Prebiotic Extracts from Thai Edible Plants

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ชื่อวิทยานิพนธ์ สารสกัดพรีไบโอติกจากพืชที่รับประทานได้ของไทย

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บทคัดย่อ

เมื่อนำสารสกัดด้วยน้ำและเอทานอล 50 เปอร์เซ็นต์ ของพืชที่รับประทานได้จำนวน 9 ชนิด ได้แก่ ข่า (*Alpinia galanga* Sw.), ขมิ้นขาว (*Curcuma mangga* Valeton & van Zijp.), ผักบุ้งจีน (*Ipomoea asarifolia* (Desr.) Roem. & Schult.), ผักบุ้งไทย (*Ipomoea aquatica* Forsk.), บัวบก (*Centella asiatica* Urban), มะเขือยาว (*Solanum melongena* Linn.), มะเขือเปราะ (*Solanum aculeatissimum* Jacq.), มะเขือพวง (*Solanum torvum* Sw.) และมะเขือเทศ (*Lycopersicon esculentum* Mill.) มาคัดเลือกคุณสมบัติการเป็น พรีไบโอติก โดยการคัดเลือกขั้นต้นเป็นการทดสอบการทนต่อสภาวะความเป็นกรดที่พีเอช 1 เป็นเวลา 4 ชั่วโมง และการทนต่อการย่อยด้วยเอนไซม์ human pancreatic α-amylase เป็นเวลา 6 ชั่วโมง พบว่าสารสกัดทั้งหมดมีศักยภาพเป็นสารพรีไบโอติกที่ดี คือยังมีปริมาณคาร์โบไฮเดรทที่ไม่ถูกย่อยหลังผ่านการย่อยด้วยกรดและเอนไซม์เหลืออยู่มากกว่า 80.44 เปอร์เซ็นต์ และเมื่อนำสารสกัดมาศึกษาผลการส่งเสริมการเจริญของแบคทีเรียโปรไบโอติกสามสายพันธุ์ คือ *Lactobacillus acidophilus* TISTR 875, *Lactobacillus plantarum* TISTR 450 และ *Bifidobacterium bifidum* DSM 20456 พบว่าเมื่อใช้สารสกัดน้ำของข่า และสารสกัดเอทานอลของขมิ้นขาวเป็นแหล่งคาร์บอนสามารถส่งเสริมการเจริญของแบคทีเรียโปรไบโอติกทั้งสามสายพันธุ์ได้ดีที่สุดอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) เมื่อเทียบกับชุดควบคุมที่ใช้กลูโคสเป็นแหล่งคาร์บอน ซึ่งให้ค่าการเจริญเติบโตจำเพาะสูงสุด (μ_{max}) เป็น 0.162, 0.205 และ 0.095 h^{-1} ตามลำดับ โดยเมื่อใช้สารสกัดจากข่าเป็นแหล่งคาร์บอนมีค่า μ_{max} เป็น 0.195, 0.379 และ 0.129 h^{-1} ตามลำดับ และเมื่อใช้สารสกัดจากขมิ้นขาวเป็นแหล่งคาร์บอนมีค่า μ_{max} เป็น 0.171, 0.294 และ 0.121 h^{-1} ตามลำดับ สารสกัดน้ำของข่า และสารสกัดเอทานอลของขมิ้นขาวจะถูกทำให้บริสุทธิ์บางส่วนโดยการตกตะกอนด้วยเอทานอล 80 เปอร์เซ็นต์ หลังจากนั้นสารสกัดที่ผ่านการทำบริสุทธิ์บางส่วนจะถูกใช้เป็นแหล่งคาร์บอนในการศึกษาผลของการเจริญร่วมกันของแบคทีเรียโปรไบโอติกทั้งสามสายพันธุ์ ร่วมกับแบคทีเรียก่อโรคสองสายพันธุ์คือ *Escherichia coli* และ *Staphylococcus aureus* พบว่า แบคทีเรียโปรไบโอติก *Lactobacillus acidophilus* TISTR 875 และ *Lactobacillus plantarum* TISTR 450 สามารถ

ยับยั้งแบคทีเรียก่อโรคทั้งสองสายพันธุ์ โดยที่ *Staphylococcus aureus* จะถูกยับยั้งอย่างสมบูรณ์เมื่อเลี้ยงร่วมกันกับ *L. plantarum* เป็นเวลา 48 ชั่วโมงในอาหารเลี้ยงเชื้อที่มีสารสกัดน้ำของข่าเป็นแหล่งคาร์บอน แต่ไม่พบการยับยั้งแบคทีเรียก่อโรคทั้งสองสายพันธุ์เมื่อเลี้ยงร่วมกันกับ *B. bifidum* เมื่อวิเคราะห์น้ำหนักโมเลกุลเฉลี่ยของสารสกัดทั้งสองชนิดที่ผ่านการทำบริสุทธิ์บางส่วน (ส่วนตะกอน) พบว่าสารสกัดน้ำจากข่ามีน้ำหนักโมเลกุลเฉลี่ยอยู่ในช่วง 1524 ถึง 135 ดาลตัน ส่วนสารสกัดเอทานอลจากขมิ้นขาวมีน้ำหนักโมเลกุลเฉลี่ยอยู่ในช่วง 2600 ถึง 135 ดาลตัน และเมื่อทำการวิเคราะห์หาองค์ประกอบน้ำตาลที่มีอยู่ในสารสกัดด้วยวิธี TLC พบว่าในสารสกัดทั้งสองชนิดนั้นมีน้ำตาลกลูโคส และฟรุคโทสเป็นองค์ประกอบ

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ABSTRACT

Water and ethanol extracts from nine edible plants, including galanga (Kha) (*Alpinia galanga* Sw.), white turmeric (Kha-min Khao) (*Curcuma mangga* Valeton & van Zijp.), water spinach (Phak Boong Jeen) (*Ipomoea asarifolia* (Desr.) Roem. & Schult.), red water spinach (Phak Boong Thai) (*Ipomoea aquatica* Forsk.), asiatic pennywort (Bua Bok) (*Centella asiatica* Urban), eggplant (Ma-khue Yao) (*Solanum melongena* Linn.), cockroach berry (Ma-Khue Prao) (*Solanum aculeatissimum* Jacq.), common asiatic weed (Ma-Khue Poung) (*Solanum torvum* Sw.) and tomato (Ma-Khue Tead) (*Lycopersicon esculentum* Mill.) were screened and characterized for prebiotic properties. Primary screening was based on the resistance of acid and enzymatic digestion of the extracts as well as probiotic growth in the presence of extracts as carbon sources. The results showed that all crude extracts resisted to the digestion of both acid (pH = 1 for 4 h) and human pancreatic α-amylase with the indigestible carbohydrate content more than 80.44%. Only water extract of galanga and ethanolic extract of white turmeric were the best carbon source for growth of *Lactobacillus acidophilus* TISTR 875, *Lactobacillus plantarum* TISTR 450 and *Bifidobacterium bifidum* DSM 20456 with the highest maximum specific growth rate (μ_{max}) of 0.195, 0.379 and 0.129 h^{-1}, respectively with galanga extract and μ_{max} were 0.171, 0.294 and 0.121 h^{-1}, respectively with white turmeric extract, which were significantly different ($p < 0.05$) from μ_{max} of these probiotic grew in the control carbon source (glucose) that were 0.162, 0.205 and 0.095 h^{-1}, respectively. Water extract of galanga and ethanolic extract of white turmeric were partially purified by precipitating with 80% ethanol and were added as carbon source in co-culture study to evaluate the competition between probiotics and pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus*. Both pathogens were inhibited by *L. plantarum* and *L. acidophilus* but not inhibited by *B. bifidum*, when they were cultivated in the presence of galanga and white turmeric extracts. Growth of *S. aureus* was completely inhibited in the presence of galanga extract at 48 h. The average molecular weight of galanga (sediment part) was in the range of 1524 to 135 dalton and the average molecular

weight of white turmeric (sediment part) was in the range of 2600 to 135 dalton. The major sugar compositions of water extract from galanga and ethanolic extract from white turmeric were glucose and fructose.

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List of Abbreviations and Symbols

°C	= Degree Celsius
CFU	= Colony-forming unit
DNS	= Dinitrosalicylic acid
DP	= Degree of polymerization
FOS	= Fructo-oligosaccharides
Fru	= Fructose
g	= Gram
Gal	= Galactose
Gal*p*A	= 1,4-linked α-D-galactosyluronic acid
GI	= Gastrointestinal tract
Glc	= Glucose
GOS	= Galacto-oligosaccharides
GPC	= Gel permeation chromatography
h	= Hour
IMO	= Isomalto-oligosaccharides
In	= Inulin
kj	= Kilo joule
LACT	= Lactulose
MHB	= Mueller Hinton Broth
ml	= Milliliter
MRS	= Lactobacillus MRS Broth
N	= Normal
nm	= Nanometer
OD_{600}	= Optical density at 600 nm
OF	= Oligofructose
PHGG	= Partially hydrolyzed guar gum
SCFAs	= Short chain fatty acids
ScFOS	= Short-chain Fructo-oligosaccharides
SHIME	= Simulator of the Human Intestinal Microbial Ecosystem
SOS	= Soybean oligosaccharides
t_2, t_2	= Time
TLC	= Thin layer chromatography

List of Abbreviations and Symbols (Continued)

TOS	= Transgalacto-oligosaccharides
v/v	= Volume by volume
w/v	= Weight by volume
X_1, X_2	= Cell density
XOS	= Xylo-oligosaccharides
%	= Percent
μ	= Specific growth rate
μ_{max}	= Maximum specific growth rate
μl	= Microliter
μm	= Micrometer

CHAPTER 1

GENERAL INTRODUCTION

Introduction

Nowadays, many people are neglecting their concerns about foods taking in each meal because they suppose that it does not worth thinking about. Nevertheless, modern consumers are changing their attitudes and increasingly interested in their personal health, and expect the foods that they eat to be safe and healthy apart from food nutritional value. As interests in linkage between diet and health extended, many consumers seek ways to feel well and stay healthy by seeking foods with extra functionality apart from the nutritional concern. Numbers of researches revealed the functional mechanisms of these foods on improving human health in many ways. Prebiotics with their benificial effects are one of the answer to better health. There is currently a great deal of interest in prebiotic oligosaccharides as functional food ingredients to manipulate the composition of colonic microflora in order to improve health (Gibson and Roberfroid, 1995; Roberfroid, 2000; Gibson and Fuller, 2000; Losada and Olleros, 2002 and Hammes and Hertel, 2002).

Prebiotic stimulates the growth and colonisation of probiotic bacteria, which are, non-pathogenic organisms and are beneficial to health (Rastall and Maitin, 2002). Prebiotic enhancement can cause growth repression of pathogens and change the colonic environment due to the formation of bacteriocins, the successful competition for substrates or adhesion sites on the gut epithelium, and stimulation of the immune system (Miller-Catchpole, 1989 and Gibson *et al.*, 1995). Increase of the lactic acid producing bacteria in host gut by feeding certain prebiotics reduced the activities of enzyme implicated in carcinogenesis (Rowland *et al.*, 1998) and decreased the incidence of tumor development after exposure to known carcinogens (Kato, 2000). Additionally, usage of prebiotic is a way of maintaining mucosal growth, mucosal function and water electrolyte balance, providing the host with energy and nutrients (Bengmark, 2003).

Naturally occurring prebiotic oligosaccharides are found in many sources such as plants (fructo-oligosaccharides, inulin, soybean oligosaccharides, xylo-

oligosaccharides, pectins, pectic-oligosaccharides and gums), animal skeleton (chitin, chitosan and chitosan oligosaccharides), human's and cow's milk (galacto-oligosaccharides). For the reason that more than 36,000 plants worldwide containing fructo-oligosaccharides and inulin, most popular substances using as food supplements in various human diets, which are usually stored in organs such as bulbs, tubers and tuberous roots of monocotyledonous and dicotyledonous families such as *Liliaceae*, *Amaryllidaceae*, and *Compositae* (Kaur and Gupta, 2002), therefore, this study aims to evaluate prebiotic properties of the extract from edible plants available in southern Thailand.

Literature reviews

1 Prebiotic

Gibson and Roberfroid (1995) first described prebiotic as a non-digestible food ingredient that beneficially affects host by selectively stimulating growth and/or activity of one or a limited number of beneficial bacteria in the colon, and subsequently inhibits growth of pathogens, therefore enhances overall health benefit (Gibson, 2004).

Prebiotic can be precisely defined as undigestible but fermentable dietary carbohydrates which selectively stimulate growth of particular gut (colon) flora such as bifidobacteria and lactobacilli considered beneficial to human health (Cummings *et al.*, 2001). To be effective, prebiotics should escape digestion and absorption in the upper part of the gastrointestinal tract, reach the large bowel, and be utilised selectively by beneficial micro-organisms meanwhile inhibit growth of pathogenic (Macfarlane and Cummings, 1998 and Gibson and Angus, 2000).

Most food oligosaccharides and polysaccharides (including dietary fibre contained in regular diet) have been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotics. Prebiotics are characterized base on their properties as followings:

- resist host digestion, and adsorption processes in the stomach or small intestine and provide low energy value (<9 kJ/g) (Hozapfel and Schillinger, 2002; Gibson, 2004)
- modulation of the colonic flora (Hozapfel and Schillinger, 2002) by:
  - selective stimulation of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus* and *Eubacterium* spp.
  - inhibition of "undesirable" bacteria such as *Clostridium* and *Bacteroides*

- products generated from prebiotic fermentation induce beneficial luminal/systemic effects within the host (Manning and Gibson, 2004)
- increase stool volume (Hozapfel and Schillinger, 2002)

Any dietary material that enters large intestine is a candidate prebiotic. This includes non-digestible carbohydrates such as resistant starch and dietary fiber. However, current study on prebiotics seems to be confined to oligosaccharides that are non-digestible in the upper gut, and confer the degree of fermentation selectivity (Gibson and Angus, 2000).

According to the IUB-IUPAC nomenclature, oligosaccharides are defined as sugars consisting of 2 and 10 saccharide units (Voragen, 1998; Gibson and Angus, 2000 and Gibson, 2004). Oligosaccharides are water soluble and typically 0.3-0.6 times as sweet as sucrose. The sweetness of prebiotic mixture depends on chemical structure, degree of polymerization of the oligosaccharides and the levels of mono- and disaccharides present (Crittenden and Playne, 1996). Some oligosaccharides occur naturally in several fruits and vegetables such as artichoke, onion, chicory, garlic, leek, and, to a lesser extent in cereals, whilst others can be commercially produced through the hydrolysis of polysaccharides (e.g. dietary fibers, starch) or through enzymatic generation (Macfarlane and Cummings, 1998; Gibson and Angus, 2000).

2 Types of oligosaccharides as prebiotics

A range of oligosaccharides have been tested using various *in vitro* methods, animal models and human clinical trials. The following oligomers have been reported with prebiotic potential:

2.1 Naturally occurring oligosaccharides

2.1.1 Fructo-oligosaccharides

Fructo-oligosaccharides or FOS typically refer to short-chain oligosaccharides comprised of D-fructose and D-glucose, containing from three to five monosaccharide units. FOS, also called neosugar and short-chain FOS (scFOS), are

produced on a commercial scale from sucrose using a fungal fructosyltransferase enzyme. Fructo-oligosaccharides have an energy value of 6 kJ/g; they have no genotoxic, carcinogenic, or toxicological effects; and they are mildly laxative, although flatulence is often a complaint when large doses are taken. FOS are resistant to digestion in the upper gastrointestinal tract. They act to stimulate the growth of *Bifidobacterium* species in the large intestine. FOS are marketed in the United States in combination with probiotic bacteria and in some functional food products (Macfarlane and Cummings, 1998).

2.1.2 Inulin and Oligofructose

Inulin and oligofructose are natural food ingredients commonly found in varying percentages in dietary foods. They are present in 36,000 plant species (Carpita *et al.* 1989). Inulin is a term applied to a heterogeneous blend of fructose polymers found widely distributed in nature as plant storage carbohydrates. Inulin refers to a group of naturally-occurring fructose-containing oligosaccharides. Inulin belongs to a class of carbohydrates known as fructans. They are derived from the roots of chicory (*Cichorium intybus*), Jerusalem artichokes (*Helianthus tuberosus*) and can be found in many plant varieties. Inulin (Figure 1) is a polydisperse β(2-1) fructan composed of a mixture of oligo- and polysaccharides (Glc α 1-2 [Fru β 1-2]$_n$ where n>10), which are almost all linear chains of fructose having the structure GF$_n$ (with G=glucosyl unit, F=fructosyl unit and n=number of β-D-fructofuranose units linked to one another). In native chicory inulin, the number of fructose units linked together ranges from 2 to more than 60, with an average degree of polymerization (DP) in the order of 10 to 12 is marketed as nutritional supplements. Oligofructose (Figure 1) is a subgroup of inulin, consisting of GF$_n$ and F$_n$ chains with n average ranging from 2 to 7 (DP ≤10) (Niness, 1999; Gibson and Angus, 2000). Inulin stimulates growth of *Bifidobacterium* species in the large intestine and is considered important prebiotic substrates. They are produced in large quantities in several countries and are added to various products such as biscuits, drinks, yoghurts, breakfast cereals, table spreads and sweeteners.

Inulin and fructo-oligosaccharides containing plant species are found in a number of mono and dicotyledonous families such as *Liliaceae*, *Amaryllidaceae*, *Gramineae*, and *Compositae*. Parts of various fructan containing plant species are often eaten as vegetables (Van Loo *et al.*, 1995). Some important sources of inulin (together

with their average inulin content) and oligofructose are given in Table 1. However, only a limited number of plant species are suitable for industrial food and non food applications. Despite the high fructan content of the aerial parts of many *gramineae* (e.g. wheat, rye and barley), particularly of young seedlings (up to 70% of their dry weight), grasses and cereals are not used for industrial extraction and processing of fructans. Conversely, in *Liliaceae*, *Amaryllidaceae* and *Compositae*, fructans (most exclusively inulins) are usually stored in organs such as bulbs, tubers and tuberous roots which because of the absence of interfering components, can be easily extracted and processed to purified products (Kaur and Gupta, 2002).

