CHAPTER 2

Materials and methods

2.1 Chemicals and instruments are shown in Appendices A and B

2.2 Scope of study

The scope of study is summarized in Figure 2.1.

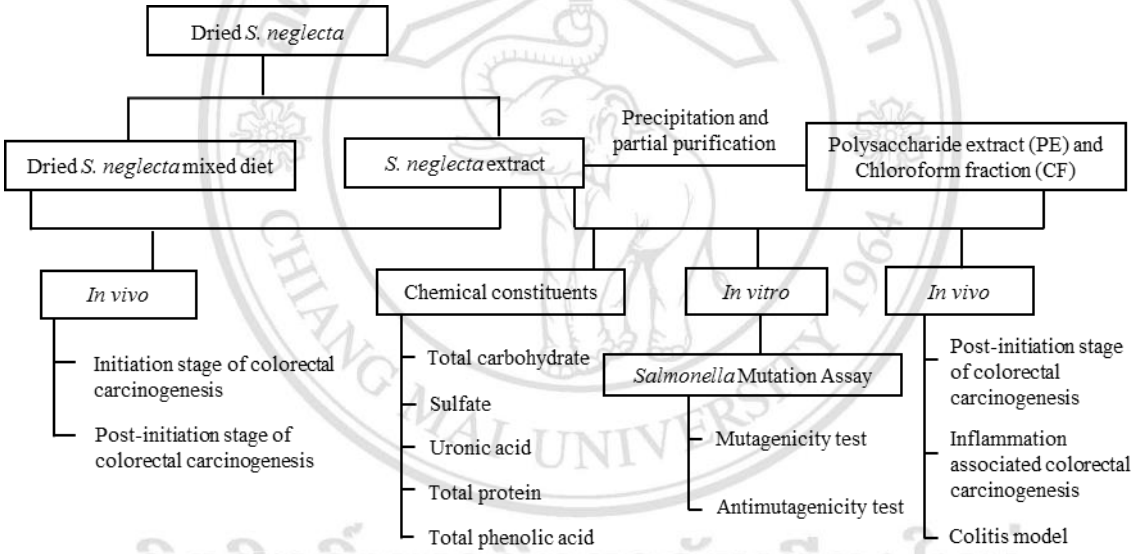

Figure 2.1 Scope of study

2.3 Algae material

The freshwater green macroalgae (*Spirogyra neglecta*) was collected from the cultivation pool of Na Kuha village, Saun Kuen Sub district, Muang District, Phrae Province, Thailand. These algae were identified and authenticated according to the morphology of vegetative cells, sex cells, conjugation tubes and zygotes by the method of John *et al*. It was then washed with fresh water, air dried and stored at -20 ^{o}C before extraction.

2.4 Preparation of dried *S. neglecta* mixed diet and *S. neglecta* extract

The algae were dried at 50-60 ^{o}C for 48 hr in a hot-air oven. Dried algae were ground into powder and mixed with a basal diet at amounts comprising 1% and 4 % for *S. neglecta* mixed diet (SND). The *S. neglecta* extract (SNE) was prepared by boiling the algae in distilled water for 2 hr under continuous stirring. The residue was removed by filtration and centrifugation. The supernatant was collected and dried using a freeze-dryer to obtain *S. neglecta* hot water extract powder. The SNE was stored at -20 ^{o}C before use.

2.5 Preparation of crude polysaccharide from *S. neglecta* extract

Fifty mg/ml of *S. neglecta* extract was defatted and decolorized by chloroform and methanol at the ratio of 3:1 (v/v). The lower part, chloroform fraction, was collected and evaporated. This dried extract was called "CF". The upper part was then added into 4 volumes of absolute ethanol. The mixture was then allowed to stand at 4 ^{o}C for 24 hr and the polysaccharide precipitate was collected by centrifugation, washed with absolute ethanol two times, and freeze-dried. The resulting polysaccharide extract was referred as "PE" throughout this thesis.

2.6 Fractionation of sulfated polysaccharide by anion exchange chromatography

The polysaccharide extract (PE) was dissolved in 0.5 M sodium acetate buffer, pH 6.0 and filtered through a Whatman filter paper no 1. The PE was fractionated using ion-exchange chromatography on DEAE Sepharose fast flow column. The column was equilibrated with 0.5 M sodium acetate buffer, pH 6.0. The 50 mg/ml of PE were loaded onto the DEAE Sepharose column (3x30 cm) and fractionated with a step gradient of NaCl from 0.1 to 3.0 M at a flow rate of 2 ml/min. The major fractions were pooled based on the phenol-sulfuric acid assay. These fractions were dialyzed and freeze-dried.

2.7 Chemical composition analysis

2.7.1 Total phenolic compounds determination

The total phenolic compounds were measured using the Folin-Ciocalteau method, as described by Inboot *et al* (Inboot *et al.*, 2012). Briefly, the reaction mixture was composed by mixing of the extract or standard with Folin-ciocalteu's working reagent

and incubated at room temperature for 10 min. After incubation, 7% sodium carbonate was added and incubated at 45 ^{o}C for 15 min. The absorption at 765 nm was measured using an UV/VIS Spectrophotometer. The total phenolic compounds were expressed as mg gallic acid equivalents per g of extract (mg GAE/g extract) using a calibration curve. All measurements were carried out in triplicate.

2.7.2 Phenolic acid determination

The analysis of the phenolic acids was performed by Reverse-phase HPLC. The column was a Zorbax C18 column (4.6x250 mm, 5 µm). The HPLC method conditions were described elsewhere (Punvittayagul *et al.*, 2014). The injection volume was 10 µl, with a flow rate of 1 ml/min. The HPLC grade methanol and 3% acetic acid in deionized water were used as mobile phases A and B, respectively. The gradient was performed as follows: 0-15 min, 10-15% A; 15-40 min, 15-30% A; 40-45 min, 30-70% A; 45-50 min, 70-90% A; 50-60 min and 90-10% A. Detection wavelengths were 260, 280 and 320 nm and extracts were analyzed in duplicate. Peaks were identified by comparing their retention time and UV-Vis spectra with the reference compounds, and data were quantified using the corresponding curves of the reference compounds as standards.

2.7.3 Total carbohydrate determination

Total carbohydrate content was determined according to the phenol-sulfuric method with minor modifications (Masuko *et al.*, 2005) using glucose as standard. The extract or standard was mixed with concentrated sulfuric acid and incubated at 90 ^{o}C for 15 min. Subsequently, 5% phenol was added to the mixture and shook at room temperature for 5 min. The absorption at 490 nm was measured using an UV-Vis spectrophotometer. The total carbohydrate was expressed as mg glucose per g of extract using a calibration curve.

