CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals and materials

The names of chemicals and reagents, and instruments are shown in Appendix A and B, respectively. The detail of reagents and/or buffers used in this study is shown in Appendix C. Purified γ-tocotrienol and phytic aid sodium salt hydrate from rice were purchased from Sigma (St. Louis, MO). The six lines of Thai rice including, Khao Dawk Mali 105, Suphan Buri 1, Pathum Thani 1, RD31, RD41, and RD47, were collected from local markets in the northern and middle regions of Thailand.

2.2 Preparation of fermented brown rice and rice bran (FBRA)

Fermented brown rice and rice bran or FBRA was supplied by Genmai Koso Co., Ltd. (Sapporo, Japan). The final composition of FBRA is shown in Table 1 (270). The manufacturing process of FBRA is briefly descripted as following. Briefly, fermentation base was made by steaming of brown rice and rice bran. *Aspergillus Oryzae*, a filamentous fungus for food fermentation, was then seeded into the fermentation base and the first fermentation process was provided for 18-24 hours. Subsequently, the second fermentation process was continued for additional 12-24 hours for aging purpose. Finally, fermented product was dried and powdered.

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Table 1 Final composition of FBRA (270)

	Amount/100 grams of FBRA
Protein	23.8 g
Fat	20.5 g
Ash	9.0 g
Hydrocarbon	22.4 g
Fiber	21.0 g
Phytic acid	3.86 g
Vitamin A	0.03 mg
Vitamin B group	4.97 mg
Vitamin E group (Tocopherols & Tocotrienols)	15 mg
Vitamin K group	63 mg
Sodium	8.1 mg
Phosphorus	1.96 g
Calcium	308 mg
Iron	9.9 mg
Magnesium	810 mg
Copper	936 mg
Zinc	5.99 mg
Manganese	15.6 mg
Selenium	8 µg

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2.3 Animals

The animal experiments were performed under protocols approved by the Institutional Ethics Review Committee for animal experiments at the Gifu University. Female A/J mice, 3 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). The *Apc*$^{Min/+}$ mice (expressing truncated Apc protein) were purchased from The Jackson Laboratory (Bar Harbor, ME). The *Apc*$^{Min/+}$ pedigree was maintained by mating C57BL/6J females with *Apc*$^{Min/+}$ males. The *Apc*$^{Min/+}$ mice were identified by allele-specific PCR on DNA isolated from tail. Male C57BL/6J mice (4 weeks of age) were purchase from SLC (Hamamatsu, Japan). The mice were divided and housed in isolated ventilation cages (3 or 4 mice/cage). All mice were maintained under specific pathogen-free conditions, including 50±10% of humidity, 12-hours light/dark cycle of lighting, and 23±2°C of temperature. All mice were bred and maintained on a basal diet, CE-2 (CLEA Japan Inc., Tokyo, Japan) with free access to water until experiments.

2.4 Cell lines

A549, human alveolar epithelial adenocarcinoma cell line, and **SW480,** human colorectal cancer cell line were purchased from the American Type Culture Collection (ATCC). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Carlsbad, CA, USA) supplement with 10% heat inactivated fetal bovine serum (FBS), 10,000 Units/mL penicillin, 10,000 µg/mL streptomycin in a humidified incubator with 5% CO_2, and 37°C. The cells were plated and allowed to grow to approximately 70 – 80% before experimentation.

2.5 Experimental designs for *in vivo* study

2.5.1 Chemopreventive effects of FBRA against NNK-induced lung tumorigenesis in female A/J mice

Female A/J mice were maintained on basal diet (CE-2) until 6 weeks of age. At week 6th, all mice were randomly divided into 8 groups as shown in Figure 23. Briefly, mice in group 1 and 6 were still maintained on basal diet throughout the experiment. Mice in group 2 and 3 received 5% and 10% FBRA in basal diet, respectively, until week 8th and then received basal diet throughout the experiment. Mice in group 4 and 5 were still maintained on basal diet until week 8th and then received 5% and 10% FBRA in basal diet, respectively, throughout the experiment.

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Finally, mice in group 7 and 8 received 5% and 10% FBRA in basal diet, respectively, throughout the experiment. At week 7th, the mice in groups 1-5 were intra-peritoneal (i.p.) injected with 10 µmol/L of tobacco-derived nitrosamine (NNK). All mice were measured their body weights and sacrificed at week 21st. Liver and kidney of all mice were collected and measured the weight for toxicity analysis. Lung were measured the weights and collected. Then, the lung were infused with 10% neutral buffered formalin and carefully inspected grossly. All of the macroscopically detected lung nodules were counted and histopathologically examined. Lung lesions, hyperplasia, and adenomas were diagnosed according to the criteria of "International Classification of Rodent Tumors: The Mouse" (291).

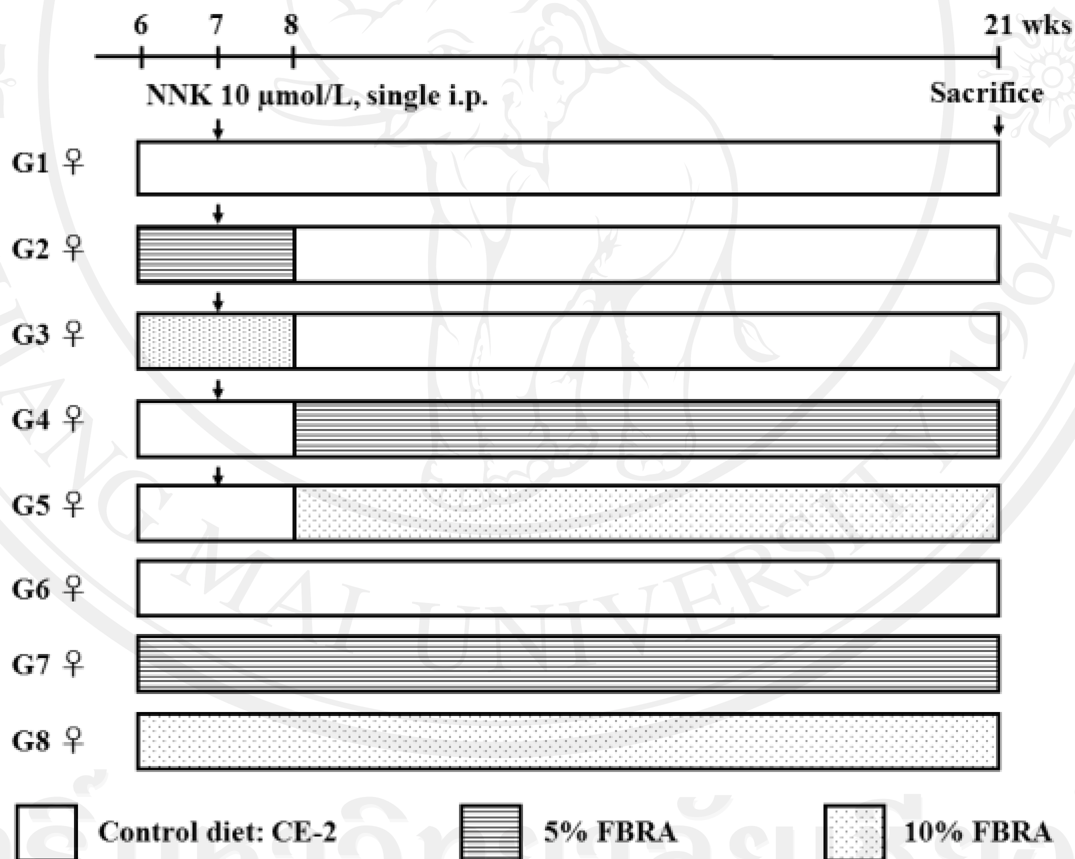

Figure 23 Experimental design for study the effects of FBRA against NNK-induced lung tumorigenesis in female A/J mice. All mice in group 1 to 8 (G1 to G8) were female A/J mice. The experiment were started at week 6th and finished (sacrifice) at week 21st. Mice in group 1 to 5 were intra-peritoneal injection with 10µmol/L of NNK at week 7th.

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2.5.2 Chemopreventive effects of FBRA against colorectal tumorigenesis in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

The *Apc*$^{Min/+}$ (expressing truncated Apc protein) mice and *Apc*$^{+/+}$ (expressing wild type Apc protein) mice were maintained on basal diet (CE-2) until 5 weeks of age. At week 5th, all mice were randomly divided into 6 groups. Group 1, 2, and 3 were *Apc*$^{Min/+}$ mice and group 4, 5, and 6 were *Apc*$^{+/+}$ mice as shown in Figure 24. Briefly, mice in group 1 and 4 were maintained on basal diet (CE-2). Mice in group 2 and 5 received 5% FBRA in basal diet. Finally, mice in group 3 and 6 received 10% FBRA in basal diet. At week 20th, all surviving mice were measured the body weights and sacrificed. Liver and kidney of all mice were collected and measured the weight for toxicity analysis. The small intestines and large bowels were flushed with PBS and excised. The large bowel were cut open longitudinally along the main axis, and washed with PBS. The large bowels were fixed in 10% buffered formalin for at least 24 hours. The colon tumors were counted macroscopically method. To identify the tumors in small intestine and large bowel, the small intestines and large bowels were dripped into a 0.2% methylene blue solution for 30 seconds, washed in saline, and counted by using a light microscope at a magnification of ×40.