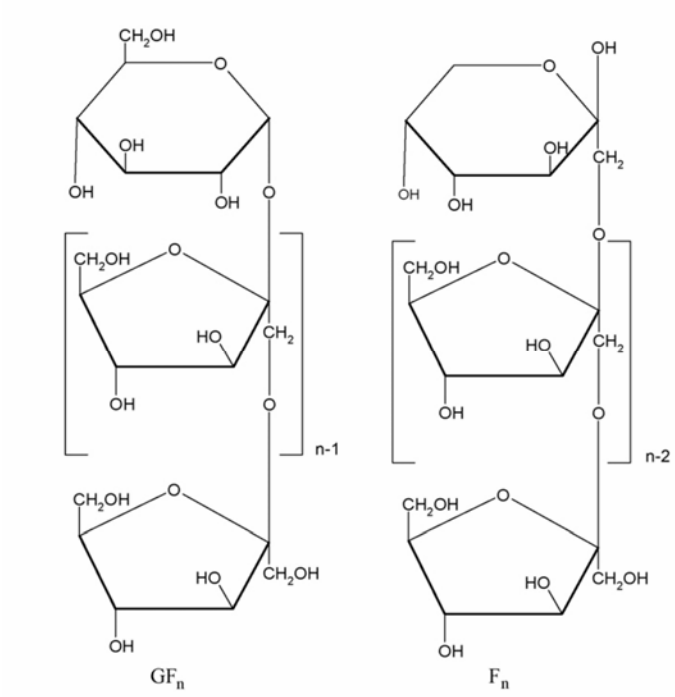


Figure 1. Chemical structure of the inulin and oligofructose. GF_n (α-D-glucopyranosyl-[β-D-fructofuranosyl]$_{n-1}$-D-fructofuranoside) and F_n (β-D-fructopyranosyl-[α-D-fructofuranosyl]$_{n-1}$-D-fructofuranoside) molecules. n is the degree of polymerization or the number of β-D-fructofuranose units; G and F stand for glucose and fructose, respectively.

Source: Ronkart *et al.* (2007)

Table 1. Inulin and oligofructose content (% on fresh weight) found in edible plants.

Source	Edible part	Inulin content (%)	Oligofructose content (%)
Jerusalem artichoke	tuber	14-19	10-15
Artichoke	leaves-heart	3-10	<1
Chicory	root	15-20	5-10
Yacon	root	3-19	3-19
Salsify	root	4-11	4-11
Murnong	root	8-13	not available
Burdock	root	3.5-4.0	not available
Garlic	bulb	9-16	3-6
Leek	bulb	3-10	2-5
Camas	bulb	12-22	not available
Banana	fruit	0.3-0.7	0.3-0.7
Wheat	cereal	1-4	1-4
Barley	cereal	0.5-1.5	0.5-1.5
Rye	cereal	0.5-1.0	0.5-1.0

Source: Modified from Van Loo *et al.* (1995)

2.1.3 Soybean oligosaccharides

Soybean oligosaccharides refer to oligosaccharides found in soybeans and also in other beans and peas. Soybean oligosaccharides are galactosyl sucrose derivatives and are non-reducing oligosaccharides. Soybean oligosaccharides are isolated from the soybean whey resulting from soy protein manufacture, and concentrated to a syrup in the commercial product. The two principal types of sugar from soybean oligosaccharides are the trisaccharides raffinose and the tetrasaccharides stachyose. Raffinose is comprised of one molecule each of D-galactose, D-glucose and D-fructose. Stachyose is comprised of two molecules of D-galactose, one molecule of D-glucose and one molecule of D-fructose. Soybean oligosaccharides act to stimulate the growth of *Bifidobacterium* species in the large intestine. They are marketed in Japan as dietary supplements and in functional foods. They

are being developed in the United States for similar uses (Macfarlane and Cummings, 1998 and Gibson and Angus, 2000).

2.1.4 Xylo-oligosaccharides

Xylo-oligosaccharides are low molecular weight reducing oligosaccharides which comprised of oligosaccharides containing β (1-4) linked xylose residues and mainly consist of xylobiose, xylotriose and xylo-tetraose. The degree of polymerization of xylo-oligosaccharides is from two to four. Xylo-oligosaccharides are manufactured by enzymatic hydrolysis of the polysaccharide xylan from corns cobs, oats or wheat arabinoxylan. The commercial products are predominantly composed of the disaccharides xylobiose, with small amounts of higher oligosaccharides. Xylose and high molecular weight components are removed by membrane processing (Gibson and Angus, 2000). They are marketed in Japan as prebiotics and are being developed for similar use in the United States (Macfarlane and Cummings, 1998).

2.1.5 Chitin, chitosan and chitosan oligosaccharides

Chitin is the second most abundant natural biopolymer after cellulose. The chemical structure of chitin is similar to that of cellulose with 2-acetamido-2-deoxy-β-D-glucose monomers attached via β (1-4) linkages. Chitosan is the name used for low acetyl substituted forms of chitin which is soluble in acidic solutions and is composed primarily of glucosamine, (1-4)-2-amino-2-deoxy-β-D-glucose. Chitosan has three types of reactive functional groups, an amino group as well as both primary and secondary hydroxyl groups at the C-2, C-3 and C-6 positions, respectively (Shahidi *et al.*, 1999). Chitosan oligosaccharides are prepared by enzymatic hydrolysis of fully deacetylated chitosan polymer. They were found to be composed of dimer (33.6%), trimer (16.9%), tetramer (15.8%), pentamer (12.4%), hexamer (8.3%), heptamer (7.1%), and octamer (5.9%). It has been reported that chitosan oligosaccharides is an excellent growth stimulator of *B. bifidium* and most of the *Lactobacillus* sp. tested (Lee *et al.* 2002).

2.1.6 Galacto-oligosaccharides

Galacto-oligosaccharides are present naturally in human's and cow's milk and are also produced from lactose by galactosidase. They are galactose-containing oligosaccharides of the form Glc α 1-4 [Gal β 1-6]$_n$ where n=2-5, and are produced from lactose syrup using the trans-galactosylase activity of the enzyme β-galactosidase. Feeding 2.5 g, 5 g, or 10 g of galacto-oligosaccharides to volunteers resulted in a dose related increase in faecal bifidobacterial excretion, although stool weight and frequency did not change noticeably (Macfarlane and Cummings, 1998).

2.1.7 Pectins and pectic-oligosaccharides

Pectin is considered a soluble dietary fibre. A generally accepted primary structure of pectin has emerged from cumulative studies spanning the last 70 years. The pectins were typically isolated from plants of economic importance (e.g. citrus, tomato, sugar beet, apple) or from suspension-cultured cells (e.g. sycamore, carrot, spinach, and rose). However, these plants represent only a small fraction of the total number of known flowering plants (~235,000 species), and little is known about the pectin of green algae, liverworts, mosses, and ferns. Pectins are a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid (Gal*p*A) residues (Ridley *et al.*, 2001). Both of the pectins and the pectic-oligosaccharides also increased the number of *bifidobacteria* (Olano-Martin *et al.*, 2002).

2.1.8 Gums

There are many naturally occurring gums such as Gum Tragacanth from *Astragalus microcephalus*, Guar Gum from the seed of the leguminous shrub *Cyamopsis tetragonoloba*, Locust Bean Gum from the carob tree (*Ceratonia siliqua*) and Gum Arabic from Acacia tree (*Acacia Nilotica* Linn.) that are processed in commercial production, but only some of these gums have been confirmed for prebiotic effect.

2.1.8.1 Guar Gum

Guar Gum is composed of galacto-mannan β (1-4)-D-manno-pyranosyl, α (1-6)-D-galacto-pyranosyl polymer Guar Gum. Partially hydrolyzed guar gum (PHGG) is a natural, water-soluble dietary fiber. The guar plant, *Cyamoposis tetragonolobus*, has been grown in India and Pakistan since ancient times. Since the 1950s, the seeds of the guar plant have been processed into guar gum and used as a food additive. Guar gum is the galactomannan of the endosperm of guar seeds. Galactomannans are present in a variety of sources in nature. Other plants that contain galactomannans are locust bean gum, alfalfa seeds, coconut meat, soybeans, pineapple, sugar beets, and coffee beans. In the food industry, guar gum is used as a thickening and stabilizing agent in a wide variety of foods, usually in amounts less than 1% of food weight. Consumption of PHGG increases the concentration of *Bifidobacterium* in the human's gut. PHGG also decreased β-glucuronidase activity, putrefactive products, and ammonia content of feces (Slavin and Greenberg, 2003).

2.1.8.2 Gum Arabic (Acacia)

Derived from the sap of the African acacia tree, Gum Arabic is one of the world's most common gums with the longest history. Also known as Gum Acacia, it was used in ancient times for purposes as varied as mummification and inks for hieroglyphics. Today, after harvest in the nations in the gum belt of Africa such as Chad, Eritrea, Kenya, Mali, Mauritania, Niger, Nigeria, Senegal, and Sudan, Gum Arabic is dried or aged for one year before being processed into spray dried powder. Defined as cold water-soluble polysaccharides, Gum Arabic is a multi-functional hydrocolloid that features a highly branched arabino-galactan-protein complex. Acacia gums can exert putatively beneficial effects on host health through both the improvement of the composition of the large intestine microflora and short chain fatty acids formation (Michel *et al.*, 1998).

2.2 Synthetic oligosaccharides

2.2.1 Isomalto-oligosaccharides

Isomalto-oligosaccharides comprise a mixture of α (1-6)-D-linked glucose oligomers, including isomaltose, panose, isomaltotetraose, isomaltopentaose, nigerose, kojibiose, isopanose and higher branched oligo-saccharides. Isomalto-oligosaccharides are produced by a two-stage enzymatic process. Starch is first hydrolysed to malto-oligosaccharides by the combined action of α-amylase and pullulanase. The resultant oligosaccharides are then acted upon by α-glucosidase. This enzyme catalyses a transfer reaction converting the α 1-4 linked malto-oligosaccharides into α 1-6 linked isomalto-oligosaccharides. Glucose is removed by chromatography to give products with high oligosaccharide contents. Isomalto-oligosaccharides act to stimulate the growth of *Bifidobacterium* species and *Lactobacillus* species in the large intestine. Isomalto-oligosaccharides are marketed in Japan as dietary supplements and functional foods. They are being developed in the United States for similar uses (Gibson and Angus, 2000 and Gibson, 2004).

2.2.2 Lactulose

Lactulose is a semi-synthetic disaccharide comprised of D-lactose and D-fructose. The sugars are joined by a β-glycosidic linkage (Gal β 1-4 Fru), making it resistant to hydrolysis by human digestive enzymes. Lactulose is, however, fermented by a limited number of colonic bacteria. This can lead to changes in the colonic ecosystem in favor of bacteria, such as lactobacilli and bifidobacteria, which may confer some health benefits. Lactulose is a prescription drug in the United States for the treatment of constipation and hepatic encephalopathy. It is marketed in Japan for use as a dietary supplement and in functional foods. Its use in the United States as a prebiotic substance is still experimental (Gibson, 2004).

2.2.3 Lactitol

Lactitol is a disaccharides analogue of lactulose. Its pharmaceutical use is in the treatment of constipation and hepatic encephalopathy. Lactitol is also used in Japan as a prebiotic. It is resistant to digestion in the upper gastrointestinal tract and it is fermented by a limited number of colonic bacteria, resulting in an increase in the biomass of bifidobacteria and lactobacilli in the colon. Lactitol is known chemically as 4-*o*-(β-D-galactopyranosyl)-D-glucitol. Lactitol is not approved for the treatment of hepatic encephalopathy or constipation in the U.S., and its use as a prebiotic is considered experimental. Lactitol is used in Europe as a food sweetener (Gibson, 2004).

2.2.4 Lactosucrose

Lactosucrose is a trisaccharides comprised of D-galactose, D-glucose and D-fructose (β 1-4 Gal α 1-2 Glc β Fru). Lactosucrose is produced enzymatically by the enzymatic transfer of the galactosyl residue in lactose to sucrose, using β-fructofuranosidase. Lactosucrose is resistant to digestion in the stomach and small intestine. It is selectively utilized by intestinal *Bifidobacterium* species resulting in significant induction of growth of these bacteria in the colon. Therefore, under physiological conditions, lactosucrose acts on the intestinal microflora as a growth factor for *Bifidobacterium* species. Lactosucrose is also known as β-4-D-galactosylsucrose. It is widely used in Japan as a dietary supplement and in functional foods, including yogurt. Lactosucrose is being developed in the United States for similar uses (Gibson and Angus, 2000 and Gibson, 2004).

2.2.5 Gluco-oligosaccharides

An oligosaccharide preparation has been enzymatically synthesized, using a glucosyl-transferase from *Leuconostoc mesenteroides*, to transfer glucose molecules from the sucrose donor to an acceptor, namely maltose. Gluco-oligosaccharides are not yet used in food (Gibson, 2004).

2.2.6 Transgalacto-oligosaccharides

Transgalacto-oligosaccharides (TOS) are a mixture of oligosaccharides consisting of D-glucose and D-galactose (Glc α 1-4 [Gal β 1-6]$_n$ where n=1-4 average 2). TOS are produced from D-lactose via the action of the enzyme beta-galactosidase obtained from *Aspergillus oryzae*. TOS are resistant to digestion in the upper gastrointestinal tract and stimulate the growth of bifidobacteria in the large intestine. TOS are marketed in Japan and Europe as dietary supplements and are used in functional foods. They are being developed for similar use in the United States (Gibson, 2004 and Rycroft *et al.*, 2001).

2.2.7 Pyrodextrins

Pyrodextrins comprise a mixture of glucose-containing oligosaccharides that is derived from the hydrolysis of starch. Pyrodextrins have been found to promote the proliferation of *Bifidobacterium* species in the large intestine. They are resistant to digestion in the upper gastrointestinal tract. Pyrodextrins are being developed for the nutritional supplement market place (Macfarlane and Cummings, 1999 and Gibson, 2004).

In general, food-grade oligosaccharides are not pure products, but are mixtures containing oligosaccharides with different degrees of polymerization, also including the parent polysaccharides or disaccharides and monomeric sugars. Often they are available in several grades of purity (Voragen, 1998). Table 2 shows the chemical composition and characteristics of numerous candidate prebiotic carbohydrates.

3. Microecosystem in gastrointestinal tract

The gastrointestinal tract (GI) associates a resident microbiota and cells of various phenotypes lining the epithelial wall. The term microbiota has been defined as the collective societies of bacteria assembled on the mucosal surfaces of an individual (Servin, 2004). Mammals are born without any such microorganisms (Mackie *et al.*, 1999). The colonization of gastrointestinal tract starts immediately at birth. The first bacteria colonized in the gut originate from the birth canal, and these include aerobic and anaerobic bacteria such as *Escherichia coli*, *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp.,

Bacteroides spp., and *Bifidobacterium* spp. The resident microbiota in the digestive tract is a heterogeneous microbial ecosystem containing up to $1x10^{14}$ colony-forming units (CFUs) of bacteria (Berg, 1996; Morelli *et al.*, 1998 and Vaughan *et al.*, 2000). The microbiota differs quantitatively and qualitatively at different points along the gastrointestinal tract. The stomach has few resident microorganisms, however lactobacilli have been isolated from the microbiota resident in the stomach (Roach *et al.*, 1977). The microbial profile of the digestive tract is typified by the absence of anaerobic microorganisms in the stomach and conversely their overwhelming predominance in the distal colon. Moreover, different microbial communities may be located in the intestinal lumen, in the mucus covering the epithelium, in the crypt spaces or in the different cells lining the epithelium, and some species adhere whereas others do not. The number of bacteria in the intestinal microbiota has been estimated to be about 400 species. The proportion of anaerobic bacteria gradually increases from the proximal to distal regions, and 99% of the inhabitants located in the large intestine are anaerobes. Currently, only 20-40% of bacterial species present in the gastrointestinal tract have been cultured or characterized.

The human gastrointestinal tract is a kinetic microecosystem that enables normal physiological functions of the host organism unless harmful and potentially pathogenic bacteria dominate it. Maintaining a proper equilibrium of the microflora may be ensured by systematic supplementation of the diet with prebiotic (Bielecka *et al.*, 2002).

The different factors influencing the gastrointestinal microflora are listed in Table 3. Oxygen is a limiting factor for the growth of colonic microflora (Vandamme *et al.*, 2002), the metabolic activity of which can be affected by factors such as age, gastrointestinal diseases, the administration of drugs, the fermentation of food residues.

Table 2. Chemical composition and characteristics of candidate prebiotic carbohydrates

Oligosaccharides	Chemical composition
Fructo-oligosaccharides (Raftilose P95)	95% oligosaccharides β (2-1) fructan; 60% glucose, fructose$_{(n)}$, 40% fructose$_{(n)}$ DP 2-8, average 4-5
Inulin	>99% oligosaccharides β (2-1) fructan; average DP 10-12
Pyrodextrins	Complex mixture of glucose-containing oligosaccharides
Transgalactosylated oligosaccharides (Oligomate 55)	Consist of galactosyl derivertives of lactose with Glc α 1-4 [β Gal 1-6]$_n$ linkages [mainly 6' galactosyllactose where n=1-4 average 2; 55% pure
Galacto-oligosaccharides	85% oligogalactose, Glc α 1-4 [β Gal 1-6]$_n$ where n=2-5, small amounts of glucose, galactose, and lactose
Soybean oligosaccharides	Stachyose (Gal α 1-6 Gal α 1-6 Glc α 1-2 β Fru) and raffinose (Gal α 1-6 Glc α 1-2 β Fru), DP 3-4
Xylo-oligosaccharides	Xyl β 1-4 [Xyl]$_n$ where n=2-7; 70% pure
Isomalto-oligosaccharides	Mixture of α (1-6) linked glucose oligomers (isomaltose, panose, isomaltotriose)
Lactulose	Galactose and fructose-containing disaccharide (Gal β 1-4 Fru)

DP =degree of polymerisation.

Sources: Adapted from Macfarlane and Cummings (1999); Fooks *et al.* (1999) and Rycroft *et al.* (2001)

Table 3. Factors influencing the composition of gastrointestinal microflora.

1. Host factors
a. Species, strain and individual differences due to:
Acid and alkali secretion
Intestinal motality and intestinal transit time
Intestinal structure
Level of endogenous nutrients (mucin, gut proteins, bile secretions, sloughed mucosal cells)
Availability of colonisation sites
Immunological interactions
Redox potential
Bile salts
Antibodies
b. Age
c. Gastrointestinal disorders
2. Environmental factors
a. Drugs
b. Diet
c. Xenobiotic compounds
d. Amount, chemical composition and availability of growth substrates
e. Availability of inorganic electron acceptors
3. Bacterial factors
a. Bacterial metabolites (short chain fatty acid, bacteriocin)
b. Bacterial interactions (competition)
c. pH

Sources: Fook *et al.* (1999) and Vandamme *et al.* (2002)

4. Utilisation of prebiotics in the colon

The resident gut microbiota ferments substances that cannot be digested by the host in the upper GI, these include resistant starch, non-digestible carbohydrates, oligosaccharides, proteins and mucins. The two main types of fermentation that are carried out in the gut are saccharolytic and proteolytic. Saccharolytic activity is more favourable than a proteolytic fermentation due to the type of metabolic end products that are formed. The main end products of saccharolytic fermentation are the short chain fatty acids (SCFAs), acetate, propionate and butyrate. All contribute towards the host's daily energy requirements. Acetate is metabolised in systemic areas like muscle, while propionate is transported to the liver and used to generate ATP. Butyrate is an important source of energy for the colonocytes and is thought to have anti-tumor properties. The end products of proteolytic fermentation on the other hand, include nitrogenous metabolites (such as amines and ammonia) some of which are carcinogens.