2.7.4 Total sulfate determination

The total sulfate was determined according to a colorimetric dye binding assay (Pothacharoen *et al.*, 2014). Chondroitin 6-sulfate (CS-C) standard or extract was mixed with DMMB dye solution and absorbance was measured at 620 nm. A standard curve of CS-C concentration was plotted. The concentration of sulfate in the extract was calculated from the standard curve and expressed as mg chondroitin sulfate C per g extract.

2.7.5 Uronic acid determination

Uronic acid is widely determined as the representative of glycosaminoglycans. Uronic acid content was assayed by the carbazole-sulfuric acid method using glucuronic acid lactone as standard (Phitak *et al.*, 2012). The mixture was composed by the mixing of glucuronic acid lactone standard or extract and concentrated sulfuric acid-borate reagent, incubated at 100 ^{o}C for 15 min and cooled to room temperature in ice bath. Subsequently, the solution of carbazole in absolute ethanol was added. The reaction mixture was incubated at 100 ^{o}C for 15 min and cooled down to room temperature in ice bath. The absorbance of the pink color was measured by spectrophotometer at 540 nm. The uronic acid content was expressed as mg glucuronic acid lactone per g of extract using a calibration curve.

2.7.6 Total protein determination

The amount of protein was estimated by the Bradford protein assay (Bradford, 1976) using bovine serum albumin as standard. Briefly, the extract or standard and Bradford working reagent were mixed and incubated at room temperature for 5 min. The absorption at 570 nm was measured using a microplate reader. The total protein in the extract was calculated from the standard curve and expressed as mg per g extract.

2.8 Monosaccharide composition analysis by thin layer chromatography

The *S. neglecta* extract and polysaccharide extract were hydrolyzed in 6% H_2SO_4 and centrifuged after neutralization by $CaCO_3$. The monosaccharides in extracts were separated by the thin layer chromatogram. D-Glucose, L-fucose, D-galactose, L-arabinose L-rhamnose, D-mannose, D-xylose and D-fructose were used as standard sugars. TLC was performed on thin layer plate (silica gel 60) with solvent system of dichloromethane, methanol, acetic acid and distilled water at the ratio of 8:2:2.5:1 (v/v/v/v) (Skalska-Kamińska *et al.*, 2009). After the layer was developed and evaporated of mobile phase, the spots on TLC plate were detected with 5% H_2SO_4 in methanol. The layer was immersed for 5 s in developing reagent and then dried out at room temperature. The resulting spots appeared after 5 min of chromogenic reaction at 150 ^{o}C. The R_f values of samples and standard panels were measured.

2.9 Mutagenicity and antimutagenicity using *Salmonella* mutation assay

The Ames test uses histidine-requiring strains of *Salmonella typhimurium* used in Ames test to detect frameshift and point mutations. The principle of this test is to detect compounds that mutate mutant bacteria back to the wild type and thereby restores the functional capability of the bacteria to synthesize histidine. The revertant bacteria are detected by their ability to grow in the absence of histidine required by the mutant strain.

The mutagenicity test was estimated by the *Salmonella* mutation assay, with the *S. typhimurium* strains TA98 and TA100, kindly provided by Dr. Kei-ichi Sugiyama, National Institute of Health, Tokyo, Japan, with (+S9) and without (-S9) activations by the pre-incubation method (Maron *et al.*, 1983). The bacteria tester strains were taken from frozen cultures and grown overnight for 14 hr in Oxoid Nutrient Broth. The standard mutagens used as positive controls in experiments were AF-2 in the condition of absence of metabolic activation and 2-AA in the condition of presence of metabolic activation. DMSO or distilled water was served as the negative control. The various concentrations of extracts or standard mutagens were added into phosphate buffer, or S9 mixture and bacterial culture, and then incubated at 37 ^{o}C for 20 min. Next, top agar containing minimal amounts of L-histidine and D-biotin was added and the mixture was poured on to a plate containing minimal agar. The plates were incubated at 37 ^{o}C for 48 hr and the His^{+} revertant colonies were counted. All experiments were carried out in triplicate. The mutagenic index (MI) was also calculated for each concentration tested. If the MI is equal or more than 2, the extract will be identified as a possible mutagen.

The antimutagenicity test was performed using the pre-incubation method described above. The extracts were tested against the mutations induced by various environmental mutagens on *S. typhimurium* strains TA98 and TA100 with S9 activation. The antimutagenic potential of the extracts was evaluated against mutagens. IQ and MeIQ were tested in strain TA100 and PhIP was used for strain TA98. The inhibition of mutagenicity was calculated using the following equation.

$$\%\text{Inhibition} = \left(\frac{(M-S)-(T-S)}{(M-S)} \right) \times 100$$

M is the number of revertant colonies in the plate containing only mutagen. T is the number of revertant colonies in plate containing mutagen and test sample and S is the number of spontaneous revertant colonies. The values of percentage inhibition which are lower than 30, 31-50, 51-70, 71-90 and greater than 90% represent absent, mild, moderate, strong and very strong antimutagenicities, respectively.

2.10 Study of *S. neglecta* and its derived extracts on early stages of colorectal carcinogenesis in rats

2.10.1 Animals

Male Wistar Rats were obtained from National Laboratory Animal Center, Mahidol University (Nakorn-Prathom, Thailand). They were housed under controlled conditions of lighting (12 hr light/dark cycles) and temperature (23±2 ^{o}C) with free access to pellet diet and tap water. The animal experimental protocol was conducted after approval from the animal ethics committee of the Faculty of Medicine, Chiang Mai University, Thailand. All rats were acclimatized for 1 week before starting the experiment.

Male Crl: CD1 (ICR) mice were purchased from Charles River Japan (Tokyo, Japan) and housed five mice per cage. The experimental protocol was approved by the Animal Care and Use Committee of Osaka City University Medical School. All experimental procedures were conducted under the Guidelines set by the National Institute of Health and Public Health Service Policy on the Humane Use and Care of Laboratory Animals. Mice were housed under controlled conditions of humidity (44±5%), lighting (12 hr light/dark cycles) and temperature (23±1 ^{o}C) with free access to tap water and commercial MF diet. All mice were acclimatized for 1 week before starting the experiment.