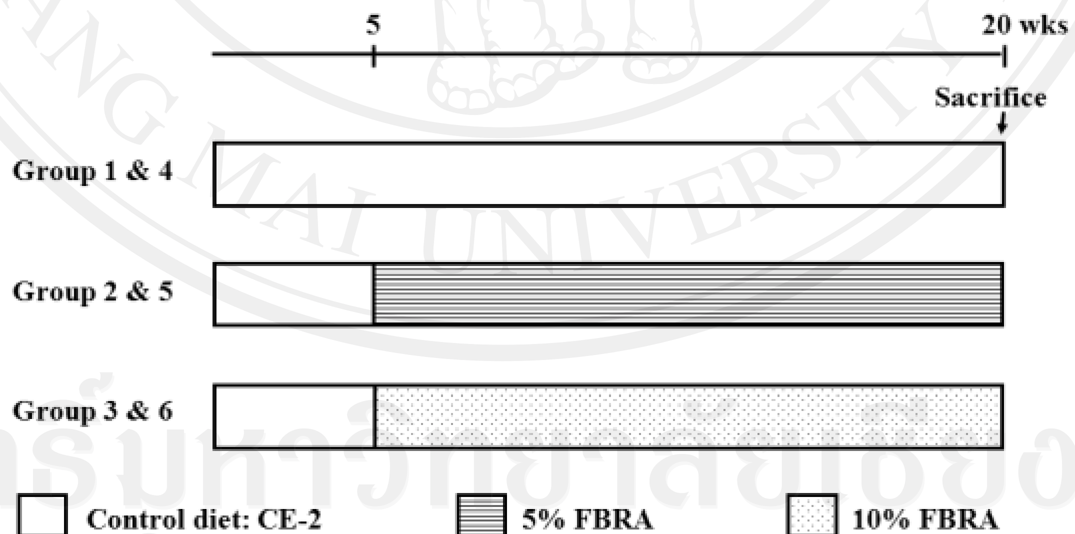

Figure 24 Experimental design for study the effects of FBRA against colorectal tumorigenesis in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice. Mice in group 1, 2, and 3 were *Apc*$^{Min/+}$, and group 4, 5, and 6 were *Apc*$^{+/+}$. The experiment were started at week 5th and finished (sacrifice) at week 20st.

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2.5.3 Chemopreventive effects of FBRA against DSS-induced colorectal tumorigenesis in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice

The *Apc*$^{Min/+}$ mice and *Apc*$^{+/+}$ mice were maintained on basal diet (CE-2) until 4 weeks of age. At week 4th, all mice were randomly divided into 6 groups. Group 1, 2, and 3 were *Apc*$^{Min/+}$ mice and group 4, 5, and 6 were *Apc*$^{+/+}$ mice as shown in Figure 25. Briefly, mice in group 1 and 4 were maintained on basal diet (CE-2). Mice in group 2 and 5 received 5% FBRA in basal diet. Finally, mice in group 3 and 6 received 10% FBRA in basal diet. At week 5th, all groups of mice were given 2% (w/v) DSS in drinking water for 1 week. At week 12th, all surviving mice were measured the body weights and sacrificed. Liver and kidney of all mice were collected and measured the weight for toxicity analysis. The large bowels were flushed with PBS and excised. The large bowels were cut open longitudinally along the main axis, and washed with PBS. The large bowels were fixed in 10% buffered formalin for at least 24 hours. The colon tumors were counted macroscopically method. To identify neoplasms in small intestine and small tumors in colon, the small intestines and colons were dripped into a 0.2% methylene blue solution for 30 seconds, washed in saline, and counted by using a light microscope at a magnification of ×40

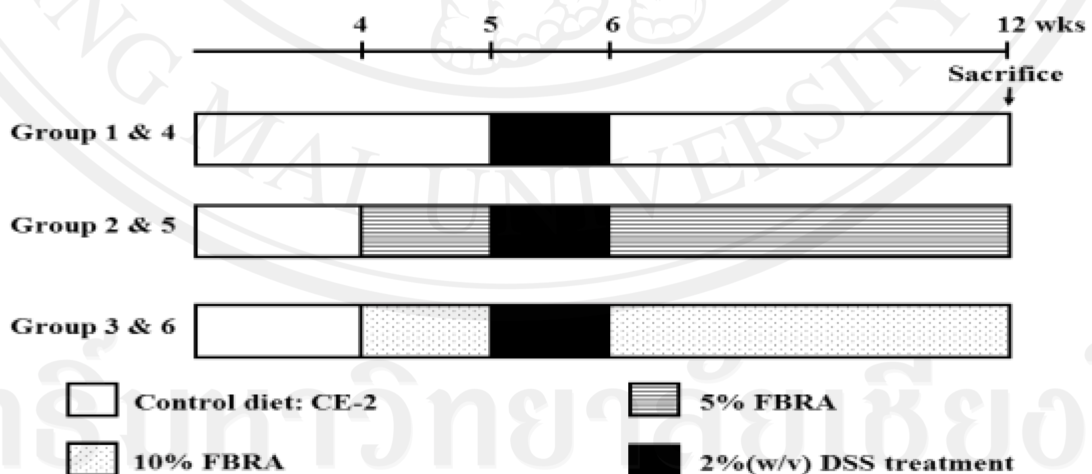

Figure 25 Experimental design for study the effects of FBRA against DSS-induced colorectal tumorigenesis in *Apc*$^{Min/+}$ and *Apc*$^{+/+}$ mice. Mice in group 1, 2, and 3 were *Apc*$^{Min/+}$, and group 4, 5, and 6 were *Apc*$^{+/+}$. The experiment were started at week 4th and finished (sacrifice) at week 12th. All mice were received 2%(w/v) of DSS in drinking water at week 5th.

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2.5.4 Chemopreventive effects of FBRA on DSS-induced colitis in *Apc*$^{+/+}$ mice

The *Apc*$^{+/+}$ mice were maintained on basal diet (CE-2) until 4 weeks of age. At week 5th, all mice were randomly divided into 4 groups as shown in Figure 26. Briefly, mice in group 1 and 3 were maintained on basal diet (CE-2). Mice in group 2 and 4 received 10% FBRA in basal diet. At week 5th, the mice in groups 1 and 2 were given 1.5% (w/v) DSS in drinking water for 1 week. At week 7th, all surviving mice were sacrificed. One centimeter of colon from the anal ring were collected and embedded in paraffin for immunohistochemistry analysis. The further 2 centimeters of colon were collected for RNA isolation and quantitative analysis of iNos, Cox2 and Tnfα mRNA expression levels by quantitative real-time RT-PCR.

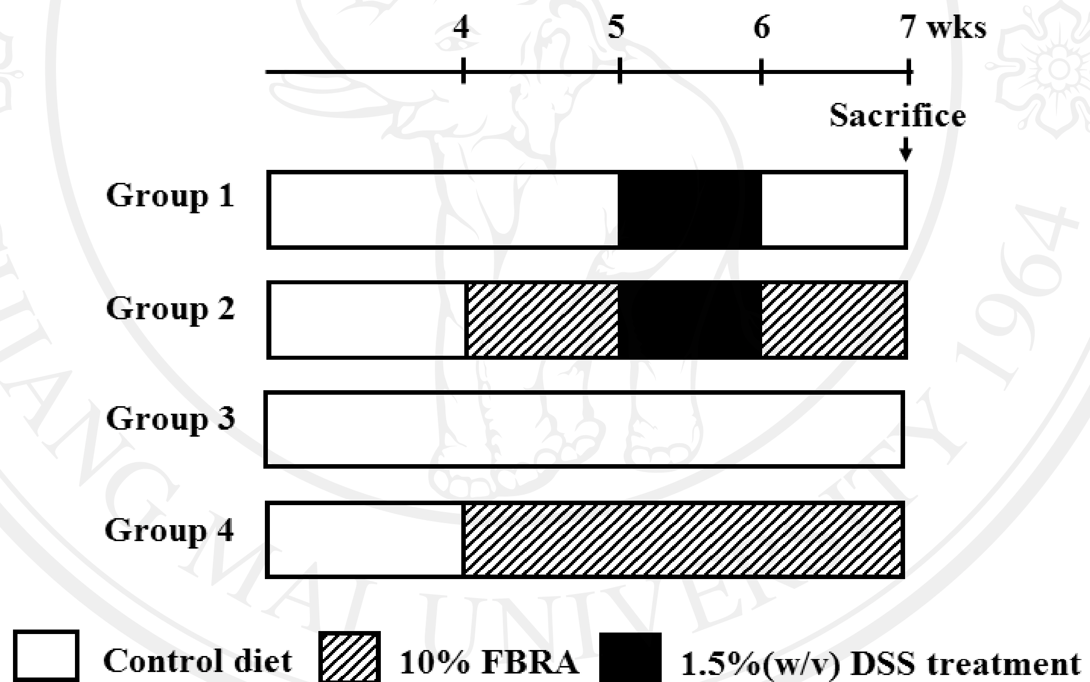

Figure 26 Experimental design for study the effects of FBRA on DSS-induced colitis in *Apc*$^{+/+}$ mice. All mice in group 1 to 4 were *Apc*$^{+/+}$. The experiment were started at week 4th and finished (sacrifice) at week 7th. Mice in group 1 and 2 were received 1.5%(w/v) of DSS in drinking water at week 5th.

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2.6 Total RNA preparation

2.6.1 RNA isolation from the lung and liver tissues of A/J mice

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, the mRNA expression levels of *Cyp2a5* gene in both liver and lung tissues were determined by quantitative RT-PCR. Lung and liver tissues from all mice were collected, extracted the RNA by using Trizol reagent (Invitrogen), and purified by RNeasy Mini Kit (Qiagen Corp.) according to the manufacturers' instructions. Briefly, fresh tissues (up to 30 mg, depending on the tissue type) was disrupted in Buffer RLT and homogenized. One volume (350 µL) of 70% ethanol was added, and mix well by pipetting. Then, 700 µL of the sample, including any precipitate that may have formed, was transferred to an RNeasy spin column in a 2 ml collection tube, centrifuged for 15 seconds at 10000 x g, and then discarded the flow-through. Next, 700µL Buffer RW1 was added to the RNeasy spin column to wash the spin column membrane by centrifugation for 15 seconds at 1000 x g, and then discarded the flow-through. The spin column membrane was washed twice with 500µL buffer RPE by centrifugation at 10000 x g for 15 seconds and 2 minutes, respectively, and then discarded the flow-through. The RNeasy spin column were place in a new 2 ml collection tube and centrifuged at 10000 x g for 1 minute to eliminate any possible carryover of buffer RPE. The RNeasy spin column was transferred to a new 1.5 mL collection tube. Finally, 30µL RNase-free water was directly added to the spin column membrane, and then centrifuge for 1 min at 10000 x g to elute the RNA. Purified RNA may be stored at –20°C or –70°C in RNase-free water. The amount and quality of RNA for each sample will be assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer. The concentration of RNA was determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer. The ratio of the readings at 260 nm and 280 nm (A260/A280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. Pure RNA has an A260/A280 ratio of 1.5–2.1.

