treated with 0-60 μmol/L of γ-tocotrienol for 12 and 24 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and expressed relative to the negative control group (0 μmol/L of γ-tocotrienol). The results represent the mean of at least three independent experiments.

Table 15 The inhibitory concentrations (IC) of γ-tocotrienol on the cell viability of SW480 cells

The inhibitory concentration (IC)	γ-Tocotrienol Concentration (μmol/L)[a]	
	12 hours	24 hours
20	30.1±2.0	22.7±3.3
50	>60	43.8±0.2
80	>60	58.6±0.1

[a] Concentration in μmol/L ± standard deviation

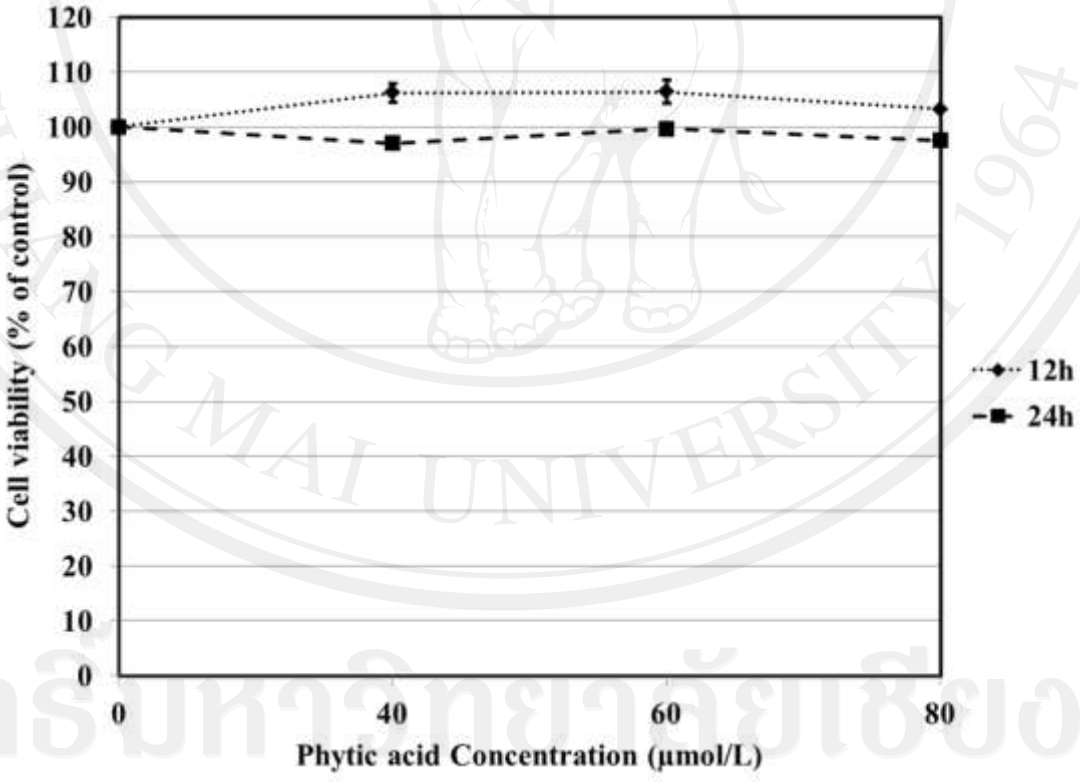

Figure 64 Effect of phytic acid on the cell viability of SW480 cells. SW480 cells were treated with phytic acid (0-80 μmol/L) for 12 and 24 hours. Each concentration was repeated in five wells. The cell viability was determined by MTT assay and expressed relative to the negative control group (0 μmol/L of phytic acid). The results represent the mean of at least three independent experiments.

