CHAPTER 3

Results

3.1 Study of *S. neglecta* on early stages of colorectal carcinogenesis in rats

3.1.1 Effect of hot water extract and dried powder of *S. neglecta* on initiation stage of colorectal carcinogenesis

The general observation of rat is shown in Table 3.1. During the experimental period of 5 weeks initiation stage, there were no significant differences in final body weights, and intakes of food and water in all groups. Furthermore, no clinical sign of toxicity was observed in all groups.

Aberrant crypt foci (ACF), preneoplastic lesions, are detected during the early stages of colorectal carcinogenesis. Table 3.2 summarizes the number of ACF, crypt multiplicity and percentage inhibition of ACF in experimental groups. At the end of the experimental period of 5 weeks, ACF found in rats treated with 1, 2-dimethylhydrazine (DMH) (Figure 3.1), with or without dried *S. neglecta* mixed diet (SND) and *S. neglecta* extract (SNE) feeding, while no ACF formation was observed in normal saline solution (NSS), SND and SNE treated groups. These result indicated dried powder and extract of green algae had no carcinogenicity in rat colon. The number of ACF in DMH-treated group was significantly increased as compared to NSS-treated group. A statistically significant reduction in total ACF was observed only in SNE-treated rats when compared to DMH-treated rats. Moreover, the percentage inhibitions of ACF in low and high doses of SNE-treated rats were 38.7 and 44.6%, respectively. However, there was no significant difference in crypt multiplicity between DMH-treated groups. These results indicated that SNE inhibited the DMH-induced ACF formation whereas SND showed no effect on ACF induced by DMH.

Table 3.1 The general observation of rats in initiation stage of colorectal carcinogenesis

Treatments	Body weight (g)		Intake	
	Initial	Final	Diet (g)	Water (ml)
DMH	77±6	282±36	18±4	29±4
DMH+1% SND	77±6	295±40	21±2	30±3
DMH+4% SND	77±4	290±23	22±1	32±7
DMH+50 mg/kg bw SNE	77±5	282±27	18±4	33±10
DMH+200 mg/kg bw SNE	77±4	286±26	18±4	31±8
NSS	78±4	298±27	19±4	36±9
NSS+4% SND	77±6	295±30	20±3	31±5
NSS+200 mg/kg bw SNE	78±3	273±49	19±4	33±8

Data represent the mean ± SD of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), ACF; aberrant crypt foci, SND; dried *S. neglecta* mixed diet, SNE; *S. neglecta* extract.

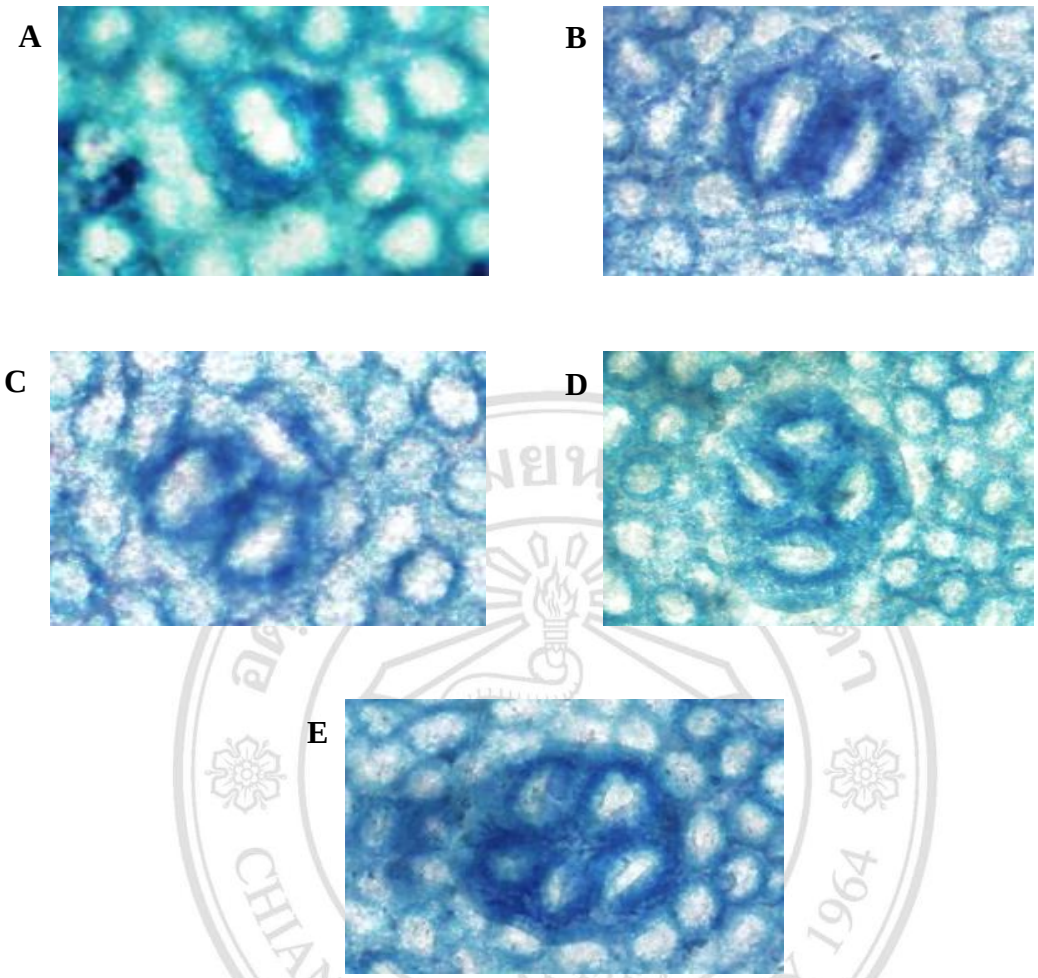


Figure 3.1 Formation of aberrant crypt foci (ACFs) in the colon of DMH-treated rats. A-E show ACFs consisting of 1 Crypt (A), 2 Crypts (B), 3 Crypts (C) 4 Crypts (D) and more than 4 crypts (E).

Table 3.2 Effect of *S. neglecta* on DMH-induced colonic ACF formation in initiation stage of colorectal carcinogenesis in rats

Treatments	Total ACF/rat			%Inhibition	Multiplicity (Aberrant crypt/focus)
	Proximal+Distal	Rectum	Total		
DMH	107.0±34.9*	14.2±6.2*	121.2±39.0*	-	2.29±0.31
DMH+1% SND	71.3±32.4	10.3±8.2	81.5±39.6	32.8	2.20±0.23
DMH+4% SND	78.5±49.1	16.0±11.8	94.5±60.1	22.0	2.18±0.27
DMH+50 mg/kg bw SNE	63.1±34.8**	11.1±8.7	74.3±41.2**	38.7	2.19±0.25
DMH+200 mg/kg bw SNE	58.9±34.1**	8.3±2.9	67.1±35.6**	44.6	2.22±0.34
NSS	0.0±0.0	0.0±0.0	0.0±0.0	-	0.0±0.0
NSS+4% SND	0.0±0.0	0.0±0.0	0.0±0.0	-	0.0±0.0
NSS+200 mg/kg bw SNE	0.0±0.0	0.0±0.0	0.0±0.0	-	0.0±0.0

Data represent the mean ± SD of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), ACF; aberrant crypt foci, SND; dried *S. neglecta* mixed diet, SNE; *S. neglecta* extract, * $p < 0.05$ compared with NSS group, ** $p < 0.05$ compared with DMH group.

1) Effect of hot water extract and dried powder of *S. neglecta* on xenobiotic metabolizing enzymes in the initiation stage of colorectal carcinogenesis

Figure 3.2 shows the effect of *S. neglecta* on the protein expression of phase I xenobiotic metabolizing enzymes. The protein expression of cytochrome P450 2E1 were significantly elevated in the liver of DMH-treated rats as compared to NSS-treated rats. However, supplementation with different doses of dried *S. neglecta* (1% and 4% SND) and *S. neglecta* extract (50 and 200 mg/kg bw) feeding exhibited no significant difference in protein expression of cytochrome P450 2E1 as compared to DMH-treated group.

Figure 3.3 illustrates the effect of *S. neglecta* on the activities of phase II xenobiotic metabolizing enzymes. The activity of glutathione *S*-transferase (GST) was significantly decreased in the liver of DMH-treated rats as compared to the NSS treated rats. Feeding with high dose of dried *S. neglecta* (4% SND) and *S. neglecta* extract (50 and 200 mg/kg bw) significantly elevated the activity of GST as compared to DMH-treated rats. Moreover, the activity of UDP-glucuronosyltransferase (UDP-GT) tended to increase in the liver of DMH-treated rats as compared to the NSS-treated rats. However, *S. neglecta* extract feeding at the dose of 50 mg/kg bw showed greater modulatory effects on phase II xenobiotic metabolizing enzymes as compared to the other treated groups. These results indicated that *S. neglecta* might prevent DMH-induced ACF formation due to modulation of phase II xenobiotic metabolizing enzymes in initiation stage of colorectal carcinogenesis.

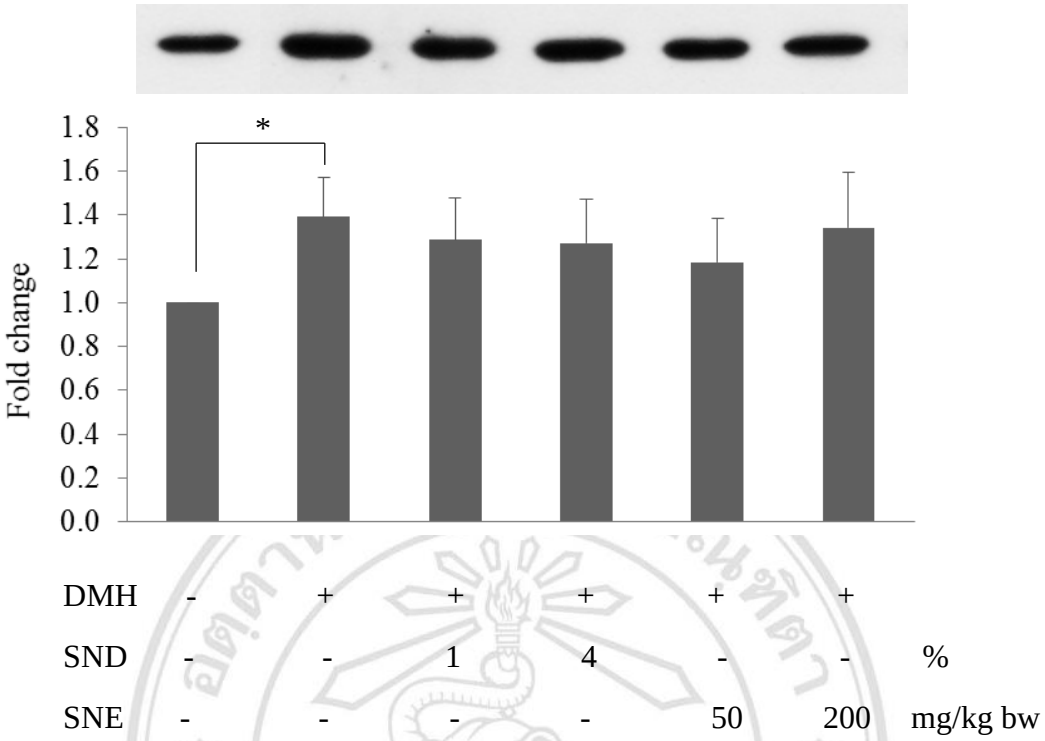


Figure 3.2 Effect of *S. neglecta* on the expression of cytochrome P450 2E1 in initiation stage of colorectal carcinogenesis in rats.

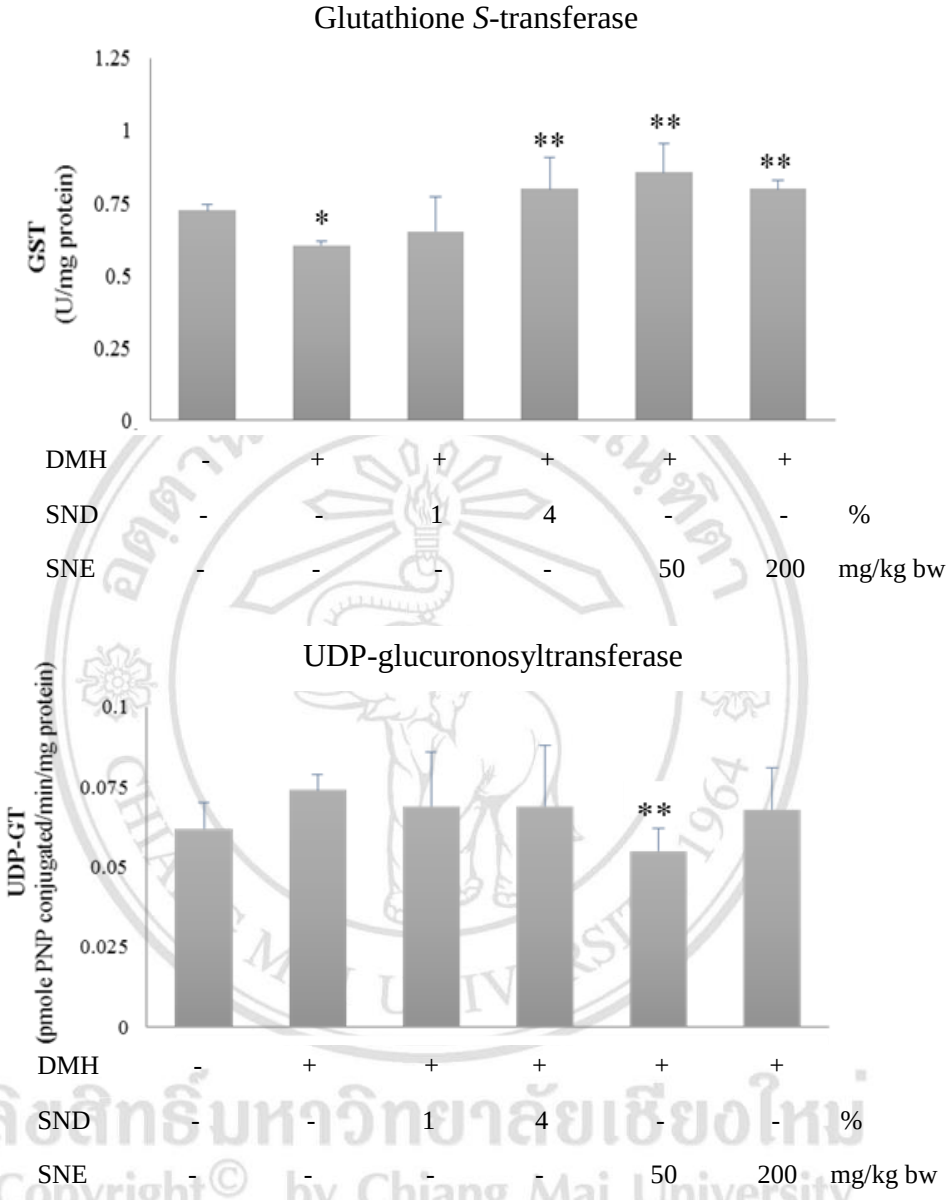


Figure 3.3 The effect of *S. neglecta* on activities of xenobiotic metabolizing enzymes in initiation stage of colorectal carcinogenesis in rats. DMH, 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.); SND, dried *S. neglecta* mixed diet; SNE, *S. neglecta* extract; * $p < 0.05$ compared with NSS group; ** $p < 0.05$ compared with DMH group.

2) Effect of hot water extract and dried powder of *S. neglecta* on antioxidant enzymes in initiation stage of colorectal carcinogenesis

The activities of hepatic antioxidant enzymes in the control and experimental rats are shown in Figure 3.4. The activities of glutathione peroxidase (GPx) and glutathione reductase (GR) tended to decrease in the liver of DMH-treated rats relative to the NSS treated rats. However, only feeding of SNE at 50 mg/kg bw significantly increased the activity of GPx as compared to DMH treated rats. The activities of catalase (CAT) and GR were not significantly different as compared to the other treated groups. These results indicated that *S. neglecta* might prevent DMH-induced ACF formation via augmentation of some glutathione dependent antioxidant system in initiation stage of colorectal carcinogenesis.