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2.6.2 RNA isolation from the colonic tissues of *Apc*$^{+/+}$ mice

To investigate the molecular mechanisms of the suppressing effect of FBRA against the inflammation-related colorectal tumorigenesis in DSS-treated mice, the colon tissues were analyzed for the mRNA expression levels of inflammation-related genes, including *Cox2*, *iNos*, and *Tnfα*, by quantitative real-time RT-PCR. The colonic tissues from the study about the effects of FBRA on DSS-induced colitis in *Apc*$^{+/+}$ mice were collected. RNA was extracted using Trizol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) and an RNeasy Mini Kit (Qiagen Corp., Hilden, Germany) according to the manufacturers' instructions. The amount and quality of RNA for each sample were assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.6.3 RNA isolation from NNK-treated A549 cells

To investigate the effect of NNK on the mRNA expression levels of the CYP2A6 and CYP2A13 genes in A549 cells, quantitative real-time RT-PCR was used to monitor the mRNA expression levels of *CYP2A6* and *CYP2A13* genes in NNK-treated A549 cells. After the A549 cells were treated with NNK (10 μmol/L) for 0-12 hours, their RNA were extracted using NucleoSpin® RNA II kit (MACHEREY-NAGEL) according to the manufacturer's instructions. Briefly, 5 x 10^6 of A549 cells were collected by centrifugation and lysed by addition of 350μL Buffer RA1 and 3.5μL β-mercaptoethanol (β-ME). The cell lysate was filtrates through NucleoSpin® Filter by centrifugation for 1 minute at 11,000 x g. The homogenized lysate was add with 350μL of 70% ethanol and mixed by pipetting up and down (5 times) to adjust the RNA binding conditions. Then, the lysate was loaded to the NucleoSpin® RNA II column and centrifuged for 30 seconds at 11,000 x g. At this step, RNA in the lysate is trapped by silica membrane of the NucleoSpin® RNA II column. The membrane was washed and dried by centrifugation at 11,000 x g for 1 minute with 350μL of Membrane Desalting Buffer. DNase reaction mixture was applied directly onto the center of the silica membrane and incubated at room temperature for 15 min to digest DNA. The membrane was washed by centrifugation for 30 seconds at 11,000 x g with Buffer RA2. Then, the membrane was washed again by centrifugation for 30 seconds and 2 minutes, respectively, at 11,000 x g with Buffer RA3. The RNA was eluted by centrifugation at 11,000 x g for 1 minute with 60 μL of

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RNase-free H_2O. Purified RNA may be stored at –20°C or –70°C. The amount and quality of RNA for each sample were assessed with spectrophotometer.

2.7 Quantitative real-time reverse transcription polymerase chain reaction (qReal-time RT-PCR)

Firstly, complementary DNA or cDNA was provided from the purified RNA by reverse transcription polymerase chain reaction (RT-PCR). The RT-PCR is a process to generate the cDNA from RNA using reverse transcriptase and an oligo dT primer. This process is very important in order to perform PCR since DNA polymerase can act only on DNA templates. The RT-PCR can be performed either in the same tube with PCR (one-step PCR) or in a separate one (two-step PCR) using a temperature between 40°C and 50°C, depending on the properties of the reverse transcriptase used (292). Last step, amounts of the cDNA was quantified by real-time PCR, which detected the amplified DNA as the reaction progresses in real time. PCR is a simple and powerful method to amplify only a target DNA of a tiny amount by cycling three incubation steps at different temperatures. Double-stranded target DNA is heat denatured (denaturation step), the two primers complementary to the target segment are annealed at low temperature (annealing step), and the annealed primers are then extended at an intermediate temperature (extension step) with a DNA polymerase. As the target copy number doubles upon each cycle, PCR can thereby amplify DNA fragments up to 106-folds in a short period.

Fluorescence detection (intercalator method) is a detection method utilizing a DNA intercalating dye that fluoresces once bound to double stranded DNA. The most commonly used dye (intercalator) is SYBR® Green I as shown in Figure 27 (293, 294). The dye is added in the reaction system and its fluorescence is detected in amplification. When an intercalator binds to double stranded DNA synthesized in PCR amplification, the fluorescence is emitted. By measuring the fluorescence intensity, the melting temperature of amplified DNA is also available as well as quantification of PCR products.

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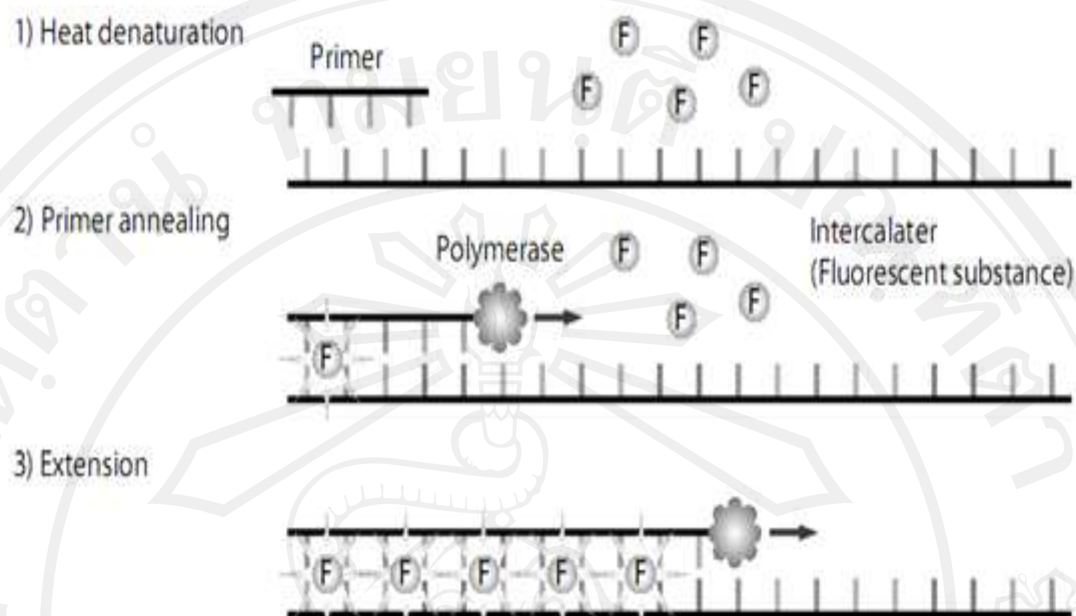

Figure 27 Detection of PCR products with SYBR® Green I

2.7.1 qReal-time RT-PCR analysis of animal tissues

The purified RNA from mouse tissues was reverse-transcribed using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Invitrogen, Carlsbad, CA). This kit has been optimized for generating first-strand cDNA for use in two-step qRT-PCR. Firstly, a master mix for RT-PCR was prepared according to the manufacturer's instructions. Next, the master mix was incubated at 25, 50, and 85ºC for 10, 30, and 5 minutes, respectively. Finally, remained RNA was digested by incubation at 37ºC for 20 minutes with 2 units of *E. coli* RNase H. Synthesized cDNA should be stored at –20°C until use.

The expression levels of Cyp2a5 and β-actin mRNA in lung and liver tissues of A/J mice, and the expression levels of iNos, Cox2, Tnfα and β-actin mRNA in colonic tissues were measured using a Light-Cycler (Roche Applied Science) and the specific sense and antisense primers. The reaction mixture contained 10.0 μL of TaKaRa SYBR Premix Ex Taq (TAKARA BIO INC), 1.0μL of 10μmol/L of each primer, 3.0μL of distilled water and 5.0μL synthesized cDNA. The amplification number of cycles was 45 and the reaction took place for 10 seconds at 95°C, 10 seconds at 60°C, and 6 seconds at 72°C, with an initial step of 95°C for 10 min (hot start). Oligonucleotide primers are shown in the Table 2.

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Table 2 Oligonucleotide primers for qReal-time RT-PCR analysis in mouse tissues

Primers	Sequence	Specification
Cyp2a5F	5'-TGGTCCTGTATTCACCATCTACC-3'	**Cyp2a5**
Cyp2a5R	5'-ACTACGCCATAGCCTTTGAAAA-3'	**Cyp2a5**
iNosF	5'-GTTCTCAGCCCAACAATACAAGA-3'	**iNos**
iNosR	5'-GTGGACGGGTCGATGTCAC-3'	**iNos**
Cox2F	5'-GCCAGGCTGAACTTCGAAACA-3'	**Cox2**
Cox2R	5'-GCTCACGAGGCCACTGATACCT-3'	**Cox2**
β-actinF	5'-CATCCGTAAAGACCTCTATGCCAAC-3'	**β-actin**
β-actinR	5'-ATGGAGCCACCGATCCACA-3'	**β-actin**

2.7.2 qReal-time RT-PCR analysis of NNK-treated A549 cells

The purified RNA from NNK-treated A549 cells was reverse-transcribed using the ReverTra Ace® qPCR RT Kit (Toyobo, Japan). This kit has been optimized for generating first-strand cDNA for use in two-step qRT-PCR. Firstly, the RNA was denatured by incubation at 65°C for 5 minutes, and kept on ice afterwards. Then, a master mix for RT-PCR was prepared according to the manufacturer's instructions. Finally, the master mix was incubated at 37 and 98ºC for 15 and 5 minutes, respectively. Synthesized cDNA should be stored at –20°C until use.

The expression levels of CYP2A6, CYP2A13 and GAPDH mRNA in NNK-treated A549 cells were measured using the Applied Biosystems 7500 and the specific sense and antisense primers. The reaction mixture was prepared using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Japan). The amplification number of cycles was 40 and the reaction took place for 60 seconds at 95°C, 15 seconds at 95°C, and 60 seconds at 60°C. Oligonucleotide primers are shown in the Table 3.

