APPENDICES

APPENDIX A

List of the chemicals and materials used in this study

Chemicals/materials	Source
β-NADPH	Oriental Yeast, Japan
1,2-Dimethylhydrazine	TCI, USA
1-Chloro-2,4-dinitrobenzene	Fluka A.G., Switzerland
2-Amino-3,4-dimethylimidazo[4,5-f]quinolone	Wako, Japan
2-Aminoanthracene	Wako, Japan
2-(2-Furyl)-3-(5-nitro-2-furyl)-acrylamide	Wako, Japan
5, 5'-Dithio-bis (2-Nitrobenzoic acid)	Sigma-Aldrich, USA
Absolute ethanol	ACI Labscan, Thailand
Acetic acid	ACI Labscan, Thailand
Acrylamide	Biorad, USA
Aluminum chloride hexahydrate	Labo Chemie, India
Ammonium persulphate	Carlo-Erba, Italy
Bacto agar	Difco, USA
Benzo(a)pyrene	Sigma-Aldrich Co., USA
Bis-acrylamide	Biorad, USA
Bovine serum albumin	Sigma-Aldrich, USA
Catechin	Sigma-Aldrich, USA

Chemicals/materials	Source
Chloroform	ACI Labscan, Thailand
Copper sulphate	Merck A.G., Germany
Dextran sodium sulfate salt	MP Biomedicals, USA
Dichloromethane	ACI Labscan, Thailand
Diethyl ether	ACI Labscan, Thailand
Dimethyl sulfoxide	ACI Labscan, Thailand
Disodium hydrogen orthophosphate	BDH, England
Dithiothreitol	Vivantis, Malaysia
Ethyl acetate	ACI Labscan, Thailand
Ethylene diamine tetraacetic acid	Sigma-Aldrich, USA
Folin&Ciocalteu's reagent	VWR BDH Prolabo, France
Gallic acid	Sigma-Aldrich, USA
Glucose-6-phosphate	Sigma-Aldrich, USA
Glutathione (Reduced form)	Wako, Japan
Glycerol	Sigma-Aldrich, USA
Glycine	Vivantis, Malaysia
Hydrochloric	BDH, England
Isopropanol	Applichem, Germany
Magnesium chloride	APS Finechem, Australia
Mercaptoethanol	Applichem, Germany
Methanol	BDH, England
n-Butanol	ACI Labscan, Thailand

Chemicals/materials	Source
Nutrient Broth No. 2	Oxoid, England
p-nitrophenol	Thermo Fisher Scientific, USA
Potassium chloride	Carlo-Erba, Italy
Potassium dihydrogen phosphate	May and Baker, England
Potassium sodium tartrate	Mallinckrodt Chemical Work, USA
RNAzol	Molecular Research Center, USA
Skim milk powder	Merck A.G., Germany
Sodium carbonate	BDH, England
Sodium chloride	BDH, England
Sodium dihydrogen phosphate	BDH, England
Sodium dodecyl sulfate	Biorad, USA
Sodium hydrogen carbonate	BDH, England
Sodium hydroxide	BDH, England
TEMED	USB, USA
Trichloroacetic acid	BDH, USA
Tris base	Vivantis, Malaysia
Tween-20	USB, USA
UDP-glucuronic acid	Sigma-Aldrich, USA

APPENDIX B

List of the instruments used in this study

Instrument	Model	Source
Analytical balance	300A	Precisa, USA
Blotting apparatus	AE-6687	Atto corp., Japan
Centrifugator	PMC-060	Tomy Seiko, Japan
	22R D-78532	Mikro, Germany
Electrophoretic apparatus	AE-6500	Atto corp., Japan
Evaporator	MX07R-20-HD2E	Heidolph, Germany
Film cassette	RPN 11649	Amersham, England
Fraction collector	FRAC-100	Amersham Biosciences, Sweden
Freezer (-80 ^{o}C)	0838	Forma Scientific, USA
Homogenizer	HS-30E	Daihan, Korea
Pellet Mixer	VWR Pallet Mixer	VWR international, USA
Hot plate&Stirrer	HPMS	Whatman, USA
HPLC	1100	Agilent Technologies, USA
Incubator	B5060	Heraeus, Germany
Light microscope	-	Olympus, Japan
Freeze Dryer	LL3000	Thermo Scientific, USA