2.10.2 The effect of *S. neglecta* on initiation stage of colorectal carcinogenesis

Rats were randomly divided into 8 groups (Figure 2.2). Groups 1-5 were subcutaneously injected with 1, 2-dimethylhydrazine (DMH 40 mg/kg bw) once a week for 2 consecutive weeks. Groups 2-5 and 7-8 were fed with *S. neglecta* mixed diet (SND) or *S. neglecta* extract (SNE) starting from a week before the first injection, and continued for 5 weeks. Group 1 was orally fed with a normal basal diet, whereas rats in groups 2 and 3 were fed with a diet supplemented with different amounts (1% and 4%) of dried *S. neglecta* and groups 4 and 5 were fed with 2 doses of SNE (50 and 200 mg/kg body weight). Groups 6-8 were subcutaneously injected with 0.9% normal saline solution (NSS) once a week for 2 consecutive weeks. Group 6 was orally given a normal basal diet, whereas group 7 was fed with a high dose (4%) of SND and group 8 was fed with 200 mg/kg body weight of SNE. At the end of the experiment, all rats were euthanized under anesthesia and the final weights were recorded. The colonic tissues were collected for aberrant crypt (AC) analysis while the liver were removed and stored at -80 °C for phases 1 and 2 xenobiotic metabolizing and antioxidant enzymatic assays.

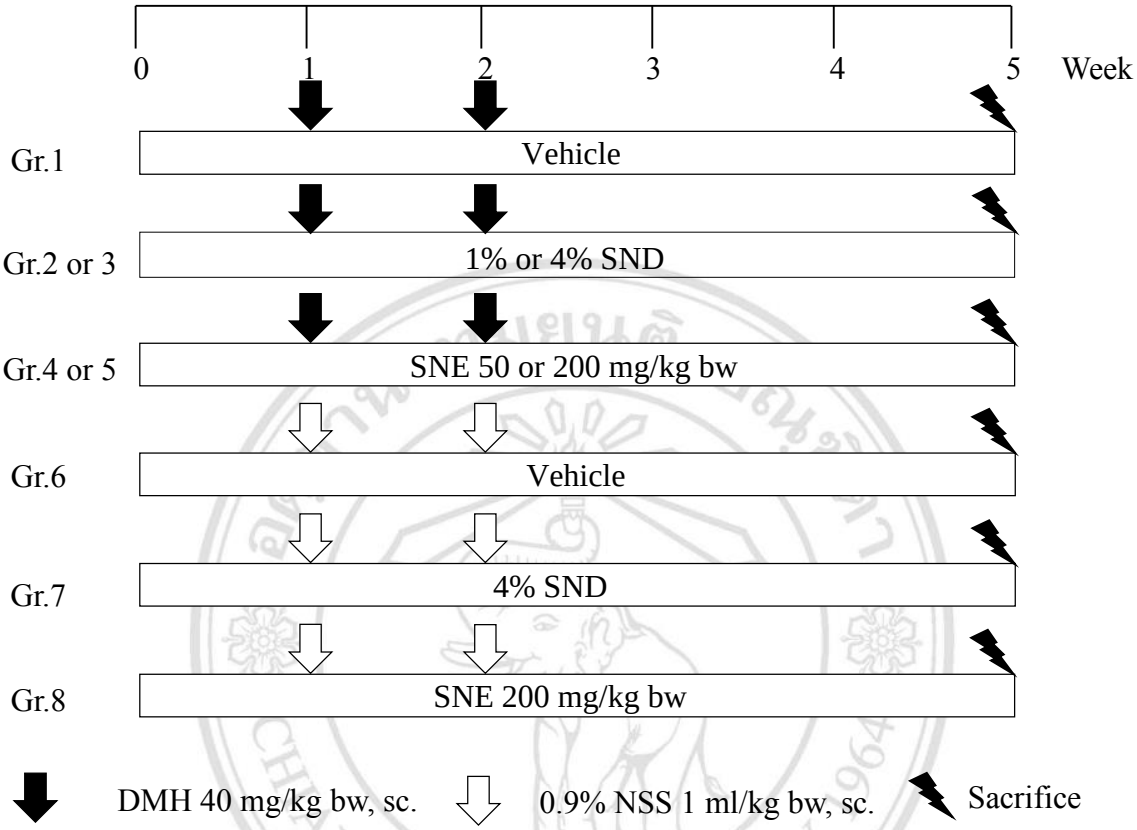

Figure 2.2 Effect of *S. neglecta* on initiation stage of colorectal carcinogenesis in rats.

2.10.3 The effect of *S. neglecta* on post-initiation stage of colorectal carcinogenesis

The experimental protocol is shown in Figure 2.3. All rats were divided into 8 groups. Groups 1-5 were subcutaneously injected with 40 mg/kg bw of 1, 2-dimethylhydrazine (DMH) once a week for 2 consecutive weeks, while groups 6 to 8 were injected with 0.9% NSS instead of DMH. Starting from week 5, the rats were administered basal diet (group 1), diet containing 0.5 and 1% dried *S. neglecta* (groups 2 and 3) and the extract of *S. neglecta* at doses 50 and 200 mg/kg body weight (groups 4 and 5) for 10 weeks. Groups 6-8 were fed basal diet (group 6), diet containing 1% dried *S. neglecta* (group 7), and the extract of *S. neglecta* at 200 mg/kg body weight (group 8) for 10 weeks. At the end of the experiment, all rats were euthanized under anesthesia and the final body weights were recorded. The number of aberrant crypt in 3 parts of the colon was counted after methylene blue staining. Some colonic tissues were dissected and processed for immunohistological analysis. The molecular mechanisms involving cell proliferation and apoptosis of *S. neglecta* on post-initiation stage of colorectal carcinogenesis were investigated.