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Table 3 Oligonucleotide primers for qReal-time RT-PCR analysis in NNK-treated A549 cells

Primers	Sequence	Specification
CYP2A6F	5’- GGGCCAAGATGCCCT -3’	**CYP2A6**
CYP2A6R	5’- AATGTCCTTAGGTGACTGGGA -3’	**CYP2A6**
CYP2A13F	5’- ACCTGGTGATGACCACCC -3’	**CYP2A13**
CYP2A13R	5’- CGTGGATCACTGCCTCTG -3’	**CYP2A13**
GAPDHF	5’- GAAGGTGAAGGTCGGAGTC -3’	**GAPDH**
GAPDHR	5’- GAAGATGGTGATGGGATTTC -3’	**GAPDH**

2.8 Tissue sections staining

2.8.1 Hematoxylin and eosin (H&E) staining

Hematoxylin-eosin (H&E) staining is the well-known and most widely used staining procedure for tissue sections. This staining uses hematoxylin solutions for nuclear staining and eosin solutions for cytoplasmic staining. In the first step of staining, hematoxylin solution was used to stain the nuclei (blue or dark violet to black). In the second step, eosin solutions such as eosin Y, eosin B or erythrosin B were used to counterstain cytoplasm, collagen, keratin and erythrocytes (red). H&E staining is a standard staining method for histological analysis. It is the most widely used stain in medical diagnosis to examine the structure of the tissue and the pathological disorders. H&E staining is used for the materials, including paraffin sections, frozen sections and clinico-cytological specimens, such as sputum, urine, and effusions.

To determine the toxicity of FBRA on liver and kidney of mice, the suspect tumors of lung in A/J mice, and the suspect tumors of colon in *Apc*$^{Min/+}$ mice, Tissues from liver, kidney, lung, and colon were collected, respectively. The fresh tissues were used to prepare 5μm thick-paraffin sections. For H&E staining, the paraffin section was deparaffinized with 3 times of xylene for 4 minutes. Then, the section

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was rehydrated in a series of ethanol rinses (99, 95, 80, and 0%, respectively). Hematoxylin was used to stain the section for 7 minutes. Then, the section was rinsed in running tap water for 20 minutes. Next, the section was counterstained with Eosin for 30 seconds, and then rinsed in running tap water for 15 seconds. A series of ethanol rinses (80, 95, 99, and 100%, respectively) were used to dehydrate the section. Finally, the section was soaked 3 times in xylene for 3 minutes and cover slipped.

2.8.2 Immunohistochemical staining or Immunohistochemistry

Immunohistochemistry (IHC) is an integral technique in many veterinary laboratories for diagnostic and research purposes. In the last decade, the ability to detect antigens (Ag) in tissue sections has dramatically improved by countering the deleterious effects of formaldehyde with antigen retrieval (AR) and increasing sensitivity of the detection systems. The basis of IHC is very simple and bridges three scientific disciplines, including immunology, histology, and chemistry. The fundamental concept behind IHC is the demonstration of antigens (Ag) within tissue sections by means of specific antibodies (Ab). Once antigen–antibody (Ag-Ab) binding occurs, it is demonstrated with a colored enzymatic reaction visible by light microscopy or fluorochromes with ultraviolet light.

To investigate the chemopreventive effect of FBRA on the cell proliferation of tumor cells in lung and colon tissues, these tissues were collected to prepare 5μm thick-paraffin sections. For IHC, the tissue section was deparaffinized and rehydrated in PBS. Then, the section was placed in 10 mmol/L citrate buffers (pH6.0) and heated in Pascal pressure cooker (Code No.S2800, Dako corp.) programmed for 1 minute at 120°C. Next, the endogenous peroxidase activity was blocked by incubation for 20 minutes in 3%H_2O_2. After washing three times with PBS, the sections were pre-incubated with a normal blocking serum for 30 minutes at room temperature, and then incubated overnight at 4°C with primary antibody, which specific for Ki67 protein (1/100 dilution). Next, the section was incubated for 30 min at room temperature with biotinylated secondary antibody, which specific for primary antibody (1/250 dilution) and followed by incubation with avidin-coupled peroxidase (VECTASTAIN® Elite ABC Kit) for 30 minutes at room temperature. Finally, the section was demonstrated a colored enzymatic reaction with 3,3V-diaminobenzidine (DAB), counterstained with hematoxylin, and mounted coverslips.

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2.9 High-performance liquid chromatography

High-performance liquid chromatography or HPLC is a chromatographic technique used to separate a mixture of compounds in analytical samples to identifying, quantifying, or purifying the individual components of the mixture. There are two variants of HPLC depending on the relative polarity of the solvent and the stationary phase, including normal phase and reversed phase HPLC. Normal phase HPLC used polar stationary phase and non-polar mobile phase. The polar or hydrophilic molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of HPLC column. Increasing the polarity of the mobile phase will subsequently decrease the adsorption and ultimately cause the sample molecules to exit the column. Reversed phase HPLC used non-polar stationary phase and polar mobile phase. The non-polar or hydrophobic molecules in the mobile phase will tend to adsorb to the surface on the inside and outside of HPLC column. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the stationary phase resulting in desorption. The more hydrophobic molecule provides the more interaction and requires the higher concentration of organic solvent to promote desorption.

HPLC analysis for γ-tocotrienol content in Thai rice

Reversed-phase HPLC was used to analyze the content of γ-tocotrienol in the six lines of Thai rice including, Khao Dawk Mali 105, Suphan Buri 1, Pathum Thani 1, RD31, RD41, and RD47 (295). Briefly, rice samples were ground and extracted three times in absolute ethanol (1:1) at 60°C for 15 min each time. The clear supernatants were collected, combined and evaporated. The rice extracts were resuspended by water and washed three times with hexane. The hexane supernatants were kept on ice, combined, and then dried under N_2. Residues were dissolved in absolute ethanol and transferred in an amber vial immediately to HPLC (Agilent). The extracts were injected on a YMC C-30 reverse-phase (RP) column (4.6 x 250 mm, 5 µm) run at 25°C and 1.0 mL min-1 with mobile phase solution (acetonitrile: methanol:water 85:12:3, v/v/v). Sample absorbance was monitored at 292 nm.

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2.10 Measurement of cell viability by MTT assay

The MTT assay, a colorimetric assay, is a main application for assessing the cell viability and also be used to determine cytotoxicity of potential medicinal agents and toxic materials. This assay is based on the ability of a mitochondrial reductase enzyme, such as succinate dehydrogenase, in living cells, which requires NADH or NADPH to catalyze the tetrazolium rings of the pale yellow tetrazole, named MTT (3-9,4,5 dimethylthiazol-2yl)-2,5 diphenyltetrazolium bromide), to be the insoluble purple formazan crystals as shown in Figure 28. The crystals are largely impermeable to cell membranes, thus resulting in its accumulation within viable cells. Then, the formazan crystals are dissolved by DMSO and quantified the absorbance of this colored solution by measuring at a certain wavelength (usually between 500 and 600 nm) using ELISA plate reader. An expansion in the number of viable cells increases the overall activity of mitochondrial reductase. This augmentation in enzyme activity leads to an increase in the amount of formazan dye and higher absorbance rate.

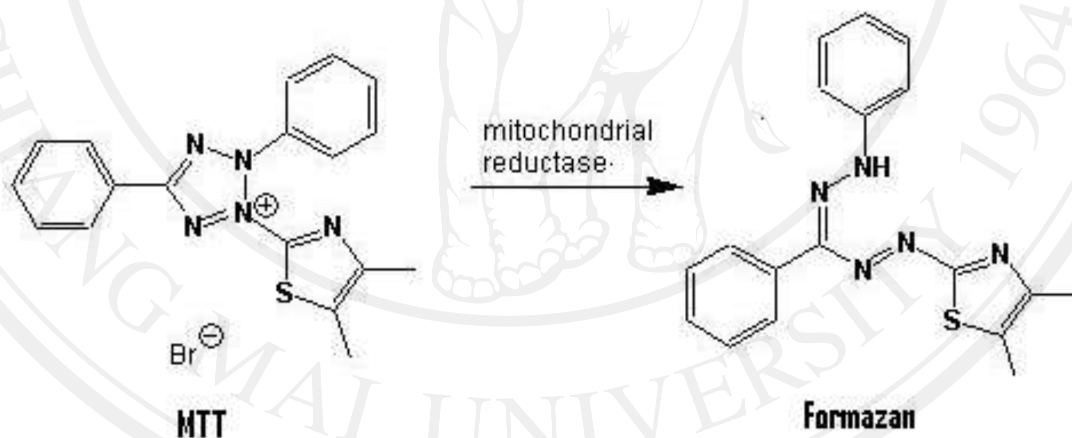

Figure 28 MTT is reduced to formazan by a mitochondrial reductase

2.10.1 Effect of NNK on the cell viability of lung cancer cell line A549

To investigate the carcinogenic effect of NNK, a carcinogen in *carcinogenic to humans (Group 1)*, on the cell viability of human lung cancer cells, MTT assay was performed to investigate its effect on A549 cells. The A549 cells were plated at 2.0×10^3 cells per well in 96-well plates in DMEM containing 10% FBS and incubated at 37°C for 24 hours. Then, NNK were added into each well to reach final concentrations of 0, 1 and 10 μmol/L and the cells were incubated with these

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conditions for 24, 48 and 72 hours, respectively. At the indicated time, the cell viability in each well was determined by MTT assay in comparison with control wells (0 μmol/L of NNK). Briefly, after 100 μL of medium removal, 20 μL of MTT dye (stocking 5 mg/ml in PBS) was added to each well and then incubated at 37°C for 4 hours. After medium removal, 200 μL of DMSO was added to dissolve the formazan crystals and mixed together by shaking for 10 minute. Finally, the absorbance rate was measured by ELISA plate reader at 540 nm with reference wavelength of 630 nm. Each concentration was repeated in five wells. The experiments were carried out in triplicate. The percentage of cell viability was calculated by following formula.

$$\%\text{Cell viability} = \frac{\text{Mean absorbance in tested wells x 100}}{\text{Mean absorbance in control wells}}$$

2.10.2 Effect of γ-tocotrienol and phytic acid on the NNK- increased cell viability of A549 cells

To investigate the preventive effect of γ-tocotrienol (γ-T_3) or phytic acid (IP_6) against the NNK-increased cell viability of A549 cells, the MTT assay was performed. The A549 cells were plated at 2.0×10^3 cells per well in 96-well plates in DMEM containing 10% FBS and incubated at 37°C for 24 hours. Next, the cells were pre-treated with 0-80 μmol/L of γ-tocotrienol or phytic acid for 12 hours, and then treated with 10 μmol/L of NNK for 24 and 48 hours. The final concentrations of γ-tocotrienol and phytic acid pre-treatment were 0, 20, 40, 60 and 80 μmol/L. After NNK treatment, the cell viability in each well was determined by MTT assay in comparison with control wells (0 μmol/L of γ-tocotrienol or phytic acid). The half maximal inhibitory concentration (IC50), 20%- and 80%-inhibitory concentration (IC20 and IC80) values were calculated using the nonlinear regression model.