The proximal colon is a saccharolytic environment with the majority of carbohydrate entering the colon being fermented in this region. As digesta moves through to the distal colon, carbohydrate availability decreases and protein and amino acids become the main metabolic energy source for bacteria in the distal colon. The principal substrates for bacterial growth are however, dietary carbohydrates. It has been estimated that about 10-60 g/day of dietary carbohydrate reaches the colon. Most research has hitherto been carried out on fibre fermentation by gut bacteria (Gibson, 2004).

5. Effects of prebiotics on gut microecosystem

A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides indicated that a significant increase in numbers of bifidobacteria was observed, when inulin and xylo-oligosaccharides (XOS) were applied (Table 4). The effect of the different prebiotics on clostridia was also comparable to fructo-oligosaccharides (FOS) and XOS caused a significant increase in numbers of clostridia after 5 h. Populations of *Bacteroides* showed very similar changes with all of the oligosaccharides, with a significant increase in numbers after 5 h fermentation. *Escherichia coli* and streptococci showed little change with most substrates although inulin caused

relatively large significant increases in both groups. Inulin caused a significantly larger increase in the total bacterial count than most of the other substrates as well as a larger increase in numbers of streptococci than lactulose.

Between 0 and 24 h, inulin gave a significantly smaller increase in numbers of bifidobacteria than isomalto-oligosaccharides (IMO) or lactulose, while FOS showed a significantly larger increase in numbers of streptococci than galacto-oligosaccharides (GOS). It is possible to identify the best prebiotic for a desired microflora change. If a maximal increase in numbers of bifidobacteria is desired, then XOS or lactulose would appear to be the best carbon sources. Maximal increases in numbers of lactobacilli were recorded on FOS, whereas the maximal decrease in numbers of clostridia was recorded on GOS (Rycroft *et al.*, 2001).

Saito *et al.* (1992) performed an *in vitro* fermentation study with monocultures of 125 strains of human intestinal bacteria of 18 different genera, including 29 strains from five species of *Bifidobacterium* on media containing five different carbohydrate substrates: refined soybean oligosaccharides, stachyose, raffinose, fructooligosaccharides or glucose. The multiple unit carbohydrate sources elicited a slower growth rate than glucose for *Lactobacillus*, *Bacteroides*, and *Enterococcus* and did not support the growth of potential pathogenic *Clostridium* species, *Veillonella*, *E. coli*, and *Klebsiella*. The growth rate of *Bifidobacterium*, except *B. bifidus*, was similar to all carbohydrate sources.

Table 4. Bacterial populations[a] in static batch culture fermentations with the various prebiotics

Group	Initial	FOS		Inulin		XOS		LACT		IMO		GOS	
		5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h
Total count	10.62	10.88	10.72	10.98*	10.60**	10.90*	10.65**	10.81	10.62	10.81	10.57	10.86	10.65
Bifidobacteria	9.61	10.28*	10.23*	10.20*	10.06*	10.42*	10.26*	10.40*	10.35*	10.37*	10.35*	10.33*	10.33*
Clostridia	9.08	9.76*	8.70	9.40	8.92	9.64*	8.90	9.47	8.70	9.04	8.79	9.26	8.43
Bacteroides	9.51	10.15*	9.77	10.01*	9.76	10.07*	9.85*	10.09*	9.72	10.05*	9.80	10.01*	9.70
Lactobacilli	9.08	9.52	9.07	9.02	9.06	8.93	8.94	8.88	8.89	9.03	8.77	9.03	8.70
Streptococci	8.46	8.75*	8.57	8.71*	8.67*	8.77*	8.48	8.34	8.44	8.61	8.52	8.56	8.43
Escherichia coli	8.25	8.57*	8.49	8.86*	8.78*	8.61*	8.26	8.69*	8.33	8.50	8.24	8.52	8.10

[a]Values are mean $\log_{10}$ cfu (g faeces)$^{-1}$

*Significantly different from initial count ($P \leq 0.05$).

**Significantly different from 5 h count ($P \leq 0.05$).

FOS, Fructo-oligosaccharides; XOS, xylo-oligosaccharides; LACT, lactulose ; IMO, isomalto-oligosaccharides; GOS, galacto-oligosaccharides.

Source: Modified from Rycroft *et al.* (2001)

Van de Wiele *et al.* (2004) investigated the effects of native chicory inulin on the composition and the fermentation activity of an *in vitro* cultured colon microbial community. Simulator of the Human Intestinal Microbial Ecosystem (SHIME), which harbours a microbial community resembling that from the human colon both in fermentation activity and in composition were applied for study model. Analysis of the microbial suspension from the SHIME colon compartments revealed that inulin administration had limited effects on the overall composition of the microbial community (Table 5). For the transverse and descending colon compartments, lactobacilli concentrations were 1.5 log CFU higher ($p < 0.01$) than the initial levels from the start-up period. Significantly higher bifidobacteria concentrations ($p < 0.05$) were observed in the ascending colon after 5 weeks of inulin supplementation. A limited decrease in staphylococci concentrations ($p < 0.05$) was observed in the descending colon whereas fecal coliforms were inhibited both in the transverse colon ($p < 0.05$) and descending colon ($p < 0.01$) (Table 6). During the control period, starch again replaced inulin in the nutrition of the SHIME reactor. This resulted in lower lactobacilli concentrations in the transverse colon and lower bifidobacteria concentrations in the ascending colon (Table 5), whereas no other significant changes compared to the startup period were found.

Besides the nutritional advantage that bifidobacteria may have from inulin compared to other intestinal microbiota, bifidobacteria together with lactobacilli also create a more acidic environment by the production of lactic acid, thus inhibiting the excessive growth of pathogens.

Table 5. Lactobacilli and bifidobacteria counts in the colon vessels of the SHIME reactor during start-up (n = 5), 5 weeks of treatment with inulin at normal concentration (n = 5) and control period (n = 3)[a]

Microbial group	Ascending colon	Transverse colon	Descending colon
Start-up			
Lactobacilli	5.57 ± 0.69	5.42 ± 0.75	5.01 ± 0.83
Bifidobacteria	6.26 ± 0.22	6.83 ± 0.84	6.75 ± 0.62
Treatment			
Lactobacilli	6.39 ± 0.21	6.91 ± 0.13**	6.64 0.05**
Bifidobacteria	6.93 ± 0.29*	6.57 ± 0.24	6.75 ± 0.56
Control			
Lactobacilli	6.77 ± 0.18	6.48 ± 0.04***	6.68 ± 0.14
Bifidobacteria	5.69 ± 0.22***	5.57 ± 0.56	5.39 ± 0.34

[a] Data are means ± SD.

* Significantly different from the start-up period, $p < 0.05$.

** Significantly different from the start-up period, $p < 0.01$.

*** Significantly different from treatment period, $p < 0.05$.

Source: Modified from Van de Wiele *et al.* (2004)

Table 6. Fecal coliforms and Staphylococci counts in the colon vessels of the SHIME reactor during start-up (n = 5), 5 weeks of treatment with inulin at normal concentration (n = 5) and control period (n = 3)[a]

Microbial group	Ascending colon	Transverse colon	Descending colon
Start-up			
Fecal coliforms	6.66 ± 0.40	7.00 ± 0.40	7.32 ± 0.28
Staphylococci	7.15 ± 0.17	6.59 ± 0.42	6.75 ± 0.08
Treatment			
Fecal coliforms	6.28 ± 0.30	6,29 ± 0.29*	6.33 ± 0.37**
Staphylococci	6.70 ± 0.28	6.53 ± 0.05	6.43 ± 0.20*
Control			
Fecal coliforms	6.45 ± 0.47	6.54 ± 0.22	6.56 ± 0.40*
Staphylococci	6.56 ± 0.69	6.40 ± 0.53	6.12 ± 0.71

[a] Data are means ± SD.

* Significantly different from the start-up period, $p < 0.05$.

** Significantly different from the start-up period, $p < 0.01$.

Source: Modified from Van de Wiele *et al.* (2004)

6. Effects of prebiotics on enhancement of probiotic growth

Bielecka *et al.* (2002) studied the influence of fructan-type oligosaccharides, such as fructo-oligosaccharides (WAKO PURE, Japan; DP 2-4), oligofructose (Raftilose P95, ORAFTI, Belgium, oligo-fructose ≥ 93.2%; DP 2-8), Raftiline HP (ORAFTI, Belgium, inulin > 99.5; average DP ≥ 23 monomers), Frutafit EXL (SENSUS, the Netherlands, inulin ≥ 99.75%; average DP ≥ 22) and Frutafit IQ (SENSUS, the Netherlands, inulin ≥ 90.0%; average DP ≥ 9) which were prebiotics for growth of *Bifidobacterium* strains as probiotics. *In vitro* studies showed that the majority of *Bifidobacterium* species utilized fructo-oligosaccharides (DP 2-4) and low-polymerized inulins (average DP ≥ 9), but only 18 out of 30 strains tested (mostly of *B. longum* and *B. animalis* species) were stimulated. Incorporation of oligofructose into the diet also

stimulated the proliferation of bifidobacteria by 1.6 log cfu/g in comparison to the control medium containing lactose. Frutafit IQ, Raftiline HP and Frutafit EXL were not utilized by bifidobacteria. This indicated that utilisation of inulin by bifidobacteria depends on the degree of polymerization of fructo-oligomer chains and purity of the preparations.

7. Effects of prebiotics on growth of intestinal food-borne pathogens

Fooks and Gibson (2002) investigated the effects of selected probiotic microorganisms (*Lactobacillus plantarum* 0407, *Lactobacillus pentosus* 905, *Lactobacillus reuteri* SD2112, *Lactobacillus acidophilus* La5 and *Bifidobacterium bifidum* Bb12) on certain human intestinal food-borne pathogens (*Escherichia coli*, *Campylobacter jejuni* and *Salmonella enteritidis*) in combination with different prebiotics such as Oligofructose (FOS), inulin, xylo-oligosaccharide (XOS), and mixtures of inulin:FOS (80:20 w/w) and FOS:XOS (50:50 w/w), as carbohydrate sources. The antimicrobial potential exhibited by each of the probiotics used here appeared to depend on the carbohydrate source used. FOS, inulin, XOS, and mixtures of FOS:XOS (50:50 w/w) and inulin:FOS (80:20 w/w) caused greater inhibition than lactulose, lactitol, starch and dextran, perhaps suggesting a structure-to-function relationship in terms of the prebiotic used (Figure 2). The type of bond linking the component monomers, in view of specific cleavage enzymes being required for fermentation of the carbohydrate, may effect fermentation rate, and thereby determine the speed at which potential inhibitory metabolic end products are released. Chain length of the carbohydrate is also likely to be a contributory factor, since long chain oligosaccharides, with multiple branching, require more enzymatic hydrolysis by the organisms before its complete fermentation.

L. plantarum combined with FOS was the most effective at inhibiting pathogen growth (Table 7). A significant, 6-log ($P<0.01$) decrease in *E. coli* numbers was observed, when FOS was used, whilst after the same time period (24 h), *C. jejuni* and *S. enteritidis* were undetectable ($P<0.001$). *B. bifidum* combined with FOS:XOS proved to be an effective synbiotic combination (Table 8). *C. jejuni* and *S. enteritidis* were decreased to below detectable levels ($P<0.001$), whereas a 2-log decrease in *E. coli* numbers was observed. This study showed that lactobacilli and bifidobacteria species can inhibit some important pathogenic species in the presence of FOS and FOS:XOS as carbon sources.

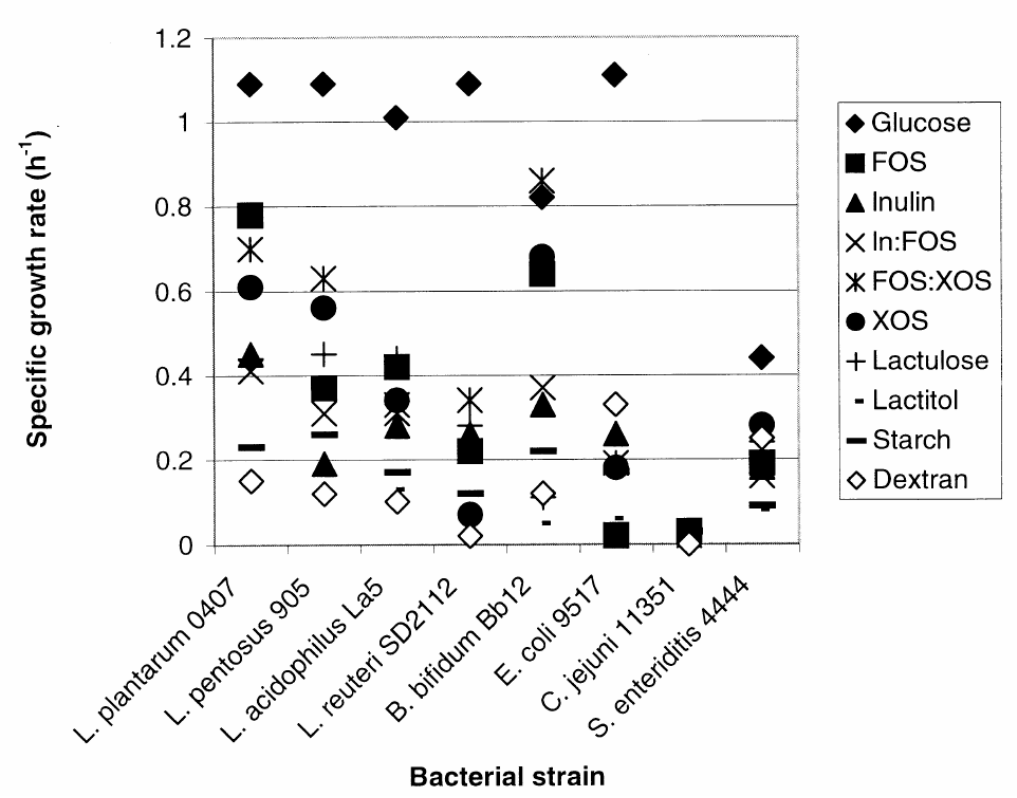


Figure 2. Specific growth rates (h^{-1}) of probiotic and enteropathogenic bacteria utilising various carbohydrate sources including Inulin (In), Oligofructose (FOS), xylo-oligosaccharides (XOS), In:FOS, mixture 80:20 w/w; FOS:XOS, mixture 50:50 w/w.

Source: Fooks and Gibson (2002)

Table 7. Inhibition of enteropathogens by probiotic *L. plantarum* 0407 in co-culture experiments

Pathogen	Starch	FOS	Inulin:FOS	FOS:XOS	XOS
E. coli	$+4.50 \times 10^{9}$	-8.25×10^{8}*	$+3.27 \times 10^{9}$	-2.37×10^{7}	-2.25×10^{8}
C. jejuni	-1.29×10^{7}	-2.09×10^{9}**	-1.74×10^{9}	-1.30×10^{9}	-1.89×10^{9}
S. enteritidis	$+7.42 \times 10^{10}$	-7.25×10^{10}**	-8.08×10^{8}	-2.68×10^{7}	-7.15×10^{6}

Data presented are changes in parameter between 0 and 24 h inoculation. Probiotic and enteropathogen numbers were enumerated at various time points after inoculation. Results are cfu ml^{-1}. + and - denote an increase or decrease respectively of the described parameter. A significant decrease of pathogen numbers from baseline (0 h fermentation) is denoted; *$P<0.01$, **$P<0.001$.

FOS, Oligosaccharides; XOS, xylo-oligosaccharides

Source: Modified from Fooks and Gibson (2002)

Table 8. Inhibition of enteropathogens by probiotic *B. bifidum* Bb12 in co-culture experiments

Pathogen	Starch	FOS	Inulin:FOS	FOS:XOS	XOS
E. coli	-2.56×10^{8}	-1.92×10^{7}	-4.80×10^{7}	-1.25×10^{6}	-6.68×10^{7}
C. jejuni	-1.26×10^{8}	-2.29×10^{8}*	-2.38×10^{8}*	-2.77×10^{8}**	-2.07×10^{8}
S. enteritidis	-1.66×10^{8}	-1.36×10^{9}	-3.19×10^{7}	-1.70×10^{9}**	-3.05×10^{8}

Data presented are changes in parameter after 24 h inoculation. Probiotic and enteropathogen numbers were enumerated at various time points after inoculation. Results are cfu ml^{-1}.

- denotes a decrease of the described parameter. A significant decrease of pathogen numbers from baseline (0 h fermentation) is denoted; *$P<0.01$, **$P<0.001$.

FOS, Oligosaccharides; XOS, xylo-oligosaccharides

Source: Modified from Fooks and Gibson (2002)

8. Effects of prebiotics on growth of probiotic bacteria

Bielecka *et al.* (2002) studied on effects of administration of bifidobacteria together with the prebiotic (as synbiotics) on bifidobacteria population *in vivo*. Daily, 5% (w/w) of oligofructose in the diet and $>10^9$ live cells of bifidobacteria strains, including *Bifidobacterium longum* KN29.1 (group OF+B1), *B. longum* KNA1 (OF+B2) and *B. animalis* KSp4 (OF+B3), were orally fed to Wistar rats everyday for 14 days. The results showed that prebiotic (oligofructose) combined with selected probiotic in this study could improve the bifidogenic effect by 1.4 log cfu/g of faeces compared to non-supplemented group (control) as shown in Table 9.

Table 9. Effect of administration of bifidobacteria together with the prebiotic (as synbiotics) on bifidobacteria population. (Count, log cfu /g faeces ±S.D.)[a]

Groups	Bifdobacteria
Control	8.892 ± 0.322
OF + B1	10.249 ± 0.227*
OF + B2	10.286 ± 0.160**
OF + B3	10.333 ± 0.580*

[a] B1, B2, B3; strains of bifidobacteria administered to rats; *B. longum* KN29.1, *B. longum* KNA1, and *B. animalis* KSp4, respectively. OF; oligofructose. Results are the average from five animals. Microbial count significantly different from control at the significance level: *P ≤ 0.01%, **P ≤ 0.001%.