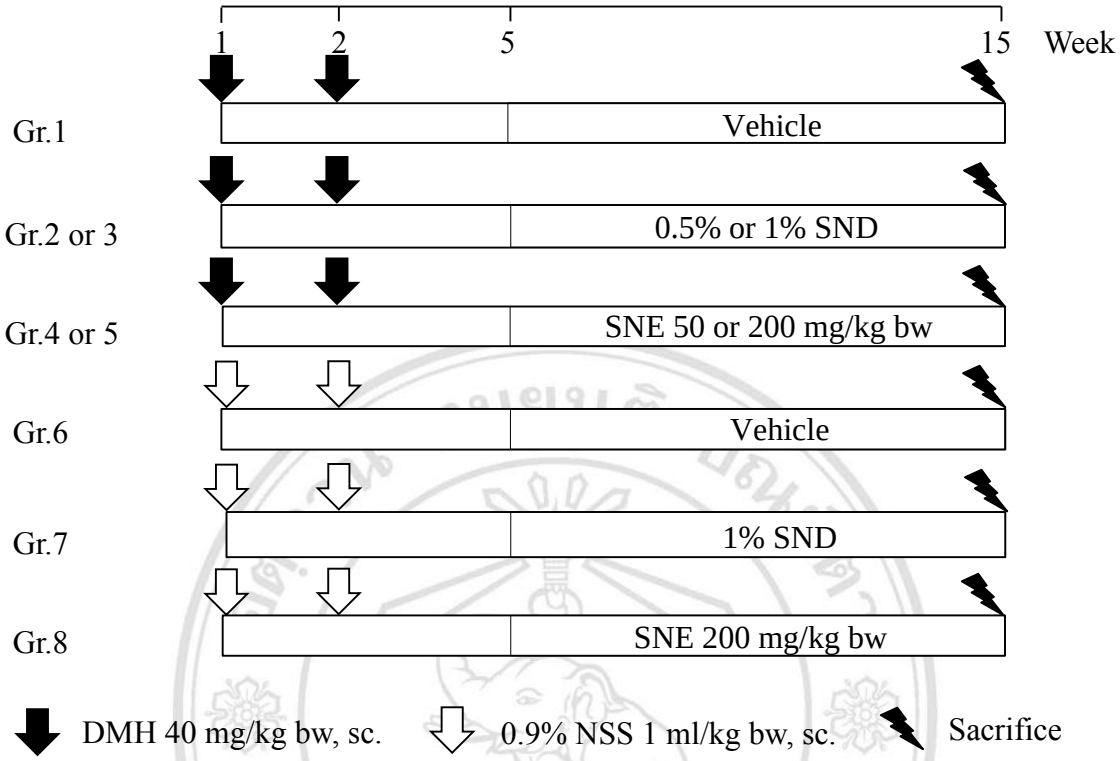

Figure 2.3 Effect of *S. neglecta* on post-initiation stage of colorectal carcinogenesis in rats.

2.10.4 The effect of *S. neglecta* extract and its derived fractions on post-initiation stage of colorectal carcinogenesis

The experimental protocol is shown in Figure 2.4. All rats were divided into 13 groups. Groups 1 to 8 were subcutaneously injected by 40 mg/kg bw of 1, 2-dimethylhydrazine once a week for 2 weeks. Groups 9 and 13 were received similar injection of NSS, a vehicle. After a week of DMH injection, groups 1 and 9 were fed with distilled water, a vehicle of SNE and PE, while groups 2 and 10 were received 1% DMSO, a vehicle of CF. Moreover, groups 3-8 were fed with various doses of SNE, PE and CF, respectively, for 10 weeks. At the end of the experiment, all rats were euthanized under anesthesia and the final body weights were recorded. The number of aberrant crypt was counted. The colonic tissues were dissected and processed for immunohistological analysis. The molecular mechanisms of *S. neglecta* extracts on post-initiation stage of colorectal carcinogenesis were investigated.

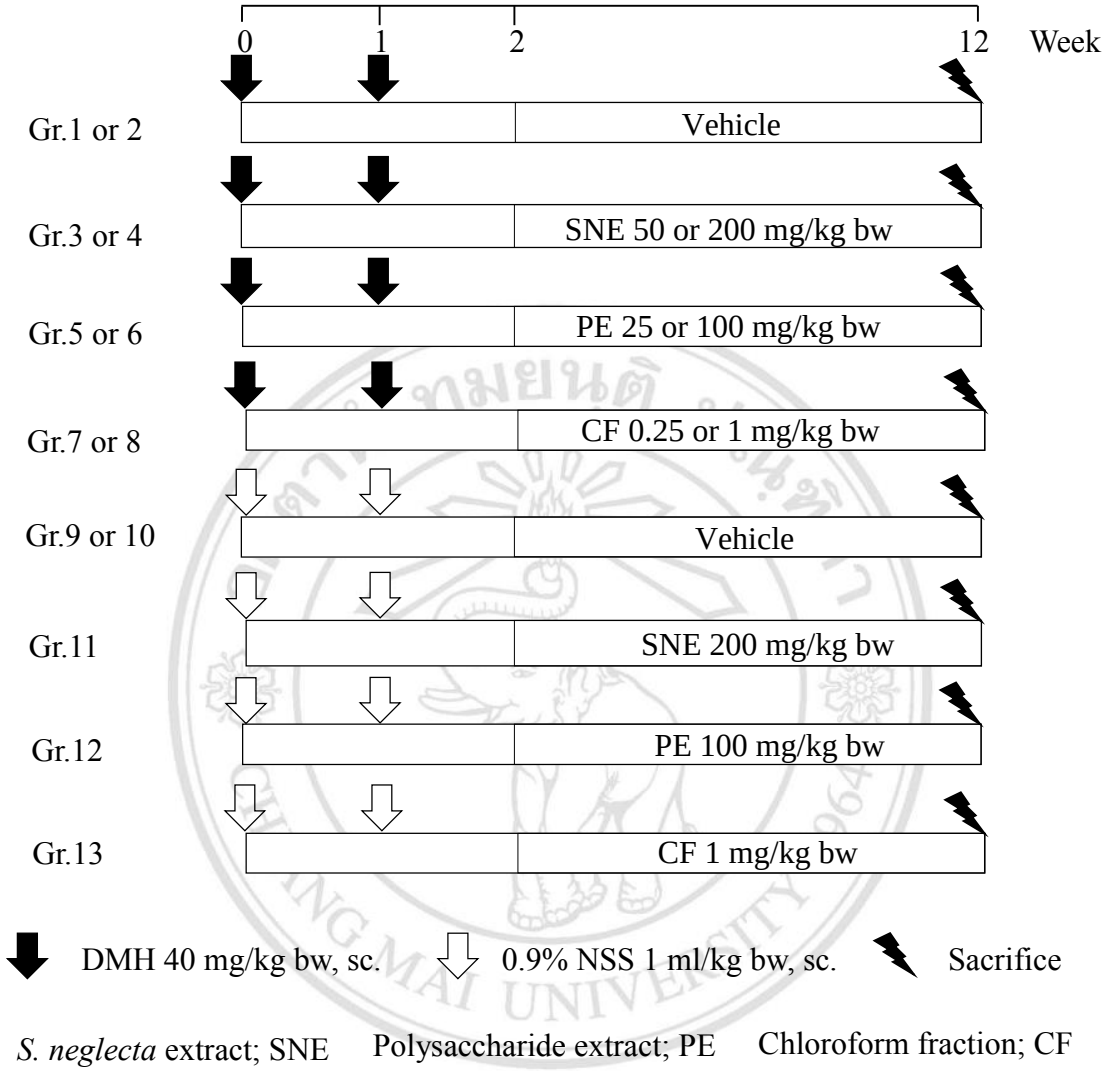

Figure 2.4 Effect of *S. neglecta* extract and its derived extracts on post-initiation stage of colorectal carcinogenesis in rats.