2.10.3 Effect of γ-tocotrienol and phytic acid on the cell viability of A549 cells

To investigate the suppressive effect of γ-tocotrienol or phytic acid on the cell viability of A549 cells, MTT assay was used to measurement the cell viability. The A549 cells were plated at 2.0×10^3 cells per well in 96-well plates in DMEM containing 10% FBS and incubated at 37°C for 24 hours. Next, the cells were treated with medium contained γ-tocotrienol or phytic acid for 12, 24, 36 and 48 hours. The final concentration of γ-tocotrienol and phytic acid were 0-90 μmol/L and 0-200 μmol/L, respectively. After treatment, the cell viability in each well was determined

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by MTT assay in comparison with control wells (0 μmol/L of γ-tocotrienol or phytic acid). The half maximal inhibitory concentration (IC50), 20%- and 80%-inhibitory concentration (IC20 and IC80) values were calculated using the nonlinear regression model.

2.10.4 Effect of γ-tocotrienol and phytic acid on the cell viability of colon cancer cell line SW480

To investigate the suppressive effect of γ-tocotrienol or phytic acid on the cell viability of SW480 cells, MTT assay was used to measurement the cell viability. The SW480 cells were plated at 4.0×10^3 cells per well in 96-well plates in DMEM containing 10% FBS and incubated at 37°C for 24 hours. Next, the cells were treated with medium contained γ-tocotrienol or phytic acid for 12 and 24 hours. The final concentration of γ-tocotrienol and phytic acid were 0-60 μmol/L and 0-80 μmol/L, respectively. After treatment, the cell viability in each well was determined by MTT assay in comparison with control wells (0 μmol/L of γ-tocotrienol or phytic acid). The half maximal inhibitory concentration (IC50), 20%- and 80%-inhibitory concentration (IC20 and IC80) values were calculated using the nonlinear regression model.

2.11 Measurement of cell proliferation by BrdU cell proliferation assay

BrdU or 5-bromo-2'-deoxyuridine is a synthetic nucleoside that is an analogue of thymidine. BrdU can be incorporated into the newly synthesized DNA of replicating cells during DNA replication in the S phase of the cell cycle by substituting for thymidine. Therefore, BrdU can be used in the detection of proliferating cells in living tissues. The BrdU cell proliferation assay is a technique to detect the incorporated BrdU in the cellular DNA of proliferating cell using an anti-BrdU antibody. When the cells are cultured with labeling medium that contains BrdU, this pyrimidine analog is incorporated in place of thymidine into the newly synthesized DNA of proliferating cells. After removing labeling medium, cells are fixed and the DNA is denatured with fixing or denaturing solution. Then, an antibody, which specific to BrdU, is added to detect the incorporated BrdU in the denaturing DNA. Next, peroxidase labeled anti-IgG antibody is used to recognize the bound detection antibody. Finally, TMB or 3,3,5,5-tetramethylbenzidine, a substrate of peroxidase, is added to develop color. The magnitude of the absorbance for the

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developed color is proportional to the quantity of BrdU incorporated into cells, which is a direct indication of cell proliferation.

2.11.1 Effect of NNK on the cell proliferation of lung cancer cell line A549

To investigate the carcinogenic effect of NNK on the cell proliferation of human lung cancer cells, BrdU cell proliferation assay was performed to investigate its effect on A549 cells. The A549 cells were plated in 96-well plates at 2×10^4 cells/ 100 µL of appropriate cell culture media for each well. Some of the wells on the plate were set aside for several controls, including blank (wells that did not receive cells) and background (wells that contain cells but will not receive any BrdU reagent). This plate is incubated at 37°C for 24 hours and then were added with medium contains NNK into each well to reach final concentrations of 0, 5 and 10 µmol/L. BrdU were added into each well at least 2 hours prior to the end of the experiments. After incubation at 37°C for 24 hours, the cell proliferation was analyzed using BrdU Cell Proliferation Assay Kit (Millipore) according to the manufacturer's instructions in comparison with control wells (0 µmol/L of NNK). Briefly, the cells were fixed and denatured their DNA by fixing solution for 30 minutes. Then, the fixing solution was aspirated and the plate was dried. After a triple washing with Wash Buffer, the anti-BrdU monoclonal was added into each well and incubated for 1 hour at room temperature. After a triple washing with Wash Buffer, the peroxidase-conjugated anti-IgG antibody was added into each well and incubated for 30 minutes at room temperature. After a triple washing with Wash Buffer, a final washing was performed by flooding the entire plate with distilled water. Next, peroxidase substrate, TMB, was added into each well and incubated for 30 minutes at room temperature in the dark. The reaction was stopped by addition of the acid Stop Solution. Finally, the absorbance rate was measured by spectrophotometer microplate reader set at dual wavelength of 450/550 nm. Each concentration was repeated in three wells. The experiments were carried out in triplicate. The percentage of cell proliferation was calculated by following formula.

$$\%\text{Cell proliferation} = \frac{\text{Mean absorbance in tested wells} \times 100}{\text{Mean absorbance in control wells}}$$

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2.11.2 Effect of γ-tocotrienol and phytic acid on the NNK- increased cell proliferation of A549 cells

To investigate the preventive effect of γ-tocotrienol (γ-T_3) or phytic acid (IP_6) against the NNK-increased cell proliferation of A549 cells, the BrdU cell proliferation assay was performed. The A549 cells were plated in 96-well plates at 2 x 10^4 cells/ 100 µL of appropriate cell culture media for each well, and incubated at 37°C for 24 hours. Next, the cells were pre-treated with 0-80 µmol/L of γ-tocotrienol or phytic acid for 12 hours, and then treated with 10 µmol/L of NNK for 24 hours. The final concentrations of γ-tocotrienol pre-treatment were 0, 30, 40 and 80 µmol/L, and phytic acid pre-treatment were 0, 40 and 80 µmol/L. After NNK treatment, the cell prolifertion in each well was determined by BrdU cell proliferation assay in comparison with control wells (0 µmol/L of γ-tocotrienol or phytic acid). The half maximal inhibitory concentration (IC50), 20%- and 80%-inhibitory concentration (IC20 and IC80) values were calculated using the nonlinear regression model.

2.11.3 Effect of γ-tocotrienol on the cell proliferation of A549 cells

To investigate the suppressive effect of γ-tocotrienol on the cell proliferation of A549 cells, BrdU cell proliferation assay was used to measurement the cell proliferation. The A549 cells were plated in 96-well plates at 2 x 10^4 cells/ 100 µL of appropriate cell culture media for each well, and incubated at 37°C for 24 hours. Next, the cells were treated with medium contained γ-tocotrienol for 24 hours. The final concentration of γ-tocotrienol was 0, 40, 60, 80 and 100 µmol/L. After treatment, the cell proliferation in each well was determined by BrdU cell proliferation assay in comparison with control wells (0 µmol/L of γ-tocotrienol). The half maximal inhibitory concentration (IC50), 20%- and 80%-inhibitory concentration (IC20 and IC80) values were calculated using the nonlinear regression model.

2.12 Cell cycle distribution analysis by flow cytometry

Flow cytometry is adapted for analysis of various cellular components, organelles or functions. The nuclear DNA content of a cell can also be quantitatively measured by flow cytometry due to its ability to stoichiometrically bind with some fluorescent dye such as propidium iodide (PI). Propidium iodide is an intercalating agent, binds to DNA, and a fluorescent molecule, fluoresces red when excited with light at a wavelength of 488 nm. The emitted fluorescence is proportional to the DNA

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content present in cells. In addition to determining the relative cellular DNA content, flow cytometry also used to identify the cell distribution during the various phases of the cell cycle, including G1-, S- (DNA synthesis phase), G2- and M-phase (mitosis). Resting cells (G0/G1 phase) contain two copies of each chromosome. As cells begin cycling, they synthesize chromosomal DNA (S phase). Fluorescence intensity from intercalating PI increases, until all chromosomal DNA has doubled (G2/M phase). At this stage, the G2/M cells fluoresce with twice the PI intensity of the G0/G1 population. The G2/M cells eventually divide into two cells. This technique permits *in vitro* characterization of drugs or compounds employed in chemotherapy to inhibit cancer cell growth and division (296, 297).

2.12.1 Effect of γ-tocotrienol on cell cycle distribution in NNK-treated A549 cells

To investigate the effect of γ-tocotrienol on cell cycle distribution in NNK-treated A549 cells, PI staining was performed to determine the cell cycle distribution. A549 cells were grown to about 60-70% confluency in a 100-mm Petri disk. Then, the cells were pre-treated with 30, 40 and 60 µmol/L of γ-tocotrienol with a negative control (0 µmol/L of γ-tocotrienol) for 12 hours, and then treated with 10 µmol/L of NNK for 24 hours. After treatment, the cells were harvested by trypsinization and centrifuged at 1500 rpm in a 15-mL tube. Harvested cells were washed twice with 1xPBS and re-suspended with 1xPBS. Then, the cells were fixed by adding 4 mL of cold 100% ethanol added in pulses of 1 ml each while being vortexed. The cells were stored -20°C for a minimum of 24 hours or up to 1 month before analysis. In the step of analysis, samples were centrifuged and removed from the ethanol. Cell pellets were washed twice with 1xPBS and re-suspended in 0.5–1 ml of propidium iodide (50µg/mL) solution in PBS plus 500 µg/mL of RNase, and incubated for 1 hour at 37°C. Cell cycle distribution was analyzed by flow cytometry (FACSCanto II; BD Biosciences, San Jose, CA).