Instrument	Model	Source
Thermocycler	Mastercycler Nexus Thermal Gradient Cycler	Eppendorf, Germany
Microplate reader	M965+	Metertech, Taiwan
pH meter	PH500B	Clean, USA
Ultracentifugator	L-100 XP	Beckman Coulter, USA
UV-Spectrophotometer	UV-1700	Shimazu, Japan
Vortex	G-560E	Scientific industries, USA
Water bath	YCW-04M	Gemmy, Taiwan
Water bath shaker	N1-13S	Bioer, China
QuantStudio Real-Time PCR	QuantStudio 6 flex	Applied Biosystems, USA

APPENDIX C

Reagents and buffers preparation

1.1 Preparation of reagents for determination of chemical constituents

1.1.1 Preparation of reagents for determination of total phenolic compounds

1) Folin Ciocalteu working reagent

Dilute with distilled water in the ratio 1:1 (v/v).

2) 7% Na_2CO_3

Dissolve 7 g of Na_2CO_3 in distilled water and make up the volume to 100 ml.

1.1.2 Preparation of reagents for determination of flavonoid content

1) 5% $NaNO_2$

Dissolve 5 g of $NaNO_2$ in distilled water and make up the volume to 100 ml.

2) 10% $AlCl_3 \cdot 6H_2O$

Dissolve 10 g of $AlCl_3 \cdot 6H_2O$ in distilled water and make up the volume to 100 ml.

3) 1 M NaOH

Dissolve 8 g of NaOH in distilled water and make up the volume to 100 ml.

1.1.3 Preparation of reagents for determination of total carbohydrate

1) 5% Phenol

Dissolve 5 g of phenol in distilled water and make up the volume to 100 ml.

1.1.4 Preparation of reagents for determination of total sulfate

1) Dimethylmethylene blue solution

Solution A: Dissolve 16 mg of dimethylmethylene blue in 5 ml of ethanol

Solution B: Dissolve 2 g of sodium formate in 2 ml of formic acid

Mix solution A and B, adjust pH to 3.5 and make up the volume to 1000 ml.

1.1.5 Preparation of reagents for determination of uronic acid content

1) Concentrated sulfuric acid-borate reagent

Dissolve 0.9535 g of $Na_2B_4O_7 \cdot 10H_2O$ in concentrated H_2SO_4 and make up the volume to 100 ml.

2) Carbazole solution

Dissolve 50 mg carbazole in ethanol and make up the volume to 40 ml.

1.2 Preparation of buffers for *Salmonella* mutation assay

1.1.1 0.2 M Sodium phosphate buffer, pH 7.4

1) Solution A (0.2 M $Na_2HPO_4 \cdot 2H_2O$)

Dissolve 17.799 g of $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up the volume to 500 ml.

2) Solution B (0.2 M $NaH_2PO_4 \cdot H_2O$)

Dissolve 2.7598 g of $NaH_2PO_4 \cdot H_2O$ in distilled water and make up the volume to 100 ml.

Use Solution B to adjust the pH of Solution A to 7.4.

1.2.2 0.85 M $MgCl_2$·1.65 M KCl

Dissolve 17.28 g of $MgCl_2 \cdot 6H_2O$ and 12.30 g of KCl in distilled water and make up the volume to 100 ml.

1.2.3 0.5mM biotin/histidine

Dissolve 105 mg of histidine and 124 mg of biotin in distilled water and make up the volume to 100 ml. After completely dissolve, filtrate with 0.45 µm of filter.

1.2.4 10X Vogel-Bonner medium E

K_2HPO_4 (anhydrous)	100	g
Citric acid monohydrate	20	g
$NH_4H_2PO_4$	19.2	g
NaOH	6.6	g
$MgSO_4 \cdot 7H_2O$	2	g

Dissolve in distilled water and make up the volume to 1000 ml.