2.11 Assessment of aberrant crypt foci

Aberrant crypt foci (ACF) were identified according to the morphological characteristics: (a) increase in size; (b) thick layer of epithelial cells; (c) more staining strength; (d) increase distance from lamina to basal surfaces of cells; and (e) abnormally lumina shape, often slit-shaped in appearance. The ACF in the colon were counted as described by Bird (Bird, 1987). The entire colon of each rat was expanded with injection of 10% buffered formalin solution. They were cut longitudinally and laid flat on Whatman paper. Then, the colon was stained with 0.2% methylene blue. The mucosal side was placed on a glass slide and examined under a microscope for investigation of ACF as well as the number of AC per focus.

2.12 Preparation of cytosolic and microsomal fractions

After sacrifice, the liver tissues were dissected out and washed with 0.9% NSS. The liver tissue was homogenized in homogenizing buffer using a Potter-Elvehjem homogenizer with a Teflon pestle. Next, the homogenate was centrifuged at 14,000 rpm for 20 min, and the supernatant was collected. The supernatant was further centrifuged at 30,000 rpm for 1 hr to obtain the cytosolic supernatant and microsomal pellet. The clear cytosolic fraction was collected and assayed for phase II xenobiotic metabolizing and antioxidant enzymes. Microsomal fraction was assayed for phase I xenobiotic metabolizing enzymes.

2.13 Molecular mechanistic studies on initiation stage of colorectal carcinogenesis

2.13.1 Determination of phases I and II xenobiotic metabolizing enzymes

Cytochrome P450 2E1 expression was described easlewhere (Umesalma *et al.*, 2011). Aliquots of the microsomal fraction were boiled in sample buffer. Twenty micrograms of protein from each group were separated by 10% gel SDS-PAGE and then transferred to a nitrocellulose membrane. The expression of CYP450 2E1 in each group was detected by rabbit anti-CYP2E1 antibody followed by anti-rabbit IgG antibody peroxidase conjugate. Chemiluminescence detection was performed using a chemiluminescence kit. The detected bands were visualized by x-ray film exposure. The intensity of each band was evaluated via the Image J program.

UDP-glucuronosyltransferase (UDP-GT) activity was determined using a colorimetric method (Suwannakul *et al.*, 2015) with minor modification. The reaction mixture contained Tris-HCl buffer (pH 8.5), *p*-nitrophenol, $MgCl_2$, UDP-glucuronic acid and 5 mg/ml microsomal fraction. The reaction was performed at 37 ^oC for 20 min and stopped by adding TCA. The mixture was then centrifuged at 10,000 rpm for 15 min. The supernatant was alkalinized with NaOH. The disappearance of *p*-nitrophenol was measured at 405 nm to evaluate the rate of *p*-nitrophenol conjugation. The activity of UDP-GT was expressed as pmoles of *p*-nitrophenol conjugate formed/minute/mg protein using the extinction coefficient 1.78x104 M^{-1} cm^{-1}.

Glutathione-*S*-transferase (GST) activity was analyzed by a spectrophotometric method (Thumvijit *et al.*, 2014). The reaction mixture contained phosphate buffer (pH 6.5), diluted cytosolic fraction and reduced glutathione. The reaction was started by adding 1-chloro 2, 4-dinitrobenzoic acid (CDNB), the absorbance measured at 340 nm. The specific activity of GST is expressed as μmoles of GSH-CDNB conjugate formed/minute/mg protein using the extinction coefficient 9.6 mM^{-1} cm^{-1}.

2.13.2 Determination of antioxidant enzymes

Glutathione reductase (GR) activity was assayed using a colorimetric method (Taya *et al.*, 2014). The activity of GR was determined by monitoring glutathione-dependent oxidation of NADPH at 340 nm. The reaction mixture contained phosphate buffer (pH 7.0) with $MgCl_2$, GSSG and NADPH and diluted cytosolic fraction. The reduction of NADPH was measured by its absorbance at 340 nm. The activity of GR was expressed as mmoles of NADPH/minute/mg protein using the extinction coefficient 6.22 mM^{-1} cm^{-1}.

Glutathione peroxidase (GPx) activity was measured by a spectrophotometric method (Taya *et al.*, 2014). The reaction mixture included Tris-HCl buffer (pH 8.0) with EDTA, GSH, NADPH, GR and cytosolic fraction. The reaction was started by adding tert-butyl hydroperoxide. The reduction of NADPH was measured at 340 nm. The activity of GPx was expressed as mmoles of NADPH/minute/mg protein using the extinction coefficient 6.22 mM^{-1} cm^{-1}.

Catalase (CAT) activity was assessed by measuring the reduction of H_2O_2 (Taya *et al.*, 2014). The reaction mixture was composed of phosphate buffer (pH 7.0), H_2O_2 and the cytosolic fraction. The reduction of H_2O_2 was measured spectrophotometrically at 240 nm. The activity of CAT was expressed as nmoles of H_2O_2/minute/mg protein using the extinction coefficient 43.6 M^{-1} cm^{-1}.

2.14 Molecular mechanistic studies on post-initiation stage of colorectal carcinogenesis

Colon epithelial homeostasis results from a balance between cell proliferation and cell death. Neoplasms arise when there is irregular accumulation of altered cells, characterized by increased proliferation and decreased cell death. Thus, both loss of cell death and increased proliferation seem to be important in the cancer development (Qiao *et al.*, 1997).

2.14.1 Detection of proliferating colonic cells

Cell proliferation plays an important role in several steps of the carcinogenic development. Proliferating cell nuclear antigen (PCNA), a highly conserved nuclear protein of DNA polymerase-delta, has been found to be a marker to evaluate tumor cell proliferation and progression (Kubben *et al.*, 1994).

Formalin-fixed and paraffin-embedded tissues were cut into 3 µm sections, deparaffinized and then rehydrated. After antigen retrieval, endogenous peroxidase activity was blocked by incubating in 3% hydrogen peroxide. To prevent nonspecific binding, the sections were incubated with normal rabbit serum. The primary antibody against PCNA was added onto the section at a dilution of 1:1000 at 4 ºC overnight. After primary antibody incubation, the tissue sections were incubated with secondary antibody for 30 min at room temperature. After secondary antibody incubation, Vectastain Elite avidin-biotin complex method kit was used following to the manufacturer's instructions. Color was developed by applying 3, 3′-diaminuteobenzidine tetrahydrochloride (DAB). Sections were counterstained with hematoxylin, dehydrated in a graded series of ethanol followed by xylene and mounted using mounting medium. The labeling indices were expressed as the percentages of cells positive for PCNA.