2.12.2 Effect of γ-tocotrienol on cell cycle distribution of A549 cells

To investigate the effect of γ-tocotrienol on cell cycle distribution of A549 cells, PI staining was performed to determine the cell cycle distribution. The A549 cells were grown to about 70% confluency in a 100-mm Petri disk and then treated with 40, 60 and 80 µmol/L of γ-tocotrienol for 24 hours with a negative control (0 µmol/L of γ-tocotrienol). After treatment, the cells were harvested by trypsinization

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and centrifuged at 1500 rpm in a 15-mL tube. Harvested cells were washed twice with 1xPBS and re-suspended with 1xPBS. Then, the cells were fixed by adding 4 mL of cold 100% ethanol added in pulses of 1 ml each while being vortexed. The cells were stored -20°C for a minimum of 24 hours or up to 1 month before analysis. In the step of analysis, samples were centrifuged and removed from the ethanol. Cell pellets were washed twice with 1xPBS and resuspended in 0.5–1 ml of propidium iodide (50μg/mL) solution in PBS plus 500 μg/mL of RNase, and incubated for 1 hour at 37°C. Cell cycle distribution was analyzed by flow cytometry (FACSCanto II; BD Biosciences, San Jose, CA).

2.13 Detection of apoptosis by flow cytometry

Apoptosis, or programmed cell death, is an important regulatory pathway of cell growth and proliferation. During apoptosis, a complex and multistep mechanisms regulate the cell propensity to respond to an environmental or intrinsic apoptosis signals and then result in physiological changes, including externalization of phosphatidylserine (PS) to the cell surface, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, and loss of membrane integrity. In the early stages of apoptosis, the translocation of phosphatidylserine or PS, a phospholipid component, from the inner side of the plasma membrane to the outer layer and stable exposure at the external surface of the cell due to decreasing activity of flippase. Then, the PS externalization mediates macrophage recognition of apoptotic cells. Annexin V is a 36 kDa phospholipid-binding protein and has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}). Annexin-V is available conjugated to a number of different fluorochromes including Alexa Fluor-647 and FITC. Therefore, detection of fluorochrome-conjugated Annexin-V, which binds to PS on the cell surface, by flow cytometry can be applied to detect apoptotic cells (298-300). As the progression of apoptotic process, cell membrane integrity is lost and the cells become more permeable to a variety of DNA-binding dyes, such as propidium iodide (PI) and 7-AAD. Therefore, it is possible to distinguish between early apoptotic, late apoptotic, and dead cells. Early apoptotic cells are stained with Annexin V but not PI. Dead cells are stained with both Annexin V and PI, whereas viable cells cannot be stained with either.

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Effect of γ-tocotrienol on the induction of apoptosis in A549 cells, characterized by the externalization of phosphatidylserine (PS)

To determine the effect of γ-tocotrienol on apoptosis induction in 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. Apoptosis detection was performed using the ApopNexin FITC apoptosis detection kit for flow cytometry analysis (Chemicon). Briefly, the A549 cells were grown to about 70% confluency in a 100-mm Petri disk and then treated with 60 μmol/L of γ-tocotrienol for 24 and 48 hours with a negative control (0 μmol/L of γ-tocotrienol). After treatment, the cells were harvested and centrifuged at 1500 rpm in a 15-mL tube. The harvested cells were washed twice with 1xPBS and resuspended in 500 μL of binding buffer. Cell staining with annexin V and PI was performed according to the manufacturer's instructions. FITC-labeled annexin V and PI were added to the cells and then incubated at room temperature for 15 minutes in the dark. The apoptosis rate was immediately determined by flow cytometry (FACSCanto II; BD Biosciences, San Jose, CA).

2.14 Scanning electron microscopy

During apoptosis, the cascade of biochemical and physiological events lead to the cellular morphology changes. Changes in nuclear morphology and in organelle structure as well as specific phenomena at the cell surface level, namely blebbing, are often considered as markers associated with apoptotic cell. The complex sequence of structural modifications of cell death can be demonstrated by light and electron microscopy techniques, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (301-303).

Scanning electron microscopy or SEM is a type of electron microscope that images a sample by scanning it with a beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties, such as electrical conductivity. A specimen for SEM is normally required to be completely dry, since the specimen chamber is at high vacuum. Therefore, living cells, tissues and whole organisms usually require chemical fixation to preserve and stabilize their structure. Fixation is usually performed by incubation in a solution

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of a buffered chemical fixative, such as glutaraldehyde, and optionally followed by post-fixation with osmium tetroxide. Then, the fixed tissue is dehydrated to replace water in the cells with organic solvents, such as ethanol, because air-drying causes cell collapse and shrinkage. Next, these solvents are replaced with a transitional fluid such as liquid carbon dioxide by critical point drying. Finally, the carbon dioxide is removed while in a supercritical state, thus no gas-liquid interface is present within the sample during drying. The dry specimen is usually mounted on a specimen stub using an adhesive such as epoxy resin or electrically conductive double-sided adhesive tape, and sputter-coated with gold or gold/palladium alloy before examination in the microscope (304, 305).

Effect of γ-tocotrienol on the induction of apoptosis in A549 cells, characterized by the cell morphology changes

A549 cells were grown to about 70% confluency on the rounded coverslip and then treated with 60 μmol/L of γ-tocotrienol for 12, 24, and 36 hours. After treatment, the cells were washed twice with 1xPBS and then fixed with 2.5% glutaraldehyde in phosphate buffer at room temperature for 2 hours. Next, the cells were washed twice with 1xPBS and then fixed with 2% OsO_4 (osmium tetroxide) in phosphate buffer at room temperature for 1 hour. The cells were dehydrated in a series of ethanol rinses, followed by critical-point drying using CO_2 as the transitional fluid. Finally, the cells were mounted on stubs and were coated with platinum, examined with a scanning electron microscope (JEOL JSM-6610LV), and photographed.

2.15 Western blotting

Western blotting is an analytical technique used to detect specific proteins in the tissue homogenate or cell extract. This technique uses gel electrophoresis to separate native proteins due to their size. Then, the proteins are transferred to a membrane (typically nitrocellulose or PVDF), where they are probed using antibodies specific to the target protein. The most common type of gel electrophoresis employs polyacrylamide gels and buffers loaded with sodium dodecyl sulfate (SDS). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis or SDS-PAGE uses SDS, strong reducing agents, to maintain proteins in a denatured state (primary structure of protein). During electrophoresis, the proteins become covered with negatively

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charged of SDS and move through the acrylamide mesh of the gel to the positively charged electrode. Smaller proteins migrate faster than the large proteins through this mesh, which allow them to separate according to their size. The concentration of acrylamide in is correlated with the resolution of proteins separation. For example, the high concentration of acrylamide gives the best resolution for small molecular weight proteins, but the low concentration of acrylamide gives the best resolution for high molecular weight proteins.

Before loading cell lysate into the gel for electrophoresis, the concentration of protein in the cell lysate were determined by Bradford protein assay. The Bradford assay is a colorimetric method, which is based on an absorbance shift of the dye, named Coomassie Brilliant Blue G-250. In acidic conditions, the red form of the dye is converted into bluer form due to its interaction with protein. Therefore, the protein concentrations are estimated by referencing the obtained absorbance with a series of standard protein dilutions, which are assayed alongside the unknown samples.

After transferring proteins from a gel to membrane, the membrane is placed in a dilute solution of protein, such as 3-5% Bovine serum albumin (BSA) or non-fat dry milk in tris buffered saline (TBS) or phosphate buffered saline (PBS), with a minute percentage of detergent, such as Tween 20 or Triton X-100, to block non-specific binding between the membrane and the antibody used for detection of the target protein. Then, the target protein is detected using specific antibodies and demonstrated by colorimetric detection, radioactive detection, fluorescently labeled probe, or enhanced chemiluminescence system or ECL.

Enhanced chemiluminescence system is a light emitting nonradioactive method to detect immobilized molecule that specifically recognizes by horseradish peroxidase-labeled antibodies. This enzyme complex then catalyzes the enhanced chemiluminescent substrate to be a sensitized reagent in the vicinity of the molecule of interest, which further oxidation by hydrogen peroxide (H_2O_2), to produce a triplet or excited carbonyl. Finally the triplet carbonyl emits light, when it decays to the singlet carbonyl. The emitted light can be detected by photographic film or by CCD cameras which capture a digital image of the western blot.

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2.15.1 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. In case of time-course effect, the A549 cells were treated with 10µmol/L of NNK for 0, 6, 12, 18 and 24 hours. In case of dose-response effect, the A549 cells were treated with 0, 1, 5 and 10µmol/L of NNK for 24 hours (to study the expression of PCNA and cyclinE proteins), and were treated with 0, 1, 5, 10 and 50µmol/L of NNK for 12 hours (to study the expression of cyclinD1 protein).

To investigate the effect of NNK on MAPK signal-transducing proteins in A549 cells, the protein expression levels of p-MEK1/2 and K-ras were also determined by western blotting. The A549 cells were treated with 10µmol/L of NNK for 0, 15, 30, 60 and 120 minutes (to study the expression of p-MEK1/2 protein), and for 0, 5, 10 and 15 minutes (to study the expression of K-ras protein).