1.3 Preparation of buffers for cytosolic and microsomal fractionation

1.3.1 Homogenizing buffer

KCl	11.5	g
EDTA·2Na	0.372	g
0.25 M PMSF	1	ml

Dissolve in deionized water and make up the volume to 1000 ml.

1.3.2 Microsome suspension buffer

KH_2PO_4	0.14	g
EDTA·2Na	0.004	g
DTT	0.002	g
Glycerol	3	ml

Dissolve in deionized water, adjuste pH to 7.4 with KOH and make up the volume to 10 ml.

1.4 Preparation of buffers and reagents for activities of xenobiotic metabolizing enzymes assay

1.4.1 Glutathione-*S*- transferase activity

1) 10 mM Glutathione reduced form

Dissolve 30.7 mg of glutathione reduced form in deionized water and make up the volume to 10 ml.

2) 10 mM 1-Chloro-2, 4-dinitrobenzene

Dissolve 102 mg of 1-chloro-2, 4-dinitrobenzene in 20 ml absolute ethanol and make up the volume to 50 ml with deionized water.

3) 0.2 M Potassium phosphate buffer, pH 6.5

Dissolve 2.722 g of KH_2PO_4 in deionized water, adjust the pH to 6.8 and make up the volume to 100 ml.

1.4.2 UDP-glucuronyl transferase activity

1) 200 mM Tris-HCl, pH 8.7

Dissolve 2.4228 g of tris in distilled water, adjust the pH to 8.7 and make up the volume to 100 ml.

2) 5 mM *p*-nitrophenol

Dissolve 70 mg of *p*-nitrophenol in distilled water and make up the volume to 100 ml.

3) 40mM $MgCl_2$

Dissolve 81.32 mg of $MgCl_2$ in distilled water and make up the volume to 100 ml.

4) 20 mM UDP-glucuronic acid

Dissolve 64.63 mg of UDP-glucuronic acid in distilled water and make up the volume to 10 ml.

5) 1 M NaOH

Dissolve 4 g of NaOH in distilled water and make up the volume to 100 ml.

6) 10% Trichloroacetic acid

Add 10 ml of 100% trichloroacetic acid and make to 100 ml with distilled water.

1.5 Preparation of buffers and reagents for activities of antioxidant enzymes assay

1.5.1 Glutathione peroxidase activity

1) 1 M Tris-HCl, 5 mM EDTA, pH 8.0

Dissolve 12.1 g of tris base and 0.19 g of $EDTA{\cdot}2H_2O$ in deionized water, adjust pH to 8.0 with concentrated HCl and make up the volume to 1000 ml.

2) 0.1 M Glutathione reduced form

Dissolve 30 mg of glutathione reduced form in 1ml of 1 M Tris-HCl.

3) 2 mM β-NADPH

Dissolve 1.8 mg of β-NADPH in 1ml of 1 M Tris-HCl.

4) 7 mM tert-butyl hydroperoxide

Add 20 µl of tert-butyl hydroperoxide and mix with 20 ml of distilled water.

1.5.2 Glutathione reductase activity

1) 0.1 M Phosphate buffer containing 1 mM $MgCl_2$, pH 7.0

Dissolve 13.61 g of KH_2PO_4 and 203.30 mg of $MgCl_2 \cdot 6H_2O$ in distilled water and make up the volume to 1000 ml.

2) 2 mM Glutathione oxidized form

Dissolve 82.07 mg of Glutathione oxidized form in 5 ml of 0.1 M phosphate buffer.

3) 0.1 mM β-NADPH

Dissolve 8.33 mg of β-NADPH in 10 ml of 0.1 M phosphate buffer.

1.5.3 Catalase activity

1) 50 mM Phosphate buffer, pH 7.0

Solution A: Dissolve 6.81 g of KH_2PO_4 in distilled water and make up the volume to 1000 ml.

Solution B: Dissolve 8.9 g of $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up the volume to 1000 ml.

Mix solution A and solution B in the ratio of 1:1.5 (v/v).

2) 30 mM H_2O_2

Add 340 µl of 30% H_2O_2 in 50 mM phosphate buffer and make up the volume to 100 ml.