2.14.2 Detection of apoptotic colonic cells

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining is a technique for staining cells that have undergone programmed cell death or apoptosis because one of the hallmarks of late stage apoptosis is the fragmentation of nuclear chromatin resulting in a multitude of 3’-hydroxyl termini of DNA ends (Bortner *et al.*, 1995). TUNEL staining relies on the ability of the enzyme terminal deoxynucleotidyl transferase to incorporate labeled dUTP into free 3'-hydroxyl termini generated by the fragmentation of genomic DNA into low molecular weight double-stranded DNA and high molecular weight single stranded DNA (Arends *et al.*, 1990).

The apoptotic cells were stained using an ApopTag® Peroxidase in Situ Apoptosis Detection Kit. The sections were treated with TdT equilibration buffer. Next, TdT enzyme was added onto the tissue sections and the reaction was terminated using stop buffer. The labeled DNA fragments were visualized by adding anti-digoxigenin conjugate and developing with DAB. The tissue sections were counterstained with 0.3% methyl green solution. Apoptotic cells were identified by the presence of a dark brown stain over the nuclei as visualized under a light microscope. The labeling indices were expressed as the percentages of cells positive for TUNEL.

2.15 The effect of *S. neglecta* and its derived extracts on dextran sodium sulfate-induced colitis in mice

Dextran sodium sulfate (DSS) is commonly used for the induction of ulcerative colitis. It leads to colon epithelial damage and robust inflammatory response, after several days of DSS treatment (Perse *et al.*, 2012). Mice were randomly divided into 13 groups. Mice in groups 3 to 10 received 3% DSS in drinking water for 7 days starting one week after the commencement. Other groups received tap water instead of DSS treatment. Distilled water and 1% DMSO were used as vehicle for SNE and PE-treated group and CF-treated group, respectively. The vehicle control groups (groups 1 and 2) and DSS positive control groups (groups 3 and 4) received distilled water or 1%DMSO, respectively. To investigate the preventive effect of *S. neglecta* and its derived fractions on DSS-induced colitis, the treatment protocol was described in Figure 2.5. Mice in groups 5 to 10 received SNE (50 and 200 mg/kg bw), PE (25 and 100 mg/kg bw) and CF (0.25 and 1 mg/kg bw) by intragastric gavage (i.g.) feeding for 7 days before DSS treatment and were continued for 7 days. The used PE and CF concentrations represent PE and CF amounts extracted from SNE. Mice in groups 11 to 13 were orally fed with SNE (50 mg/kg bw), PE (25 mg/kg bw) and CF (0.25 mg/kg bw) for 14 days. The diet and water intakes were measured twice a week. At day 14, mice were sacrificed under isofluorane anesthesia and colons were immediately removed. H&E staining was performed on paraffin-embedded sections to evaluate histological score. The Ki-67 immunohistochemical staining was to determine cell proliferation. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining kit was used to investigate cell apoptosis. The colonic mucosa was kept at -80 ºC for proteome analysis. The internal organs weights including liver, kidneys and spleen were measured.

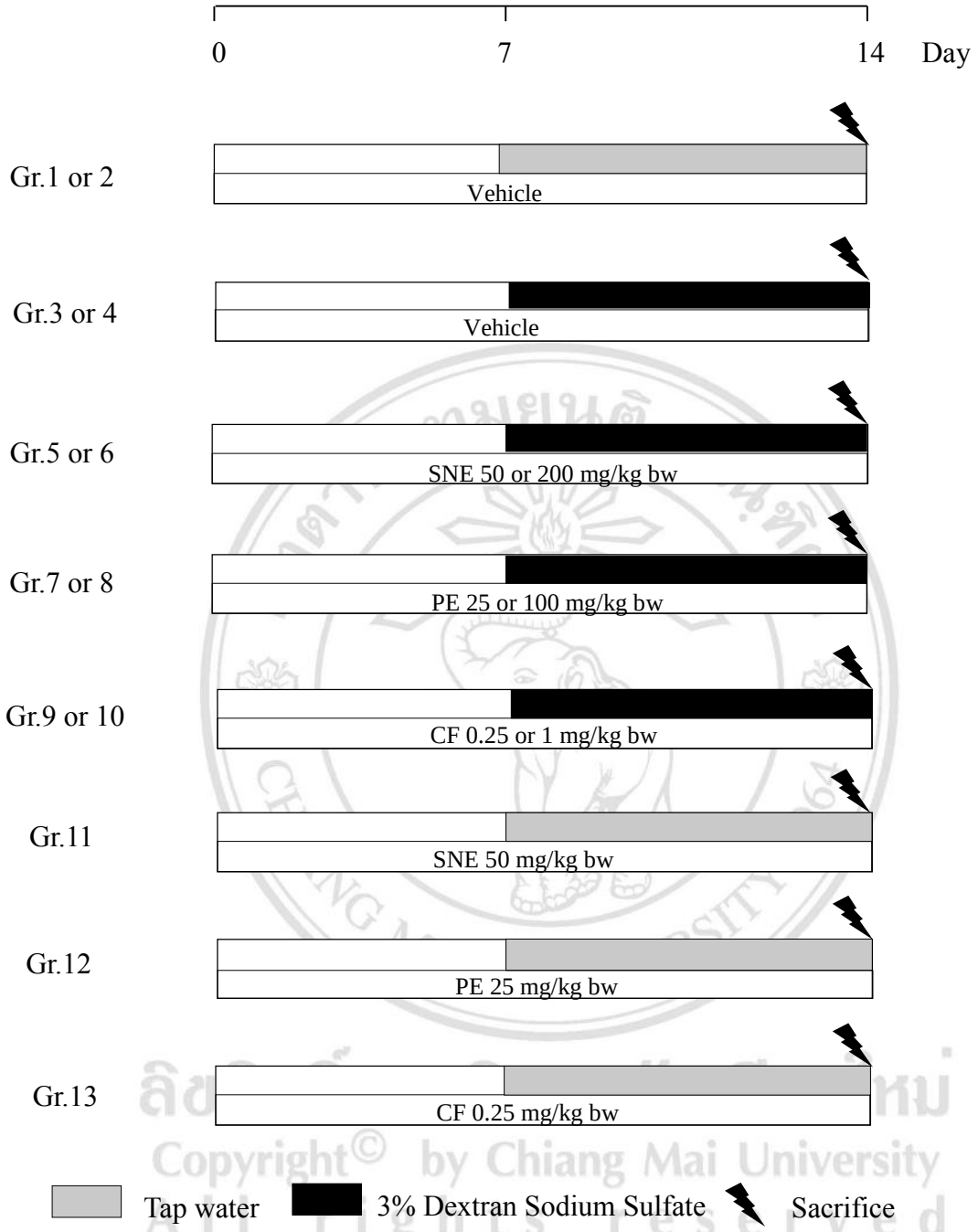

Figure 2.5 Effect of *S. neglecta* extracts on dextran sodium sulfate induced colitis in mice.