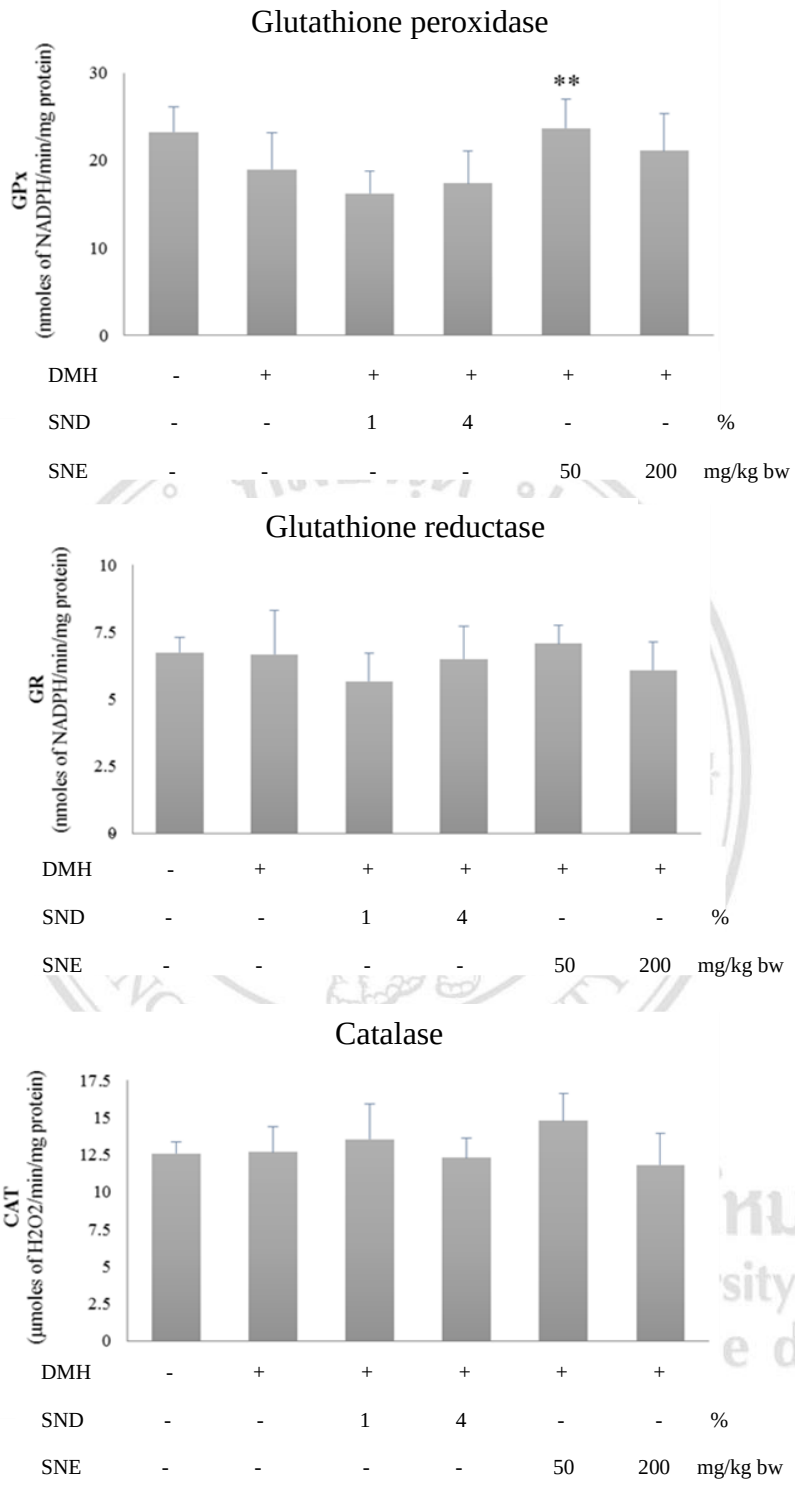


Figure 3.4 The effect of *S. neglecta* on activities of antioxidant enzymes in initiation stage of colorectal carcinogenesis in rats. DMH, 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.); SND, dried *S. neglecta* mixed diet; SNE, *S. neglecta* extract; ** $p < 0.05$ compared with DMH group.

3.1.2 Effect of hot water extract and dried powder of *S. neglecta* on post-initiation stage of colorectal carcinogenesis

The dried power or extract of green algae-treated rats observed healthy throughout the experimental period. No significant changes in body weight, food and water consumption in any of the groups (Table 3.3). Furthermore, no clinical signs of toxicity were observed in any of the groups.

The number of ACF, crypt multiplicity and percentage inhibition of ACF in experimental groups are summarized in Table 3.4. No ACF was observed in normal saline solution (NSS) group as well as in rats fed with high dose of dried *S. neglecta* mixed diet (SND) or *S. neglecta* extract (SNE). These results indicated dried powder and extract of green algae had no carcinogenicity in post-initiation model. DMH could induce the ACF formation in rats within 15 weeks treatment. Moreover, ACF which were found in 15 weeks treatment showed higher number and multiplicity compared to 5 week treatment. The total number of ACF detected in the DMH treated rats significantly increased as compared to a negative control group. Daily administration of SNE to DMH-treated rats caused a significant reduction in the total number of ACF. However, there was no significant difference in crypt multiplicity between DMH-treated groups. These results showed that SNE and SND was ineffective on crypt multiplicity.

Table 3.3 General observation of rats in post-initiation stage of colorectal carcinogenesis

Treatments	Body weight (g)		Intake	
	Initial	Final	Diet (g)	Water (ml)
DMH	78±4	465±27	19±3	34±6
DMH+0.5% SND	77±4	494±27	22±4	34±7
DMH+1% SND	78±3	492±45	22±4	34±6
DMH+50 mg/kg bw SNE	78±4	471±38	20±4	30±6
DMH+200 mg/kg bw SNE	77±6	468±26	20±3	31±5
NSS	80±9	496±17	22±3	32±5
NSS+1% SND	80±7	496±19	23±4	33±6
NSS+200 mg/kg bw SNE	80±5	497±15	22±4	33±6

Data represent the mean ± SD of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), ACF; aberrant crypt foci, AC; aberrant crypt; SND; dried *S. neglecta* mixed diet, SNE; *S. neglecta* extract.

Table 3.4 Effect of *S. neglecta* on DMH-induced colonic ACF formation in post-initiation stage of colorectal carcinogenesis in rats

Treatments	Total ACF/rat			%Inhibition	Multiplicity (Aberrant crypt/focus)
	Proximal+Distal	Rectum	Total		
DMH	235.0±91.4*	57.6±26.1	292.6±116.7*	-	3.20±0.50
DMH+0.5% SND	163.7±87.1	43.0±39.7	206.7±125.7	29.4	3.15±0.44
DMH+1% SND	155.7±69.1	40.3±21.8	196.0±86.0	33.0	3.66±0.87
DMH+50 mg/kg bw SNE	122.3±51.6**	23.0±18.0	145.3±66.5**	50.3	3.52±0.34
DMH+200 mg/kg bw SNE	214.0±121.9	40.1±39.4	254.1±156.0	13.1	3.51±0.56
NSS	0±0	0±0	0±0	-	0±0
NSS+1% SND	0±0	0±0	0±0	-	0±0
NSS+200 mg/kg bw SNE	0±0	0±0	0±0	-	0±0

Data represent the mean ± SD of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), ACF; aberrant crypt foci, AC; aberrant crypt; SND; dried *S. neglecta* mixed diet, SNE; *S. neglecta* extract, * $p < 0.05$ compared with NSS group, ** $p < 0.05$ compared with DMH group.

1) Effect of hot water extract and dried powder of *S. neglecta* on cell proliferation and apoptosis on post-initiation stage of colorectal carcinogenesis

The important events during the development of carcinogenesis are uncontrolled cell proliferation and suppressed apoptosis. This study aimed to clarify the protective effects of *S. neglecta* on post-initiation stage of colorectal carcinogenesis. Figure 3.5 shows effect of *S. neglecta* on cell proliferation and cell apoptosis in post-initiation stage of colorectal carcinogenesis. DMH-treated rats presented significantly increased number of PCNA labeling index as compared to NSS-treated rats. Administration of SNE and SND significantly decreased number of PCNA labeling index in DMH-treated rats. As shown in Figure 3.6, the apoptotic index was significantly decreased in rats treated with DMH as compared to NSS-treated rats. Administration of SNE and SND significantly increased the number of TUNEL-positive cell as compared to DMH-treated rats. These results indicated that *S. neglecta* might inhibited DMH-induced ACF formation due to inhibition of cell proliferation and inhibition of cell apoptosis.

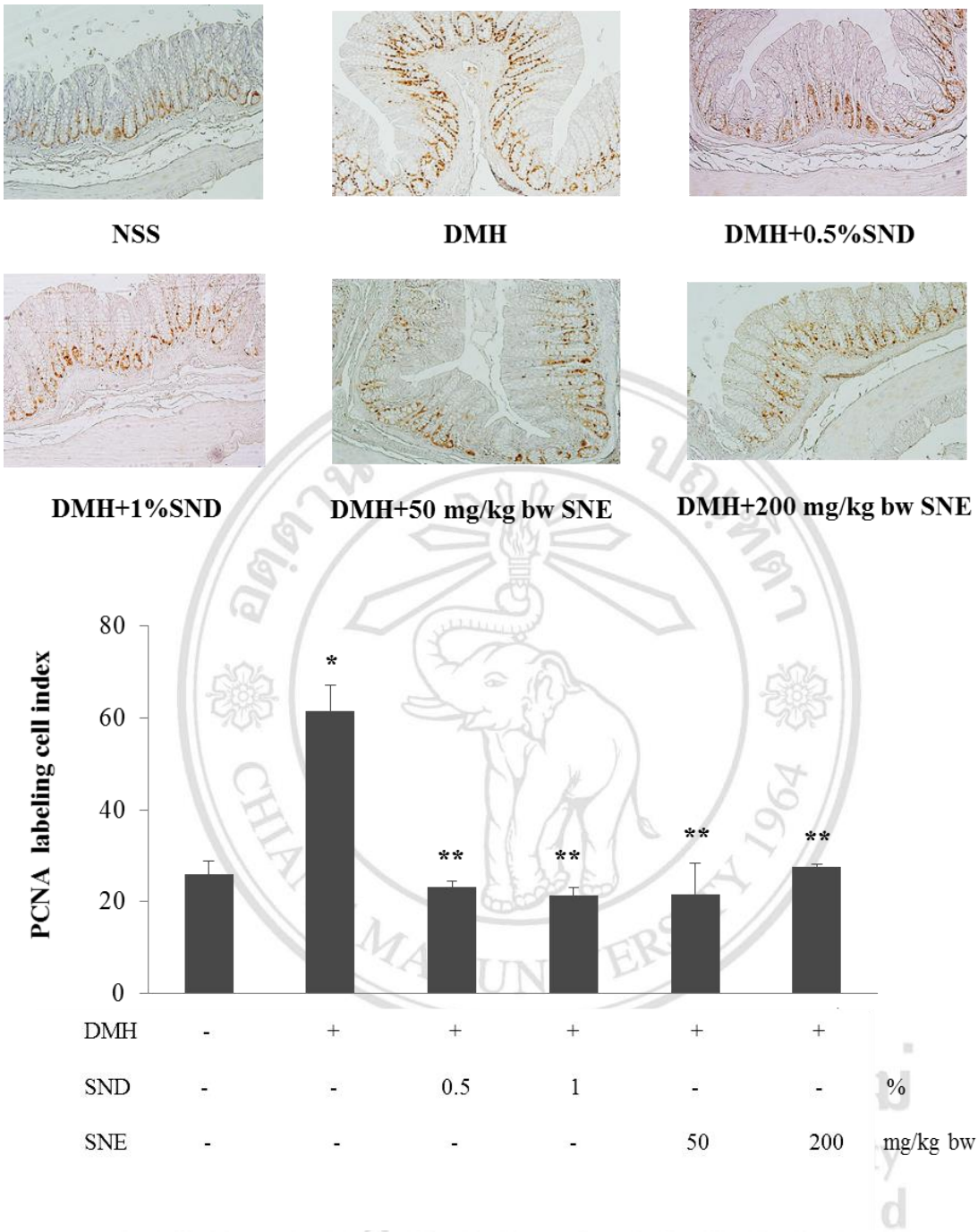


Figure 3.5 Effect of *S. neglecta* on cell proliferation in post-initiation stage of colorectal carcinogenesis in rats. Representative microphotographs of PCNA-positive cell (magnification × 100) in rat colons. * $p < 0.05$ compared with NSS group, ** $p < 0.05$ compared with DMH group.

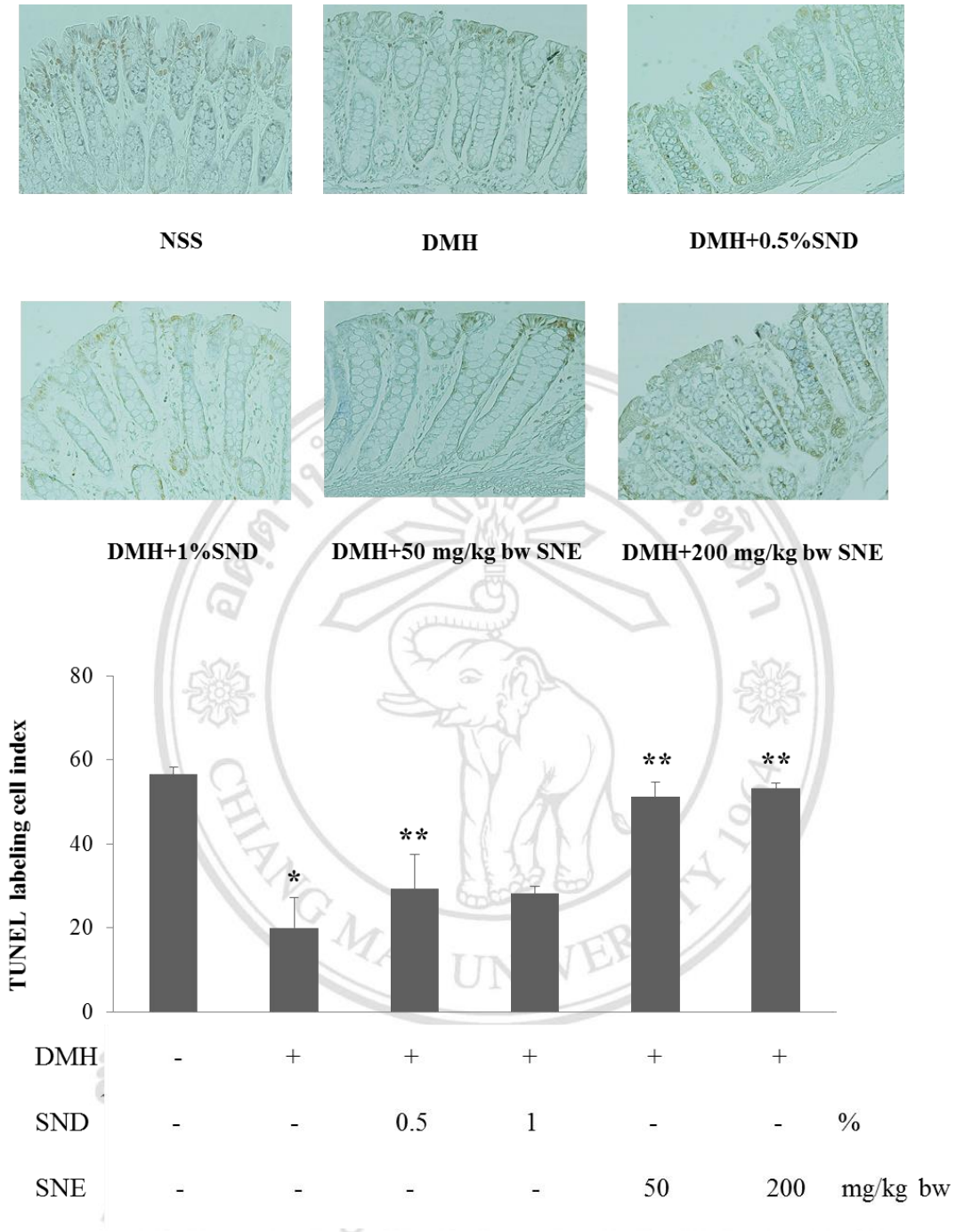


Figure 3.6 Effect of *S. neglecta* on cell apoptosis in post-initiation stage of colorectal carcinogenesis in rats. Representative microphotographs of TUNEL-positive cells (magnification × 200) in rat colons. * $p < 0.05$ compared with NSS group, ** $p < 0.05$ compared with DMH group.