1.6 Preparation of reagents and buffers for SDS-PAGE

1.6.1 30% Acrylamide-0.8%Bis-acrylamide

Dissolve 300 g of acrylamide and 8 g of bis-acrylamide in deionized water and make up the volume to 100 ml.

1.6.2 Separating gel buffer, pH 8.8

Dissolve 19.71 g of tris base and 4 g of sodium dodecyl sulfate in deionized water, adjust pH to 8.8 with concentrated HCl and make up the volume to 100 ml.

1.6.3 Stacking gel buffer, pH 6.8

Dissolve 60.57 g of tris base and 8 g of sodium dodecyl sulfate in deionized water, adjust pH to 6.8 with 1 N NaOH and make up the volume to 100 ml.

1.6.4 10% Ammonium persulfate

Dissolve 10 g of ammonium persulfate in deionized water and make up the volume to 100 ml.

1.6.5 Sample buffer, pH 6.8

Tris base	0.15	g
Sodium dodecyl sulfate	0.4	g
Glycerol	2	ml
2-Mercaptoethanol	1	ml
0.002% Bromophenol blue	5	µl

Dissolve in deionized water, adjusted pH to 6.8 with concentrated HCl and make up the volume to 1000 ml.

1.6.6 Electrode buffer

Tris base	3.04	g
Glycine	4.41	g
Sodium dodecyl sulfate	1	g

Dissolve in deionized water and make up the volume to 1000 ml.

1.6.7 Bloting buffer

Tris base	12.11	g
Glycine	14.4	g

Dissolve in deionized water, add 200 ml of methanol and make up the volume to 1000 ml.

1.7 Preparation of buffers for immunohistochemistry

1.7.1 0.5 M Tris base, 0.5% Tween 20, pH 7.6

Tris base	6.1	g
NaCl	8.1	g
Tween 20	5	ml

Dissolve in deionized water, adjust pH to 7.6 with concentrated HCl and make up the volume to 1000 ml.

1.7.2 Phosphate buffered saline, pH 7.4

Na_2HPO_4	5.5	g
NaH_2PO_4	1.4	g
NaCl	11.7	g

Dissolve in deionized water, adjust pH to 7.4 and make up the volume to 1000 ml.

1.7.3 0.1 M Sodium acetate, pH 4.0

Dissolve 1.36 g of sodium acetate in deionized water, adjust pH to 4.0 with acetic acid and make up the volume to 100 ml.

1.7.4 10 mM Citrate buffer, pH 6.0

1) Solution A

Dissolve 4.2 g of citric acid in distilled water and make up the volume to 500 ml.

2) Solution B

Dissolve 14.7 g of sodium citrate in distilled water and make up the volume to 500 ml.

Prepare working reagent, mix solution A and solution B in the ratio of 18:82 ml, adjust pH to 6.0 and make up the volume to 1000 ml.

1.7.5 Tris buffered saline, pH 7.6

Dissolve 0.057 g of tris base and 8.77 g of NaCl in distilled water, adjust pH to 7.6 with concentrated HCl and make up the volume to 1000 ml.

1.7.6 0.5% (w/v) Methyl green

Dissolve 0.5 g of methyl green in 0.1 M sodium acetate and make up the volume to 100 ml.

APPENDIX D

Supplement data

1.1 Determination of monosaccharide composition of polysaccharide extract using HPLC

The polysaccharide extract was dissolved in 2 M trifluoroacetic acid (TFA) and hydrolyzed at 120 ^{o}C for 90 min. The monosaccharide composition of polysaccharide extract was determined using high performance liquid chromatography (HPLC). The HPLC system consisted of a pump (Waters 510, Waters, Milford, MA, USA), an injection valve (Model 7010, Rheodyne, Rohnert Park, CA, USA) with a 20 ul sample loop an RI detector (Waters 2414). A carbohydrate analysis column, 4.6 x 250 mm, (Waters, Milford, MA, USA) was used as a stationary phase. 80% acetonitrile in water was used as a mobile phase at a flow rate of 2 ml/min. D-glucose, D-galactose, L-fucose, L-arabinose, L-rhamnose, D-xylose and D-mannose was used as standard monosaccharides.