After treatment, the cells were washed with ice-cold PBS and harvested by scraping. Whole-cell lysates were prepared by suspending cell pellets in whole cell lysis buffer (250mM NaCl, 20mM HEPES, and 1% NP-40) with freshly added protease inhibitors (1mM EGTA, 5mM EDTA, 5mM NaF, 1mM Na_3VO_4, 2µg/ml aprotinin, 5µg/ml leupeptin, 1mM PMSF) on ice for 30 min. 50-80 µg protein was separated in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for PCNA, cyclinE and cyclinD1 was 1/2000, for p-MEK1/2 and K-ras was 1/500, and for β-actin was 1/4000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

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2.15.2 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 were determined by western blotting. In case of time-course effect, the A549 cells were pre-treated with 30µmol/L of γ-tocotrienol for 12 hours, before 10µmol/L of NNK treatment for 0, 6, 12, 18 and 24 hours. In case of dose-response effect, the A549 cells were pre-treated with 0, 10, 20 and 30µmol/L of γ-tocotrienol for 12 hours, before 10µmol/L of NNK treatment for 24 hours (to study the expression of PCNA and cyclinE proteins), and 12 hours (to study the expression of cyclinD1 protein).

To 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 also determined by western blotting. In case of p-MEK1/2, the A549 cells were pre-treated with 0, 10, 20, 30 and 40µmol/L of γ-tocotrienol, and then treated with 10µmol/L of NNK for 120 minutes. In case of K-ras, the A549 cells were pre-treated with 0, 20, 30 and 40µmol/L of γ-tocotrienol, and then treated with 10µmol/L of NNK for 15 minutes.

After treatment, total cell lysates were harvested and analyzed the expression of PCNA, cyclinE, CyclinD1, p-MEK1/2 and K-ras proteins by western blotting as described previously.

2.15.3 Effect of NNK on the nuclear accumulation of DNMT1 protein in A549 cells

To investigate the effect of NNK on the nuclear accumulation of DNMT1 protein in A549 cells, the nuclear lysates were collected and analyzed the levels of DNMT1 protein in the nucleus by western blotting. A549 cells were treated with 10µmol/L of NNK for 0, 45, 90 and 180 minutes. After treatment, the cells were washed with ice-cold PBS and harvested by scraping. The cells were lysed with lysis buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 0.5 mg/ml benzamidine) in addition with 0.5%NP-40 on ice for 15 minutes. After centrifugation at 12,000 rpm for 30 seconds, supernatants were collected as the cytosolic lysates.

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Then, pellets were lysed with nuclear lysis buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine) on ice for 30 minutes. After centrifugation at 14,000 rpm for 12 minutes, supernatants were collected as the nuclear lysates. 60-80 μg protein was separated in 8% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for DNMT1 was 1/500, for PCNA was 1/2000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

2.15.4 Effect of γ-tocotrienol and phytic acid on the NNK-induced nuclear accumulation of DNMT1 protein in A549 cells

To investigate the chemopreventive effect of γ-tocotrienol and phytic acid on the NNK-induced nuclear accumulation of DNMT1 protein in A549 cells, the nuclear lysates were collected and analyzed the levels of DNMT1 protein in the nucleus by western blotting. The A549 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, 45, 90 and 180 minutes. After treatment, the nuclear lysates were harvested and analyzed the nuclear accumulation of DNMT1 protein by western blotting as described previously.

2.15.5 Effect of NNK on the expression of cytochrome P450 isotypes 2A6 (CYP2A6) and 2A13 (CYP2A13) proteins in A549 cells

To investigate the effect of NNK on the expression of cytochrome P450 isotypes 2A6 (CYP2A6) and 2A13 (CYP2A13) proteins in A549 cells, the protein expression levels of CYP2A6 and CYP2A13 were determined by western blotting. The A549 cells were treated with 0, 1, 5, 10 and 50μmol/L of NNK for 12 hours. After treatment, the cells were washed with ice-cold PBS and harvested by scraping.

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Whole-cell lysates were prepared by suspending cell pellets in whole cell lysis buffer (250mM NaCl, 20mM HEPES, and 1% NP-40) with freshly added protease inhibitors (1mM EGTA, 5mM EDTA, 5mM NaF, 1mM Na_3VO_4, 2µg/ml aprotinin, 5µg/ml leupeptin, 1mM PMSF) on ice for 30 min. 60-80 µg protein was separated in 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for CYP2A6 and CYP2A13 was 1/1000, and for β-actin was 1/4000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

2.15.6 Effect of γ-tocotrienol and phytic acid on the NNK-induced overexpression of CYP2A6 and CYP2A13 proteins in A549 cells

To investigate the chemopreventive effect of γ-tocotrienol and phytic acid on the NNK-induced overexpression of CYP2A6 and CYP2A13 proteins in A549 cells, the expression of CYP2A6 and CYP2A13 proteins were determined by western blotting. The A549 cells were pre-treated with 0, 10, 20 and 30µmol/L of γ-tocotrienol or with 0, 40, 60 and 80µmol/L of phytic acid for 12 hours, before 10µmol/L of NNK treatment for 12 hours. After treatment, total cell lysates were harvested and analyzed the expression of CYP2A6 and CYP2A13 proteins by western blotting as described previously.

2.15.7 Effect of γ-tocotrienol on the expression of cyclinD1 protein in A549 cells

To investigate the effect of γ-tocotrienol on the expression of cyclinD1 protein in A549 cells, the expression of cyclinD1 protein was determined by western blotting. The A549 cells were treated with 0, 20, 40, 60 and 80µmol/L of γ-tocotrienol for 24 hours. After treatment, the cells were washed with ice-cold PBS and harvested by scraping. Whole-cell lysates were prepared by suspending cell pellets in whole cell lysis buffer (250mM NaCl, 20mM HEPES, and 1% NP-40) with freshly added

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protease inhibitors (1mM EGTA, 5mM EDTA, 5mM NaF, 1mM Na3VO4, 2μg/ml aprotinin, 5μg/ml leupeptin, 1mM PMSF) on ice for 30 min. 60-80 μg protein was separated in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for cyclinD1 was 1/2000, and for β-actin was 1/4000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

2.15.8 Effect of γ-tocotrienol on the expression of apoptosis-related proteins in A549 cells

To investigate the effect of γ-tocotrienol on the induction of apoptosis in A549 cells, the expression of apoptosis-related proteins, including cleaved PARP, procaspase-3, procaspase-8, procaspase-9, bcl-XL, and bax, were analyzed by western blotting. The A549 cells were treated with 0, 20, 40, 60 and 80μmol/L of γ-tocotrienol for 24 hours. After treatment, the cells were washed with ice-cold PBS and harvested by scraping. Whole-cell lysates were prepared by suspending cell pellets in whole cell lysis buffer (250mM NaCl, 20mM HEPES, and 1% NP-40) with freshly added protease inhibitors (1mM EGTA, 5mM EDTA, 5mM NaF, 1mM Na3VO4, 2μg/ml aprotinin, 5μg/ml leupeptin, 1mM PMSF) on ice for 30 min. 80-100 μg protein was separated in 8-12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for procaspase-3, procaspase-8, procaspase-9, bcl-XL, and bax was 1/1000, for PARP was 1/4000 and for β-actin was 1/5000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was

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washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

2.15.9 Effect of LPS on the nuclear accumulation of NF-κB protein in human colon cancer cell line SW480

To investigate the effect of lipopolysaccharide (LPS) on the nuclear accumulation of nuclear factor-κB (NF-κB) protein in SW480 cells, the nuclear lysates were collected and analyzed the levels of NF-κB protein in the nucleus by western blotting. SW480 cells were treated with 1μg/mL of LPS for 0, 0.5, 1 and 3 hours. After treatment, the cells were washed with ice-cold PBS and harvested by scraping. The cells were lysed with lysis buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine) in addition with 0.5%NP-40 on ice for 15 minutes. After centrifugation at 12,000 rpm for 30 seconds, supernatants were collected as the cytosolic lysates. Then, pellets were lysed with nuclear lysis buffer B (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine) on ice for 30 minutes. After centrifugation at 14,000 rpm for 12 minutes, supernatants were collected as the nuclear lysates. 60-80 μg protein was separated in 10% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for NF-κB was 1/800, for PCNA was 1/2000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

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2.15.10 Effect of phytic acid on the NNK-induced nuclear accumulation of NF-κB protein in SW480 cells

To investigate the anti-inflammatory effect of phytic acid on the LPS-induced nuclear accumulation of NF-κB protein in SW480 cells, the nuclear lysates were collected and analyzed the levels of NF-κB protein in the nucleus by western blotting. The SW480 cells were pre-treated with 0, 40, 60 and 80μmol/L of phytic acid for 12 hours before treatment with 1μg/mL of LPS for 3 hours. After treatment, the nuclear lysates were harvested and analyzed the nuclear accumulation of NF-κB protein by western blotting as described previously.

2.15.11 Effect of LPS on the expression of iNOS and cyclinD1 proteins in SW480 cells

To investigate the effect of LPS on the expression of inflammatory-related protein, inducible nitric oxide synthase (iNOS), and the cell cycle regulatory protein, cyclinD1, in SW480 cells, the protein expression of iNOS and cyclinD1 were determined by western blotting. In case of iNOS protein, the SW480 cells were treated with 1μg/mL of LPS for 0, 2, 4 and 6 hours. In case of cyclinD1 protein, the SW480 cells were treated with 1μg/mL of LPS for 0, 0.25, 0.5 and 1 hours.

After treatment, the cells were washed with ice-cold PBS and harvested by scraping. Whole-cell lysates were prepared by suspending cell pellets in whole cell lysis buffer (250mM NaCl, 20mM HEPES, and 1% NP-40) with freshly added protease inhibitors (1mM EGTA, 5mM EDTA, 5mM NaF, 1mM Na3VO4, 2μg/ml aprotinin, 5μg/ml leupeptin, 1mM PMSF) on ice for 30 min. 60-80 μg protein was separated in 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked in a blocking buffer (3% BSA in PBST, pH 7.4) for 1 hour at room temperature, and then incubated overnight with the appropriate monoclonal or polyclonal primary antibody in the blocking buffer at 4°C. Primary antibody dilution for iNOS was 1/800, for cyclinD1 was 1/2000, and for β-actin was 1/4000. The membrane was washed five times with PBST (5 min each time) and incubated with anti-mouse or anti-rabbit secondary antibody at 37°C for 1 hour. The membrane was washed five times with PBST (5 min each time) and then washed with PBS once. After the final washing, the membrane was developed with an ECL

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substrate and visualized by the Molecular Imager ChemiDoc XRS System (Bio-Rad) or exposed to photographic film.

