CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Culture media

(1) De Man, Rogasa and Sharp agar (MRS agar)

(2) Basal medium

(3) Salmonella – Shigella agar (SS agar, HIMEDIA Company)

(4) Eosin methylene blue agar (EMB agar, HIMEDIA Company)

(5) Nutrient broth and Nutrient agar (NB and NA)

Note: the composition and preparation of these media were shown in Appendix A

3.1.2 Chemical reagents

Names of chemical regents	Company
Peptone	BIOMARK™
Beef extract	HIMEDIA
Yeast extract	BIOMARK™
Glucose	FLUKA
Lactose	MERCK
Sucrose	UNILAB
Tween-80	LABCHEM

Names of chemical regents	**Company**
K_2HPO_4	UNIVAR
KH_2PO_4	Fisher Scientific
$(NH_4)_2SO_4$	UNIVAR
$CH_3COONa.3H_2O$	UNIVAR
Triammonium citrate	PANREAS
$MgSO_4.12H_2O$	UNIVAR
$MnSO_4$	Scharlau
NaCl	UNIVAR
DNS reagent	(See Appendix B)
Ethanol	MERCK
Methanol	MERCK
Eosin	CARCO ERBA
Methylene blue	BAKER ANALYZED
Bromocresol purple	FLUKA
SS-agar powder	HIMEDIA
EMB-agar powder	HIMEDIA
MRS powder	HIMEDIA
Pectinase enzyme	FLUKA
Carbazal	FLUKA
Gluculonic acid	FLUKA
Fructose	UNIVR
Galactose	FLUKA
Manose	NAKARAI CHEMICALS.LTD
di-sodium tetraborate	BDH
1-Butanol	J.T.Baker
2-Propanol	MERCK
Phenol	MERCK
Sulfuric acid	MERCK
Agar	-

3.1.3 Equipment

Names of equipment	Company
Analytical balance (4 digits)	OERTING
Analytical balance (2 digits)	OERTING
Autoclave Model ACV-3167	IWAKE
Hot air oven	MEMMERT
Evaporator	EYELA
Magnetic stirrer	VELP SCIENTIFICA
Magnetic bar	O.V
Laminar air flow	LABCONCO
Hood	TOP LAB
Autopipette	GIBSON
Anaerobic jar	MERCK
Micro centrifuge Model 5415 C	BERUN
Refrigerated centrifuge (Model Super T21)	SORVALL
Centrifuge (Model Harmonic series)	GEMMY INDUSTRY
pH meter (Model C830)	CONSORT
Spectronic 21	GENESYS
TLC plate (silica gel aluminum sheet)	MERCK
TLC chamber	CAMAG
Vortex Mixer-2 Genie	BOHEMIA
Water bath Model 1255 PC	SHEL-LAB
Blender (Model IF-308)	IMARFLE
Test tube	PYREX
Duran	DURAN
Petri dish	PYREX
Spreader	(Hand made)

3.1.4 Microorganisms

Lactic acid bacteria (LAB) used in this experiment were including *Enterococcus faecium*, lactic bacteria isolated from pig (Wongputtisin, 2003), LABG12, lactic bacteria isolated from chicken (Niamsup, 2003) and LAB33, a newly isolated from infant feces, respectively. Normal flora and pathogenic bacteria used were *Escherichia coli* and *Salmonella havana*, respectively. Those were kindly provided from Microbiology Section, Department of Biology, Faculty of Science, Chiang Mai University. Moreover, fecal sample from healthy volunteer was used as a source of mixed cultures in fecal slurry experiment.

3.1.5 Plant material

Moo-noi leave was used as source of carbohydrate in this study from local area Chiang Mai.

3.2 Methods

3.2.1 Preparation of plant materials

The fresh Moo-noi leaves were washed with tap water and divided into two groups. The first group was kept at 4^{o}C as a fresh sample. Another group was dried at 55^{o}C for 12 hours, and stored at room temperature for using as dried Moo-noi leaves.

3.2.2 Polysaccharides extraction

The fresh and dried Moo-noi leaves were extracted under condition at room temperature (~30^{o}C) with a leaves: distilled water ratio of 1 g: 100 ml and homogenized using blender. Non-homogenized residue remained in the plant extract was removed by filtration through a thin cotton cloth and homogenized once again with 50 ml distilled water. The first and second gels obtained were

centrifugation with 6,000 rpm for 15 min at room temperature. The supernatant was concentrated to about half of its volume in a evaporator before being precipitated with 70%(v/v) ethanol. The precipitate gel was separated by The ethanol remained in the precipitate in oven at 55°C, over night and ground to obtained dry crude polysaccharides (Figure 16).