3.2 Chemical composition of *S. neglecta* extract and its derived extracts

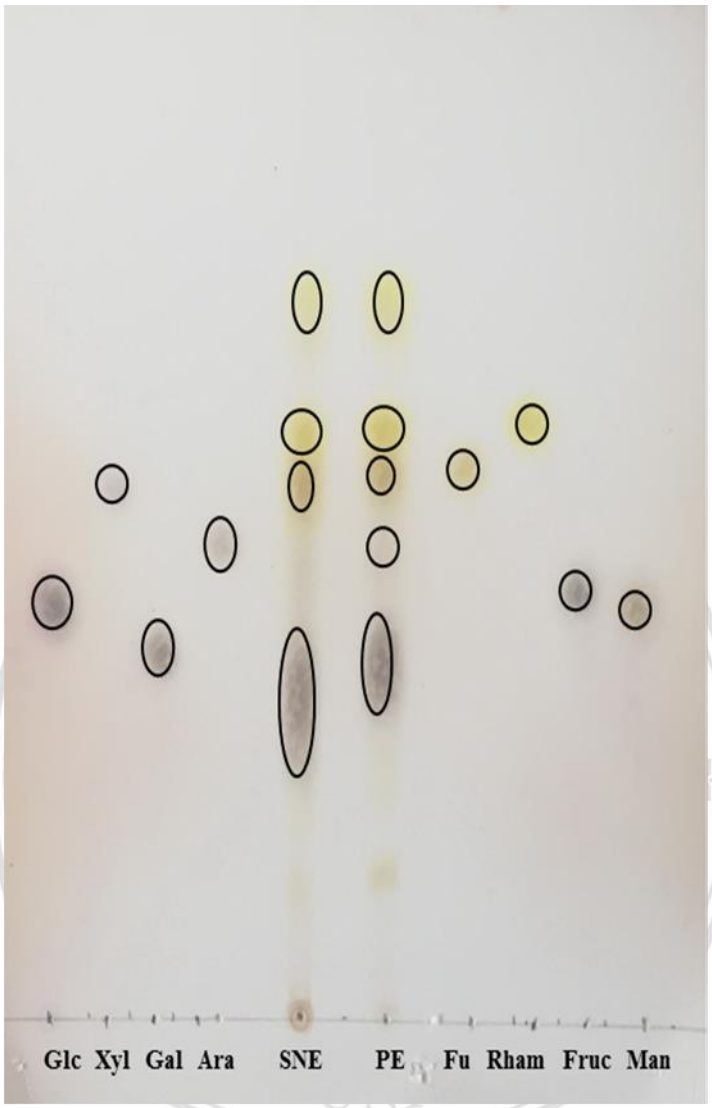
Chemical composition of *S. neglecta* extract (SNE), polysaccharide extract (PE) and chloroform fraction (CF) was determined as shown in Table 3.5. The contents of chemical constituents including total carbohydrates, sulfate and uronic acid contained in polysaccharide extract, obtaining from SNE precipitation, were found in high level when compare to SNE. However, these chemicals did not detect in CF. From these results, we suggested that PE might be a major component in SNE. In addition, the monosaccharide composition of SNE and PE was determined using TLC technique. Monosaccharides found in SNE were fucose, rhamnose and unknown sugar. While monosaccharides found in PE were galactose, arabinose, fucose, rhamnose and unknown sugar (Figure 3.7). These results indicated that SNE and PE shared the same sugars, fucose and rhamnose.

To quantity the monosaccharide compositions in PE, HPLC technique was performed. The major monosaccharides in PE were D-glucose (23.4%), L-fucose (22.6%) and D-galactose (21.4%). L-rhamnose and L-arabinose were approximately 17.1 and 11.3%. The trace amount of monosaccharides were D-mannose (2.7%) and D-xylose (1.6%) (Appendix D). These results suggested that the polysaccharide derived from *S. neglecta* might be a heteropolysaccharide. Moreover, the total phenolic contents of *S. neglecta* were determined by spectrophotometric technique as shown in Table 3.5 and HPLC technique as shown in Figure 3.8. The standard chromatogram of phenolic acids is shown in Figure 3.8A. From these results, the major phenolic acids containing in SNE were gallic acid and ellagic acid (Figure 3.8B). In addition, the phenolic acid containing in PE and CF were ellagic acid (Figure 3.8C) and gallic acid (Figure 3.8D), respectively. The flavonoid contents of SNE, PE and CF were not detected by spectrophotometry technique (data not shown). Moreover, the chlorophyll and carotenoid contents were found only in CF as shown in Table 3.5.

Table 3.5 Chemical constituents in *S. neglecta* extract and its derived extracts

Chemical constituents	*S. neglecta* extract	Polysaccharide extract	Chloroform fraction
Yield (%)	21.9±8.2	9.9±2.0	0.2±0.0
Total Phenolic compounds [a]	62.6±6.1	16.9±1.0	37.3±1.2
Total protein [b]	13.0±0.2	20.2±1.5	ND
Total carbohydrate [c]	208.9±15.8	367.1±34.0	ND
Total sulfate [d]	24.0±0.1	26.0±0.2	ND
Uronic acid [e]	60.2±32.2	82.0±18.6	ND
Chlorophyll a [f]	ND	ND	0.62±0.00
Chlorophyll b [f]	ND	ND	0.36±0.02
Carotenoids [f]	ND	ND	0.31±0.01

Data are presented as mean ± SD. [a]; mg gallic acids equivalent/g extract, [b]; mg/ g extract, [c]; mg glucose/ g extract, [d]; mg chondroitin sulfate C/ g extract, [e]; mg glucuronic acid/ g extract, [f]; µg/mg extract ND; not detected.



Standards/sample	Distance (cm)	Stationary phase (cm)	R_f
Glucose	4.9	10	0.49
Xylose	5.1	10	0.51
Galactose	3.5	10	0.35
Arabinose	4.5	10	0.45
Fucose	5.2	10	0.52
Rhamnose	5.6	10	0.56
Fructose	4.1	10	0.41
Mannose	3.9	10	0.39
SNE	3.1, 5.0, 5.6, 6.8	10	0.31, 0.50, 0.56, 0.68
PE	3.4, 4.4, 5.2, 5.6, 6.8	10	0.34, 0.44, 0.52, 0.56, 0.68

Figure 3.7 Identification of monosaccharides in *S. neglecta* extract and polysaccharide extract by thin layer chromatography. R_f values of monosaccharide standards and monosaccharides in SNE and PE separated by thin layer chromatography, using TLC silica gel G60.

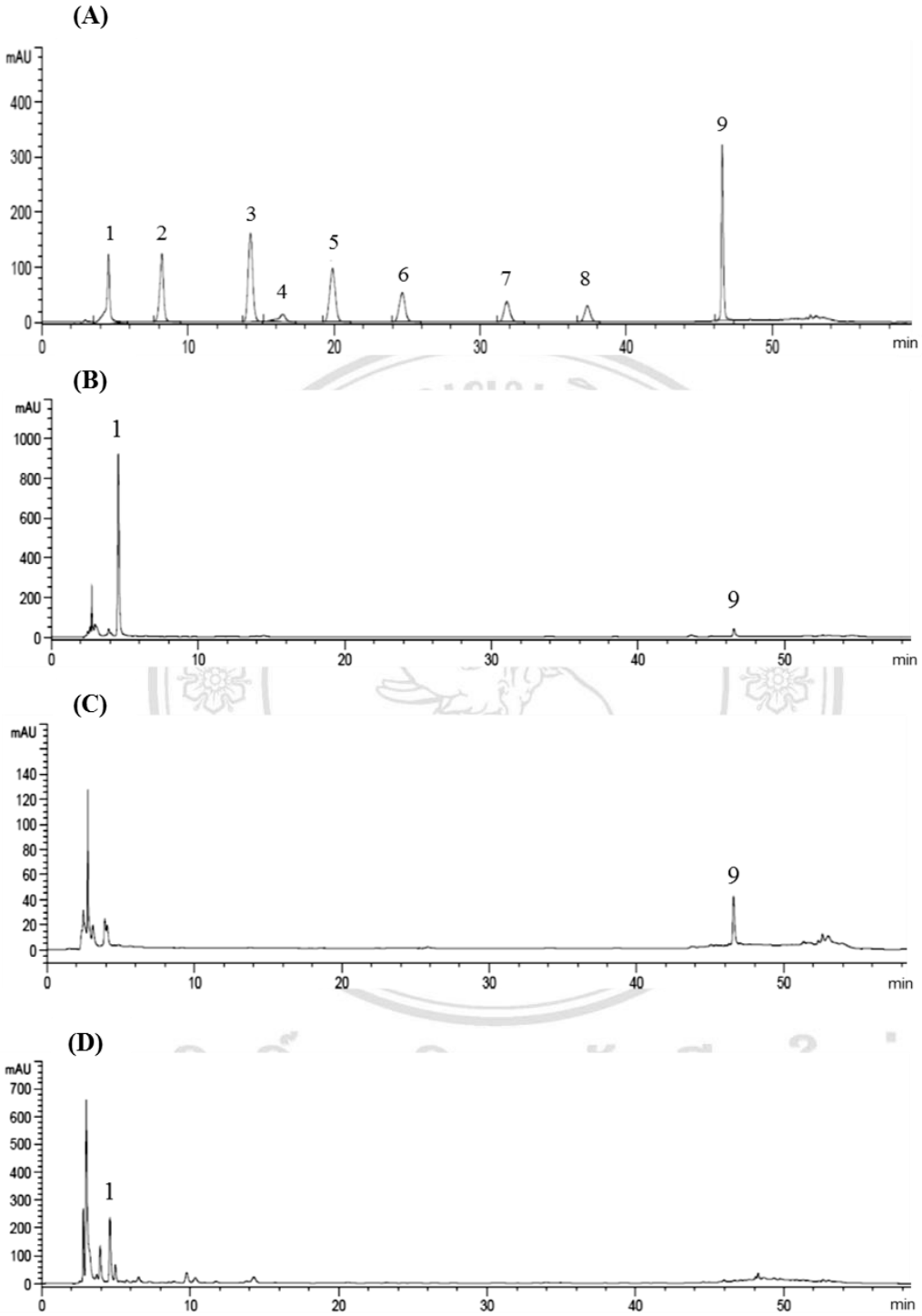


Figure 3.8 Chromatograms of standard phenolic acids and *S. neglecta* extracts. A represents gallic acid (1), protocatechuic acid (2), 4-hydroxybenzoic acid (3), chlorogenic acid (4), vanillic acid (5), syringic acid (6), *p*-coumaric acid (7), ferulic acid (8) and ellagic acid (9). B, C and D represent phenolic acids in *S. neglecta* extract, polysaccharide extract and chloroform fraction, respectively.

3.3 Chemical composition of partial polysaccharide fractions derived from crude polysaccharide extract

The crude polysaccharide was extracted from SNE with absolute ethanol and fractionated by anion-exchange chromatography. Three carbohydrate fractions, F1, F2 and F3, were obtained from DEAE-sepharose column, which were eluted by 0.25 M NaCl in sodium acetate buffer, pH 6. The carbohydrates could not be eluted when the concentration of NaCl was over than 0.25 M, 0.5-3.0 M (Figure 3.9). The chemical composition of three carbohydrate fractions are presented in Table 3.6. The amount of all chemical constituents found in F3 was lower than other fractions.

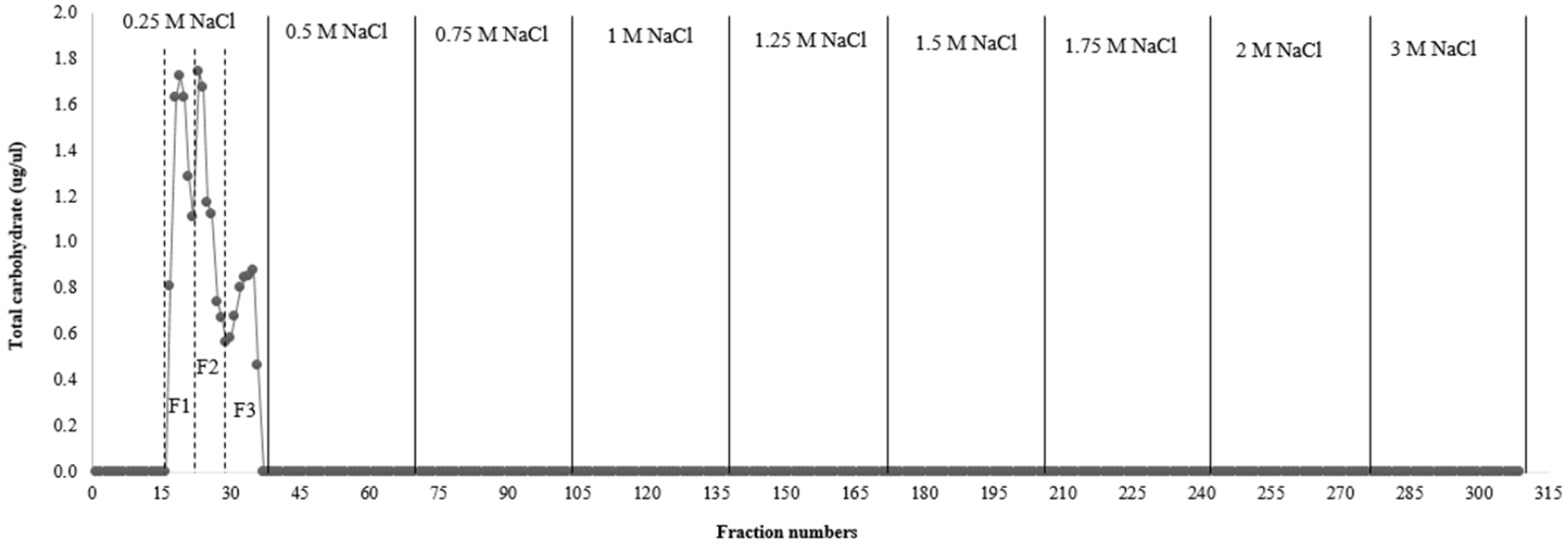


Figure 3.9 The concentration of total carbohydrates in polysaccharide extract obtained from DEAE-sepharose chromatography. The mobile phase is gradient concentration of 0-3.0 M of NaCl sodium acetate buffer, pH 6 with a flow rate of 2 ml/min.

Table 3.6 Chemical constituents in partial polysaccharide fractions of polysaccharide extract obtained from DEAE-sepharose chromatography.

Chemical constituents	Fraction 1	Fraction 2	Fraction 3
Yield (%)	4.2±2.9	6.5±0.6	3.1±1.5
Total phenolic compounds [a]	8.5±3.2	9.3±0.8	6.3±1.9
Total protein [b]	24.6±6.8	22.4±0.8	15.9±3.4
Total carbohydrate [c]	252.0±30.8	270.5±17.0	246.8±6.5
Total sulfate [d]	5.1±0.3	5.4±0.9	2.5±2.0
Uronic acid [e]	73.2±2.3	77.0±2.9	69.6±4.0

Data are presented as mean ± SD. [a]; mg gallic acids equivalent/g extract, [b]; mg/g extract, [c]; mg glucose/g extract, [d]; mg chondroitin sulfate C/g extract, [e]; mg glucuronic acid/g extract, ND; not detected.