3.27 Effect of γ-tocotrienol and phytic acid on the LPS-induced chemoattractants secretion in colorectal cancer cell line SW480

Inflammation, the response of tissue to injury, is mediated by a variety of factors. During the inflammatory processes, the inflammatory stimuli are involved in leukocyte recruitment within the injured tissue through increased expression of cellular adhesion molecules and secretion of chemoattractants in injured cell (235, 236). Lipopolysaccharide (LPS), a substance from the bacterial cell wall, has been shown to induce inflammation in various experimental models (312-314). Therefore, LPS may induce the secretion of chemoattractants in injured cells. To test this, Boyden chamber assay was used to investigate the secretion of chemoattractants from LPS-treated colorectal cancer cell line SW480. As shown in Figure 65, the number of migrated cells was increased upon the increasing concentration of LPS, suggested that chemoattractive activity was increased in SW480 culture supernatant due to the increasing concentration of LPS. These results indicated that LPS induced the secretion of chemoattractants from SW480 cells.

To investigate the suppressive effects of γ-tocotrienol or phytic acid against the LPS-induced chemoattractants secretion in SW480 cells, the SW480 cells were pre-treated with 0-40 μmol/L of γ-tocotrienol or 0-80 μmol/L of phytic acid for 12 hours before 1μg/mL of LPS treatment for 36 hours. As shown in Figure 66, pre-treatment with phytic acid but not γ-tocotrienol significantly decreased the number of migrated cells. These results indicated that phytic acid pre-treatment suppressed the LPS induced the secretion of chemoattractants from SW480 cells.