2.16 Histological examination

The excised of colon were fixed in 10% neutral formalin solution and embedded in paraffin for sectioning at 3 µm. Sections were stained with hematoxylin and eosin. The pathophysiology of the colon was assessed as described by Laroui *et al*. (Laroui *et al.*, 2012). The pathophysiology of the colon was characterized by the presence of edema, infiltration of inflammatory cells into submucosa and crypt loss. Scores were given for grades as indicated in Table 2.1. The histological score for the pathophysiology of the colon was calculated as the sum of these three parameters, giving a maximum score of 9.

Table 2.1 Histological grading of colitis (Laroui *et al.*, 2012).

Feature graded	Grade	Description
Inflammatory cell infiltration into submucosa	0	None
	1	Mild
	2	Moderate
	3	Severe
Edema	0	None
	1	Mild
	2	Moderate
	3	Severe
Crypt damage	0	None
	1	1/3 damaged
	2	2/3 damaged
	3	Entire crypt loss

2.17 Molecular mechanistic studies on dextran sodium sulfate-induced colitis in mice

2.17.1 Detection of proliferating and apoptotic colonic cells

The proliferation rate of tumors has been monitored by using various markers, such as the Ki-67 antigen (Suzuki *et al.*, 1992) and proliferating cell nuclear antigen (PCNA) (Kubben *et al.*, 1994). The Ki-67 antigen has provided estimates of the growth fraction of many normal and malignant human tissues, including the colon (Suzuki *et al.*, 1992).

Cell proliferation was analyzed by Ki-67 immunohistochemistry. From paraffin-embedded sections, 3 µm sections were cut and placed on polylysine-coated glass slides for immunohistochemistry. The sections were deparaffinized and then rehydrated. Before immunohistochemical staining, the antigen epitope were retrieved in sodium citrate buffer using microwave oven. After antigen retrieval, the endogenous peroxidase was blocked by incubating in 3% hydrogen peroxide solution. Then, tissue sections were incubated in normal serum for 15 min to inhibit non-specific binding. The primary antibody for Ki-67 was anti-Ki-67 antibody (SP6, Abcam, USA) used at a dilution of 1:500 at 4 ^{o}C overnight. After primary antibody incubation, the tissue sections were incubated with secondary antibody and then treated with VECTASTAIN Elite ABC solution for 30 min at room temperature. After each incubation step, tissue sections were treated with 3, 3′-diaminuteobenzidine tetrahydrochloride and counterstained with hematoxylin. The labeling indices were expressed as the percentages of cells positive for Ki-67.

The apoptotic cells were stained using an ApopTag® Peroxidase in Situ Apoptosis Detection Kit and were described in 2.14.2.

2.17.2 LC-MS/MS analysis

Mouse colonic mucosa were homogenized and then lysed using 9M Urea/2% CHAPS and T-PER lysis buffers with protease inhibitor. Subsequently, the lysate was centrifuged at 10,000 rpm for 10 min at 4 ^{o}C. After acetone precipitation, protein concentrations were measured by BCA Protein Assay (Pierce, IL, USA). Protein reduction, alkylation, digestion and iTRAQ labeling of 40 µg protein for each pool sample

were performed using an iTRAQ Reagent 4 Plex Kit according to standard procedures (Gluckmann *et al.*, 2007). Pooled samples of colonic mucosa of groups 1, 3, 6 and 8 were labeled with iTRAQ isobaric reagents 114, 115, 116 and 117, respectively, and then eluted as six fractions with 10, 50, 70, 100, 200 and 350 mM KCl on an ICAT cation exchange column cartridge (AB Sciex, Foster City, Calif, USA). The peptides in each fraction were desalted using a Sep-Pak C18 Plus light cartridge, evaporated and then dissolved in 0.1% (v/v) formic acid. Proteome analysis was performed with a DiNa-AI nano system coupled to a QSTAR Elite Hybrid mass spectrometer though a Nano Spray ion source. MS/MS data were searched against the Swiss Protein database (MOUSE) using ProteinPilot™ software (version 2.0, AB Sciex, Concord, ON, Canada) with trypsin set as the digestion enzyme and methyl methanethiosulfonate as the cysteine modification. The search results were further processed by ProteinPilot™ software using the Paragon Algorithm for redundant hits removal and comparative quantitation, resulting in a minimal set of justifiable identified proteins. All reported data were used at the 95% confidence cut-off limit created by the ProteinPilot™ software. The same program was used to remove the bias for proteins expressed at very low levels. Protein ratios with a *p*-value less than 0.05 were considered reliable. Previously standard deviations of the protein ratio, which stem from technical variation, were reported to be less than 0.3 in 90% of iTRAQ experimental runs (Song *et al.*, 2008). In this experiment, expression changes greater than 1.2-fold or less than 0.8-fold in normalized expression levels were considered to be outside the range of technical variability.

2.17.3 Ingenuity Pathway Analysis (IPA)

The Ingenuity program (Ingenuity Systems, Mountain View, CA) was utilized to assign biological significance to differentially labeled proteins, identify function and networks of interacting proteins, functional groups and pathways, and predict activated up-stream regulators of pool proteins obtained from DSS, DSS and SNE or DSS and PE treated groups. Transcriptional regulation was measured by the z-score. The basis for z-score predictions is related to the molecular pathways (networks) which represent experimentally observed protein expression or transcription events. A z-score of above 2 or lower than -2 was considered significant.

2.18 The effect of *S. neglecta* and polysaccharide extract on inflammation associated colorectal carcinogenesis in rats

All rats were divided into 11 experimental and control groups (Figure 2.6). Groups 1 to 5 and 9 were subcutaneously injected by 40 mg/kg body weight of 1, 2-dimethylhydrazine once a week for 2 weeks. After the last injection, groups 1 to 5 received 1% dextran sodium sulfate (DSS) in drinking water for 7 days. Groups 6 to 8 were subcutaneously injected by 0.9% NSS and received 1% DSS in drinking water after last injection for 7 days. Subsequently, they were received distilled water (vehicle), 50 or 200 mg/kg bw of *S. neglecta* extract (SNE) and 30 or 120 mg/kg bw of polysaccharide extract (PE) for 10 weeks, starting at 3 week after the cessation of DSS treatment. Group 10 was served as a negative control. Group 11 was fed with 120 mg/kg bw of PE. All rats were sacrificed at week 15 under anesthesia and the final body weights were recorded. The number of aberrant crypts was counted.