3.4 Mutagenicity of *S. neglecta* extracts in *Salmonella* mutation assay

Tables 3.7 and 3.8 show the average number of His^+ revertant colonies and the mutagenic index (MI) after the treatment with *S. neglecta* extract (SNE), polysaccharide extract (PE) and chloroform fraction (CF) observed in *S. typhimurium* strains TA 98 and TA100, in the presence and absence of metabolic activation (S9 mix). SNE and PE at the highest dose 5 mg/plate presented cytotoxic effect in TA98 strain with or without S9 mix, while no cytotoxic effect was observed in TA 100 strain. Moreover, CF at the highest dose of 2.5 mg/plate showed killing effect in both strains with or without metabolic activation. SNE, PE and CF did not increase the number of His^+ revertant colonies as compared to the number of spontaneous colonies. These results indicated that SNE, PE and CF were non-mutagenic in any of the two standard tester strains with or without metabolic activation.

Table 3.7 Mutagenicity of *S. neglecta* extracts in the *Salmonella typhimurium* strain TA98 with presence and absence of metabolic activation

Sample/Mutagen	Concentration (per plate)	Average of His^+ revertant colonies			
		-S9	MI	+S9	MI
DMSO	50 µl	22±4	-	30±8	-
Distilled water	50 µl	21±3	-	33±8	-
AF-2	0.1 µg	372±28	17.9	NA	-
2AA	0.5 µg	NA	-	828±72	28.8
S. neglecta extract	0.1 mg	21±3	1.0	NA	-
	0.25 mg	19±2	0.9	NA	-
	1 mg	19±2	0.9	31±11	0.9
	2.5 mg	k	-	31±10	0.9
	5 mg	k	-	30±12	0.9
Polysaccharide extract	0.1 mg	23±4	1.1	NA	-
	0.25 mg	22±4	1.0	NA	-
	1 mg	21±3	1.0	32±10	1.0
	2.5 mg	k	-	29±9	0.9
	5 mg	k	-	26±6	0.8
Chloroform fraction	0.01 mg	21±3	1.0	30±12	1.0
	0.1 mg	20±3	0.9	31±12	1.0
	1 mg	19±3	0.9	30±10	1.0
	2.5 mg	k	-	k	-

Data are presented as Mean ± SD of triplicate determination, MI; Mutagenicity Index, NA; not analyzed, k; killing effect, 2AA; 2-aminoanthracene, AF-2; 2-(2-furyl-3-(5-nitro-2-furyl) acrylamide.

Table 3.8 Mutagenicity of *S. neglecta* extracts in the *Salmonella typhimurium* strain TA100 with presence and absence of metabolic activation

Sample/Mutagen	Concentration (per plate)	Average of His^+ revertant colonies (MI)			
		-S9	MI	+S9	MI
DMSO	50 µl	146±21	-	146±11	-
Distilled water	50 µl	135±18	-	159±8	-
AF-2	0.01 µg	490±36	3.3	NA	-
2AA	0.5 µg	NA	-	700±74	4.8
S. neglecta extract	1 mg	134±18	1.0	151±8	0.9
	2.5 mg	135±26	1.0	153±11	1.0
	5 mg	140±26	1.0	143±10	0.9
Polysaccharide extract	1 mg	148±20	1.1	147±14	0.9
	2.5 mg	145±21	1.1	148±7	0.9
	5 mg	143±26	1.1	151±10	0.9
Chloroform fraction	0.01 mg	138±19	0.9	138±7	0.9
	0.1 mg	140±25	1.0	131±8	0.9
	1 mg	116±33	0.8	133±10	0.9
	2.5 mg	k	-	k	-

Data are presented as Mean ± SD of triplicate determination, MI; Mutagenicity Index, NA; not analyzed, k; killing effect, 2AA; 2-aminoanthracene, AF-2; 2-(2-furyl-3-(5-nitro-2-furyl) acrylamide.

3.5 Antimutagenicity of *S. neglecta* extracts in *Salmonella* mutation assay

The Ames test was used to evaluate the antimutagenic effects against heterocyclic amines including IQ, MeIQ and PhIP, which have been reported about their carcinogenicity in several organs. The antimutagenic potential of SNE, PE and CF is presented in Table 3.9 and Figure 3.10. At 1 mg/plate, only CF was effective in reducing the number of His^+ revertant colonies induced by the indirect mutagen PhIP in strain TA98, as well as by the two indirect mutagens IQ and MeIQ in strain TA100 (Figure 3.10). From this result, CF showed strong antimutagenicity against IQ, MeIQ and PhIP at dose 1 mg/plate, while SNE and PE failed to show antimutagenic effect against three heterocyclic amine mutagens (Figure 3.10). These results indicated that CF is the most potent antimutagenicity in *Salmonella* mutation assay.

Table 3.9 Antimutagenicity of *S. neglecta* extracts against various heterocyclic amines induced mutagenesis in the *Salmonella typhimurium* strains TA98 and 100

Sample/Mutagen	Concentration (per plate)	IQ (0.25 µg/plate)		MeIQ (0.025 µg/plate)		PhIP (0.5 µg/plate)	
		Average of His^+ revertant colonies	%inhibition	Average of His^+ revertant colonies	%inhibition	Average of His^+ revertant colonies	%inhibition
DMSO	100 µl	167±11	-	148±12	-	37±8	-
Distilled water	100 µl	189±30	-	161±20	-	33±9	-
Mutagen		550±99	-	523±52	-	711±80	-
S. neglecta extract	1 mg	516±41	13.8±11.4	565±87	-7.9±22.8	751±56	-6.4±5.3
	2.5 mg	349±28	51.9±9.1	463±71	19.1±23.2	717±65	-1.3±8.1
	5 mg	244±17	82.3±4.3	296±78	63.7±25.9	213±67	73.3±9.4
Polysaccharide extract	1 mg	600±58	-1.7±23	595±109	-15.5±24.8	788±53	-12.0±8.2
	2.5 mg	433±27	39.3±7.7	543±97	-2.5±21.8	726±86	-2.8±12.6
	5 mg	278±54	61.7±15.4	336±89	53.4±26.6	679±59	4.3±6.8
Chloroform fraction	0.01 mg	283±28	70.1±10.5	370±52	40.8±14.3	497±88	32.0±11.3
	0.1 mg	197±15	92.6±5.7	213±29	82.8±5.8	260±44	66.7±6.7
	1 mg	135±17	100.0±0.0	148±22	99.9±7.1	53±15	97.4±2.2

Data are presented as Mean ± SD of triplicate determination, IQ; 2-amino-3-methylimidazo[4,5-f]quinoline, MeIQ; 2-amino-3,4-dimethyl-imidazo[4,5-f]quinolone, PhIP; 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine.

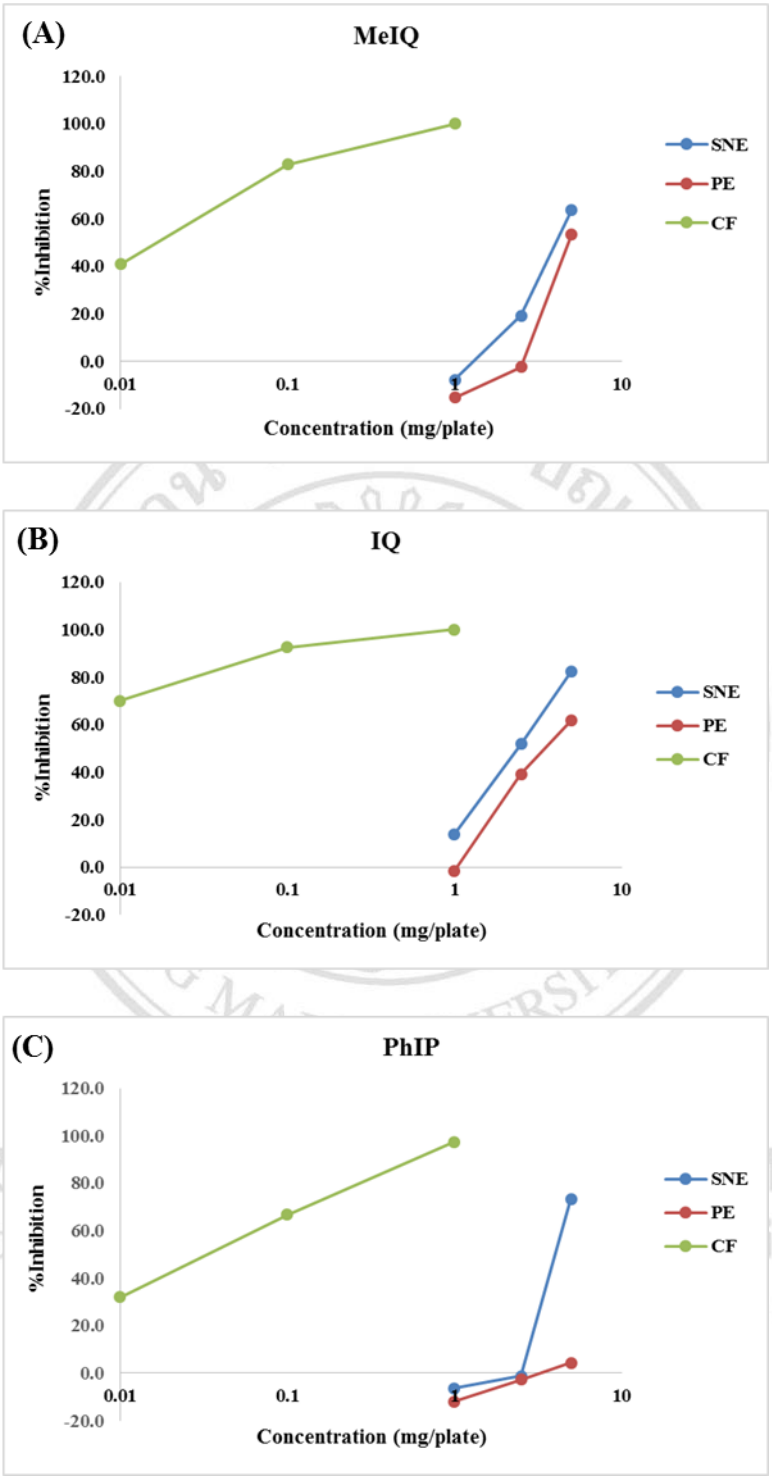


Figure 3.10 The inhibition of *S. neglecta* extracts against heterocyclic amines induced mutagenesis using *Salmonella* mutation assay.

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

According to previous results, SNE exhibited stronger anticarcinogenicity than SND. Therefore, the extracts derived from SNE including PE and CF were investigated their anticarcinogenicity on post-initiation stage of colorectal carcinogenesis in rats.

The general observation and relative internal organ weights of rat are shown in Table 3.10. There were no adverse effects on body weight gain during the experimental period of post-initiation stage. Moreover, there was no significant difference in diet and water intakes in any of the groups.

ACF were found in all rats treated with DMH. The SNE, PE or CF administration did not induce ACF formation in colon and rectum of rats (Table 3.11). However, ACF could observe in colon of only one rat treated with NSS or SNE or CF. These results indicated that PE and CF were not carcinogenic in rats. The number of ACF in DMH-treated groups was significantly increased as compared to their vehicle groups. A reduction of total ACF was observed in SNE, PE and CF treated rats with the exception of low dose SNE. Moreover, the percentage of ACF inhibition in rats treated with *S. neglecta* is presented in Table 3.11. However, the mean numbers of aberrant crypts per focus (AC/focus) were not significantly different in all DMH-treated groups. In DMH-treated rats, the ACF with 4 and more crypts per focus were observed. The amount of large ACF, equal or more than 4 crypts, was significantly reduced only in PE treated rats. However, there was no significant difference in crypt multiplicity between DMH-treated groups. These results showed that polysaccharide extracts obtained from SNE significantly inhibited DMH-induced ACF formation.

Table 3.10 General observation and organ weights of rats in post-initiation stage of colorectal carcinogenesis

Treatments	Body weight (g)		Intake		Relative weight (g per 100 g bw)		
	Initial	Final	Diet (g)	Water (ml)	liver	Spleen	Kidneys
NSS + DW	114±6	452±28	20±2	35±5	3.32±0.15	0.20±0.02	0.55±0.02
DMH + DW	115±5	413±29	18±1	32±5	3.01±0.17	0.20±0.02	0.54±0.04
DMH + SNE 50 mg/kg bw	115±11	441±24	20±2	30±4	3.14±0.21	0.18±0.02	0.53±0.04
DMH + SNE 200 mg/kg bw	116±8	415±23	18±2	32±4	3.15±0.21	0.20±0.02	0.55±0.05
DMH + PE 25 mg/kg bw	115±6	433±37	19±2	29±3	3.23±0.17	0.19±0.02	0.55±0.04
DMH + PE 100 mg/kg bw	116±7	420±21	19±2	32±5	3.15±0.27	0.20±0.03	0.55±0.03
NSS + 1% DMSO	115±8	438±25	20±4	35±5	3.47±0.27	0.20±0.02	0.56±0.06
DMH + 1% DMSO	115±9	411±34	18±2	30±4	3.15±0.11	0.21±0.03	0.56±0.03
DMH + CF 0.25 mg/kg bw	116±4	422±47	19±2	33±5	2.98±0.19	0.19±0.02	0.52±0.03
DMH + CF 1 mg/kg bw	115±7	433±42	19±2	32±5	3.12±0.17	0.20±0.03	0.53±0.04
NSS + SNE 200 mg/kg bw	114±7	453±21	20±2	36±4	3.11±0.21	0.19±0.02	0.52±0.04
NSS + PE 100 mg/kg bw	114±2	418±29	19±2	31±4	3.09±0.16	0.19±0.01	0.55±0.03
NSS + CF 1 mg/kg bw	115±8	428±30	20±2	30±5	3.21±0.31	0.19±0.02	0.56±0.03

Data represent the mean ± S.D. of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), SNE; *S. neglecta* extract, PE; polysaccharide extract, CF; chloroform fraction.

Table 3.11 The effect of *S. neglecta* extracts on number of aberrant crypt foci in post-initiation stage of colorectal carcinogenesis

Treatments	No. of total ACF/rat	%Inhibition	Aberrant crypt/focus	No. of ACF>=4 AC/focus/rat	%Inhibition	Aberrant crypt>=4/focus
NSS + DW	0±0	-	0±0	0±0	-	0±0
DMH + DW	263±90*	-	4.3±0.4*	118±36*	-	6.9±0.5*
DMH + SNE 50 mg/kg bw	233±65	11.5±24.7	4.5±0.5	114±29	3.3±24.5	6.9±0.7
DMH + SNE 200 mg/kg bw	176±32**	33.3±12.1	4.1±0.9	80±30	32.5±25.1	6.3±0.8
DMH + PE 25 mg/kg bw	137±28**	47.9±10.7	4.0±0.8	65±24**	44.9±20.6	6.2±0.8
DMH + PE 100 mg/kg bw	119±68**	54.6±25.9	4.2±0.9	56±38**	52.4±32.0	6.5±0.9
NSS + 1% DMSO	0±0	-	0±0	0±0	-	0±0
DMH + 1% DMSO	218±60$^{\#}$	-	3.9±0.7 $^{\#}$	86±27 $^{\#}$	-	6.5±0.6 $^{\#}$
DMH + CF 0.25 mg/kg bw	139±63$^{\#\#}$	36.1±28.9	4.6±0.9	73±38	14.9±44.1	6.8±0.8
DMH + CF 1 mg/kg bw	111±44$^{\#\#}$	49.0±20.1	4.2±0.7	51±25	40.3±28.6	6.7±06
NSS + SNE 200 mg/kg bw	0.2±0.4	-	1.2±2.9	0.2±0.4	-	1.2±2.9
NSS + PE 100 mg/kg bw	0±0	-	0±0	0±0	-	0±0
NSS + CF 1 mg/kg bw	0.2±0.4	-	1.0±2.5	0.2±0.4	-	1.0±2.5

Data represent the mean ± S.D. of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), SNE; *S. neglecta* extract, PE; polysaccharide extract, CF; chloroform fraction, * $p < 0.05$ compared with NSS+DW group, ** $p < 0.05$ compared with DMH+DW group, $^{\#}$ $p < 0.05$ compared with NSS+1%DMSO group, $^{\#\#}$ $p < 0.05$ compared with DMH+1%DMSO group.