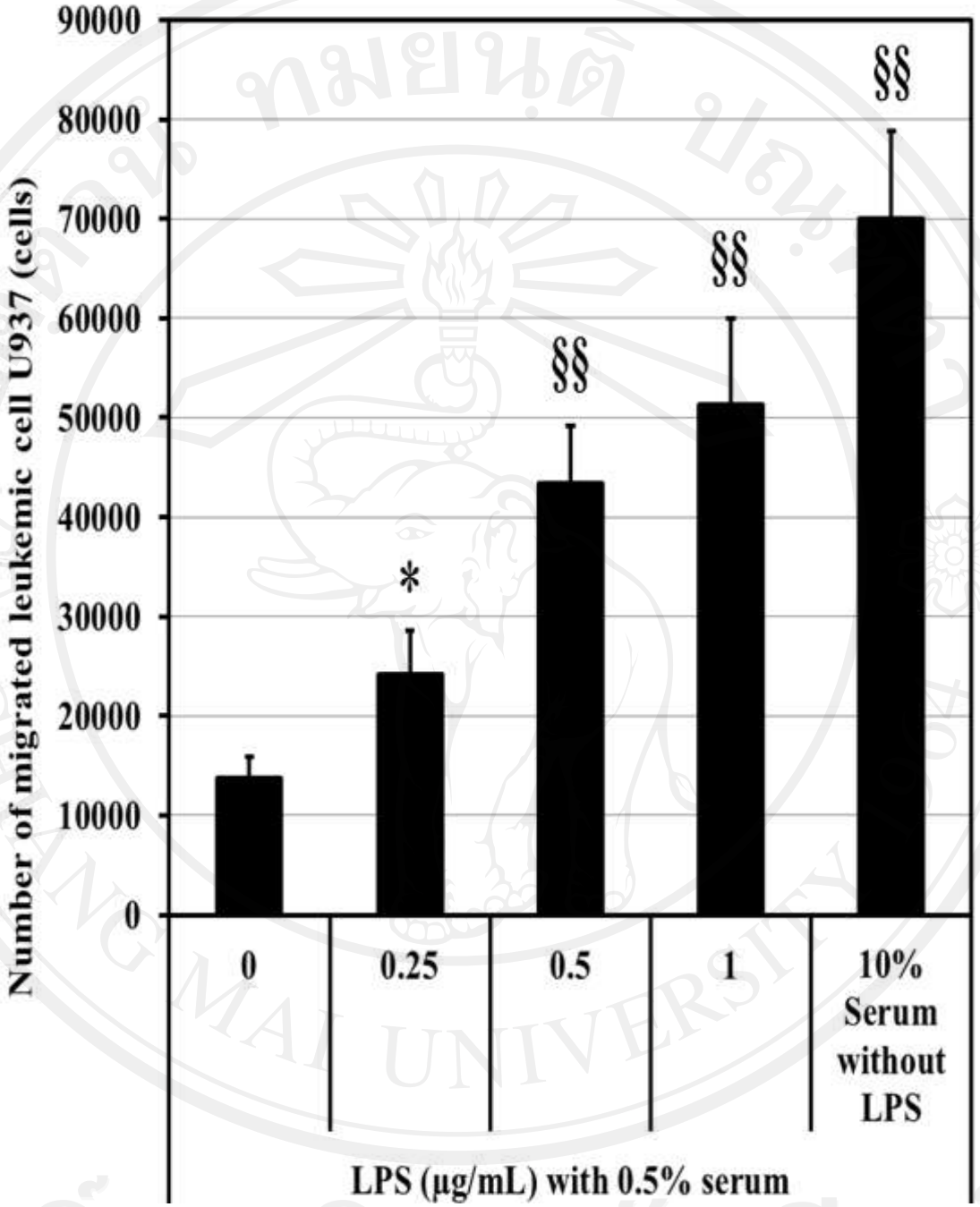

Figure 65 Effect of lipopolysaccharide (LPS) on the leukemic cell migration due to chemoattractants secretion from human colorectal cancer cell line SW480 cells. SW480 cells were treated with 0-1 µg/mL of LPS with 0.5% serum for 36 hours. The cultured supernatants were collected and monitored for chemoattractive activities by Boyden chamber. These results represent the mean of at least three independent experiments. 10% serum was used as positive control. * P<0.05, and §§ P<0.001, vs. control group (0µg/mL of LPS).