Source: Modified from Bielecka *et al.* (2002)

One physiological function of the oligosaccharides was as reserve carbohydrates in storage organs such as seeds and tubers. They were mobilized during the early stages of germination. They were therefore synthesized and were deposited in these organs during the maturation process. It was also conceivable that a fraction of these oligosaccharides arrive at this location as translocated material. However, in some plants they continue the mature soluble carbohydrates that were translocated from leaves, the site of their biosynthesis (Dey, 1990).

This studies aimed to extraction the extract expected to having prebiotic properties from different families (Zingiberaceae, Umbelliferae, Convolvulaceae and Solanaceae) and extracted parts (rhizome, leaf, shoot tip, whole stem and fruit) of Thai herbal plants; Galanga (*Alpinia galanga* Sw.), White turmeric (*Curcuma mangga* Valeton & van Zijp.), Asiatic pennywort (*Centella asiatica* (Linn.) Urban), Chinese water spinach (*Ipomoea asarifolia* (Desr.) Roem. & Schult.), Red water spinach (*Ipomoea aquatica* Forsk.), Eggplant (*Solanum melongena* Linn.), Cockroach berry (*Solanum aculeatissimum* Jacq.), Common asiatic weed (*Solanum torvum* Sw.) and Tomato (*Lycopersicon esculentum* Mill.). The reasons of selected these herbal plants were:

- Plants in Umbelliferae, Convolvulaceae and Solanaceae families had been investigated that consist of trisaccharides (umbelliferose and plantenose) which were oligosaccharides placing in different parts (leaf, storage organ or fruit) of plants.
- Oligosaccharides were as reserve carbohydrates in storage organs. Therefore, rhizome part of edible plants is interested for this study.

Objectives

1. To screen edible plants extracts based on prebiotic properties
2. To study on inhibition of in *vitro* growth of pathogens by probiotic strains
3. To characterize the prebiotic compounds of the selected extract

Scope of this study

This study will cover the extraction of crude extracts from Thai edible plants and herbs that expected to have prebiotic properties, selection of the crude extracts based on their prebiotic properties, and inhibition of in *vitro* pathogens by probiotic strains in the presence of the extract as well as characterization of the prebiotic compounds.

Outcomes

1. The extract from the selected edible plants and herbs which expected to use as a prebiotic.
2. The potential of the extracts from the selected edible plants and herbs as a prebiotic will be known.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Plant Materials

(1) Galanga (*Alpinia galanga* Sw.) (Kha)

(2) White turmeric (*Curcuma mangga* Valeton & van Zijp.) (Kha-min Khao)

(3) Asiatic pennywort (big leaf) (*Centella asiatica* (Linn.) Urban) (Bua Bok)

(4) Asiatic pennywort (small leaf) (*Centella asiatica* (Linn.) Urban) (Bua Bok)

(5) Water spinach (*Ipomoea asarifolia* (Desr.) Roem. & Schult.) (Phak Boong Jeen)

(6) Red water spinach (*Ipomoea aquatica* Forsk.) (Phak Boong Thai)

(7) Eggplant (*Solanum melongena* Linn.) (Ma-khue Yao)

(8) Cockroach berry (*Solanum aculeatissimum* Jacq.) (Ma-Khue Prao)

(9) Common asiatic weed (*Solanum torvum* Sw.) (Ma-Khue Poung)

(10) Tomato (*Lycopersicon esculentum* Mill.) (Ma-Khue Tead)

1.2 Microorganisms

1.2.1 Probiotic strains

(1) *Lactobacillus acidophilus* TISTR 450

(2) *Lactobacillus plantarum* TISTR 875

(3) *Bifidobacterium bifidum* DSM 20456

Lactobacillus acidophilus and *Lactobacillus plantarum* were obtained from Microbiological Resources Centre (MIRCEN), Thailand. *Bifidobacterium bifidum* was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany.

1.2.2 Indicator strains

(1) *Escherichia coli*

(2) *Staphylococcus aureus*

Escherichia coli and *Staphylococcus aureus* were obtained from Microbiology laboratory of Faculty of Agro-Industry, Prince of Songkla University, Thailand.

1.3 Chemicals and mediums

List of chemicals and mediums	Grade/company/country
1. HCl	Analytical/Lab scan/Thailand
2. NaOH	Analytical/Merck/Germany
3. H_2SO_4	Analytical/Merck/Germany
4. 3,5-Dinitrosalicylic acid	Analytical/Fluka/Switzerland
5. Phenol	Analytical/Fisher Scientific/England
6. α-Amylase from human pancreas	Analytical/Sigma®/Germany
7. $Na_2HPO_4.2H_2O$	Analytical/Ajax Finechem/Australia
8. NaH_2PO_4	Analytical/Ajax Finechem/Australia
9. Lactobacillus MRS Broth (MRS)	Analytical/Himedia/India
10. Peptone water	Analytical/Merck/Germany
11. Yeast extract	Analytical/Himedia/India
12. NaCl	Analytical/Merck/Germany
13. K_2HPO_4	Analytical/Ajax Finechem/Australia
14. KH_2PO_4	Analytical/Ajax Finechem/Australia
15. $CaCl_2.2H_2O$	Analytical/Ajax Finechem/Australia
16. $MgSO_4.7H_2O$	Analytical/Ajax Finechem/Australia
17. $NaHCO_3$	Analytical/Merck/Germany
18. Tween 80	Analytical/Ajax Finechem/Australia
19. L-cysteine HCl	Analytical/Fluka/Germany
20. Bile salts	Analytical/Himedia/India
21. Mueller Hinton Broth (MHB)	Analytical/Merck/Germany
22. Mueller Hinton Agar (MHA)	Analytical/Merck/Germany
23. Bromocresol purple	Analytical/Ajax Finechem/Australia
24. 95% Ethanol	Commercial/Thailand

List of chemicals and mediums	Grade/company/country
25. Trifluroacetic acid	Analytical/Fluka/Germany
26. Methanol	Commercial/Thailand
27. D-Glucose	Analytical/Sigma®/USA
28. D-Fructose	Analytical/Sigma®/USA
29. L-Arabinose	Analytical/Sigma®/Germany
30. D-Galactose	Analytical/Sigma®/USA
31. D-Xylose	Analytical/Sigma®/USA
32. $CHCl_3$	Analytical/Merck/Germany
33. Ethyl acetate	Commercial/Thailand
34. Acetic acid	Analytical/Fluka/Germany
35. Isopropyl alcohol	Analytical/Merck/Germany

1.4 Equipments

List of equipments	Company/Country
1. Filtering flask, Erlenmeyer shape, with glass hose connection for vacuum use (1,000 ml)	Pyrex/Germany
2. Porcelain büchner funnel sealed with rubber filter aid	-
3. Filter paper #41 (diameter 90 mm, pore size 20-25 μm, catalogue number: 1441-090)	Whatman®/England
4. Rotary evaporator	Büchi Rotavapor® R-200/205/Switzerland
5. Flask, round bottom narrow neck (1,000 ml)	Büchi/Switzerland
6. Digital weighing electronic scale Model: TE1502S	Satorius/USA
7. Digital weighing electronic scale Model: BP221S	Satorius/USA
8. Water bath Model: WB14	Memmert/USA
9. Larminar Air flow Model: 527044	Hotpack/USA
10. Autoclave Model: SS-325	Tomy Seiko/Japan
11. Centrifuge Model: Universal 32R	Hettich Zentrifugen/USA
12. Microplate reader Model: Powerwave X	Biotek/UK
13. Microtiter plate 96 flat bottom WI	NUNC™/Denmark
14. Micropipette (5-20 μl)	Eppendof/USA

List of equipments	Company/Country
15. Micropipette (10-100 µl)	Labmate/USA
16. Micropipette (100-1000 µl)	Labmate/USA
17. Multichannel micropipette (20-200 µl)	Labmate/USA
18. Microtubes (1.5 ml) Cat. No. MCT-150-C	Axygen Scientific/USA
19. Microtubes (2.0 ml) Cat. No. MCT-200-C	Axygen Scientific/USA
20. Pipette tips	Axygen Scientific/USA
21. pH meter Model: Mettler Toledo 320	Mettler Toledo/USA
22. Vortex mixer	Labnet/USA
23. Vial (20 ml)	-

2. Methods

2.1 Preparation of plant materials

Edible parts of the plants as indicated in Table 10 were sliced and dried at temperature of 50 °C for 12 to 24 hours or until moisture content reached 10-14%. However, galanga and white turmeric rhizome were peeled before slicing and drying. The dried materials were stored under vacuum plastic bags at 4 °C until required.

Table 10. The edible parts of each plant.

Type of plants	Edible parts
Galanga (*Alpinia galanga* Sw.)	Rhizome
White turmeric (*Curcuma mangga* Valeton & van Zijp.)	Rhizome
Asiatic pennywort (big leaf) (*Centella asiatica* (Linn.) Urban)	Leaf and whole stem
Asiatic pennywort (small leaf) (*Centella asiatica* (Linn.) Urban)	Leaf and whole stem
Water spinach (*Ipomoea asarifolia* (Desr.) Roem.&Schult.)	Leaf and whole stem
Red water spinach (*Ipomoea aquatica* Forsk.)	Shoot tip and Stem
Eggplant (*Solanum melongena* Linn.)	Fruit
Cockroach berry (*Solanum aculeatissimum* Jacq.)	Fruit
Common asiatic weed (*Solanum torvum* Sw.)	Fruit
Tomato (*Lycopersicon esculentum* Mill.)	Fruit

2.2 Preparation of plant extracts

Each plant sample was blended using a blender into fine pieces and then was separated into two parts for extraction in two different mixture conditions with the ratio 1 to 6. The first part was extracted twice in hot water (80°C) for 1 hour and another part was extracted twice in ethanol (50%, v/v) at 80 °C for 1 hour. Subsequently the plant slurry was filtered, and the residue was discarded. The filtrate was freeze-dried, and stored under desiccated condition at -20 °C. In the case of using the 50% ethanol mixture, ethanol was removed from the filtrate by vacuum-evaporator at 45 °C before freezed-drying. The crude extracts were determined for total sugar and reducing sugar contents by phenol-sulfuric method (Fox and Robyt, 1991) and dinitrosalicylic acid (DNS) method (Robertson *et al.*, 2001), respectively using glucose as a standard as described in Appendix B.

2.3 Primary screening

2.3.1 Screening for plant extracts resistant to acid hydrolysis

The crude extracts from 2.2 were dissolved with deionized (DI) water to obtain 10% (w/v) concentration. Each extract solution was diluted to 1% (w/v) using HCl buffer solution at pH 1 in a 24-well plate. The mixture with total volume of 1.5 ml was shaken gently for mixing and was incubated on shaker with a speed of 150 rpm at 37 °C for 4 hours. Each 100 µl of sample was collected at 1, 2, 3 and 4 h. The reaction of mixture was stopped with 1 N NaOH. The sampling mixture was diluted into a 96-well plate with the proper ratio and analyzed for reducing sugar released using modified DNS method (Robertson *et al.*, 2001) for with glucose as the standard as described in Appendix B. Percentage of hydrolysis was calculated based on reducing sugar liberated and the total sugar content as the equation below (adapted from Korakli *et al.*, 2002).

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar released (Final - Initial sugar)}}{\text{(Total sugar content - Initial reducing sugar)}} \times 100$$

2.3.2 The susceptibility of the selected crude extracts to α-amylase digestion

The sensitivity of the selected crude extracts to α-amylase digestion was determined using α-amylase (EC. 3.2.1.1) obtained from human pancreas. Each extract was prepared to 10% (w/v) solution and was digested with HCl buffer solution pH 1 for 4 hours. Enzyme solution was added to extract solution into a 96-well plate with a final concentration 1 unit/ml and shaken mildly by hand for mixing before incubated on shaker at 37 °C for 6 hours. The reducing sugar released was analyzed using modified DNS method (Robertson *et al.*, 2001) for with glucose as the standard as described in Appendix B. Percentage of hydrolysis was calculated based on reducing sugar liberated using the DNS colorimetric method and the total sugar content of the sample using the modified phenol-sulfuric method with glucose as standard.

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar released (Final - Initial sugar)}}{\text{(Total sugar content - Initial reducing sugar)}} \times 100$$

From these above procedures, the crude extracts that showed high level of total sugar content with low total reducing sugar content as well as high tolerance to an acidic hydrolysis and α-amylase digestion (high level of indigestible part) were selected for the secondary screening step.

2.4 Secondary screening

2.4.1 Antibacterial activity of the extracts

Using broth microdilution assay to test the antimicrobial activity of each plant crude extract. This assay was performed in a 96-well plate, containing 180 µl of each probiotic bacteria (*Lactobacillus acidophilus* TISTR 450, *Lactobacillus plantarum* TISTR 875 and *Bifidobacterium bifidum* DSM 20456) with a final concentration in a range of $5x10^5$ to $9x10^5$ CFU/ml in each well. 10% (w/v) of each crude extract (based on the concentration of total sugar) was added to each well 20 µl to obtain final concentration of 1% (w/v). Positive control contained the basal medium plus 1% crude extract without probiotic strain and negative control contained the basal medium plus 1%

crude extract with probiotic strain. The lid and base of tested plates were wrapped with parafilm and incubated at 37 °C for 24 to 48 hours in plastic zip bag filled with N_2 gas to obtain the anaerobic condition. Antimicrobial assay was measured by a turbidity using microplate reader at OD_{600}. If the culture in well was clear (the absorbance value at 600 nm of culture was not higher than 0.05 nm when comparing to the positive control), the crude extract had an antimicrobial activity.

2.4.2 Effects of the extracts on probiotic growth

In order to study the effect of the extract on growth of 3 strains of probiotic bacteria (*Lactobacillus acidophilus* TISTR 450, *Lactobacillus plantarum* TISTR 875 and *Bifidobacterium bifidum* DSM 20456), each of glycerol stock bacteria was cultured overnight (24 h) in 10 ml of MRS broth contained in injection vial, which was sealed with rubber stopper and secured by aluminium cap, after being filled with N_2 gas to obtain an anaerobic condition at 37 °C. After that, the culture was inoculated to 10 ml of fresh MRS medium and incubated at the same condition for 18 h, then 10 % of each culture was then inoculated with the final concentration of $1.5x10^5$ to $2x10^5$ CFU/ml into basal medium containing in g/l, peptone water, 2.0; yeast extract, 2.0; NaCl, 0.1; K_2HPO_4, 0.04; KH_2PO_4, 0.04; $CaCl_2.6H_2O$, 0.01; $MgSO_4.7H_2O$, 0.01; $NaHCO_3$, 2.0; Tween 80; Cysteine-HCl, 0.5; bile salts, 0.5; pH 7.0 with each extract in the concentration of total sugar 1.0 % (w/v) as a carbon source (modified from Fooks and Gibson, 2003). The bacteria were cultivated at 37 °C under anaerobic condition as described above. The cell growth was measured the absorbance at 600 nm at 0, 3, 6, 9, 12, 15, 18, 24 and 48 h. All measurements were determined for specific growth rate (μ) to obtain maximum specific growth rate (μ_{max}) and the μ_{max} values were compared with the control, which μ_{max} values were obtained from cell growth of each probiotic strain in basal medium with glucose as a carbon source. Specific growth rate (μ) for each culture was calculated using the following equation:

$$\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1),$$

where X_2 and X_1 are the cell density at time t_2 and t_1.

The selected crude extract that obtained from the secondary screening (section 2.4) were partially purified by precipitation using chilled 80% ethanol sedimentation for overnight. In this procedure, monosaccharide dissolved in supernatant and the longer chain of saccharides such as oligosaccharides precipitated in the solution. After centrifugation, the supernatant was discarded for eliminating the monosaccharide molecules before using in the next experiment. The precipitate was resuspensed in distilled water and evaporated to liberate the existence ethanol before freeze-dried. The partial purified freeze-dried extract was stored under plastic bags, which contained in a vacuum plastic box with silica gel beads at -20 °C until required.

2.5 Inhibition of *in vitro* growth of pathogens by probiotic strains

The interference of lactobacilli and bifidobacteria to the growth of pathogenic strains was evaluated by co-incubating two strains of pathogens (*E. coli* and *S. aureus)* individually with each probiotic strain. For each experiment, one loop of each indicator strain was picked from a subculture on Mueller Hinton Agar (MHA) slant and streaked on a MHA plate, after that the plate was incubated inoculated for 24 h at 37 °C under aerobic condition. One colony of the culture was picked into five ml Mueller Hinton Broth (MHB) containing in the test tube and incubated for 24 h at 37 °C with shaking under aerobic condition. After that, 0.5 ml of a culture broth was inoculated to 4.5 ml fresh MHB and incubated under the same condition as describe above. One ml of the culture broth was inoculated into nine ml fresh MHB and incubated at 37 °C with shaking under aerobic condition for 18 h. The probiotic strains were prepared as indicated in section 2.4.2. Each pre-cultured strain was diluted with their culture medium and added to obtain final concentration of $1x10^5$ to $2.5x10^5$ CFU/ml into basal medium, containing 1% (w/v) of each selected extract from secondary screening. Pure cultures of each strain were subjected to the same treatments and used as controls (pathogen plus minimal medium with 1% (w/v) extract; probiotic plus minimal medium with 1% (w/v) extract). All series of experiments were incubated at 37 °C in sealed bottle filled with N_2 gas to obtain the anaerobic condition. Samples were taken at 0, 12, 24, 48 and 72 hours for enumerating bacteria by spreading on plate (or pouring plate for *B. bifidum*) with the proper medium. In this study, MRS agar was added 0.02 g/l bromocresol purple as indicator for detecting probiotic bacteria, which could change the color from purple to yellow due to acid production (modified by Drago *et al.*, 1997). Colonies grown on each medium agar plate

(MRS plus indicator for probiotic strains or MHA for pathogen strains) from co-cultured were compared to the colonies of each pure probiotic or pathogen strain which grew on the same medium agar and also on another medium (MRS plus indicator or MHA) to proofed that they could not grow on another medium or could grow on another medium with the different appearance which could see and screen by eyes.