3.7 Inhibitory mechanisms of *S. neglecta* extracts against post-initiation stage of colorectal carcinogenesis

The expression of cell proliferative index (PCNA-positive cells) in the colonic tissues of all experimental groups is illustrated in Figure 3.11. The PCNA positive cells were significantly increase in DMH-treated rats as compared to NSS-treated rats. They were also dramatically reduced in the rats treated with SNE, PE and CF when compared to DMH-treated group while only low dose of PE showed no significant difference. In addition, Figure 3.12 shows apoptotic index (TUNEL-positive cells) in the colonic tissues of all experimental groups were no significant different as compared to the other treated groups.

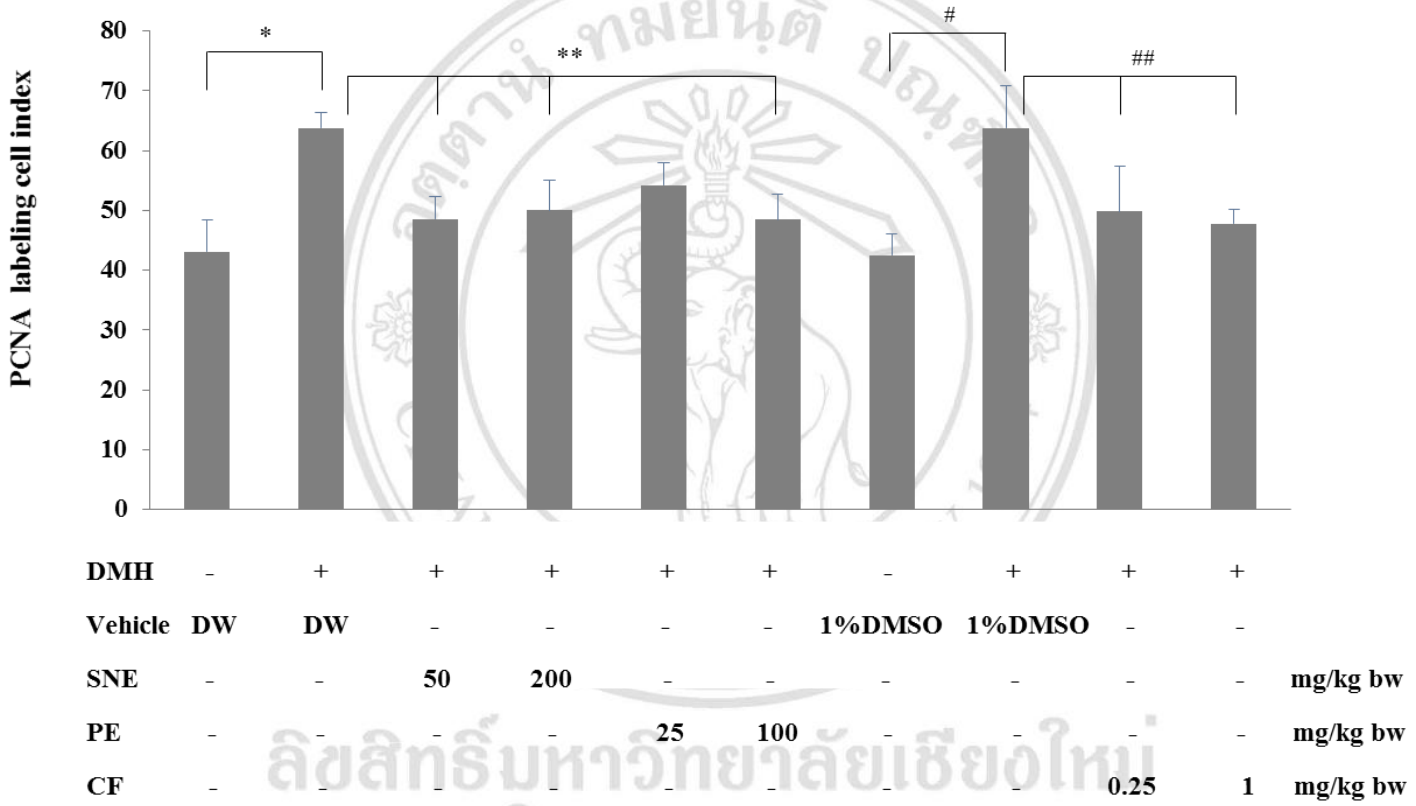


Figure 3.11 Effect of *S. neglecta* extracts on cell proliferation in post-initiation stage of colorectal carcinogenesis in rats.

* $p < 0.05$ compared with NSS+DW group, ** $p < 0.05$ compared with DMH+DW group,

$^{\#}$ $p < 0.05$ compared with NSS+1%DMSO group, $^{\#\#}$ $p < 0.05$ compared with DMH+1%DMSO group.

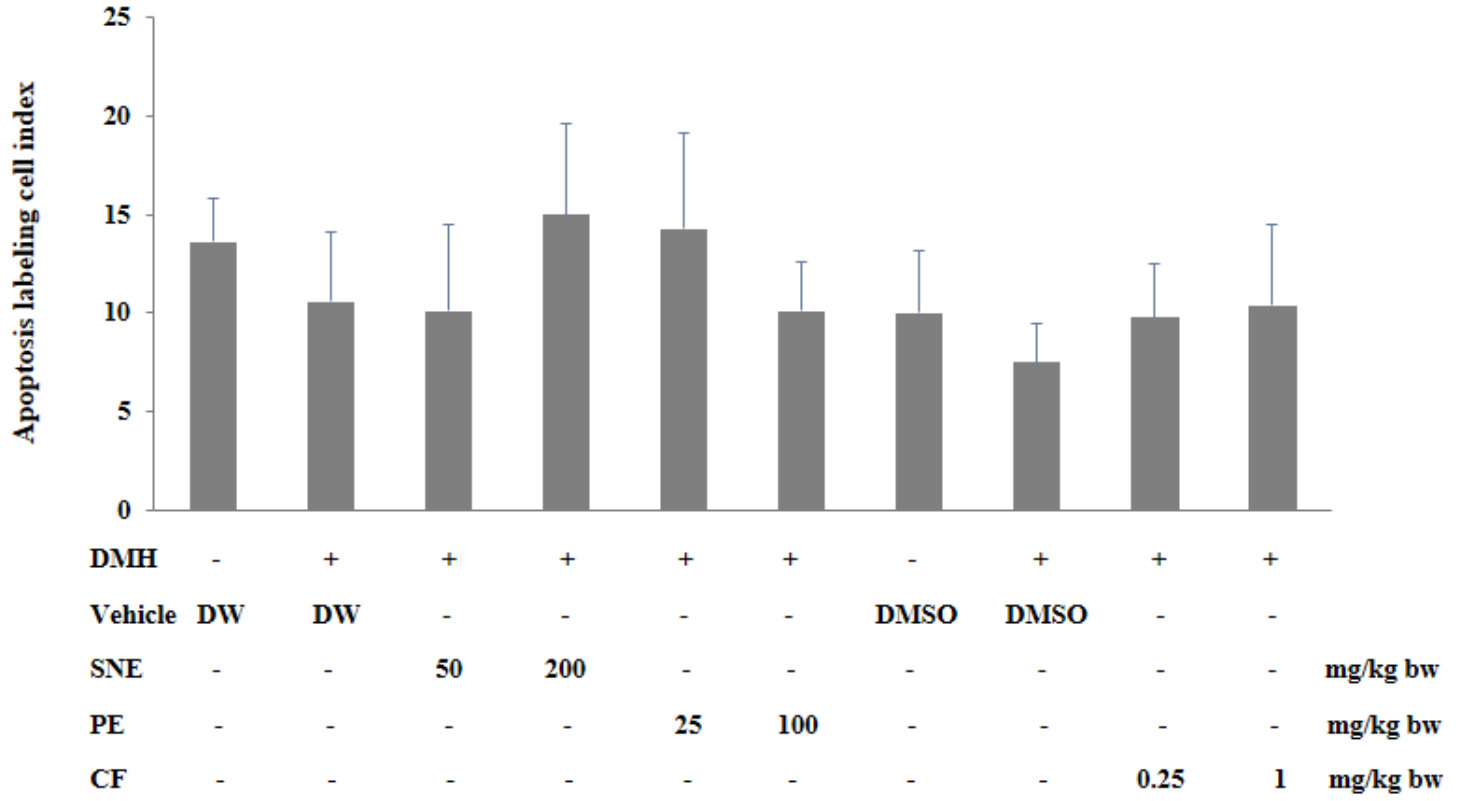


Figure 3.12 Effect of *S. neglecta* extracts on cell apoptosis in post-initiation stage of colorectal carcinogenesis in rats.

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

The general observation and relative internal organ weights of mice are shown in Table 3.12. There were no significant differences in the weights of whole body, kidneys and spleen between the groups (Table 3.12). Furthermore, no significant body weight changes were found in mice of vehicle control, SNE, PE and CF alone groups. As the results in Table 3.12, DSS treatment significantly increase in relative liver weight when compared to a vehicle control group.

Figure 3.13 summarizes the histological score of pathological changes in colonic mucosa of mice. Histology sections of colon in mice treated with DSS and combined with SNE, PE and CF are presented in Figures 3.14 and 3.15.

The vehicle control groups showed normal colonic architecture including intact surface epithelium, cryptal glands and submucosa (Figures 3.14A and 3.15A), whereas epithelial erosion and ulceration, cryptal glands disruption, submucosal edema and infiltration of inflammatory cells to submucosa were observed in the DSS-treated groups (Figures 3.14B and 3.15B). Interestingly, treatment of SNE, PE and CF combined with DSS improved those pathological changes such as a quite intact surface epithelium and cryptal glands (Figure 3.14C, D, E and F and 3.15C and D). Moreover, a lower degree of infiltration of inflammatory cells to submucosa was also detected. These results indicated that crypt structures were preserved and the numbers of infiltrated inflammatory cells to submucosa were decreased, resulting in the low histological scores for colons of SNE, PE and CF-treated mice (Figure 3.13).

Table 3.12 The body weights and relative internal organ weights of mice in dextran sodium sulfate-induced colitis in mice

Treatment	Body weight (g)		Relative organ weight (g per 100 g bw)		
	Initial	Final	Liver	Kidneys	Spleen
Tap water + DW	31.1±1.7	32.9±1.4	3.56±0.24	1.41±0.11	0.31±0.05
Tap water + 1% DMSO	31.9±1.8	32.6±1.7	3.70±0.26	1.25±0.11	0.30±0.03
3% DSS + DW	31.9±0.7	31.9±1.3	4.58±0.48*	1.33±0.13	0.73±0.37
3% DSS + 1% DMSO	31.6±1.0	31.4±1.4	3.92±0.43	1.34±0.11	0.43±0.11
3% DSS + SNE 50 mg/kg bw	31.3±2.4	30.1±2.8	4.14±0.30	1.28±0.16	0.76±0.20
3% DSS + SNE 200 mg/kg bw	31.4±1.1	31.6±1.2	4.06±0.54	1.37±0.12	0.64±0.33
3% DSS + PE 25 mg/kg bw	31.6±1.5	30.5±1.5	3.90±0.28	1.50±0.16	0.63±0.25
3% DSS + PE 100 mg/kg bw	32.1±0.5	31.8±0.6	3.89±0.23	1.43±0.15	0.59±0.11
3% DSS + CF 0.25 mg/kg bw in 1% DMSO	32.9±1.2	32.3±1.4	3.85±0.29	1.37±0.15	0.65±0.23
3% DSS + CF 1 mg/kg bw in 1% DMSO	31.4±1.4	31.1±1.4	3.77±0.48	1.36±0.07	0.42±0.17
Tap water + SNE 50 mg/kg bw	31.0±1.4	32.4±0.9	3.55±0.17	1.39±0.09	0.28±0.05
Tap water + PE 25 mg/kg bw	31.2±1.8	31.0±2.1	3.72±0.44	1.39±0.05	0.33±0.20
Tap water + CF 0.25 mg/kg bw in 1% DMSO	31.0±1.9	32.4±0.8	3.54±0.20	1.31±0.06	0.26±0.04

Data are mean ± SD; * $p < 0.05$ compared with Tap water and DW-treated group.

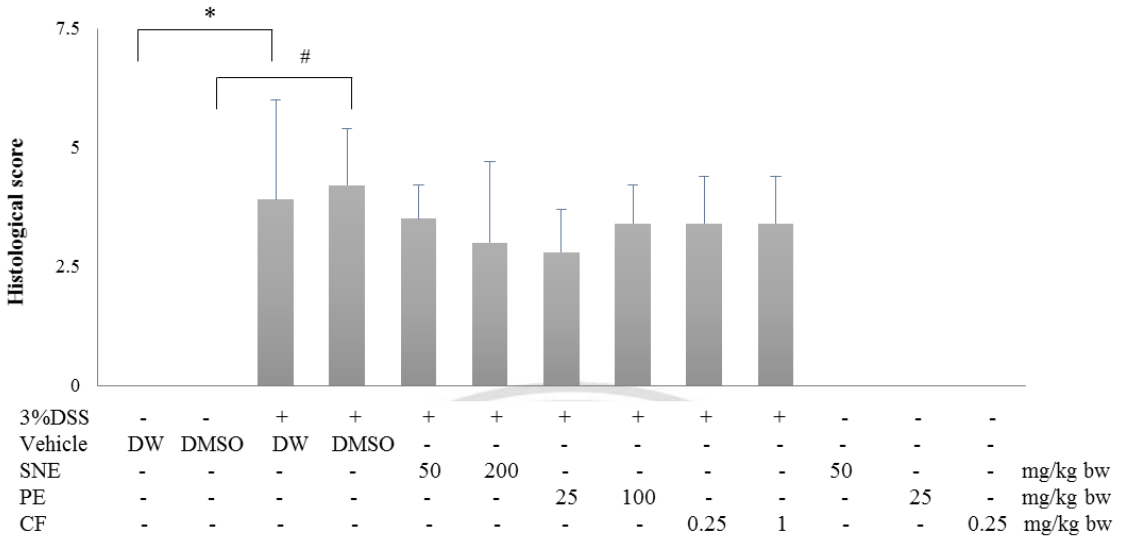


Figure 3.13 Effect of *S. neglecta* extracts on histopathological changes in the colons of mice treated with DSS. Data are mean ± SD. * $p < 0.05$ compared with DW vehicle control group, # $p < 0.05$ compared with DMSO vehicle control group.