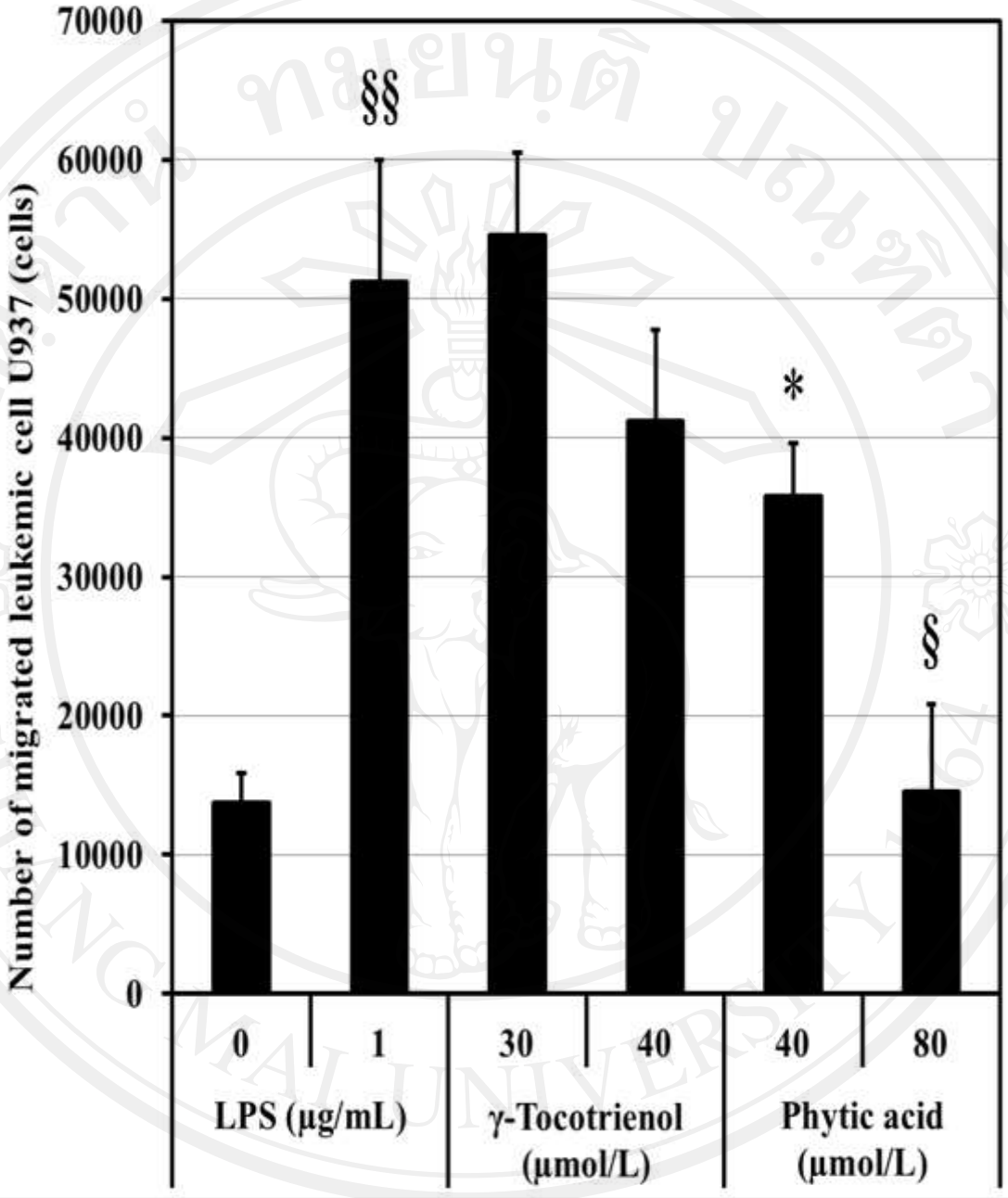

Figure 66 Effect of γ-tocotrienol and phytic acid on the LPS-induced chemo-attractants secretion in colorectal cancer cell line SW480. SW480 cells were pre-treated with 0-40 µmol/L of γ-tocotrienol or 0-80 µmol/L of phytic acid for 12 hours before 1µg/mL of LPS treatment for 36 hours. The cultured supernatants were collected and monitored for chemoattractive activities by Boyden chamber assay. These results represent the mean of at least three independent experiments. * P<0.05, and § P<0.005, vs. control group (1µg/mL of LPS) and §§ P<0.001, vs. control group (0µg/mL of LPS).

3.28 Effect of phytic acid on the LPS-induced NF-κB nuclear accumulation in SW480 cells

Nuclear factor-κB, a transcription factor, plays an important role in the onset of inflammation (315). To examine the effect of LPS on the translocation of NF-κB into nucleus of SW480 cells, western blotting was used to examine the NF-κB nuclear accumulation in LPS-treated SW480 cells. Treatment of LPS at concentrations of 1 µg/mL in SW480 cells induced the nuclear translocation of NF-κB protein in time-dependent manner as shown in Figure 67.

To investigate the effect of phytic acid on the LPS-induced NF-κB nuclear accumulation in SW480 cells, the SW480 cells were pre-treated with 0-80 µmol/L of phytic acid for 12 hours before 1 µg/mL of LPS treatment for 3 hours. As shown in Figure 68, pre-treatment with phytic acid prevented the LPS-induced NF-κB nuclear accumulation in dose-response manner.

3.29 Effect of phytic acid on the LPS-induced overexpression of iNOS protein in SW480 cells

NF-κB involves in the expression of many pro-inflammatory genes, including inducible nitric oxide synthase (iNOS) (316). To investigate whether LPS mediated the overexpression of iNOS protein, western blotting was used to examine the expression of iNOS protein in LPS-treated SW480 cells. Treatment of LPS at concentrations of 1µg/mL induced the expression of iNOS protein in time-dependent manner as shown in Figure 69.

To investigate the effect of phytic acid on the LPS-induced iNOS protein overexpression in SW480 cells, the SW480 cells were pre-treated with 0-80 µmol/L of phytic acid for 12 hours before 1 µg/mL of LPS treatment for 6 hours. As shown in Figure 70, phytic acid pre-treatment prevented the LPS-induced overexpression of iNOS protein in dose-response manner.