2.6 Characterization of the of prebiotic compounds in the selected extracts

2.6.1 Molecular weight distribution of the selected extracts

The partialy purified extracts were analyzed for the molecular weight distribution by gel permeation chromatography (GPC) model: Waters 600E (Waters Corporation, USA.) at National Metal and Materials Technology Center (Bangkok, Thailand). The extract was dissolved in 0.1 M $NaNO_3$ to obtain final concentration of 0.1 % (w/v). The sample solution was filtered with nylon 66 membrane (pore size 0.45 μm) before injected into the Ultrahydrogel 120 (MW resolving range = 100-5,000) + guard column and the injection volume was 20 μl. The flow rate was 0.6 ml/min and the operating temperature was 30 °C. Pullulans were used as standard to determine the molecular weight of the extract. The result was analyzed using PL Logical GPC software (England).

2.6.2 Determination of sugar compositions

Each selected extract was diluted into 1 % (w/v) with distilled water. The trifluroacetic acid was added to the extract solution (to achieve final concentration of 2 N). After that, the mixture was autoclaved for 1 h at 121 °C 15 min for hydrolysis reaction. The mixture solution (0.5 μl) was characterized for its sugar composition using thin layer chromatography method (TLC) on a thin-layer silica gel 60 (MERCK, Germany). Three solvent systems, including, ethyl acetate : isopropyl alcohol : water (3:1:1 v/v/v), $CHCl_3$: methanol : water (6.5:4:1 v/v/v) and ethyl acetate : methanol : water : acetic acid (6.5:2:1.5:1.5 v/v/v), were applied. The thin-layer chromatography plate was dried and visualized by dipping into a solution containing H_2SO_4 : methanol (1:3 v/v) and then heating on hot plate until the brown spot appeared on the sheet. The R_f values of sugar

composition from the extract were compared to those of standard sugars such as D-glucose, D-fructose, L-Arabinose, D-Galactose and D-Xylose (modified by Yang *et al.*, 2004).

2.7 Statistic analysis

Differences between treatments were tested for significance using paired-samples *t*-test, assuming equal variance and considering both sides of the distribution (two-tailed distribution), and one-way ANOVA. Differences were considered at 95% significance if $P < 0.05$ using SPSS for Windows software.

CHAPTER 3

RESULTS AND DISCUSSION

1. Preparation of plant extracts

Extraction of dried plants using two different extraction solvents showed the dissimilar percent yield of crude extracts. The highest percent yield of water extract was obtained from tomato (61.03 %dry weight) followed by ethanolic extract from tomato, ethanolic extract from cockroach berry, water extract from red water spinach (shoot tip), ethanolic extract from asiatic pennywort (small leaf), ethanolic extract from eggplant, ethanolic extract from common asiatic weed, ethanolic extract from red water spinach (shoot tip), which yielded 43.55, 30.84, 30.60, 27.20, 26.82, 26.00 and 24.68 %dry weight respectively (Figure 3). Water extract from cockroach berry, water extract from red water spinach (stem), ethanolic extract from asiatic pennywort (big leaf), water extract from galanga, ethanolic extract from red water spinach (stem), water extract from water spinach, ethanolic extract from galanga, ethanolic extract from water spinach, water extract from asiatic pennywort (big leaf), water extract from white turmeric, water extract from asiatic pennywort (small leaf), water extract from common asiatic weed, water extract from eggplant and ethanolic extract from white turmeric had percent yield of crude extracts as 21.27, 20.80, 20.35, 19.67, 19.60, 19.39, 16.42, 15.85, 14.84, 13.58, 12.75, 12.62, 11.81 and 11.03 %dry weight, respectively (Figure 3).

Extraction of oligosaccharides from natural resources had not been fully developed due to the complexity of these substances and their connections with other macromolecules, particularly proteins (Charalampopoulos *et al.*, 2002). In this experiment, high temperature (80 °C) was used in extraction processes, which was related to the commercial production of oligosaccharides, such as inulin and oligofructose, from chicory (*Cichorium intybus*) root (contained 15-20% inulin and 5-10% oligofructose). The manufacturing process for inulin was rather similar to that of sugar extracted from sugar beets. The roots were typically harvested, sliced and washed. Inulin was then extracted from the root by using a hot water diffusion process, then purified and dried. The resulting

product had an average degree of polymerization (DP) of 10-12 and a distribution of molecules with chain lengths from 2-60 units. The finished inulin powder typically contains 6-10% sugars represented as glucose, fructose and sucrose. These are native to the chicory root; they were not added after extraction (Niness, 1999). In addition, Ronkart *et al.* 2007 also extracted inulin from globe artichoke using 80 °C distilled water as a solvent.

From yield percentage of the extracts obtained from two different solvent extraction (Figure 3), the different parts of edible plant showed the different yield values. The highest yield was found in the water extract from tomato fruit, classified in family Solanaceae, up to 61.03%, which opposited to another plant in this family (eggplant, cockroach berry and common asiatic weed) as well as ethanolic extracts from plant in family Umbelliferae; asiatic pennywort (big leaf) and asiatic pennywort (small leaf) that obtained higher yield when extracted with 50% ethanol. Other water extracts from water spinach and red water spinach (family Convolvulaceae) including galanga rhizome and white turmeric (family Zingiberaceae) were also gave higher yield than ethanolic extraction. The occurring results in this study were concurred to the experiment conducted by Johansen *et al.* (1996) who studied on the effect of extraction solvents and temperatures on extraction yields of monosaccharides, sucrose, and raffinose oligosaccharides from plant materials, which were extracted in either water, 50% (v/v), or 80% (v/v) aqueous methanol or ethanol at 20 or 50 °C or at the boiling point of the solvent. They found that extraction for 1 h either in water or in 50% (v/v) alcohol (methanol or ethanol) was sufficient for most applications. Boiling provided the maximum yield, but the loss by reducing the temperature to 50 °C was the lowest acceptance temperature to obtaining maximum yield in most cases. Complete extraction was not obtained when using 80% alcohol unless boiling is included in the extraction procedure. Aqueous ethanol (50%, v/v) is as effective as methanol as an extraction medium, whereas lower yields are observed at higher alcohol strength. Since ethanol is less hazardous compared to methanol, it was recommended to use the former at the strength of 50% (v/v). There was no consistent difference in the use of reflux with constant stirring compared to extraction in water bath with occasional mixing. They suggested that it might be due to water was the optimal extraction solvent for the low molecular weight sugars. Unfortunately, it was also an excellent solvent for interfering hydrophilic components such as polysaccharides proteins, etc. Extraction in aqueous alcohol minimized these problems, but alcohol strength, extraction temperature, and method varied considerably among the extraction methods.

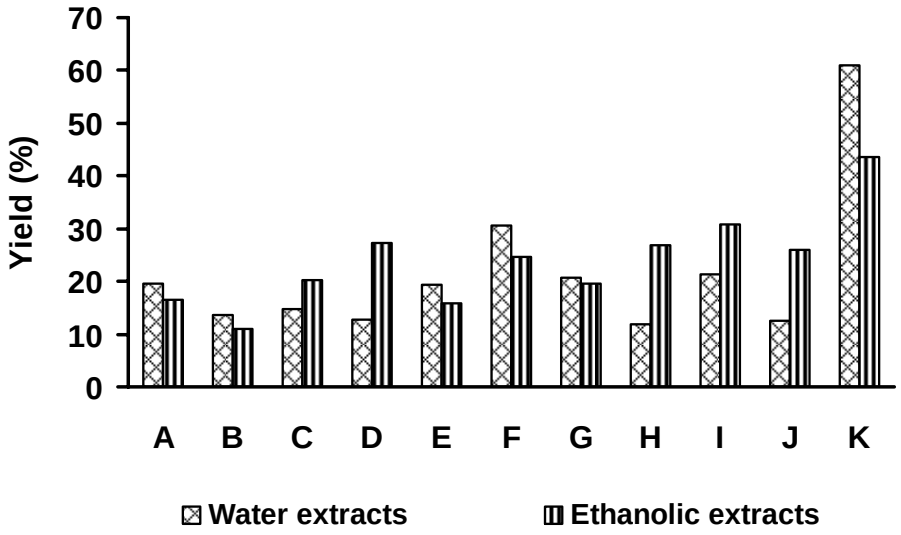


Figure 3. Percent yield of water extracts and ethanolic extracts from galanga (A), white turmeric (B), big leaf asiatic pennywort (C), small leaf asiatic pennywort (D), water spinach (E), shoot tip of red water spinach (F), stem of red water spinach (G), eggplant (H), cockroach berry (I), common asiatic weed (J) and tomato (K).

All twenty-two extracts were determined for total sugar content and found that the water extract from cockroach berry had the highest total sugar content (1,152.89 mg glucose/g extract) followed by water extract from galanga, ethanolic extract from tomato, ethanolic extract from galanga, water extract from tomato and water extract from eggplant which were 1,016.00, 955.56, 927.56, 926.22 and 924.89 mg glucose/g extract respectively (Figure 4a and 4b). Ethanolic extract from cockroach berry, ethanolic extract from asiatic pennywort (small leaf), water extract from red water spinach (stem), ethanolic extract from red water spinach (stem), water extract from common asiatic weed, ethanolic extract from asiatic pennywort (big leaf), ethanolic extract from white turmeric, ethanolic extract from eggplant, water extract from red water spinach (shoot tip), water extract from asiatic pennywort (small leaf), ethanolic extract from common asiatic weed, water extract from white turmeric, water extract from asiatic pennywort (big leaf), ethanolic extract from red water spinach (shoot tip), water extract from water spinach and ethanolic extract from water spinach contained total sugar content as 895.33, 845.33, 791.11, 772.00, 761.53, 756.44, 744.44, 726.67, 673.78, 614.22, 590.22, 579.33, 499.56, 420.44, 211.33 and 170.00 mg glucose/g extract respectively (Figure 4a and

4b). Total sugar content indicated total carbohydrate content in each extract by comparing to glucose sugar. This could be conclude that the water extract from cockroach berry and the water extract from galanga with total sugar content as 1,152.89 and 1,016.00 mg glucose/g extract contained a hundred percent of carbohydrate. In the other hand, extracts with total sugar content less than one hundred mg glucose/g extract, contained other non-carbohydrate compositions. After reducing sugar content determination was performed, the highest initial reducing sugar content was found in the ethanolic extract from tomato (443.56 mg glucose/g extract) followed by water extract from tomato, water extract from cockroach berry and water extract from galanga which were 399.18, 288.59 and 224.13 mg glucose/g extract respectively. The extract containing the lowest initial reducing sugar content was the ethanolic extract from water spinach (12.43 mg glucose/g extract) followed by ethanolic extract from common asiatic weed, water extract from water spinach, ethanolic extract from red water spinach (stem) and ethanolic extract from red water spinach (shoot tip) which were 12.78, 20.65, 21.71 and 21.94 mg glucose/g extract respectively. It could be noticed that high reducing sugar content was mostly found in the extracts containing high total sugar content.

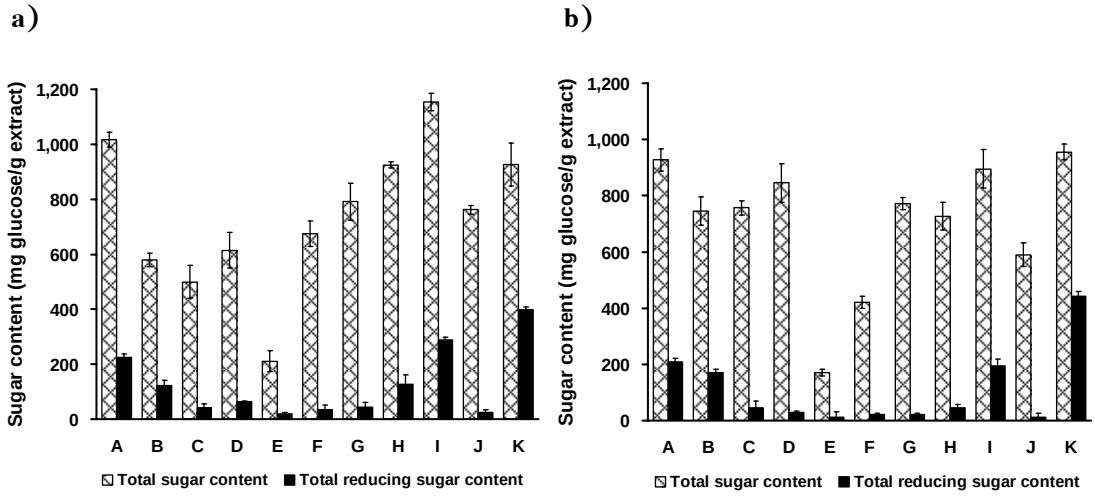


Figure 4. Total sugar and total reducing sugar contents of water extracts (a) and ethanolic extracts (b) from galanga (A), white turmeric (B), big leaf asiatic pennywort (C), small leaf asiatic pennywort (D), water spinach (E), shoot tip of red water spinach (F), stem of red water spinach (G), eggplant (H), cockroach berry (I), common asiatic weed (J) and tomato (K)

High reducing sugar content indicated high content of simple sugar (monosaccharide, disaccharides) in the extracts. These simple sugars could be absorbed in

the upper gastrointestinal tract, and were not functioned as prebiotic. Therefore, the extracts contained high percentage of reducing sugar content were not accounted as suitable prebiotic in the colon, at which probiotics could be proliferated. Calculating for non-reducing carbohydrate content in the extracts would indicate the carbohydrate content that might have prebiotic property (resist digestion and adsorption processes in host stomach or small intestine). After initial sugar content was minused by total sugar content, the highest non-reducing carbohydrate content was found in water extract from cockroach berry (864.30 mg glucose/g extract) followed by ethanolic extract from asiatic pennywort (small leaf), water extract from eggplant and water extract from galanga which were 816.08, 796.91 and 791.87 mg glucose/g extract respectively (Figure 5). Ethanolic extract from red water spinach (stem), water extract from red water spinach (stem), water extract from common asiatic weed, ethanolic extract from galanga, ethanolic extract from asiatic pennywort (big leaf), ethanolic extract from cockroach berry, ethanolic extract from eggplant, water extract from red water spinach (shoot tip), ethanolic extract from common asiatic weed, ethanolic extract from white turmeric, water extract from asiatic pennywort (small leaf), water extract from tomato, ethanolic extract from tomato, water extract from asiatic pennywort (big leaf), water extract from white turmeric, ethanolic extract from red water spinach (shoot tip), water extract from water spinach and ethanolic extract from water spinach contained non-reducing carbohydrate content which were 750.29, 747.30, 736.85, 717.24, 711.19, 700.74, 680.06, 640.22, 577.44, 573.35, 549.77, 527.04, 512.00, 458.47, 456.43, 398.50, 190.68 and 157.57 respectively (Figure 5).

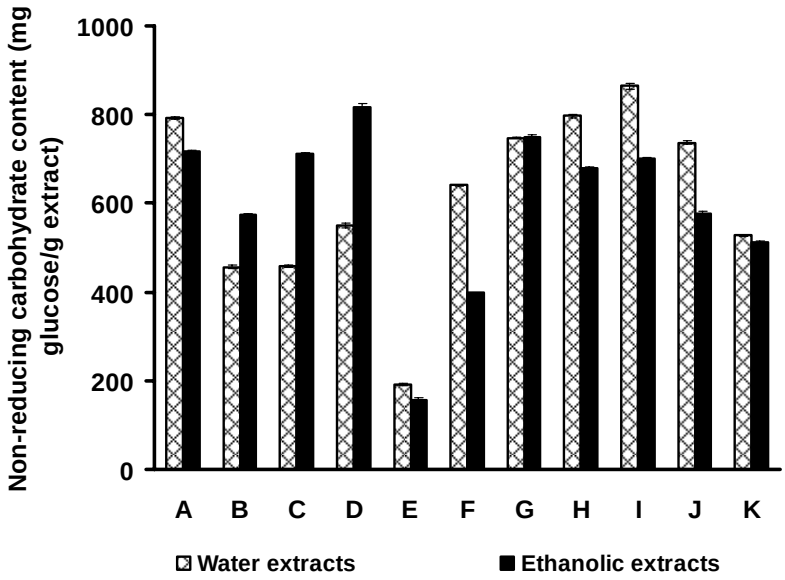


Figure 5. Non-reducing carbohydrate contents (Total sugar content - initial reducing sugar content) of water extracts and ethanolic extracts from galanga (A), white turmeric (B), big leaf asiatic pennywort (C), small leaf asiatic pennywort (D), water spinach (E), shoot tip of red water spinach (F), stem of red water spinach (G), eggplant (H), cockroach berry (I), common asiatic weed (J) and tomato (K).

2. Primary screening for acid and enzymatic resistance of the extracts

Existing of the extracts was considered to be important in order to ensure their optimal functionality. After ingestion, these extracts must overcome two main biological barriers, the acidic environment of the stomach and human enzyme secreted in the small intestine. To guarantee their stability during passage through the upper gastrointestinal tract, the extracts were primarily screened for their tolerance to acidic pH and enzymatic hydrolysis.

2.1 Effect of acid hydrolysis on the extracts

Acid hydrolysis of the extracts prepared from galanga, white turmeric, asiatic pennywort (big leaf), asiatic pennywort (small leaf), water spinach, red water spinach (shoot tip), red water spinach (stem), eggplant, cockroach berry, common asiatic

weed and tomato in HCl buffer pH 1 (the lowest value occurred in stomach). The range of percent hydrolysis of the water extracts was between 0.59 and 12.07, whereas ethanolic extracts showed the hydrolysis percentage of 3.81-15.51%. Most ethanolic extracts excepting ethanolic extract from asiatic pennywort (big leaf) were more susceptible to acid hydrolysis than water extracts (Figure 6a). The highest hydrolysis was found in ethanolic extract from white turmeric (15.51±0.81%) followed by the ethanolic extracts from asiatic pennywort (small leaf), ethanolic extracts from tomato and ethanolic extracts from eggplant which were 14.92±0.76%, 14.41±1.78% and 13.16±2.11% respectively (Figure 6a). Water extract from white turmeric, water extract from asiatic pennywort (small leaf), red water spinach (stem), asiatic pennywort (big leaf) and red water spinach (shoot tip) were hydrolysed at the percentage of 12.07±1.21%, 10.10±0.18%, 9.61±0.60%, 7.07± 0.34% and 7.04±0.34% respectively (Figure 6a). Normally, the potential prebiotic carbohydrate must resisted to acid hydrolysis in the pH range of 1 to 3, and must be remain to transferred to the colon more than 60% (Cummings and Englyst, 1995). The indigestible carbohydrate content of all extracts remained more than 85%. All extracts were carried on the next screening step based on their resistance to enzymatic hydrolysis.