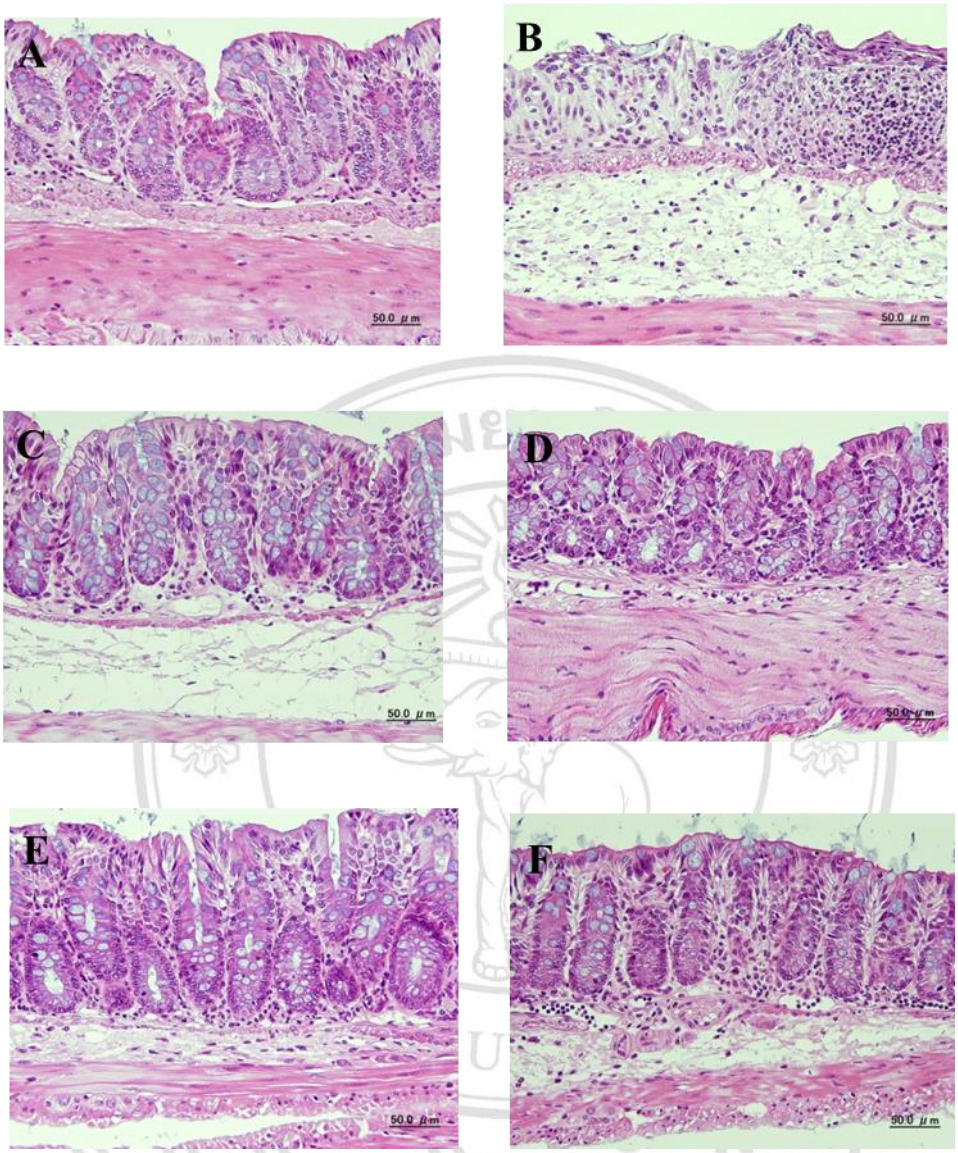


Figure 3.14 Cross section of the mouse colon stained with haematoxylin and eosin. Histology sections of colon in mice treated with DSS and combined to *S. neglecta* extracts. (A-F) Representative microphotographs of H&E stained mice colons from DW vehicle control (A), DSS-DW control (B), DSS-SNE (50 mg/kg b.w.) (C), DSS-SNE (200 mg/kg b.w.) (D), DSS-PE (25 mg/kg b.w.) (E) and DSS-PE (100 mg/kg b.w.) (F) groups.

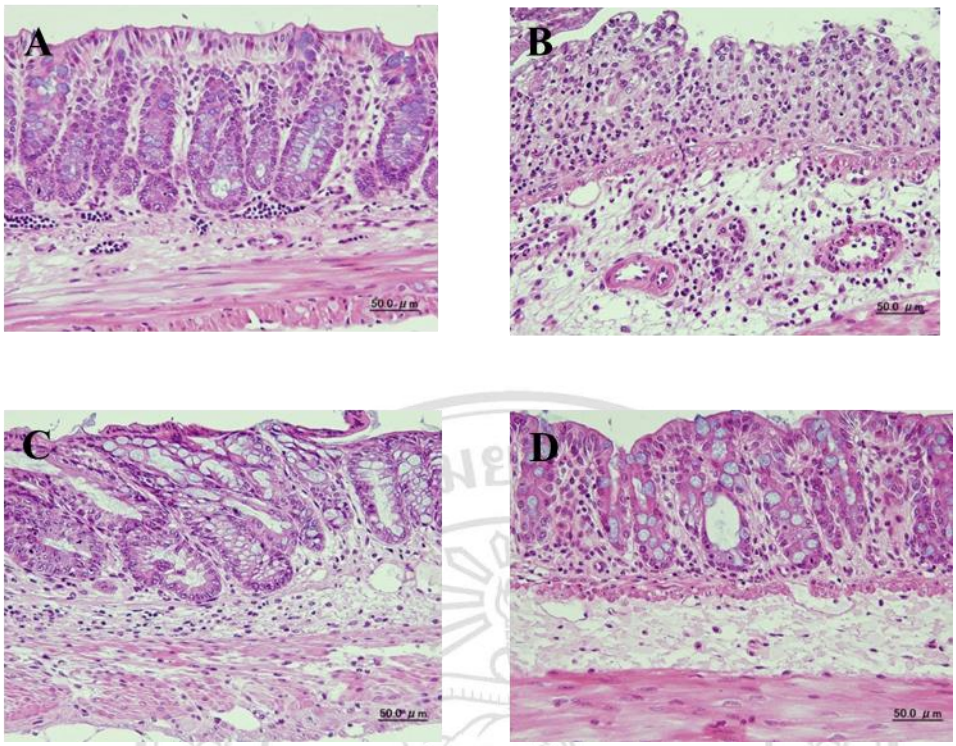


Figure 3.15 Cross section of the mouse colon stained with haematoxylin and eosin. Histology sections of colon in mice treated with DSS and combined to chloroform fraction. (A-D) Representative microphotographs of H&E stained mice colons from DMSO vehicle control (A), DSS-DMSO control (B), DSS-CF (0.25 mg/kg b.w.) (C) and DSS-CF (1 mg/kg b.w.) (D) groups.

3.8.1 The inhibitory effects of *S. neglecta* extracts on cell proliferation and apoptosis in mice colitis model

The expression of cell proliferative index (Ki-67-positive cells) in the colonic tissues of all experimental groups is illustrated in Figures 3.16, 3.17 and 3.18. DSS tended to reduce the number of Ki-67 positive cells, indicating induction of cell cycle arrest due to significant colon epithelial cell damage. Nevertheless, administrations of SNE and PE significantly recovered the number of Ki-67 positive cells to the normal level observed in the vehicle control group. However, administration of CF showed no significant difference in number of Ki-67 positive cells as compared to DSS-treated group.

Figure 3.19 shows TUNEL-positive cell in colonic mucosa and Figure 3.20 illustrates the effect of *S. neglecta* extracts on DSS-induced cell apoptosis in mice colitis model. There were a few apoptotic cells presented in colon sections of vehicle control group. However, the number of apoptotic cells were increased in colonic sections of DSS-treated group. The administrations of SNE, PE and CF significantly recovered the number of apoptotic cells to the normal level observed in the vehicle control group.

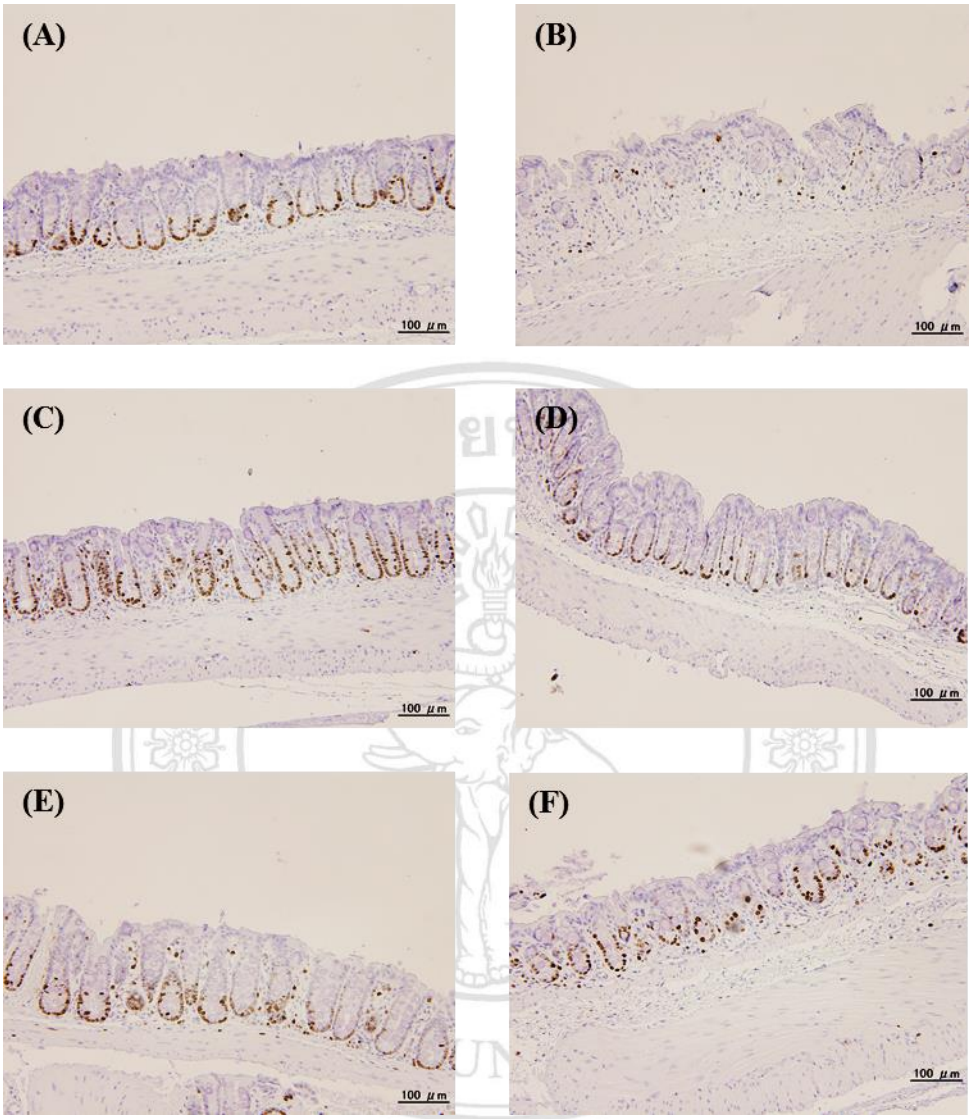


Figure 3.16 Immunohistochemical staining of Ki-67 in mice treated with DSS and combined to *S. neglecta* extracts. (A-F) Representative microphotographs of Ki67 expression in mice colons from DW vehicle control (A), DSS-DW control (B), DSS-SNE (50 mg/kg b.w.) (C), DSS-SNE (200 mg/kg b.w.) (D), DSS-PE (25 mg/kg b.w.) (E) and DSS-PE (100 mg/kg b.w.) (F) groups.

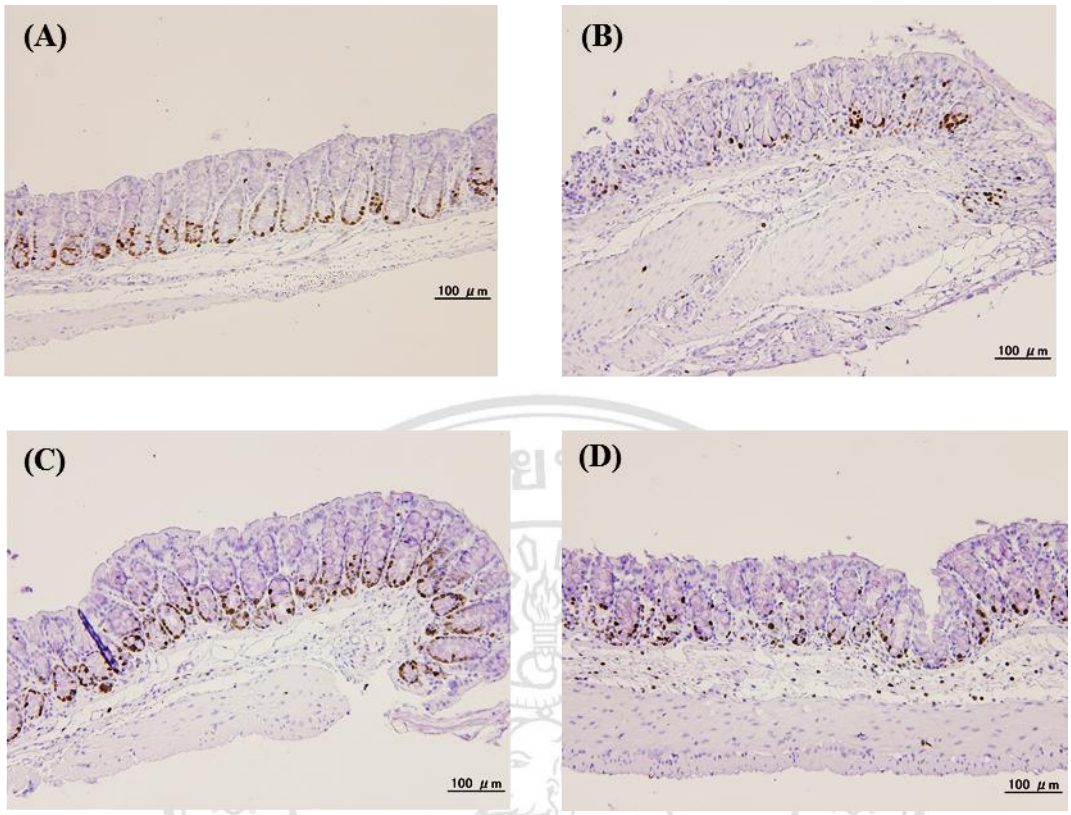


Figure 3.17 Immunohistochemical staining of Ki-67 in mice treated with DSS and combined to chloroform fraction. (A-D) Representative microphotographs of Ki67 expression in mice colons from DMSO vehicle control (A), DSS-DMSO control (B), DSS-CF (0.25 mg/kg b.w.) (C) and DSS-CF (1 mg/kg b.w.) (D) groups.

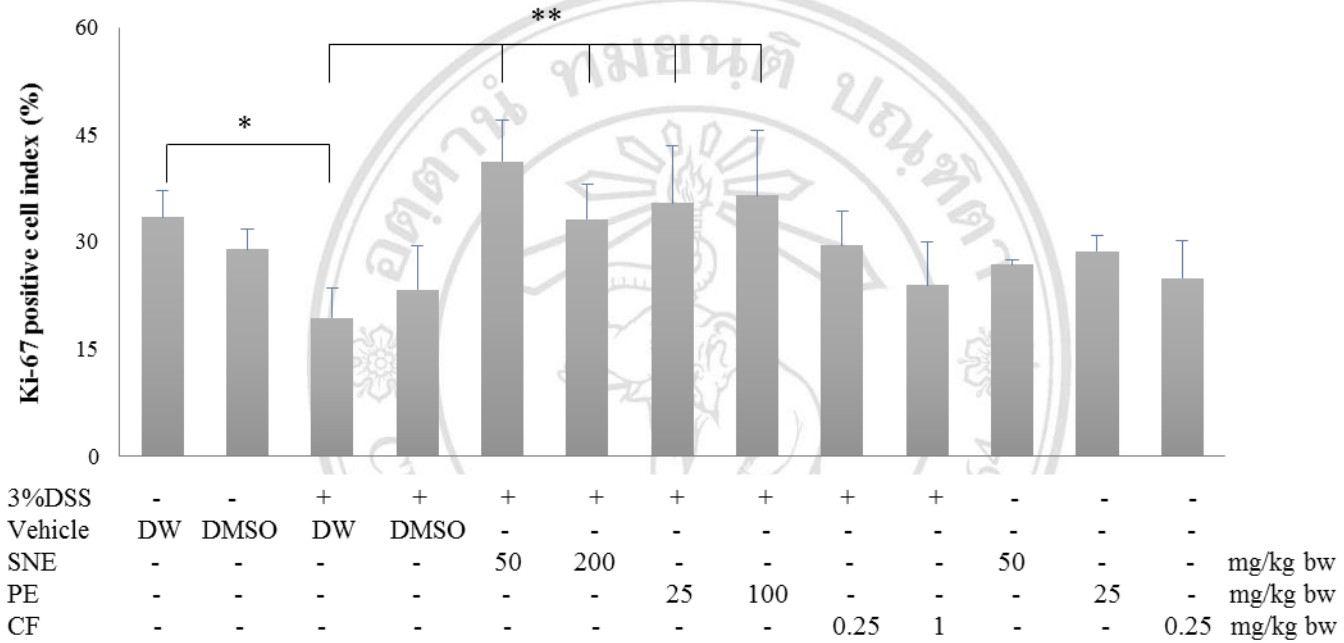


Figure 3.18 Effect of *S. neglecta* extracts on cell proliferation in the colons of mice treated with DSS. Data are mean ± SD. * $p<0.05$ vs. negative control group. ** $p<0.05$ vs. the positive control group.