2.15.12 Effect of phytic acid on the LPS-induced overexpression of iNOS and cyclinD1 proteins in SW480 cells

To investigate the anti-inflammatory effect of phytic acid on the LPS-induced overexpression of iNOS and cyclinD1 proteins in SW480 cells, the expression of iNOS and cyclinD1 proteins were determined by western blotting. In case of iNOS protein, the SW480 cells were pre-treated with 0, 40, 60 and 80μmol/L of phytic acid for 12 hours, before 1μg/mL of LPS treatment for 6 hours. In case of cyclinD1 protein, the SW480 cells were pre-treated with 0, 40, 60 and 80μmol/L of phytic acid for 12 hours, before 1μg/mL of LPS treatment for 12 hours. After treatment, total cell lysates were harvested and analyzed the expression of iNOS and CyclinD1 proteins by western blotting as described previously.

2.16 DNA damage analysis by Comet assay

The Comet assay or single-cell gel electrophoresis is a simple and sensitive technique for measuring deoxyribonucleic acid (DNA) strand breaks or DNA damage in eukaryotic cells (306-308). Firstly, the cells are embedded in agarose on a microscope slide. Then, the cells are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix and electrophoresed at high pH. Under an electrophoretic field, damaged DNA (containing fragments and strand breaks) is separated from intact DNA, yielding a comet tail shape under the microscope. Finally, the comet tails were observed by fluorescence microscopy. The intensity of the comet tail relative to the head reflects the number of DNA breaks. The basis for this is that loops containing a break lose their supercoiling and become free to extend toward the anode. DNA migration is a function of both size and the number of broken ends of the DNA. Tail length increases with damage initially and then reaches a maximum depending on the electrophoretic conditions, not the size of fragments.

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

To examine the effect of γ-tocotrienol and phyic acid on the NNK-induced DNA damage in A549 cells, OxiSelect™ Comet assay kit 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. After treatment, the cells were harvested by scraping and washed with ice-cold PBS without Mg^{2+} and Ca^{2+}. The collected cells were resuspend with ice-cold PBS without Mg^{2+} and Ca^{2+} to be 1 x 10^5 cells/mL. Then, the cells were combined at 1:10 ratio (v/v) with Comet Agarose, which heated at 90-95ºC in a water bath for 20 minutes and cooled down to 37ºC for 20 minutes, and immediately pipette onto the slide. After incubation at 4ºC for 15 minutes in the dark, the slide was immersed in pre-chilled lysis buffer at 4ºC for 30-60 minutes in the dark, and then replaced with pre-chilled alkaline solution at 4ºC for 30 minutes in the dark. Alkaline electrophoresis was performed for 15-30 minutes with voltage at 1 volt/centimeter. After electrophoresis, the slide was immersed in pre-chilled distilled water for 2 minutes, and then repeated twice more. Next, the slide was immersed in cold 70% ethanol for 5 minutes and then allowed to air dry. Once it was completely dry, the Vista Green DNA Dye was added and incubated at room temperature for 15 minutes. Finally, comet tail was visualized with fluorescence microscope.

2.17 Cell migration analysis by Boyden chamber assay

The Boyden chamber assay is a useful tool to study cell migration and cell invasion. This technique is based on a chamber of two medium-filled compartments separated by a microporous membrane (309, 310). In general, cells are placed in the upper compartment and are allowed to migrate through the pores of the membrane into the lower compartment, in which chemotactic agents are present. After an appropriate incubation time, the membrane between the two compartments is fixed and stained (to study the cell invasion), and the number of cells that have migrated to the lower side of the membrane is determined (to study the cell migration).

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2.17.1 Effect of LPS on the secretion of chemoattractants from colorectal cancer cell line SW480

To investigate the effect of lipopolysaccharide (LPS) on the secretion of chemoattractants, which induced the migration of leukocytes into its secreting tissues, the Boyden chamber assay was used to examine the secretion of chemoattractants in LPS-treated SW480 cells. The SW480 cells were treated with 0, 0.25, 0.5 and 1µg/mL of LPS in 0.5% fetal bovine serum-contained medium for 36 hours. After treatment, the supernatant was collected and placed into the lower compartment of Boyden chamber. Then, membrane filters type 8.0 µm was placed in between the lower and upper compartments of Boyden chamber. Next, U937 cells, a human leukemic monocyte lymphoma cell line, were re-suspended to 1.5×10^5cells/mL and placed into the upper compartments of Boyden chamber. Finally, the Boyden chamber was incubated in CO_2 incubator at 37^oC, 5% CO_2 for 24 hours, and immediately determined the number of U937 cells that had migrated to the lower compartment of Boyden chamber.

2.17.2 Effect of γ-tocotrienol and phytic acid on the LPS-induced secretion of chemoattractants in SW480 cells

To investigate the suppressive effects of γ-tocotrienol or phytic acid against the LPS-induced chemoattractants secretion in SW480 cells, the Boyden chamber assay was performed. The SW480 cells were pre-treated with 30 and 40µmol/L of γ-tocotrienol or 40 and 80µmol/L of phytic acid in 10% fetal bovine serum-contained medium for 12 hours before treatment with 1µg/mL of LPS in 0.5% fetal bovine serum-contained medium for 36 hours. After treatment, the Boyden chamber assay was performed as described previously.

2.18 Statistical analysis

Statistical analyses were expressed as means ± SD. The data for liver, kidney, lung, or final body weights were analyzed by the Student's *t*-test. The incidence, multiplicity and size of lung lesions and colonic tumors were also analyzed by Student's *t*-test. The relative mRNA expression of *Cyp2a5, iNos*, *Cox2* and *Tnfα* genes in mouse tissues was analyzed by Mann—Whitney *U*-test. The cell proliferative index (Ki67 staining) was analyzed by Welch's *t*-test. All *in vitro* experiments were performed at least in triplicate to confirm reproducibility.

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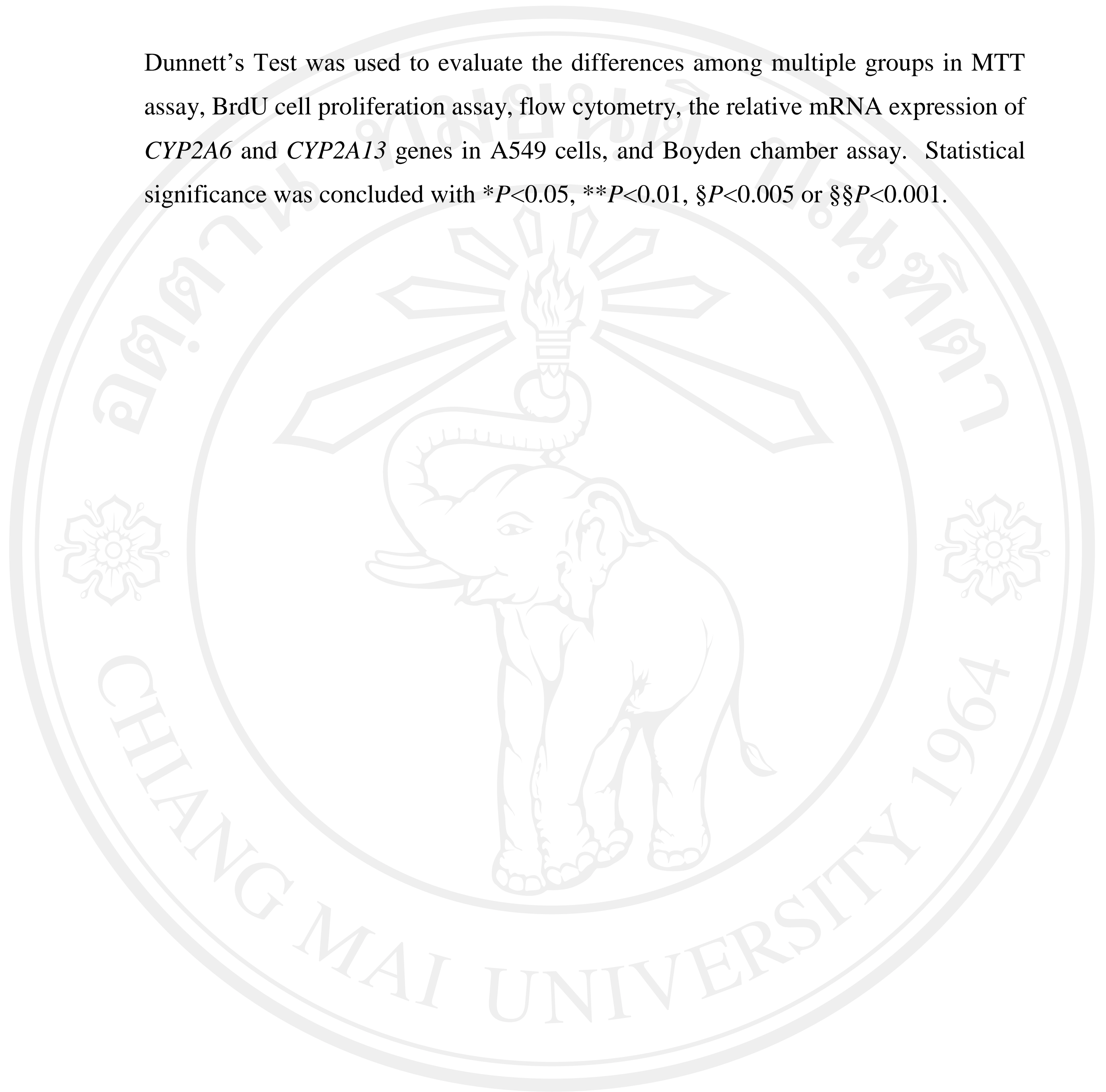
Dunnett’s Test was used to evaluate the differences among multiple groups in MTT assay, BrdU cell proliferation assay, flow cytometry, the relative mRNA expression of *CYP2A6* and *CYP2A13* genes in A549 cells, and Boyden chamber assay. Statistical significance was concluded with $^{*}P<0.05$, $^{**}P<0.01$, §$P<0.005$ or §§$P<0.001$.



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