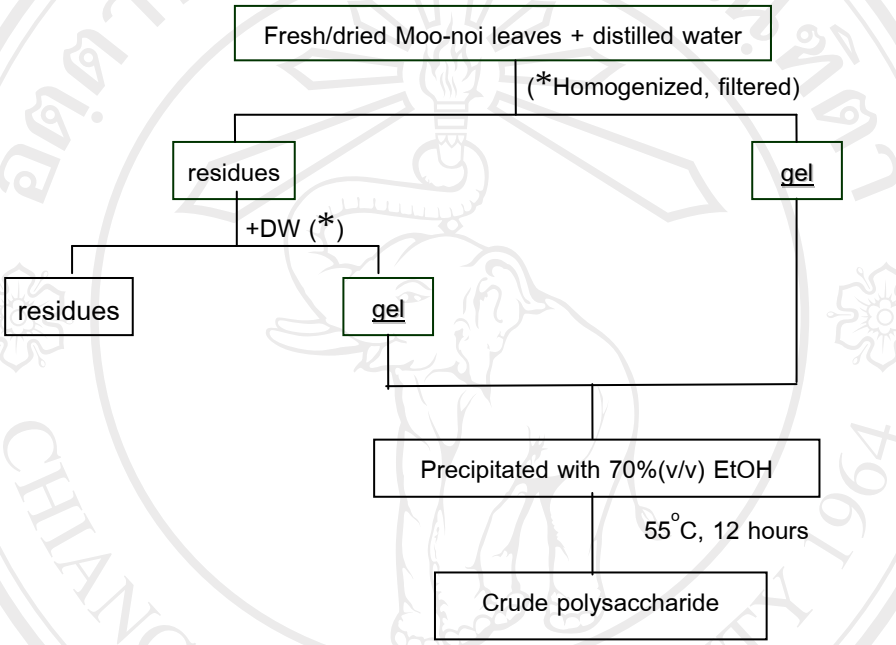

Figure 16 Flow chart demonstrated the extraction of polysaccharides from Moo-noi leaves

3.2.3 Size determination of polysaccharides

Size determination of polysaccharides was estimated by using degree of polymerization value (DP) that was calculated by using the formula described below.

$$DP = \frac{\text{Amount of total sugar}}{\text{Amount of reducing sugar}}$$

Total sugar was determined by using Phenol-sulfuric method (Dubois *et al.*, 1956) using glucuronic acid as the standard. Reducing sugar was determined by dinitrosalicylic acid method (DNS) (Miller, 1959). Details of those analytical methods were explained in Appendix B. Uronic acid content were determined using the method of carbazole assay (Dische, 1947)

3.2.4 Analysis of carbohydrates by thin layer chromatography (TLC)

Prepared oligosaccharides solutions containing 10 mg total sugar was added 120 µl of conc.H_2SO_4, followed by heating at 121^oC for 15 min. After cooling, the hydrolyzed solution was neutralized with excess $CaCO_3$ salt and the precipitates were removed by centrifugation at 10,000 rpm for 10 min. The 15 µl of samples and standard sugar were spotted on silica gel aluminum sheet (Merck Company) with the smallest spot size. Spotted TLC plate was developed in closed chamber containing mobile phase mixture of isopropanol: 1-butanol: water with 12: 5: 4 ratio (Sa-nguansook, 2002). The separated sugar spots were visualized by dipping in 5%(v/v) H_2SO_4 in methanol and heating at 150^oC in hot air oven for 10 min. Standard sugars used in this experiment were 0.1%(w/v) of glucose, fructose, arabinose, raffinose, mannose, galactose, and glucuronic acid.

3.2.5 Preparation of pectic oligosaccharides (POS) by enzymatic method

3.2.5.1 Effect of enzymes quantity on oligosaccharide hydrolysis by pectinase

Each 5 ml of 0.2%(w/v) extracted polysaccharide dissolved in 0.2 M of acetate buffer pH 4.4 was mixed with 10, 20, 40 and 60 unit of commercial pectinase (Fluka Company). The reaction mixture was incubated in water bath at 37^OC. The 200 µl of reaction mixture was sampling by sterile autopipette at 10, 20, 30 and 60 minute and determined for total sugar and reducing sugar by phenol-sulfuric and DNS method as described previously. DP was calculated as described in 3.2.3.