2.2 Effect of enzymatic hydrolysis on the extracts

Both water extracts and ethanolic extracts of all plants were low susceptible to human pancreas α-amylase with the percentage of hydrolysis between 0.45 to 2.10% and 0.63 to 4.51% respectively (Figure 6b). The highest hydrolysis was found in ethanolic extract from water spinach (4.51±0.32%) followed by ethanolic extracts from white turmeric, ethanolic extracts from galanga and water extract from asiatic pennywort (big leaf) which were 4.04±0.81%, 2.91±0.14% and 2.10±1.58% respectively. Water extracts from asiatic pennywort (small leaf), galanga, water spinach and ethanolic extract from cockroach berry were hydrolysed with the highest percentage value of 1.99±0.28%, 1.82±0.22% and 1.82±0.91% and 1.71±0.72% respectively (Figure 6b). Pancreatic α-amylase was presented in high activity in the lumen of the small intestine and it has been widely assumed that the enzyme does not contribute significantly to the rate-limiting process governing carbohydrate digestion and absorption. Because of differences in the assay systems and in the definition of a unit of activity, calculations of amylase concentration based on modern knowledge of the turnover numbers of homogeneous enzyme preparations cannot be made precisely (Slaughter *et al.*, 2001). This enzyme was specific

to α-1,4 glycosidic linkages, and the knowledge that botanical oligosaccharides and starches linked with different linkage types such as α-1,2, α-1,4, α-1,6, β-2,1, β-2,6 and β-1,6 (Mandalari *et al.*, 2006). The lower amount hydrolyzed of extracts in enzymatic hydrolysis could implied that the extracts might be composed of β-linked such as β-1,2, β-1,6 and β-2,6 (highly resistant towards the enzyme activity) with the low level of α-linkage especially α-1,4 in the saccharides chains.

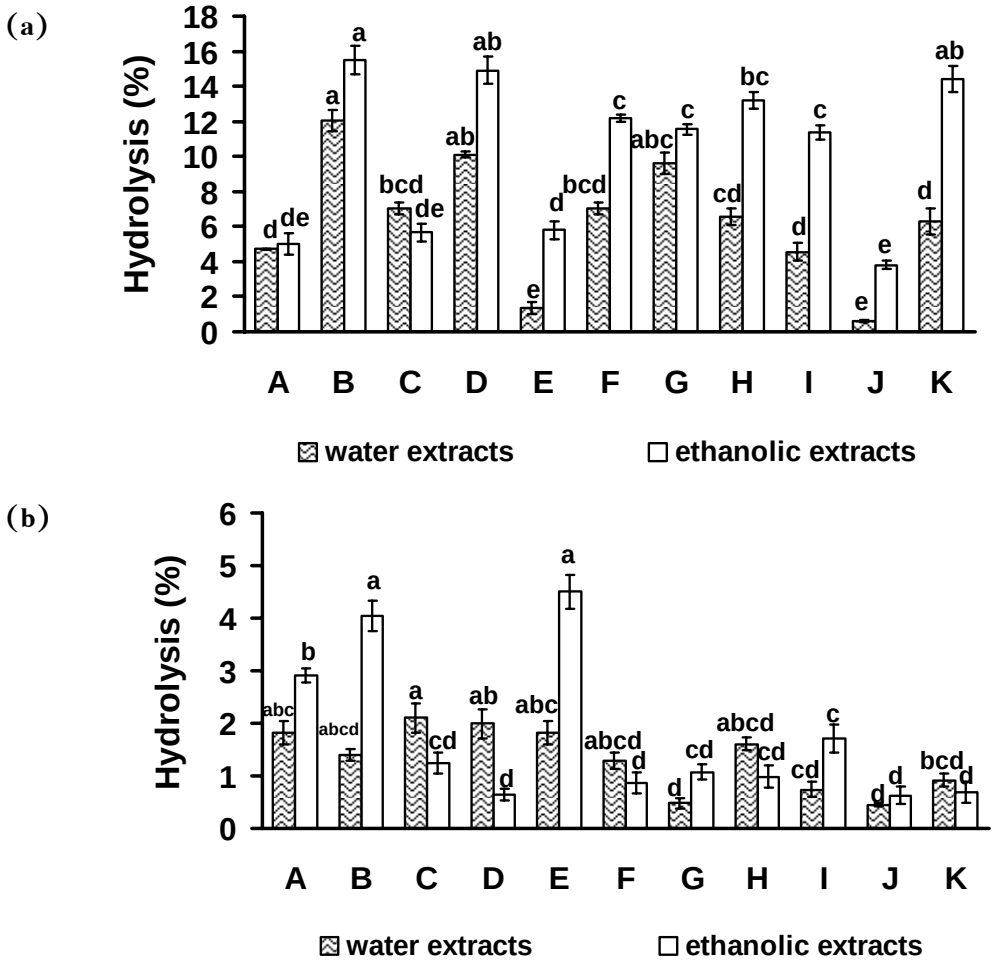


Figure 6. Hydrolysis percentage of crude extracts from galanga (A), white turmeric (B), big leaf asiatic pennywort (C), small leaf asiatic pennywort (D), water spinach (E), shoot tip of red water spinach (F), stem of red water spinach (G), eggplant (H), cockroach berry (I), common asiatic weed (J) and tomato (K) by HCl pH 1 (a) and human pancreatic α-amylase (b). Bars with different superscripts (a,b,c,d,e) indicate significant differences ($P < 0.05$).

From the primary screening experiments, the results showed that all the crude extracts resisted to both acid and enzymatic hydrolysis with the indigestible carbohydrate content more than 80% (Figure 7). Therefore, all extracts were carried on to the secondary screening.

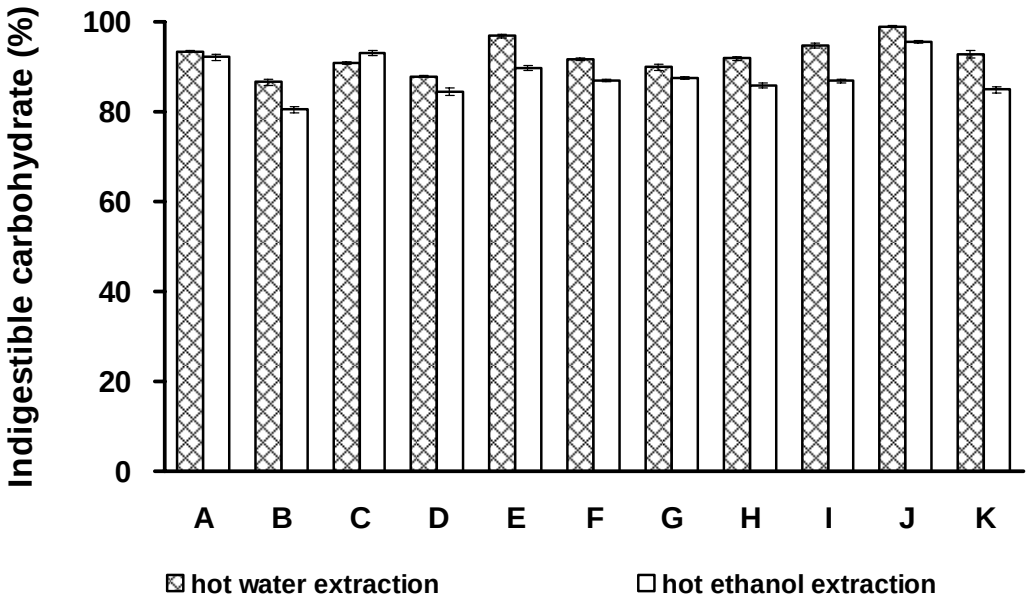


Figure 7. Percentage indigestible carbohydrate content of crude extracts from galanga (A), white turmeric (B), big leaf asiatic pennywort (C), small leaf asiatic pennywort (D), water spinach (E), shoot tip of red water spinach (F), stem of red water spinach (G), eggplant (H), cockroach berry (I), common asiatic weed (J) and tomato (K).

3. Secondary screening

3.1 Antibacterial activity of the extracts

The antibacterial activities of the extracts against probiotics (*L. acidophilus* TISTR 450, *L. plantarum* TISTR 875 and *B. bifidum* DSM 20456) were evaluated. All extracts did not inhibit growth of three strains of probiotic bacteria, when they were applied at the level of 1% of total sugar content. These outcomes were not same as the relative researches due to the difference of extraction; water and 50% ethanol extraction with high temperature (80 °C) versus 100% ethanol extraction at room temperature as used in the research work of Oonmetta-aree and co-workers (2006). These results might be due to the difference type and concentration of solvent gave the different soluble substances and

their functionalilies. They found that many extracts from edible plants and herbs usually have been reported to contain antimicrobial substances. Essential oils from both fresh and dried rhizomes of galanga have antimicrobial activities against bacteria, fungi, yeast and parasite. The major constituent of galanga extracted with 100% ethanol at room temperature for overnight was essential oil D,L-1'-ACA (Acetoxychavicol acetate), a compound isolated from an n-pentane/diethyl ether-soluble extract of dried rhizomes, is active against some bacteria and many dermatophyte species, which was found in some plants in the Zingiberaceae family, especially galangal. This galanga extract had the greatest inhibitory effect against *S. aureus* among ginger, turmeric and krachai. This report related with the previous report, which found that some Gram-positive bacteria and yeast were susceptible to the extract, especially Staphylococcal species. Terpinen-4-ol, one of the monoterpenes in the essential oil from fresh galangal rhizomes, contains an antimicrobial activity against *Trichophyton mentagrophytes* (Janssen and Scheffer, 1985).

3.2 Effects of the extracts on probiotic growth

This step was performed to screen for the extracts which had the best results in promoting growth of all three probiotic strains (*L. acidophilus* TISTR 450, *L. plantarum* TISTR 875 and *B. bifidum* DSM 20456). The growth of three probiotic strains in minimal medium containing 1% (w/v) based on total sugar of water and ethanolic extract (as carbon sources) were expressed as the maximum specific growth rate (μ_{max}) compared to growth occurred in the presence of 1% glucose as a control treatment.

Growth of *Lactobacillus acidophilus* TISTR 450 in the presence of water extract from galanga as a carbon source exhibited highest μ_{max} of 0.195 h^{-1} ($p < 0.05$) followed by ethanolic extract from white turmeric, water extract from white turmeric and ethanolic extract from cockroach berry which exhibited μ_{max} of 0.171, o.143 and 0.135 h^{-1} ($p < 0.05$) respectively. The lowest μ_{max} (0.036 h^{-1}) was obtained in ethanolic extract from red water spinach (stem), whereas water extract from red water spinach (shoot tip) and ethanolic extract from common asiatic weed did not promote growth of *L. acidophilus* (Table 14).

Growth of *Lactobacillus plantarum* TISTR 875 in the presence of water extract from galanga exhibited highest μ_{max} of 0.379 h^{-1} ($p < 0.05$) followed by ethanolic

extract from asiatic pennywort (big leaf), ethanolic extract from cockroach berry and ethanolic extract from white turmeric which the μ_{max} of 0.303, 0.292 and 0.294 h^{-1}($p < 0.05$) respectively. The lowest μ_{max} was found in ethanolic extract from eggplant which the μ_{max} of 0.046 h^{-1}, while water extract from red water spinach (shoot tip) and ethanolic extract from common asiatic weed did not promote growth of *L. plantarum* (Table 14).

Growth of *Bifidobacterium bifidum* DSM 20456 in the presence of water extract from galanga exhibited highest μ_{max} of 0.129 h^{-1} ($p < 0.05$) followed by water extract from red water spinach (stem part) and ethanolic extract from white turmeric which the μ_{max} of 0.127 and 0.121 h^{-1}($p < 0.05$) respectively. The lowest μ_{max} was found in water extract from asiatic pennywort (big leaf) which the μ_{max} of 0.025 h^{-1}, whereas ethanolic extract from galanga, water extract from white turmeric, water extract from red water spinach (shoot tip), ethanolic extract from eggplant, ethanolic extract from common asiatic weed and water extract from tomato did not promote growth of *B. bifidum* (Table 14). The results of probiotic on *B. bifidum* growth were similar to the experimentation of Hopkins *et al.* (1998). They used eight commercial prebiotic products as carbon sources, which were Galacto-oligosaccharides: 85% oligogalactose, small amounts of lactose, glucose and galactose; Oligomate 55: predominantly galactosyllactose, degree of polymerization (DP) 2-5 (55% pure); Raftilose LS: inulin DP 10-12 (99% oligosaccharides); Raftilose L60: fructo-oligosaccharides (60%); Raftilose P95: fructo-oligosaccharides, DP 2-8 (95%); Pyrodextrins: complex mixture of glucose oligosaccharides; Soya-oligosaccharides: mixture of raffinose (fructose, galactose, glucose) and stachyose (fructose, galactose, galactose, glucose); Xylo-oligosaccharides: xylose containing oligosaccharides, DP 2-4 (70% pure) to promote growth of *B. bifidum* NCFB 2203. Results showed that Oligomate 55, Xylo-oligosaccharides, Raftilose P95, Galacto-oligosaccharides, Raftilose L60 and Soya-oligosaccharides supported the best growth of the test bacteria with the specific growth rate as 0.29, 0.24, 0.22, 0.20, 0.19 and 0.17 h^{-1} respectively. While pyrodextrins and Raftilose LS did not promoted growth of the test strain. This might be due to *B. bifidum* sequentially utilized the carbohydrates in the order glucose > galactose > mannose, with inhibition of pentose uptake. In addition, growth was often found to be better on oligosaccharides in comparison to their component monosaccharides, supporting previous suggestions that some bifidobacterial species may be particularly adapted to growth on oligosaccharides in the large gut (Macfarlane *et al.*, 2008).

The water extract from galanga and the ethanolic extract from white turmeric were the best carbon sources for growth of all probiotic strains tested with the significant μ_{max} values ($p < 0.05$) compared to glucose as shown in Table 14.

From overall probiotic growths of using each extract as a carbon source, water extract from galanga showed the best carbon source for all probiotic strains, whereas ethanolic extract from white turmeric was also considered as the best utilized substrate due to its property on enhancing the probiotic growth. The reason why *L. plantarum* TISTR 875 capable to grow well in many extracts when compared to other two probiotic strains might be due to *L. plantarum* had the coding capacity for the uptake and utilization of many different sugars. *L. plantarum* exhibited various enzymes correlated with the activities on carbohydrate catabolism such as α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase and α-mannosidase. The large number of surface anchored proteins suggested that *L. plantarum* had the potential to associate with many different surfaces and potential substrates for growth. In addition, the relatively high number of genes encoding regulatory functions indicated the ability to adapt too many different conditions (de Vries *et al.*, 2006; Papamanoli *et al.*, 2003). All together this reflected the potential of *L. plantarum* to grow in a large range of environmental niches. The results of low growth and non-growth promoting when using each extract as carbon sources in the experiments indicated that these maybe due to the incapability of this probiotic to used the extracts as carbon sources.

Table 11. Maximum Specific Growth Rate for *L. acidophilus* TISTR 450, *L. plantarum* TISTR 875 and *B. bifidum* DSM 20456 (h^{-1}) on medium containing 1% (w/v) of different carbon sources.

Carbon sources	Maximum specific growth rate (h^{-1})					
	L. acidophilus		*L. plantarum*		*B. bifidum*	
	Water	50% EtOH	Water	50% EtOH	Water	50% EtOH
Galanga	0.195*+	0.108	0.379*+	0.167	0.129*+	nd
White turmeric	0.143*	0.171*+	0.179	0.294*+	nd	0.121*+
Asiatic pennywort (big leaf)	0.073*	0.050	0.103*	0.303*+	0.025*	0.071
Asiatic pennywort (small leaf)	0.086*	0.038	0.210	0.273*+	0.098	0.066
Red water spinach (shoot tip part)	nd	0.062	nd	0.194	nd	0.052*
Red water spinach (stem part)	0.115	0.036	0.085*	0.167	0.127*+	nd
Eggplant	0.109	0.133*	0.211	0.046*	0.057*	0.031*
Cockroach berry	0.129	0.135*	0.240	0.292*+	0.066	0.029*
Common asiatic weed	0.138	nd	0.164	nd	0.029*	nd
Tomato	0.111	0.089	0.201	0.155	nd	0.039*
Glucose (control)	0.162		0.205		0.095	

[a]Not determined

*Indicates significantly different ($p < 0.05$) from the control (comparisons are made only with the control).

+Indicates significantly different ($p < 0.05$) from the same extraction treatment and probiotic bacteria.

4. Effects of partially purified extracts on pathogen inhibition by probiotic bacteria

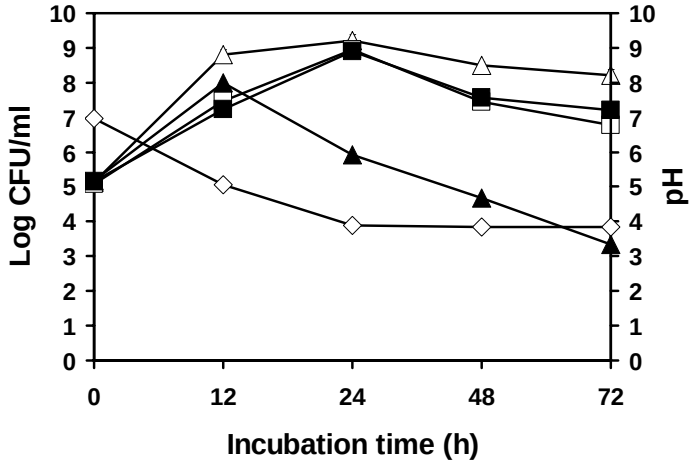
Inhibitory effects of *L. acidophilus* TISTR 450, *L. plantarum* TISTR 875 and *B. bifidum* DSM 20456 was evaluated by co-incubating the probiotic strains together with either *E. coli* or *S. aureus* in the presence of 1% (w/v) partially purified water extract from galanga and ethanolic extract from white turmeric as carbon sources. The probiotic strain was inoculated simultaneously with the pathogen at initial cell concentration of 10^5 CFU/ml. The bacterial counts of both pathogens and probiotic were evaluated at 0, 12, 24, 48 and 72 h of co-incubation.

In mixed culture system, water extract from galanga and ethanolic extract from white turmeric enhanced the inhibitory effects of *L. acidophilus* and *L. plantarum* against *E. coli* and *S. aureus* (Figure 8 and 9). *S. aureus* was completely inhibited when cultivated together with *L. plantarum* after 48 h (Figure 9a). In contrast, no significant difference of reduction in pathogen population (compared to pathogen alone) was observed for both *E. coli* and *S. aureus* when co-cultivation with *B. bifidum* was performed.