Table S1 shows monosaccharide composition of polysaccharide extract (PE). The chromatogram of monosaccharides in polysaccharide extract is illustrated in Figure S1. 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% in PE, respectively. The trace amount of moosaccharides in PE were D-mannose (2.7%) and D-xylose (1.6%). These results suggested that the polysaccharide derived from *S. neglecta* might be a heteropolysaccharide.

1.2 Infrared spectroscopy of polysaccharide extract

The IR spectrum of the polysaccharide extract was determined by Fourier transform infrared spectrometer (FTIR) (Bruker Vertex 70 FTIR spectrometer). The polysaccharide extract was milled with potassium bromide (KBr) powder and then pressed into pellets for FTIR measurement in the wavenumber range of 600 and 4000 cm^{-1}. Characterization

of polysaccharide rich extract by FT-IR showed typical absorption bands of sulfated polysaccharides especially a band at 1248 cm^{-1} attributed to S=O stretching vibration and it could suggest that the presence of sulfate esters in crude polysaccharide. Moreover, the band at 1642 and 1412 cm^{-1} is allocated to asymmetric and symmetric stretching vibration of -COO- of uronic acid (Ananthi et al., 2010). The absorption bands at 1078 and 1045 cm^{-1} in the range of 1200-1000 cm^{-1} in FT-IR spectrum suggested that the monosaccharides in polysacchararide rich extract has a pyranose ring (Ding et al., 2010a). Moreover, the absorption band at 914 cm^{-1} are typical for D-Glucose in the pyranose form (Liu et al., 2013). The band at 874 cm^{-1} indicated the presence of β-glycosidic linkages (Ding et al., 2010a). Based on the FT-IR spectrum it could be expected that polysaccharide rich extract might be a sulfated polysaccharide which displayed pyran type sugar rings, and the polysaccharide might be connected to β-glycosidic bond.

Table S1 The composition of monosaccharide in polysaccharide extract.

Sample	Monosaccharide content (%)						
	L-Fucose	**D-Glucose**	**D-Galactose**	**L-Rhamnose**	**L-Arabinose**	**D-Mannose**	**D-Xylose**
Polysaccharide extract	22.6±1.5	23.4±1.5	21.4±0.5	17.1±1.4	11.3±0.8	2.7±0.1	1.6±0.0