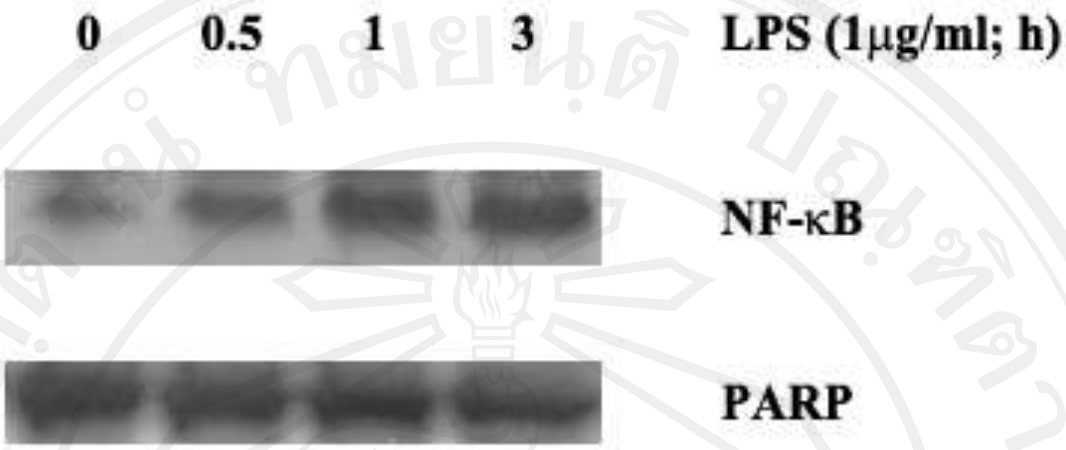

Figure 67 Effect of LPS on the NF-κB nuclear accumulation in SW480 cells. SW480 cells were treated with 1 μg/mL of LPS for 0-3 hours. The nuclear lysates were collected and analysed the levels of NF-κB protein in the nucleus by western blotting. These results were representative of 3 independent experiments with similar results and PARP was used as a loading control.

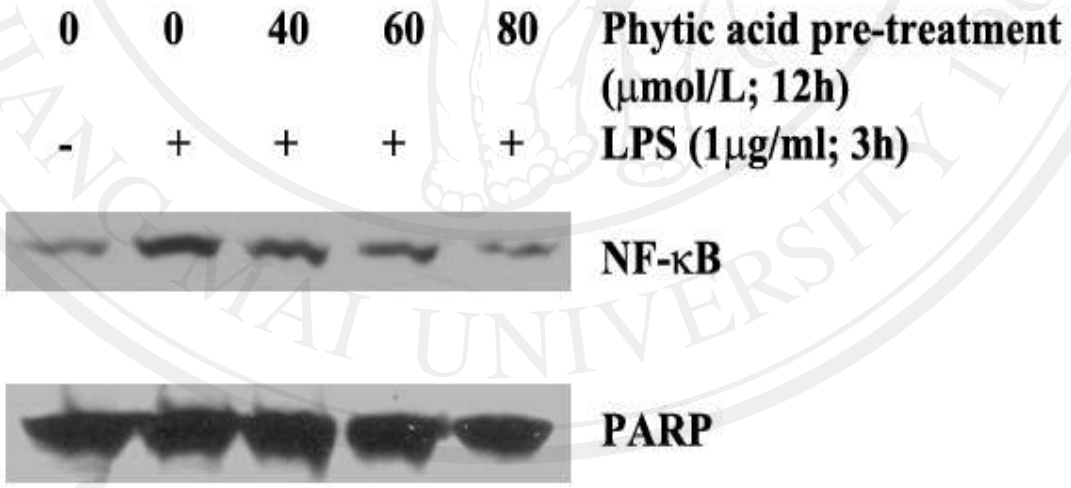

Figure 68 Suppressive effect of phytic acid on the LPS-induced NF-κB nuclear accumulation in SW480 cells. SW480 cells were pre-treated with 0-80 μmol/L of phytic acid for 12 hours before 1 μg/mL of LPS treatment for 3 hours. The nuclear lysates were collected and analysed the levels of NF-κB protein in the nucleus by western blotting. These results were representative of 3 independent experiments with similar results and PARP was used as a loading control.