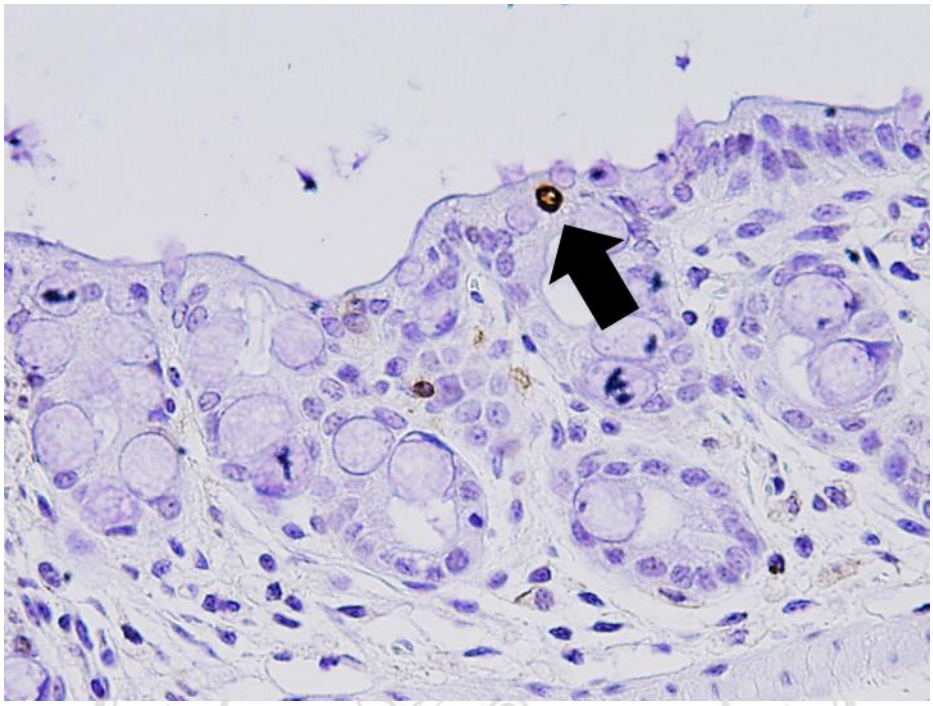


Figure 3.19 TUNEL-positive cell in colonic mucosa. Black arrow indicated apoptotic cell (magnification × 400).

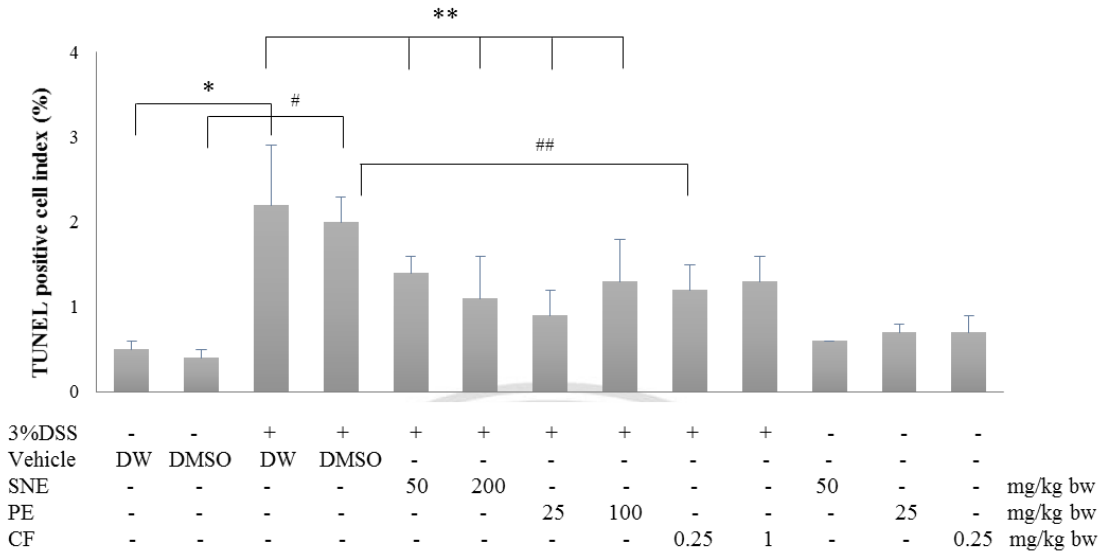


Figure 3.20 Effect of *S. neglecta* extracts on DSS-induced cell apoptosis in mice colitis model. * p<0.05 vs. a negative control group. ** p<0.05 vs. a positive control group, $^{\#}$ p < 0.05 compared with NSS+1%DMSO group, $^{\#\#}$ p < 0.05 compared with DMH+1%DMSO group.

3.8.2 The inhibitory effects of *S. neglecta* extracts on differentially expressed proteins in the mice colon mucosa of dextran sodium sulfate treatment

From the previous study, SNE and PE improved the pathological changes induced by DSS and as well inhibited cell cycle arrest and induced apoptosis. It was indicated that administrations of SNE and PE have higher potential preventive effect than CF treatment. To clarify the mechanisms of DSS induced colitis and further study the preventive mechanisms of SNE and PE on DSS induced colitis in mice, we examined alteration of protein expressions in colon mucosa. The results obtained from QSTAR Elite LC-MS/MS and Ingenuity Pathway Analysis (IPA) are showed in Table 3.13. A total of 49 differentially expressed proteins were identified in 3 sample pairs for comparison including, DSS control vs. vehicle control, SNE→DSS vs. vehicle control and PE→DSS vs. vehicle control. Table 3.13 showed the differentially expressed proteins in mice treated with DSS were involved in various functions including xenobiotic metabolism, oxidation-reduction, glutathione and lipid metabolism, mitochondrial function, calcium metabolism, transcription, protein synthesis, protein peroxisome proliferation, cytoskeleton organization, cell proliferation and apoptosis processes.

Table 3.14 summarizes the IPA upstream regulator analysis of SNE and PE treatment and combined with DSS treatment in mice colon. According to the IPA upstream regulator analysis, proteins regulated by c-Myc, n-MYC, TNF-α and transcriptional factor X-box-binding protein 1 (XBP1) associated with cell proliferation and angiogenesis were up-regulated. However, the proteins that promoted apoptosis such as a cellular tumor antigen p53 (p53) were down-regulated. These results indicated the adaptive response to the severe cellular damage, cell cycle arrest and cell death induced by DSS. Interestingly, IPA upstream regulator analysis showed that SNE and PE pretreatment prevented activation of several up-stream regulators such as TNF-α, nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1), heat shock transcription factor 2 (HSF2), XBP1 and pancreatic and duodenal homeobox 1 (PDX1). Moreover, SNE and PE pretreatment suppressed activity of several protein kinases involved in cell

proliferation such as SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4 (SMARCA4), cAMP responsive element binding protein 1 (CREB1), erb-b2 receptor tyrosine kinase 4 (ERBB4), prohibitin 2 (PHB2), insulin-like growth factor 1 receptor (IGF1R), presenilin 2 (PSEN2), Raf kinase 1 (RAF1), mitogen-activated protein kinases (MAPK) p38 MAPK, ERK1/2 and MAP2K1, peroxisome proliferator-activated receptors alpha (PPARA), gamma, coactivator 1 alpha (PPARGC1A) and beta (PPARGC1B). Additionally, DSS inhibited the expression of cytoskeleton proteins including keratins 8 (KRT8) and 18 (KRT18) and microfilament-associated proteins such as tropomyosin 1, alpha (Tpm 1) and tropomyosin 2, beta (Tpm 2) in the mice colons. Administration of SNE and PE improved down-regulation of these proteins that involved in cytoskeleton organization including KRT8, KRT18, Tpm 1 and Tpm 2. These results indicated that SNE and PE might play a role in maintenance of colonic epithelial structure in DSS induced colitis.

Table 3.15 illustrates the altered biological functions in DSS control, SNE and PE pretreated mice detected by IPA. Importantly, DSS treatment induced activation of nuclear receptor subfamily 1, group I, member 2 (PXR) which could result in quantity of reactive oxygen species (ROS) in the colon mucosa. Moreover, SNE and PE pretreatments blocked the activation of PXR and only PE treatment suppressed the up-regulation of various proteins that involved in generation of oxidative stress such as cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9) (Table 3.13). In addition, the down-regulation of cytochrome c oxidase subunit Va (COX5A) and glutathione reductase (GSR) enzymes, which associated with maintenance of normal mitochondrial function and antioxidant response were detected in DSS-treated mice (Table 3.13). However, PE and SNE ameliorated the down-regulation of COX5A and GR by reduction of ROS generation in mouse colons. These results showed that the down-regulation of GSR and COX5A might recover in mice supplemented with SNE and PE, which could be related to improvement of mitochondria function and antioxidant effects.

Table 3.13 Effect of *S. neglecta* extracts on differentially expressed proteins in the mice colon mucosa of dextran sodium sulfate treatment, identified by QSTAR Elite LC-MS/MS and Ingenuity Pathway Analysis

Protein Name (Symbol)	GI Number	DSS vs vehicle		SNE→DSS vs vehicle		PE→DSS vs vehicle		Location	Type	Function	Up-stream regulator
		FC	*p*-value	FC	*p*-value	FC	*p*-value				
Carboxylesterase 2A (Ces2a)	19527178	NC		NC		-1.43	0.022	C	E	M	NR1/2, NR1/3
Carboxylesterase 2C (Ces2c)	21704206	-1.17	0.0008	-1.27	0.0000	-1.62	0.0000	C	E	M	NR1/2, NR1/3
Aldehyde dehydrogenase 1 family, member A1 (ALDH1A1)	85861182	-1.32	0.0000	NC		-1.73	0.0000	C	E	M	N1/2, N1/3
Adenosylhomocysteinase (AHCY)	262263372	-1.09	0.047	-1.19	0.0021	-1.22	0.034	C	E	M	N1/2
Cytochrome P450, fam. 2, subfam. C, polypep. 9 (CYP2C9)	268607516	1.20	0.031	1.09	0.0001	-1.20	0.022	C	E	XM, OR	N1/2, N1/3,PPARGC1A
Cytochrome P450, fam. 2, subfam. C, polypep. 18 (CYP2C18)	13386282	-1.10	0.0005	NC		-2.12	0.0000	C	E	XM, OR	
Glutathione S-transferase, alpha 4 (Gsta4)	160298217	1.15	0.048	NC		-1.24	0.0013	O	E	GM	N1/2, PPARA
Glutathione S-transferase mu 1 (GSTM1)	6680121	-1.36	0.0009	-1.67	0.0003	-1.63	0.0000	C	E	GM	N1/2, N1/3, TP53
Glutathione S-transferase mu 5 (GSTM5)	6754084	-1.95	0.0000	-2.10	0.0000	-2.40	0.0000	C	E	GM	N1/2, SRF, TP53
Sulfotransferase family 1A, phenol-pref., member 1 (Sult1a1)	19526822	NC		-1.36	0.0007	-2.24	0.0020	C	E	XM	N1/3
3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2)	61098088	1.14	0.0013	1.22	0.0012	NC	C		E	SM	N1/3
Calnexin (CANX)	6671664	1.37	0.0000	1.42	0.0007	1.38	0.0004	C	O	CaM	c-MYC
Cytochrome c oxidase subunit Va (COX5A)	112181182	-1.54	0.0000	-1.32	0.0000	-1.34	0.0002	C	E	MitF	
Glutathione reductase (GSR)	160298213	-1.85	0.0000	-1.62	0.0000	NC	C		E	MitF	
Prohibitin 2 (PHB2)	126723336	NC		-1.18	0.022	-1.45	0.020	C	TR	TRA	c-MYC
Nucleophosmin (NPM1)	6679108	1.31	0.001	2.09	0.0000	1.95	0.0000	N	TR	TRA	c-MYC, MYCN, SRF
Nucleolin (NCL)	84875537	1.22	0.0000	1.37	0.0000	1.50	0.0000	N	O	CP	c-MYC, MYCN,ERBB4
Ribosomal protein L10a (RPL10A)	255003735	2.44	0.0000	2.84	0.000	2.94	0.0000	N	SCR	PS	c-MYC, MYCN
Ribosomal protein L13 (RPL13)	33186863	2.19	0.0022	2.58	0.0043	2.45	0.0076	N	SCR	PS	c-MYC, MYCN
Ribosomal protein L14 (RPL14)	13385472	1.95	0.0000	2.79	0.0000	2.91	0.0000	C	SCR	PS	c-MYC, MYCN
Ribosomal protein L24 (RPL24)	18250296	1.84	0.0007	2.42	0.0001	2.58	0.0001	C	SCR	PS, AP	c-MYC, MYCN
Ribosomal protein L28 (RPL28)	6677779	2.41	0.0028	3.09	0.0024	3.20	0.0012	C	SCR	PS	c-MYC, MYCN
Ribosomal protein L32 (Rpl32)	25742730	2.20	0.0018	2.20	0.0012	2.55	0.0016	C	SCR	PS	c-MYC, MYCN
Ribosomal protein L4 (RPL4)	30794450	2.13	0.0000	2.29	0.0000	2.24	0.0000	C	E	PS	c-MYC, MYCN
Ribosomal protein L6 (RPL6)	84662736	2.46	0.0000	2.67	0.0000	2.56	0.0000	N	SCR	PS	c-MYC, MYCN
Ribosomal protein L8 (RPL8)	6755358	2.00	0.003	2.11	0.0039	2.07	0.0076	O	SCR	PS, PM	c-MYC, MYCN
Ribosomal protein S24 (RPS24)	46519158	2.21	0.0005	2.35	0.0004	2.41	0.0004	C	SCR	PS	c-MYC, MYCN
Ribosomal protein S3A1 (Rps3a1)	254553321	2.07	0.0000	2.30	0.0000	2.60	0.0000	C	SCR	PS	c-MYC, MYCN
Ribosomal protein S6, pseudogene 4 (Rps6-ps4)	94367038	3.09	0.0006	3.82	0.0044	4.23	0.0027	O	SCR	PS	c-MYC, MYCN
S100 calcium binding protein A10 (S100A10)	6677833	1.45	0.013	1.78	0.0009	1.37	0.0013	C	O	CaM	c-MYC, MYCN
Eukaryotic translation elongation factor 1 gamma (EEF1G)	110625979	1.47	0.0000	1.60	0.0000	1.57	0.0000	C	TR	TRA	MYCN
Peroxiredoxin 6 (PRDX6)	6671549	-1.42	0.0000	-2.11	0.0000	-1.93	0.0000	C	E	AP	TP53
4-aminobutyrate aminotransferase (ABAT)	37202121	-1.64	0.0011	-1.86	0.0001	-2.24	0.0003	C	E	AP	TP53
Calreticulin (CALR)	6680836	1.16	0.0000	1.41	0.0003	1.51	0.0003	C	TR	TRA	TNF, XBP1

Table 3.13 Effect of *S. neglecta* extracts on differentially expressed proteins in the mice colon mucosa of dextran sodium sulfate treatment, identified by QSTAR Elite LC-MS/MS and Ingenuity Pathway Analysis (Cont.)