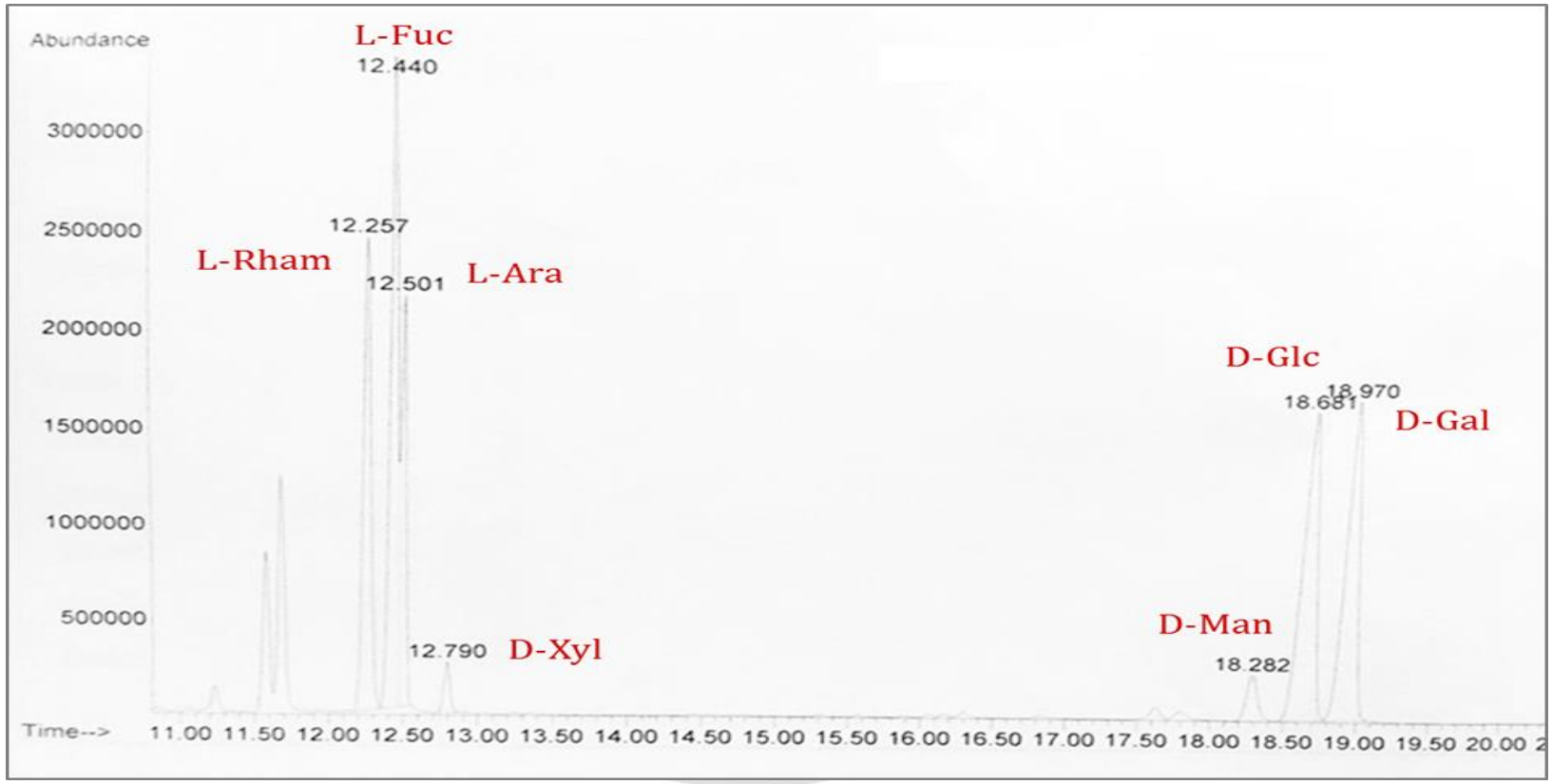

Figure S1 Chromatogram of monosaccharide composition in polysaccharide extract.