3.2.5.2 Effect of extracted polysaccharide concentration on enzymatic hydrolysis of Moo-noi polysaccharide by pectinases

Various amounts of extracted polysaccharides including 5, 25, 50, 75 and 100 ml of 0.2%(w/v) were mixed with 10 units of commercial pectinase with the pH control by 0.1 M acetate buffer pH 5.5. The reaction mixture was incubated in water bath at 37^{O}C. The reaction mixture (1 ml) was sampling at 30, 60, 90 and 120 minute, respectively. Total sugar and reducing sugar were determined as described above and the DP of pectic oligosaccharide products was calculated.

3.2.6 Study of prebiotic properties of extracted polysaccharides

3.2.6.1 *In vitro* study with defined microorganisms in pure culture

The extracted polysaccharides assumed as pectin and POS obtained by the enzymatic hydrolysis were used as a sole carbon source in basal medium at 1%(w/v) for culturing each of LAB, *E. coli* and *S. havana*. The inoculums were prepared in MRS broth for LAB and NB for *E. coli* and *S. havana*. All inoculums preparations were incubated at 37^{o}C for 15 hours. The inoculum size of each strains adjusted to 10^{8} cfu by steriled 0.85%(w/v) NaCl were transferred to the basal mediums containing different carbon sources and static incubated at 37^{o}C for 42 hours. The culture was aseptically sampling every 6 hours for determination of viable cell and pH. Addition of glucose and no carbon source were set as the control experiments.

Table 3 Treatments of *In vitro* study on prebiotic property of Moo-noi pectin and pectic oligosaccharide in pure culture

C-sources / Strains	pectin	POS	Control*	Control**
Ent. faecium	T1	T2	T3	T4
LABG12	T5	T6	T7	T8
LAB33	T9	T10	T11	T12
S. havana	T13	T14	T15	T16
E. coli	T17	T18	T19	T20

Control* = with glucose, Control ** = without carbon source

3.2.6.2 *In vitro* study with defined microorganisms by mixed cultures

Basal medium contained 1%(w/v) of different carbon sources pH 7.0 was inoculated with LAB, *S. havana* and *E. coli* together as Table 2. Totally intitial number of LAB was fixed at 10^8 cfu the same as those of *E. coli* and *S. Havana* inoculated in the same culture and static incubated at 37^{o}C. Sampling with 4 hours interval was performed. For viable cell enumeration of LAB, *S. havana* and *E. coli*, the serial decimal dilution with pour plate or spread plate techniques were performed using MRS, SS and EMB agar, respectively. Cultures were subsequently incubated at 37^{o}C for 24 hours. LAB colonies were visualized as the oval shape colony with yellow zone around colony on MRS agar. *S. havana* showed the black colony on SS agar, while the metallic sheen forming colonies of *E. coli* were observed on EMB agar.

Table 4 Treatments of *In vitro* study on prebiotic property of Moo-noi pectin and pectic oligosaccharide in mixed culture

C-sources / **Strains**	**pectin**	**POS**	**Control***	**Control****
Ent. faecium + *E. coli* + *S. havana*	T1	T2	T3	T4
LABG12 + *E. coli* + *S. havana*	T5	T6	T7	T8
LAB33 + *E. coli* + *S. havana*	T9	T10	T11	T12

Control* = with glucose, Control ** = without carbon source

3.2.6.3 Mixed culture test in fecal slurry medium

Fecal slurry was used as source of natural mixed cultures. Fresh human feces was collected immediately from healthy volunteer who had no preceding history of gastrointestinal disorder and had not been prescribed of antibiotics for at least three months before the study. Specimens were kept in anaerobic jar during transportation. Fecal slurry (5% w/v) was prepared in 0.1 M sodium phosphate buffer pH 7.0 and each oligosaccharide were added to give a final concentration of 0.7%(w/v) as described by Wang and Gibson (1994). Adding glucose as carbon source was performed as control group. The ferments were statically incubated at 37^{o}C in anaerobic jar. The difference in bacterial number at 0, 12 and 24 h incubation were determined as total lactic acid bacteria in MRS agar, *E. coli* on EMB agar and *Salmonella-Shigella* spp. on SS agar.