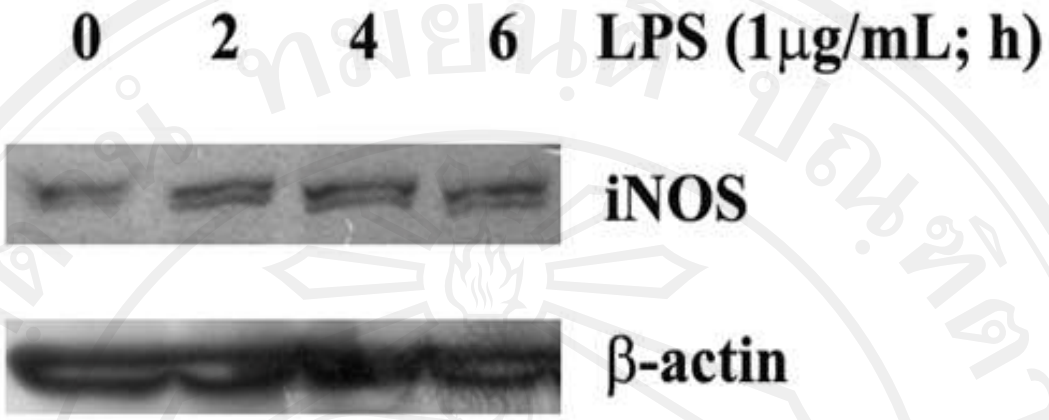

Figure 69 Effect of LPS on the expression of iNOS protein in SW480 cells. SW480 cells were treated with 0-1 μg/mL of LPS for 12 hours. The total cell lysates were corrected and analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

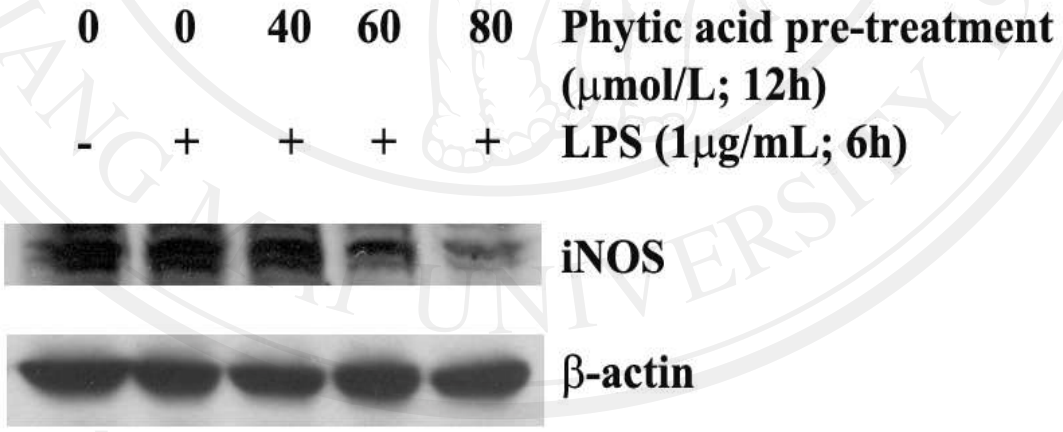

Figure 70 Effect of phytic acid on the overexpression of iNOS protein in LPS-treated SW480 cells. SW480 cells were pre-treated with 0-80 μmol/L of phytic acid for 12 hours before 12 hours treatment of LPS at concentrations of 1 μg/mL. The total cell lysates were corrected and analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

3.30 Effect of phytic acid on the LPS-induced cyclinD1 overexpression in SW480 cells

Cyclin D1 protein plays a central role in the regulation of the G1 phase restriction point. Recently, it has been clear that cyclin D1 is a direct target gene for NF-κB (317). Therefore, LPS may induce the overexpression of cyclinD1 protein. To test this, western blottting was used to examine the expression of cyclinD1 protein in LPS-treated SW480 cells. As shown in Figure 71, treatment of SW480 cells with increasing concentration of LPS induced the overexpression of cyclinD1.

To investigate the effect of phytic acid on the LPS-induced overexpression of cyclinD1 in SW480 cells, the SW480 cells were pre-treated with 0-80 μmol/L of phytic acid for 12 hours before 1 μg/mL of LPS treatment for 12 hours. As shown in Figure 72, phytic acid pre-treatment prevented the LPS-induced cyclinD1 overexpression in dose-response manner.