L. acidophilus reduced numbers of *E. coli* and *S. aureus* from 9.207 log CFU/ml to 5.927 log CFU/ml and from 9.067 log CFU/ml to 5.503 log CFU/ml, respectively when water extract from galanga was applied as carbon source after 24 h incubating: a decrease of 3.280 log CFU/ml and 3.564 log CFU/ml was observed for *E. coli* and *S. aureus*, respectively (Figure 8a, 9a and Appendix A; Table 17). After 48 h, *E. coli* and *S. aureus* were reduced by 3.836 log CFU/ml and 3.148 log CFU/ml compared to the controls (*E. coli* and *S. aureus* alone). 4.873 log CFU/ml and 3.513 log CFU/ml of *E. coli* and *S. aureus* decline in growth compared to controls (*E. coli* and *S. aureus* alone) was observed at 72 h. The co-incubating in minimal medium containing ethanolic extract from white turmeric demonstrated inhibiting capability of *L. acidophilus* to the growth of the pathogens. After 24 h, *E. coli* and *S. aureus* were diminished by 3.117 log CFU/ml and 3.323 log CFU/ml respectively (Figure 10a and 11a and Appendix A; Table 17). *E. coli* and *S. aureus* count had decreased by about 4.430 log CFU/ml and 3.618 log CFU/ml values at 72 h which had the inhibitory effect better than using glucose as a carbon source (Figure 12a and 13a and Appendix A; Table 17). This is in agreement with the research work of Drago *et al.* (1997) which exhibited the capability of *L. acidophilus* B21190 to inhibit pathogens in co-culture experiments. They observed that

Lactobacillus strain B21190 had an effective to inhibit the growth of *E. coli* inoculated simultaneously.

a)



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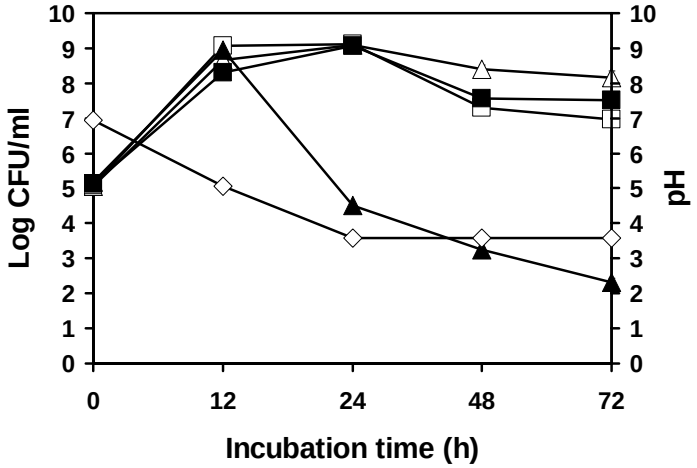

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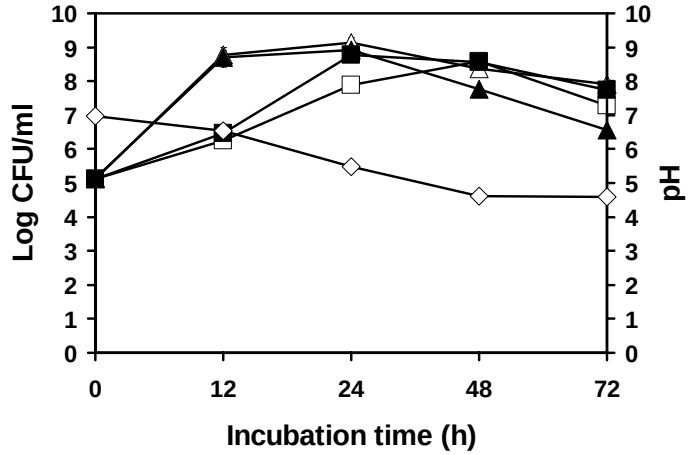


Figure 8. Growth of *E. coli* co-cultivated with probiotics; a) *L. acidophilus* TISTR 450; b) *L. plantarum* TISTR 875 and c) *B. bifidum* DSM 20456 in the presence of water extract from galanga as a carbon source (△ *E. coli* alone, ▲ *E. coli* in co-culture, □ probiotic alone, ■ probiotic in co-culture and ◇ pH)

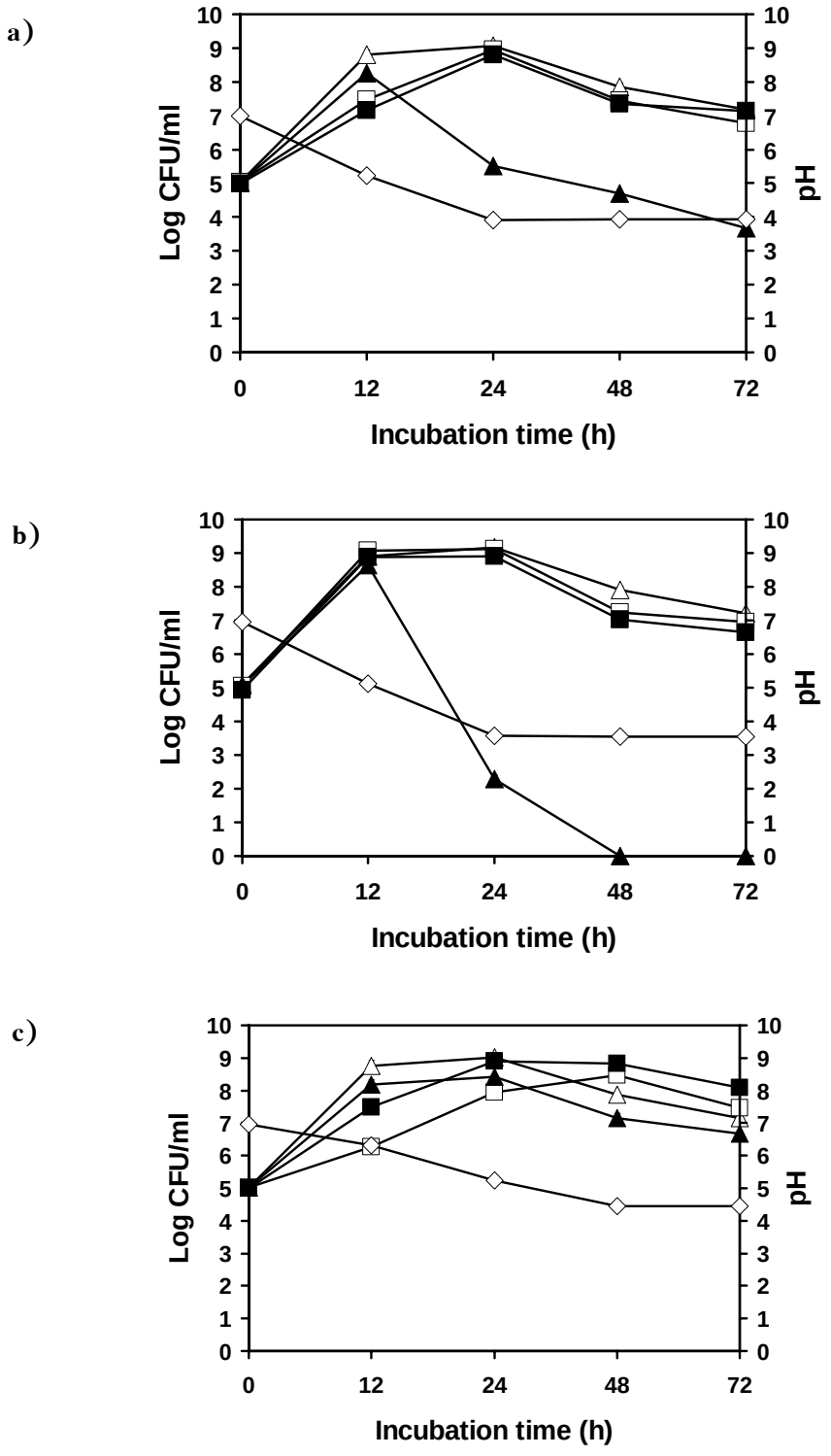
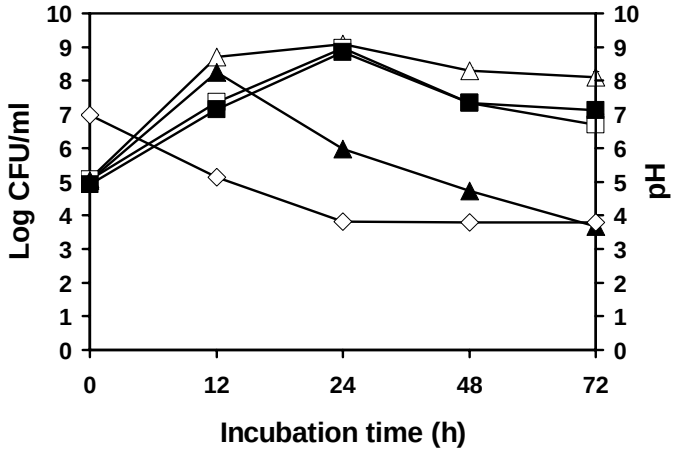


Figure 9. Growth of *S. aureus* co-cultivated with probiotics; a) *L. acidophilus* TISTR 450; b) *L. plantarum* TISTR 875 and c) *B. bifidum* DSM 20456 in the presence of water extract from galanga as a carbon source (△ *S. aureus* alone, ▲ *S. aureus* in co-culture, □ probiotic alone, ■ probiotic in co-culture and ◇ pH).

a)



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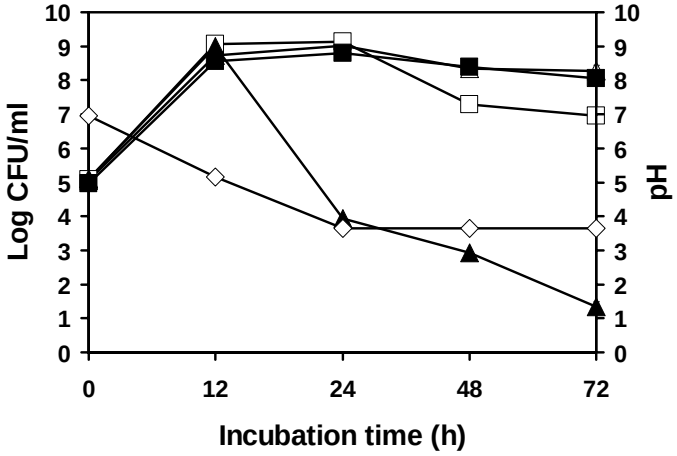

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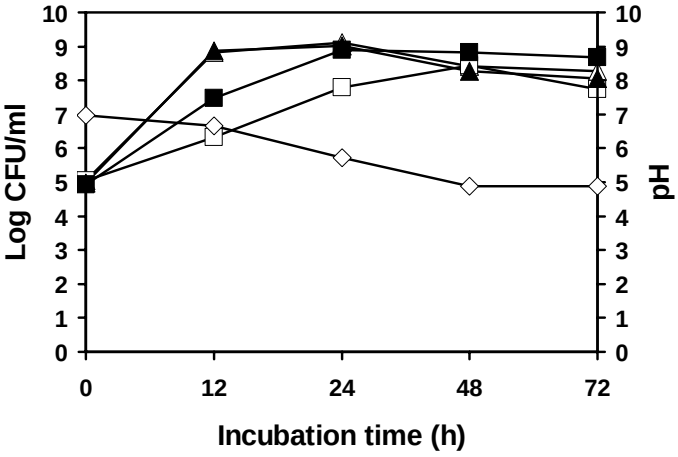
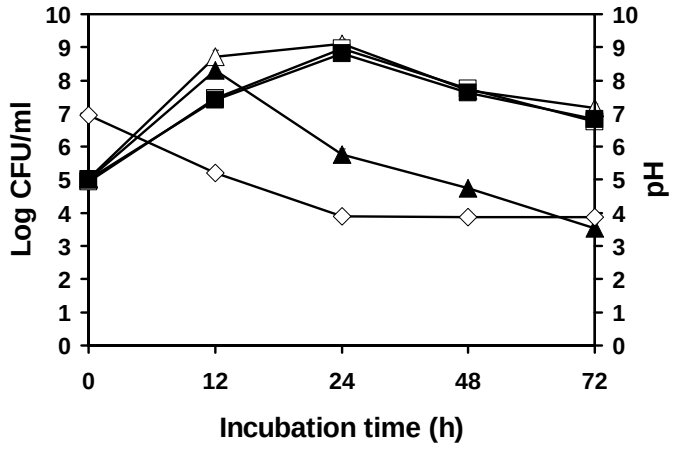


Figure 10. Growth of *E. coli* co-cultivated with probiotics; a) *L. acidophilus* TISTR 450; b) *L. plantarum* TISTR 875 and c) *B. bifidum* DSM 20456 in the presence of ethanolic extract from white turmeric as a carbon source (△ *E. coli* alone, ▲ *E. coli* in co-culture, □ probiotic alone, ■ probiotic in co-culture and ◇ pH).

a)



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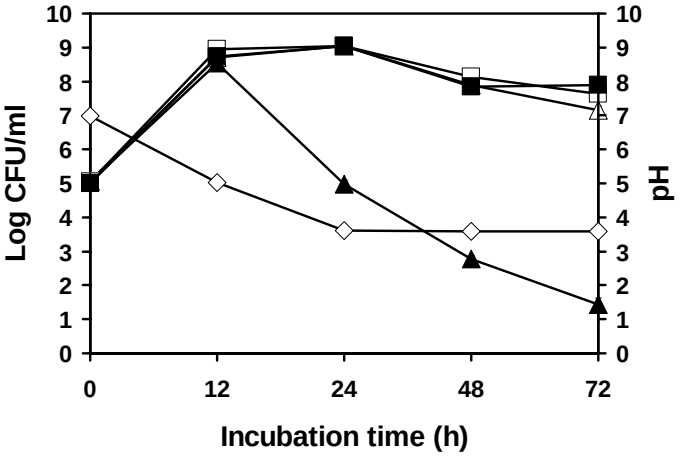

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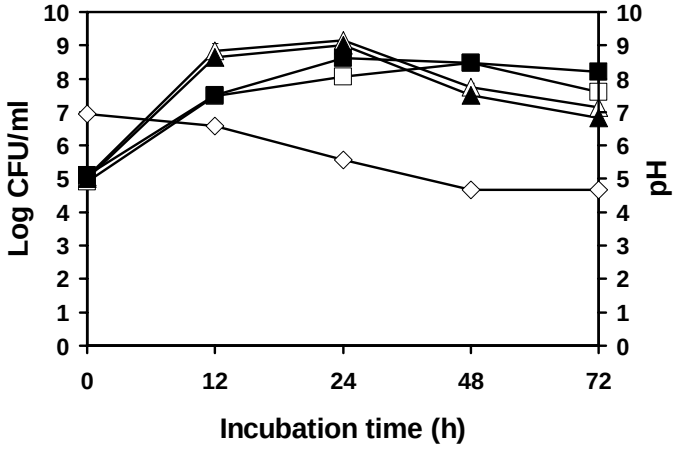


Figure 11. Growth of *S. aureus* co-cultivated with probiotics; a) *L. acidophilus* TISTR 450; b) *L. plantarum* TISTR 875 and c) *B. bifidum* DSM 20456 in the presence of ethanolic extract from white turmeric as a carbon source (△ *S. aureus* alone, ▲ *S. aureus* in co-culture, □ probiotic alone, ■ probiotic in co-culture and ◇ pH).

L. plantarum showed effective in inhibiting the growth of the harmful microorganisms with water extract from galanga as carbon source. Growth of *E. coli* and *S. aureus* was reduced by 4.583 log CFU/ml and 6.878 log CFU/ml compared to the controls (*E. coli* and *S. aureus* alone) after 24 h (Figure 8b and 9b and Appendix A; Table 18). *L. plantarum* was able to completely inhibit the growth of *S. aureus* at 48 h which had the inhibitory effect better than using glucose, non-complex substrate, as a carbon source (Figure 12b and 13b and Appendix A; Table 18). This result showed the concurrence to the recent experiment conducted by Millette *et al.* (2007) suggesting that 84.7% of *S. aureus* was inhibited due to the antimicrobial effect of both organic acids and bacteriocin-like substances produced by *L. acidophilus* and *L. casei* in fermented milk. The co-culturing in minimal medium containing ethanolic extract from white turmeric revealed the competence of *L. plantarum* to inhibit the growth of *E. coli* and *S. aureus*. At 24 h, *E. coli* and *S. aureus* decreased 5.080 log CFU/ml and 4.067 log CFU/ml respectively (Figure 9b and 10b and Appendix A; Table 18). The reduction of 6.923 log CFU/ml and 5.733 log CFU/ml of *E. coli* and *S. aureus* were shown at 72 h of incubation. The growth of pathogen in co-cultured between *L. plantarum* and *E. coli* were similar to the experimentation of Fook and Gibson (2003). They monitored the inhibition of *E. coli* inoculated with *L. plantarum* 0407 in minimal medium containing 1% (w/v) of oligofructose (FOS) and found that *E. coli* count had decreased by about 5 log CFU/ml after 24 h, where pH control was not employed.

In contrast, no significant difference ($P < 0.05$) was observed in growth of both *E. coli* and *S. aureus* compared to the control (*E. coli* and *S. aureus* alone) when co-incubating with *B. bifidum* DSM 20456 in minimal medium containing with galangal extract and white turmeric extract (Figure 8c, 9c, 10c and 11c and Appendix A; Table 19). The growth of *E. coli* and *S. aureus* was reduced slightly by only 0.212 log CFU/ml and 0.683 log CFU/ml compared to the controls (*E. coli* and *S. aureus* alone) when water extract from galanga was applied as a carbon source after 24 h (Figure 8c and 9c and Appendix A; Table 19). The highest decreasing were found in *S. aureus* (0.722 log CFU/ml) and *E. coli* (1.337 log CFU/ml) after 48 h and 72 h of inoculation respectively. As well as, using ethanolic extract from white turmeric as a carbon source, The highest reducing were found in *E. coli* (0.210 log CFU/ml) and *S. aureus* (0.317 log CFU/ml) after 72 h of inoculation (Figure 10c and 11c and Appendix A; Table 19). The results were related to the studies of Fook and Gibson (2003). They observed that the *E.*

coli count reduced by 1 log value in the presence of *B. bifidum* Bb12 and a mixture of 1% oligofructose and xylo-oligosaccharides (50:50 w/w) in non-pH controlled which was a small reduction. The inability to inhibit *E. coli* may be attributed to being species dependent, since a study by Gibson and Wang (1994) established other bifidobacterial species, such as *B. longum* and *B. infantis* as being more inhibitory than *B. bifidum.*

From the results of this study, the successful of lactic acid bacteria (*L. acidophilus* TISTR 450 and *L. plantarum* TISTR 875) on inhibition of pathogens (*E. coli* and *S. aureus*) in this study, as shown in Figure 8 to 11 and Appendix A; Table 17 to 18, might be due to many mechanisms e.g., production of antibacterial compounds, competition for nutrients and competition for colonization sites (Nousiainen and Setala, 1993). The production of antibacterial substances such as lactic acid and hydrogen peroxide by all lactic acid bacteria (homofermentative and heterofermentative), which have been reported as well as other antimicrobial substances, for examples, acetic acid, alcohols and carbon dioxide, were by-products of heterofermentative lactic acid bacteria (Axelsson, 1993; Salminen, *et al.,* 1993). Weak organic acids, particularly lactic acid and acetic acid, were important compounds, inhibiting a broad range of microorganisms. The inhibitory effect of undissociated organic acids was 10-600 times stronger than that of their dissociated forms; the extent of dissociation was directly determined by the pH. Undissociated acetic acid, citric acid and lactic acid possed high antimicrobial activity. The synergistic inhibitory effects of mixtures of lactic acid and acetic acid have been ascribed to the potentiation of acetic acid at the lower pH produced by lactic acid, shifting the equilibrium to favour the undissociated form of the acids. Acid production is one of the oldest methods used to influence the growth of bacteria (Helander *et al*., 1997).