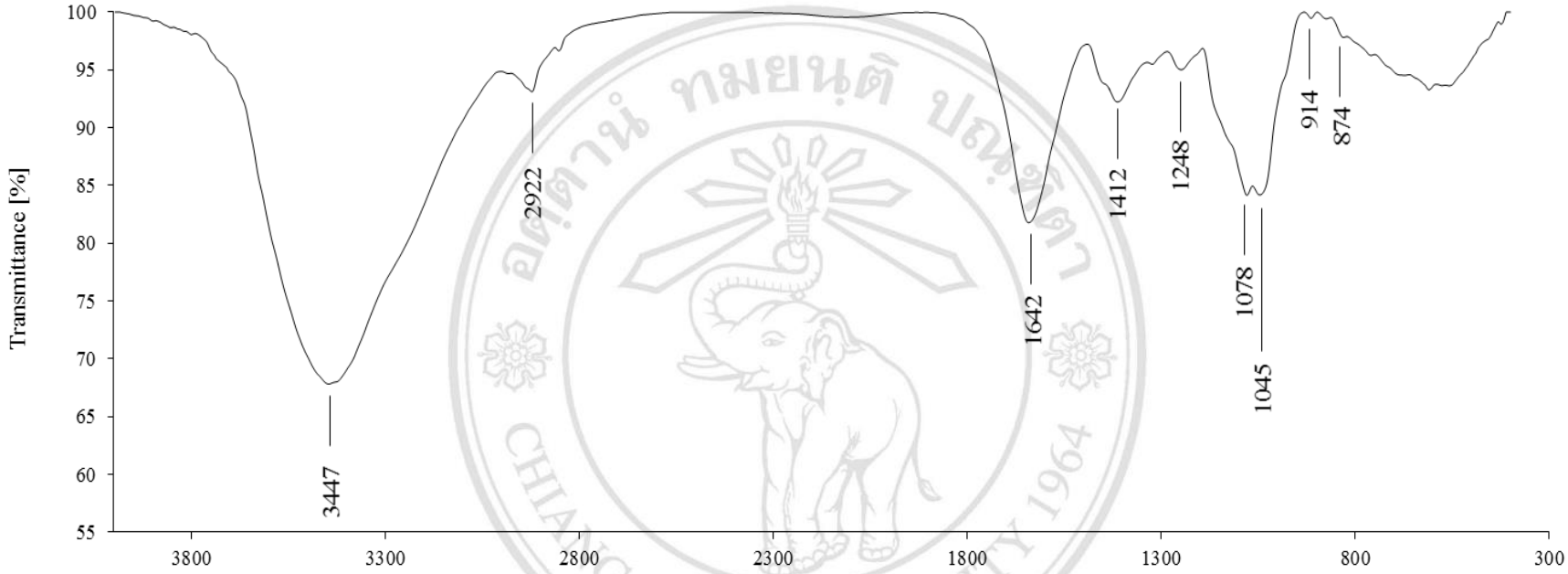

Figure S2 FT-IR spectrum of polysaccharide extract from *S. neglecta*.

Table S2 FT-IR analysis of polysaccharide extract from *S. neglecta*.

Wavelength (cm^{-1})	**Characterization of functional groups**
3447	O-H stretching vibration
2922	C-H stretching vibration
1642	Asymmetric stretching of –COO- of uronic acid
1412	Symmetric stretching of –COO- of uronic acid
1248	S=O stretching vibration of sulfate group
1078	Monosaccharide in pyranose ring
1045	Monosaccharide in pyranose ring
914	A typical for D-glucose in pyranose form
874	B-glucopyranose linkage

CURRICULUM VITAE

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Publications	1. Taya S., Thumvijit T., Chewonarin T., Punvittayagul C., Wongpoomchai R., Effect of *Spirogyra neglecta* on the early stages of 1, 2-dimethylhydrazine-induced colon carcinogenesis in rats, European Journal of Cancer Prevention, (in press).	
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1. Taya S, Chewonarin T, Thumvijit T, Wongpoomchai, R. Chemoprevention of 1, 2-dimethylhydrazine-induced aberrant crypt foci by crude polysaccharide isolated from *Spirogyra neglecta* extract. Oral presentation at The 14th Annual Biochemical Research Meeting. July 28-29, 2016, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, page 40.

2. Taya S, Chewonarin T, Thumvijit T, Wongpoomchai, R. Modifying effect of *Spirogyra neglecta* on 1, 2-dimethylhydrazine-induced aberrant crypt foci in rats. Oral presentation at The 7th Biennial Meeting of Society for Free Radical Research-Asia "Advanced Oxidative Stress Research for Health Benefits and Well beings". November 29- December 2, 2015, Chiang Mai, Thailand, page 37.

3. Taya S., Kakehashi A., Wongpoomchai R., Wei M., Ishii N. Wanibuchi H. Protective effect of *Spirogyra neglecta* in colitis- induced by dextran sodium sulfate in mice. Oral presentation at The 13th Annual Biochemical Research Meeting. July 28-29, 2015, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, page 18.

4. Taya S., Pothacharoen P., Chewonarin T., Thumvijit T., Wongpoomchai R. Effect of *Spirogyra neglecta* on inflammation-associated colorectal carcinogenesis in rats. Oral presentation at The 12th Annual Biochemical Research Meeting. July 8-9, 2014, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, page 16.

5. Taya S., Pothacharoen P., Thumvijit T., Wongpoomchai R. *Spirogyra neglecta* extract inhibited inflammation induced by dextran sulfate sodium via downregulation of TNF-α and iNOS expressions. Poster presentation at the The 4th International Biochemistry and Molecular Biology Conference 2014 "Bridging ASEAN Biochemical Research Communities". April 2-3, 2014, Bangkok, Thailand. Page 230.

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10. Taya S., Chewonarin T., Thumvijit T., Amornlerdpison D., Wongpoomchai R. Effect of *Spirogyra neglecta* on 1, 2-dimethylhydrazine-induced early stages of colon carcinogenesis in rats. Poster presentation at 1st Malaysian Congress of Toxicology. October 2-3, 2012, Kuala Lumpur, page 37.