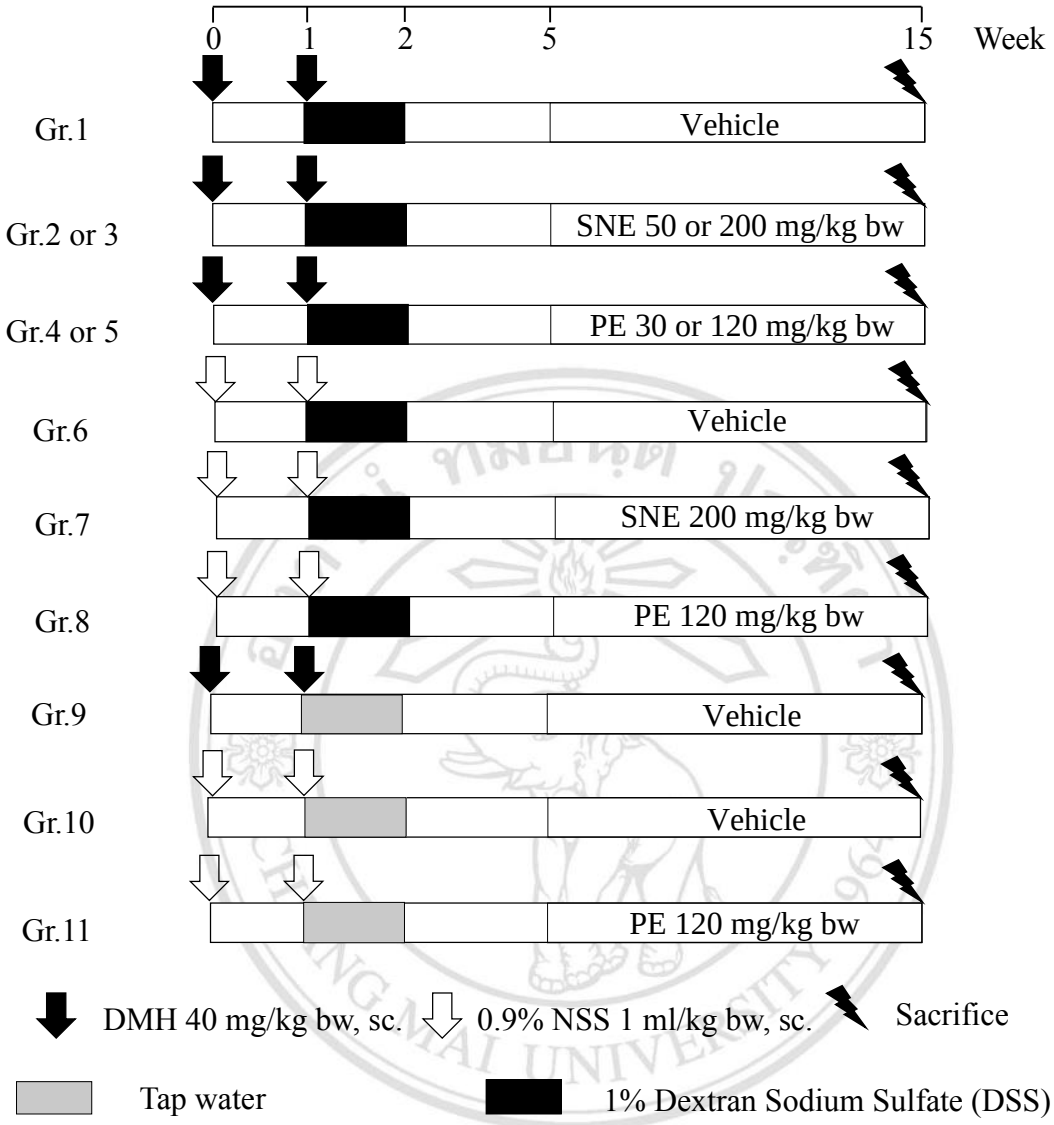

Figure 2.6 Effect of *S. neglecta* extracts on 1, 2-dimethylhydrazine and dextran sodium sulfate induced colorectal carcinogenesis in rats.

2.19 Molecular mechanistic studies on inflammation associated colorectal carcinogenesis in rats

The inflammatory mRNA levels of colonic mucosa of groups 6, 8, 10 and 11 in a previous protocol (Figure 2.6) were determined by real-time reverse transcriptase polymerase chain reaction.

The RNA extraction was performed by using RNAzol Reagent according to the manufacturer's instructions. The quality and quantity of the total RNA was measured using a nanodrop machine. Synthesis of cDNA was performed by reverse transcription using total RNA as a template. Real-time polymerase chain reaction (PCR) was performed using a quantstudio 6 flex system with the SensiFast^{TM} SYBR Lo-ROX kit, primers (Table 2.2), and complementary DNA (cDNA). Each target and standard β-actin cDNA was analyzed in duplicate and three independent times of real-time reverse transcriptase (RT)-PCR assay. Thermal cycling was initiated with an activation step of 95°C for 2 min, and this step was followed by 40 cycles of 95°C for 5 s and 60°C for 10s immediately after amplification, melting curve protocols were used to confirm that the primer dimers and other nonspecific products were minimized. The relative expression of the target genes was analyzed by the $^{\Delta\Delta}$Ct method.

Table 2.2 List of primers used in real-time polymerase chain reaction.

Gene	Primer sequence	Primer length
TNF- α	Forward-5-AAATGGGCTCCCTCTCATCAGTTC-3	24
	Reverse-5-TCTGCTTGGTGGTTTGCTACGAC-3	23
COX-2	Forward-5-TGTATGCTACCATCTGGCTTCGG-3	23
	Reverse-5-GTTTGGAACAGTCGCTCGTCATC-3	23
iNOS	Forward-5-CAGGTGCTATTCCCAGCCCAACA-3	23
	Reverse-5-CATTCTGTGCAGTCCCAGTGAGGAA-3	25
IL-6	Forward-5-TCCTACCCCAACTTCCAATGCTC-3	23
	Reverse-5-TTGGATGGTCTTGGTCCTTAGCC-3	23
IL-1β	Forward-5-CACCTCTCAAGCAGAGCACAG-3	21
	Reverse-5-GGGTTCCATGGTGAAGTCAAC-3	21
NF-κB	Forward-5-GGCATGCGTTTCCGTTACAA-3	20
	Reverse-5-TGATCTTGATGGTGGGGTGC-3	20
β-actin	Forward-5-ACAGGATGCAGAAGGAGATTAC-3	22
	Reverse-5-ACAGTGAGGCCAGGATAGA-3	19

2.20 Statistical analysis

The results are presented as mean value ± standard deviation (SD). The significance of differences for each parameter were analyzed by one-way analysis of variance (ANOVA) and evaluated at the level of a) $p < 0.05$.