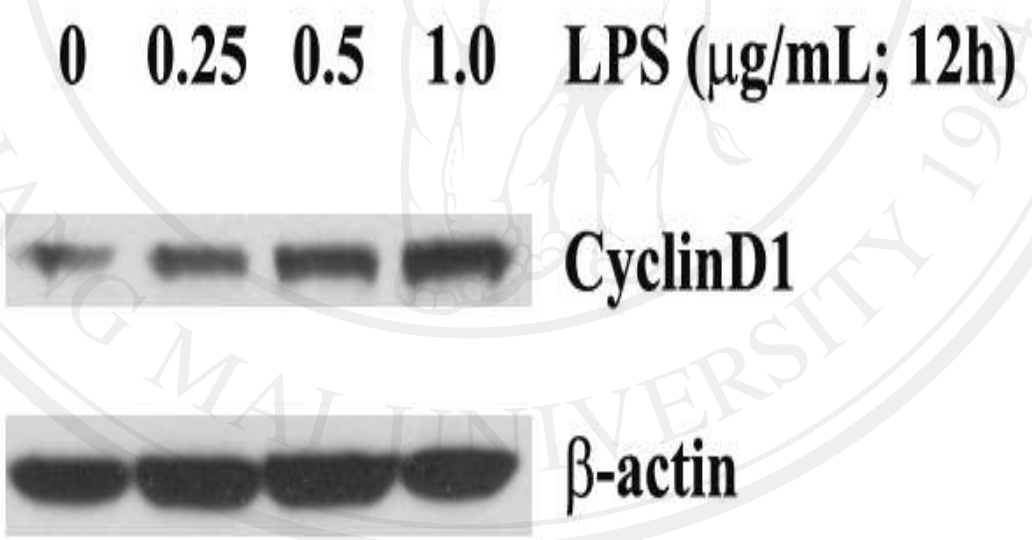

Figure 71 Effect of LPS on the expression of cyclin D1 protein in SW480 cells. SW480 cells were treated with 0-1 μg/mL of LPS for 12 hours. The total cell lysates were corrected and analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.

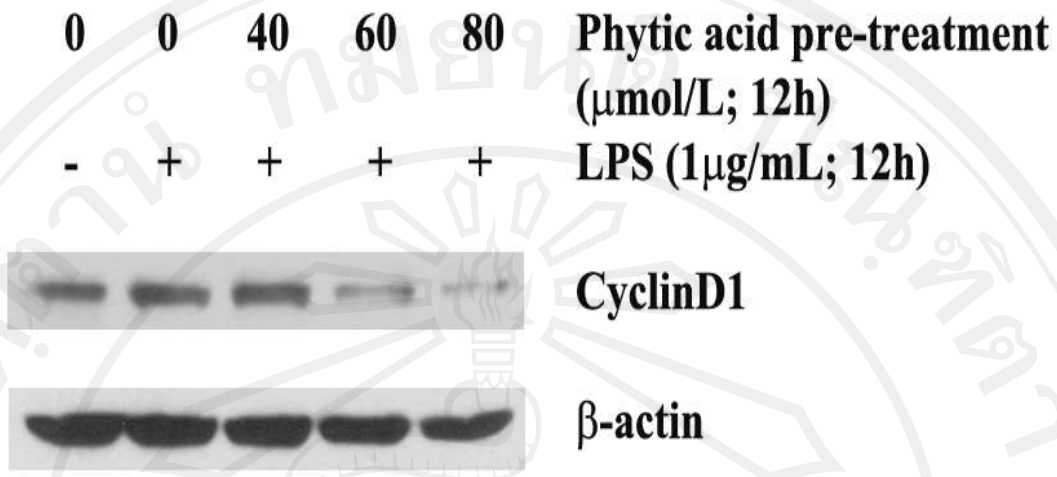

Figure 72 Effect of phytic acid on the overexpression of cyclin D1 protein in LPS-treated SW480 cells. SW480 cells were pre-treated with 0-80 μmol/L of phytic acid for 12 hours before 12 hours treatment of LPS at concentrations of 1 μg/mL. The total cell lysates were corrected and analyzed by western blotting. These results were representative of 3 independent experiments with similar results and β-actin was used as a loading control.