Protein Name (Symbol)	GI Number	DSS vs vehicle		SNE→DSS vs vehicle	PE→DSS vs vehicle		Location	Type	Function	Up-stream regulator
		FC	*p*-value	FC	FC	*p*-value	FC				
Actinin, alpha 1 (ACTN1)	61097906	-1.13	0.011	-1.28	0.0006	-1.41	0.0000	C	TR	TRA	TP53, c-MYC
Actin, alpha 2, smooth muscle, aorta (ACTA2)	6671507	-1.48	0.0001	-1.56	0.0004	-2.67	0.0000	C	CS	CO	TP53, SP1, ERBB4
Transgelin (TAGLN)	6755714	-1.98	0.0000	-2.13	0.0000	-2.59	0.0000	C	CS	CO	c-MYC, MYCN
Transgelin 2 (TAGLN2)	30519911	-1.19	0.0098	-1.19	0.0009	-1.44	0.0000	C	CS	CO	c-MYC, MYCN,TP53
Tropomyosin 1, alpha (Tpm1)	31560030	-2.84	0.0000	-2.18	0.0000	-2.23	0.0000	PM	CS	CO	c-MYC, SRF
Tropomyosin 2, beta (Tpm2)	482677666	-3.02	0.0000	-2.65	0.0000	-2.15	0.0000	C	CS	CO	c-MYC,RAF, SRF
Vimentin (VIM)	31982755	-1.22	0.0000	-1.61	0.0000	-1.23	0.0002	C	CS	CO	c-MYC, MYCN, TP53
Keratin 8, type II (KRT8)	114145561	-1.73	0.0000	-1.23	0.0000	-1.64	0.0000	C	CS	CO, AP	TP53, PPARA
Keratin 18, type I (KRT18)	254540068	-2.06	0.0001	-1.93	0.0000	-2.23	0.0000	C	CS	CO	TP53, SP1, SMARCA4
Keratin 19, type I (KRT19)	6680606	NC		-1.32	0.0000	-1.84	0.0000	C	CS	CO	OSM, IGFR1, SP1
Myosin, light chain 9, regulatory (MYL9)	198278553	-2.59	0.0003	-2.54	0.0001	-2.67	0.0017	C	CS	CO	c-MYC
Myosin, heavy chain 11, smooth muscle (MYH11)	241982718	-1.42	0.0000	-1.52	0.0000	-2.17	0.0000	C	CS	CO	SRF
Anterior gradient 2 (AGR2)	6753010	-1.52	0.0000	-1.61	0.0000	-1.91	0.0000	EM	O	PP	SMARCA4, ERBB2
Acetyl-CoA acyltransferase 2 (ACAA2)	29126205	-1.21	0.0012	-1.15	0.022	-1.53	0.0000	C	E	PP, LM	PPARA, PPARGC1A
Acyl-CoA dehydrogenase, C-4 to C-12 str. chain (ACADM)	6680618	NC		-1.14	0.026	-1.69	0.01	C	E	PP, LM	PPARA, PPARGC1A

1.C: cytoplasm; ES: extracellular space; EPR: endoplasmic reticulum; G: Golgi apparatus; Mi: mitochondria; N: nucleus; P: peroxisome; PM: plasma membrane; 2. E: enzyme; IC, ion channel; K: kinase; Pe: peptidase; Ph: phosphatase; SCR: structural constituent of ribosome; T: transporter; TR: transcriptional regulator; O: other; 3. AP: apoptotic process; CaM: calcium metabolism; CM: cellular migration; CO: cytoskeleton organization; CP: cell proliferation; GM: glutathione metabolism; LM: lipid metabolism; M: metabolism; PP: protein peroxisome proliferation; PS: protein synthesis; OR: oxidation-reduction process; SM: sulfur metabolism; TRA: transcription; XM: xenobiotic metabolism; Ratio: ratio to vehicle control group; NC: no change.

Table 3.14 Effect of *S. neglecta* extracts on upstream regulator in the mice colon mucosa of dextran sodium sulfate treatment, identified by Ingenuity Pathway Analysis

Up-stream regulators	DSS vs. NSS	SNE→DSS vs. NSS	PE→DSS vs. NSS	Function
NR1/2 (PXR)	2.00	-0.05	-0.66	XMS, OR
NR1/3 (CAR)	-0.24	-0.54	-2.04	XMS, OR
PPARA	-1.76	-1.48	-2.33	PP
PPARGC1A	-3.03	-3.25	-4.17	PP
PPARGC1B	-1.46	-2.59	-1.63	PP
MYC (c-Myc)	1.70	2.28	1.78	P
MYCN	4.87	4.54	4.82	P
ERBB4	2.40	1.71	1.18	P
TNF-α	2.00	1.49	-0.14	P, A
NR3C1	2.00	NC	NC	P
HSF2	2.24	0.76	NC	P
XBP1	2.59	-0.79	3.34	P
PDX1	2.14	1.09	0.71	P
SMARCA4	-1.79	-2.18	-2.54	CCP
CREB1	NC	-2.71	0.40	P
IGF1R	-1.72	-3.50	-1.99	P
P38 MAPK	NC	-2.22	NC	P
ERK1/2	NC	-2.17	NC	P
MAP2K1	NC	-2.20	NC	P
RAF1	NC	-2.00	-2.00	P
PRKG1	NC	-2.00	NC	P
PSEN2	NC	-2.00	NC	P
TP53	-3.02	-3.25	-2.88	A
SP1	-2.03	-2.13	-1.20	A, TRA
SRF	-2.34	-2.78	-2.68	ACO

NC: no change; A: apoptosis; ACO: actin cytoskeleton organization; CCP: cell cycle progression; D: differentiation; OR: oxidation-reduction process; P: proliferation; PP: peroxisome proliferation; TRA: transcription; XMS: xenobiotic metabolism signaling; z-score: <-2.0 → regulator significantly inhibited; >2.0 → regulator significantly activated.

Table 3.15 Altered biological functions in DSS-induced and *S. neglecta* extracts pretreated mice

Biological Functions	DSS vs NSS	SNE→DSS vs NSS	PE→DSS vs NSS
quantity of reactive oxygen species	2.39	0.00	1.29
generation of reactive oxygen species	0.91	0.23	0.00
formation of filaments	0.00	-1.75	-1.90
formation of actin filaments	0.00	-1.53	0.00
disruption of cell-cell contacts	0.00	-2.22	-1.98
disruption of plasma membrane	0.00	-1.67	-2.00
processing of RNA	2.00	0.69	-1.17
cell death	2.75	2.24	1.17
apoptosis	2.49	1.39	0.30
aggregation of cells	0.50	-1.51	-1.34
tumorigenesis of tissue	1.63	1.09	1.28
neoplasia of epithelial tissue	1.85	0.00	0.00
cell spreading of endothelial cells	0.00	-2.00	0.00
inflammation of organ	0.70	-0.46	-0.70
metabolism of nucleotide	-0.79	-1.27	-1.21
glycolysis	1.43	0.71	0.24
transport of molecule	2.65	2.93	2.37

Data are z-score. NC: no change.

3.9 Effect of *S. neglecta* extracts on 1, 2-dimethylhydrazine/dextran sodium sulfate induced inflammation associated colorectal carcinogenesis in rats

The general observation of rats is shown in Table 3.16. During the experimental period, there were no significant differences in the initial and final body weights, and intakes of food and water in all groups. Furthermore, no sign of toxicity was observed in all groups.

Table 3.17 summarizes the number of ACF and crypt multiplicity in experimental groups. The colonic ACF were not found in rats treated by *S. neglecta* extracts. However, some rats in a negative control group and PE treated group found some foci containing one aberrant crypt. ACF were found in all DMH-treated rats. The number of ACF in DMH-treated rats was significantly increased as compared to a negative control group. However, the number of ACF in rats treated with DMH alone was higher than DMH/DSS-treated rats. No significant reduction in total ACF was observed in SNE and PE-treated groups. In addition, the mean numbers of aberrant crypts per focus (AC/focus) were not significantly different in any DMH/DSS-treated groups. In DMH/DSS-treated rats, the ACF containing 4 or more crypts per focus was observed. The amount of large ACF (>=4 crypts) was no significantly reduced in SNE and PE-treated rats. Furthermore, there was no significant difference in crypt multiplicity between DMH/DSS-treated groups. These results showed that SNE and PE from *S. neglecta* in this condition were not anticarcinogenic in 1, 2-dimethylhydrazine-initiated and dextran sodium sulfate-promoted colonic preneoplastic lesion in rats.

Table 3.16 The general observation in rats treated by either 1, 2-dimethylhydrazine or dextran sodium sulfate

Group	Body weight (g)		Intake	
	Initial	Final	Diet (g)	Water (ml)
DMH+ 1%DSS	106±5	511±42	22±4	31±5
DMH+ 1%DSS+ 50 mg/kg bw SNE	105±5	484±33	21±3	29±4
DMH+ 1%DSS+ 200 mg/kg bw SNE	105±5	493±20	21±3	27±3
DMH+ 1%DSS+ 30 mg/kg bw PE	105±4	477±17	21±2	28±6
DMH+ 1%DSS+ 120 mg/kg bw PE	105±6	497±33	21±2	24±4
1%DSS	108±8	494±40	21±3	28±4
1%DSS+ 200 mg/kg bw SNE	109±9	528±33	23±4	30±5
1%DSS+ 120 mg/kg bw PE	109±4	496±34	22±4	28±5
DMH	105±4	484±44	21±3	30±6
NSS	107±9	484±34	21±3	30±4
120 mg/kg bw of PE	108±9	517±49	22±4	30±4

Data represent the mean ± S.D. of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), DSS; dextran sodium sulfate, SNE; *S. neglecta* extract, PE; polysaccharide extract.

Table 3.17 The formation of aberrant crypt foci in 1, 2-dimethylhydrazine and dextran sodium sulfate-induced colorectal carcinogenesis

Treatment	No. of total ACF/rat		AC/focus	
	Total	ACF>=4 AC/focus	Total	Aberrant crypt>=4/focus
DMH+ 1%DSS	63.4±50.8**	35.6±31.7**	4.8±0.6**	6.9±0.7**
DMH+ 1%DSS+ 50mg/kg bw SNE	83.8±44.8	45.6±24.7	5.1±0.6	7.4±0.4
DMH+ 1%DSS+ 200mg/kg bw SNE	104.6±43.0	51.8±25.3	4.6±0.5	7.2±0.6
DMH+ 1%DSS+ 30mg/kg bw PE	76.2±43.3	40.8±28.0	4.7±0.9	6.9±0.8
DMH+ 1%DSS+ 120mg/kg bw PE	85.6±34.5	50.6±26.3	4.9±0.3	6.8±0.7
1%DSS	0±0	0±0	0±0	0±0
DMH	243.6±101.7*	128.0±56.9*	4.6±0.9*	6.7±0.8*
NSS	0.3±0.5	0±0	0.5±1.0	0±0
120 mg/kg bw of PE	0.3±0.5	0.3±0.5	1.5±3.0	1.5±3.0

Data represent the mean ± S.D. of each group, DMH; 1, 2-dimethylhydrazine (40 mg/kg bw x 2 times, s.c.), NSS; 0.9% normal sodium saline (1 ml/kg bw x 2 times, s.c.), DSS; dextran sodium sulfate, ACF; aberrant crypt foci, SNE; *S. neglecta* extract, PE; polysaccharide extract. * $p < 0.05$ compared with NSS group, ** $p < 0.05$ compared with DMH group

3.9.1 Effect of *S. neglecta* extract and polysaccharide extract on dextran sodium sulfate induced inflammation in rats.

Various proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α, are involved in the inflammatory bowel disease (IBD) development. Figure 3.21 summarizes the effect of *S. neglecta* extract and polysaccharide extract on inflammatory related gene expression in dextran sodium sulfate (DSS)-treated rats. At week 15 of an experiment, the relative mRNA expressions of iNOS and IL-1β were significantly increased in the DSS-treated rats (Figure 3.21). The administration of PE (120 mg/kg bw) significantly decreased the mRNA expressions of IL-1β, while it did not ameliorate iNOS mRNA expression. In contrast, the mRNA level of other inflammatory-related genes such as TNF-α, IL-6 and COX-2 as well as transcription factors that mediate the regulatory effects on gene expression in DSS-treated rats, NF-κB, significantly decreased in DSS-treated rats as compared to a control group. Moreover, the administration of PE (120 mg/kg bw) significantly increased mRNA expressions of IL-6, COX-2 and NF-κB as compared to DSS-treated rats. Remarkably, PE treatment significantly decreased mRNA expression of inflammation-related genes, IL-1β, iNOS, TNF-α, IL-6, COX-2 and NF-κB as compared to a control group.

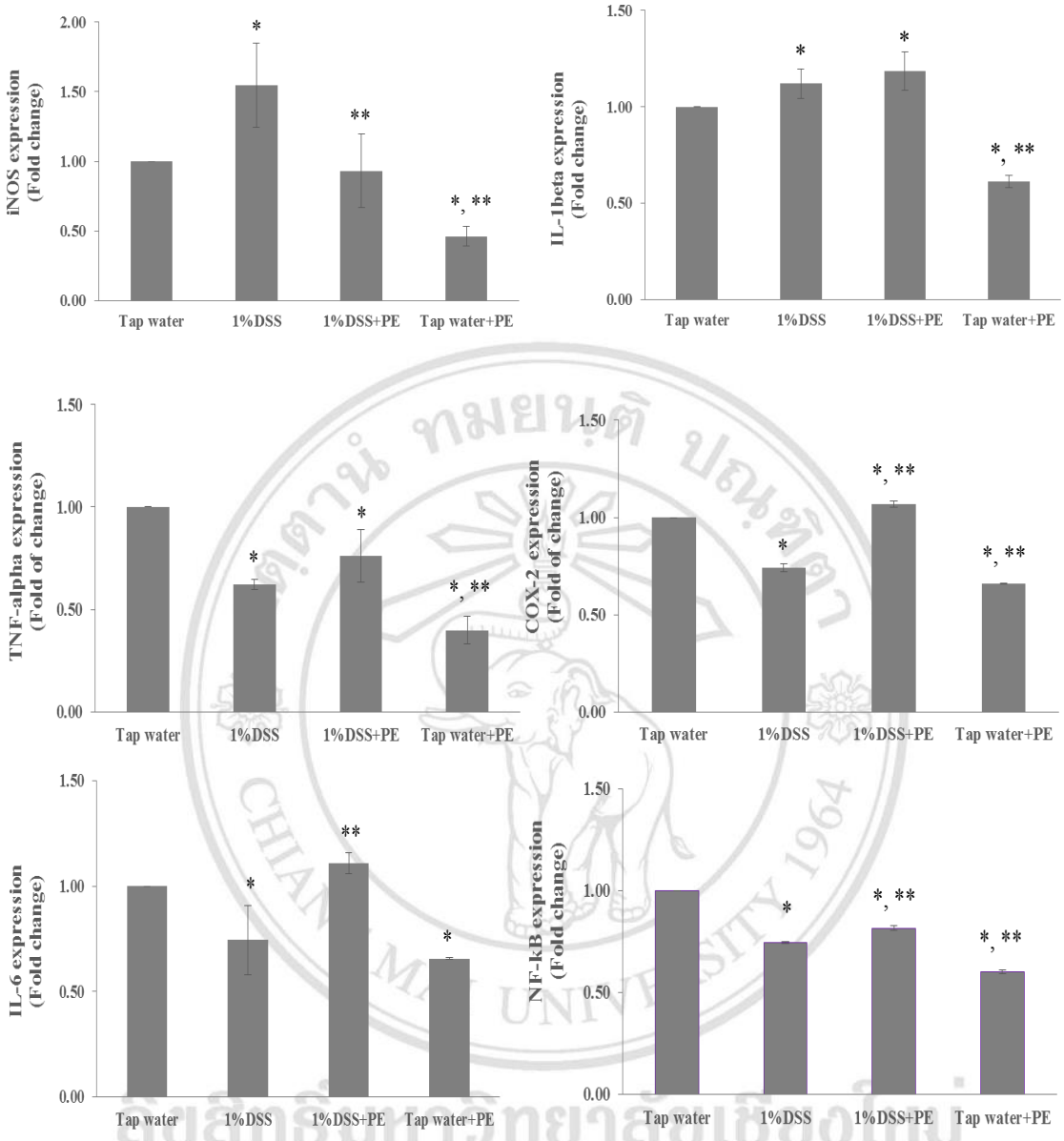


Figure 3.21 Effect of polysaccharide extract on inflammatory related gene expression in dextran sodium sulfate-treated rats. *$p < 0.05$ compared with a negative control group, ** $p < 0.05$ compared with a positive control group.