In this study, the co-cultured of pathogens (*E. coli* and *S. aureus*) with *L. acidophilus* TISTR 450 were also measured lactic acid production which was determined from dropping of pH values. pH in the co-cultivated medium declined slightly at first 12 h after incubation and dropped dramatically as 3.81 to 3.92 at 24 h. After that, pH values were steady until 72 h. The pattern of pH decrease in the media containing water extract from galanga was similar to that in the media containing ethanolic extract from white turmeric as shown in Figure 8a, 9a, 10a and 11a. *L. acidophilus* TISTR 450, classified as

an obligately homofermentative (Axelsson, 1993), showed an effective on inhibition pathogen by producing lactic acid and hydrogen peroxide as antibacterial substances.

In the co-incubating medium with *E. coli* and *S. aureus*, acids production was determined from the pH drop, which decreased gradually at first 12 h and fell rapidly as 3.56 to 3.64 at 24 h. After that, pH values were stable until 72 h. The pattern of pH decline in the media containing water extract from galanga was similar to that in the media containing ethanolic extract from white turmeric (Figure 8b, 9b, 10b and 11b). The successful on pathogen inhibition of *L. plantarum* TISTR 875, classified as an facultatively heterofermentative (Axelsson, 1993) caused by it produced many antimicrobial compounds such as lactic acid, acetic acid, alcohol, carbon dioxide, hydrogen peroxide, benzoic acid, mevalonic acid, methylhydantoin and lactone (Helander *et al*., 1997). Acetic acid, which was also produced, was a stronger antimicrobial agent than lactic acid and might play a role in the ability of *L. plantarum* to combat harmful microorganisms more efficient than *L. acidophilus*. This difference appeared to be due more to the quantity of undissociated acid than to the type of acid. The pK_a of acetic acid was 4.76, whereas that of lactic acid was 3.86. Acetic acid 8.4% and lactic acid 1.1% were presented in an undissociated form at an intestinal pH of 5.8 (Mogensen, 2000; Ballongue, 1993). The undissociated form of the organic acid enters the bacterial cell and dissociates inside its cytoplasm. The eventual lowering of the intracellular pH or the intracellular accumulation of the ionized form of the organic acid lead to the death of the pathogen (Makras and De Vuyst, 2006). As a result, *L. plantarum* co-incubated with pathogens illustrated more inhibition of pathogens than *L. acidophilus*.

On the other hand, the co-culturing between *B. bifidum* DSM 20456, heterofermentative bacteria, and two strains of pathogens found that pH values declined slightly at first 12 h after incubation and dropped as 5.71 to 5.24 at 24 h. After that, pH values were dropped leastwise until 72 h. The pattern of pH drop in the media containing water extract from galanga was similar to that in the media containing ethanolic extract from white turmeric as demonstrated in Figure 8c, 9c, 10c and 11c.

These experiments also gave information on the capability of intestinal species of bacteria to utilize the oligosaccharides. However, this study could not predict

about the co-cultured *in vivo*, since this would depend on various factors such as the availability of other substrates, growth factors, intestinal pH, actual number of bacteria, and the interactions between the different species of bacteria present. *E. coli* and *S. aureus*, representative of Gram-negative and Gram-positive pathogens in intestinal in this study, were also able to utilize non-complex substrate such as monosaccharide residue from both galanga and white turmeric extracts as carbon sources. Lactobacilli and bifidobacteria were known that they were able to utilize the structurally complex oligosaccharides as composed in the extracts. When monosaccharide components of the extracts were completely utilized, they were capable to compete with *E. coli* and *S. aureus* for limiting nutrients, produce antibacterial substances, especially organic acids to inhibit the pathogens and thus capable to survive even in low pH condition. It might be due to their resistance to low pH ability (Kaplan and Hutkins, 2000). The comparisons between using glucose, non-complex substrate, as a carbon source and the extracts, which included complex substrates, containing in medium for co-culturing studies were shown in Figure 12 to 15. From the results, it could concluded that both probiotic and pathogens could utilized glucose, but only probiotic were capable to utilized the complex substrate, oligosaccharides, thus probiotic in the co-incubating with galanga and white turmeric extracts had more effectiveness to reduce the growth of pathogens than the co-incubating with glucose as a carbon source. This experiment related with the previous experiment of Van Laere *et al.* (2000), which found that the *Bacteroides* spp., predominant intestinal bacteria, were also able to utilize plant cell wall derived oligosaccharides besides their reported activity toward plant polysaccharides. Bifidobacteria were able to utilize the more complex structure of oligosaccharides derived from plant cell wall polysaccharides in addition to the bifidogenic oligosaccharides such as fructooligosaccharides and xylooligosaccharides. Furthermore, they suggested that fermentation of a specific substrate was not always accompanied with a drop in pH, which could explain why the pH dropped not much in the co-cultured of *B. bifidum* and pathogens, although the probiotic was able to grow well in the medium containing galanga and white turmeric extracts as carbon sources. This supported the conclusions of Barry *et al.* (1995) that a pH drop should not be used as an index for fermentation.

From the studies on inhibition of *E. coli* or *S. aureus* in the presence of *L. acidophilus* TISTR 450, *L. plantarum* TISTR 875 and *B. bifidum* DSM 20456 in medium broth contained 1% (w/v) water extract from galanga and ethanolic extract from white turmeric as carbon sources, the results showed that the co-incubating of *S. aureus* and *L.*

plantarum TISTR 875 with galanga extract as a carbon source exhibited the completely inhibition of *S. aureus* after 48 h. As this result, the combination between *L. plantarum* TISTR 875 and galanga extract as a synbiotic would be interesting to study on the inhibiting of gram positive pathogens especially *S. aureus* in the further experiment.

5. Characterization of the of prebiotic compounds in the selected extracts

5.1 Molecular weight distribution of the selected extracts using GPC (Gel Permeation Chromatography)

Sediment parts and supernatant parts from partial purification by 80% chilled ethanol of water extract from galanga and ethanolic extract from white turmeric were determined molecular weight components. The sediment parts from galanga had components of average molecular weights (Mw) of 1524, 791, 549, 230 and 135 dalton with the percent area of 2.4, 8.55, 5.20, 62.87 and 20.63% respectively (Table 15). The composition in supernatant parts from galanga also had molecular weights of 755, 528, 260, 183 and 134 dalton with the percent area of 1.00, 0.98, 36.35, 28.62 and 33.06% respectively (Table 15). From the results, it could be concluded that water extract from galanga after partial purification was composed of the oligosaccharides with the highest degree of polymerization (DP) of 9. The sediment parts from white turmeric had average molecular weights of 2600, 1527, 1030, 751, 493, 279, 186 and 135 dalton with the percent area of 2.83, 27.10, 11.75, 18.34, 7.31, 13.90, 7.90 and 10.87% respectively (Table 15). The composition in supernatant parts from white turmeric also had average molecular weight of 1336, 770, 528, 273, 185 and 134 dalton with the percent area of 0.86, 3.42, 6.37, 31.02, 24.62 and 33.70% respectively (Table 15). It might be summarized that ethanolic extract from white turmeric after partial purification was composed of the oligosaccharides with the DP up to 15 (Mw 2600). These results showed that high ethanol concentration (80%) precipitation at low temperature of galanga extract obtained the DP range between 2 to 10, which was classified the as oligosaccharides. The sediment part of white turmeric extract also exhibited the low levels of high-molecular-mass (DP > 10). The observations were in agreement with the experiments of Gelders *et al.* (2003). They were able to fractionate starch hydrolysates with ethanol (final concentration 30-80% at 6 °C) into dextrin fractions with a relatively narrow molecular

mass distribution, which allow for detection of low levels of high-molecular-mass dextrins. In contrast, high portion of small molecular weight substances in the extracts of this study might be due to partial purification of extracts by 80% chilled ethanol was performed in one time without dissolution the sediment again for partial purification. Therefore, small molecular weight substances such as monosaccharide and disaccharides remained in the extract with the high amount.

Table 12. Molecular weight distribution of the selected extracts

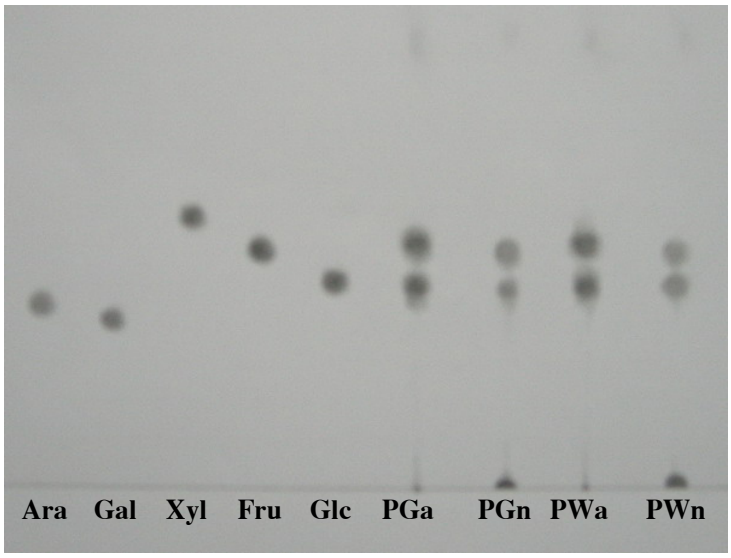
Extracts	Mw (Dalton)	Area (%)
Galanga (sediment part)	1524	2.74
	791	8.55
	549	5.20
	230	62.87
	135	20.63
Galanga (supernatant part)	755	1.00
	528	0.98
	260	36.35
	183	28.62
	134	33.06
White turmeric (sediment part)	2600	2.83
	1527	27.10
	1030	11.75
	751	18.34
	493	7.31
	279	13.90
	186	7.90
	135	10.87
White turmeric (supernatant part)	1336	0.86
	770	3.42
	528	6.37
	273	31.02
	185	24.62

Table 12. Molecular weight distribution of the selected extracts (cont.)

Extracts	Mw (Dalton)	Area (%)
White turmeric (supernatant part)	134	33.70

5.2 Determination of sugar composition

Two sediment parts of extracts (galangal and white turmeric) hydrolyzed with 2 N TFA and two non-hydrolyzed sediment parts of extracts were performed TLC test which was applied with three solvent systems; ethyl acetate : isopropyl alcohol : water (3:1:1 v/v/v), $CHCl_3$: methanol : water (6.5:4:1 v/v/v) and ethyl acetate : methanol : water : acetic acid (6.5:2:1.5:1.5 v/v/v). From Figure 12, R_f values of sediment part of hydrolyzed galanga extract (PGa), sediment part of galanga extract (PGn), sediment part of hydrolyzed white turmeric extract (PWa) and sediment part of white turmeric extract (PWn) after applying three solvent systems were 0.39 and 0.48, 0.39 and 0.47, 0.40 and 0.48, 0.40 and 0.47, respectively. R_f values from these extracts were compared with standard sugars (arabinose, galactose, xylose, glucose and fructose) which had the R_f values of 0.36, 0.33, 0.53, 0.47 and 0.40, respectively. The R_f values of PGa and PWa were closed to the R_f values of glucose and fructose, therefore, it might be concluded that glucose and fructose were major sugar components present in the partially purified extracts from galangal and white turmeric.



R_f of galactose (Ara) = 0.36 R_f of xylose (Xyl) = 0.53

R_f of arabinose (Gal) = 0.33 R_f of fructose (Fru) = 0.47

R_f of glucose (Glc) = 0.40

R_f of sediment part of galanga extract hydrolyzed with 2 N TFA (PGa) = 0.39 and 0.48

R_f of sediment part of galanga extract (PGn) = 0.39 and 0.47

R_f of sediment part of white turmeric extract hydrolyzed with 2 N TFA (PWa) = 0.40 and 0.48

R_f of sediment part of white turmeric extract (PWn) = 0.40 and 0.47

Figure 12. TLC chromatograms of sugar composition after applied with three solvent systems; ethyl acetate : isopropyl alcohol : water (3:1:1 v/v/v), $CHCl_3$: methanol : water (6.5:4:1 v/v/v) and ethyl acetate : methanol : water : acetic acid (6.5:2:1.5:1.5 v/v/v), from sediment part of galanga extract hydrolyzed with 2 N TFA (PGa), sediment part of galanga extract (PGn), sediment part of ethanolic extract from white turmeric hydrolyzed with 2 N TFA (PWa) and sediment part of white turmeric extract (PWn) used glucose (Glc), fructose (Fru), xylose (Xyl), arabinose (Ara) and Galactose (Gal) and as standard sugars.

CHAPTER 4

CONCLUSION

Nine edible plants were dried at 50 C° and then were separated into two parts for extraction in two different mixture conditions. The first part was extracted twice in hot water (80°C) and the another part was extracted twice in (50%, v/v) ethanol at 80 °C. After freeze-drying, the highest yield was found in water extract from tomato (61.03 %dry weight) followed by ethanolic extract from tomato, ethanolic extract from cockroach berry and water extract from red water spinach (shoot tip) which were 43.55, 30.84 and 30.60 %dry weight respectively. Ethanolic extract from white turmeric had a lowest percent yield of crude extract which was 11.03 %dry weight. All twenty-two extracts were highly resisted to the digestion of both acid and human pancreatic α-amylase with the indigestible carbohydrate content more than 80.0%. Only water extract of galanga and ethanolic extract of white turmeric were the best carbon source for growth of *Lactobacillus acidophilus* TISTR 450, *Lactobacillus plantarum* TISTR 875 and *Bifidobacterium bifidum* DSM 20456 with the highest maximum specific growth rate (μ_{max}) of 0.195, 0.379 and 0.129 h^{-1}, respectively (in the presence of galanga extract) and 0.171, 0.294 and 0.121 h^{-1}, respectively (in the presence of white turmeric extract), whereas μ_{max} of these probiotic grown in glucose were 0.162, 0.205 and 0.095 h^{-1} respectively. *Escherichia coli* and *Staphylococcus aureus* were inhibited by *L. plantarum* and *L. acidophilus* but not *B. bifidum* in the presence of partially purified galanga and white turmeric extracts. At 48 h, growth of *S. aureus* was completely inhibited by *Lactobacillus plantarum* TISTR 875 in the presence of galanga extract. The combination between *L. plantarum* TISTR 875 and galanga extract as a synbiotic would be a potential approach in the inhibition of Gram-positive pathogens especially *S. aureus*. The average molecular weight of galanga (sediment part) was in the range of 1524 to 135 dalton and the average molecular weight of white turmeric (sediment part) was in the range of 2600 to 135 dalton. Both partially purified of water extract from galanga and ethanolic extract from white turmeric were composed of glucose and fructose.

The results from this study might be useful to other researchers to study more about the potential of combination between the selected probiotic strain (*Lactobacillus*

plantarum TISTR 875) and selected extract (water extract from galanga) into symbiotic form. Furthermore, if this synbiotic has been proofed that it has a potential to promoting the better health in human, it could up-scale to produce in mass production and contribution to the global world. This may benefit to both producers to add value on the raw material (edible plant) and the consumer's health. This study may be magnetizing people to seek and study on plenty of plants all around the world for their prebiotic properties.

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APPENDIX A

Table 13. Percent yield of water extracts and ethanolic extracts.

Type of plants	Yield (% dry weight)	
	Hot water	Hot 50% EtOH
Galanga	19.67	16.42
White turmeric	13.58	11.03
Asiatic pennywort (big leaf)	14.84	20.35
Asiatic pennywort (small leaf)	12.75	27.20
Water spinach	19.39	15.85
Red water spinach (shoot tip part)	30.60	24.68
Red water spinach (stem part)	20.80	19.60
Eggplant	11.81	26.82
Cockroach berry	21.27	30.84
Common asiatic weed	12.62	26.00
Tomato	61.03	43.55

Table 14. The remained indigestible carbohydrate of water and ethanolic extracts from galanga, white turmeric, asiatic pennywort (big leaf), asiatic pennywort (small leaf), water spinach, red water spinach (shoot tip part), red water spinach (stem part), eggplant, cockroach berry, common asiatic weed and tomato.

	Indigestible carbohydrate (%)	
Extracts	Hot water	Hot 50% EtOH
Galanga	93.46±0.04	92.11±0.62
White turmeric	86.54±0.62	80.44±0.81
Asiatic pennywort (big leaf)	90.83±0.34	93.09±0.49
Asiatic pennywort (small leaf)	87.91±0.18	84.44±0.76
Water spinach	96.85±0.34	89.70±0.52
Red water spinach (shoot tip part)	91.67±0.34	86.96±0.18
Red water spinach (stem part)	89.91±0.60	87.39±0.29
Eggplant	91.81±0.49	85.85±0.47
Cockroach berry	94.72±0.51	86.91±0.42
Common asiatic weed	98.96±0.08	95.55±0.24
Tomato	92.77±0.73	84.90±0.78

Table 15. Growth of probiotic used water extract from galanga and ethanolic extract from white turmeric as carbon sources in minimal medium

Probiotic	Extract	Time (h)	Viable count (Log CFU/ml)	
			E. coli	*S. aureus*
L. acidophilus	Galanga	0	5.166±0.127	4.994±0.017
		12	7.233±0.102	7.149±0.083
		24	8.892±0.008	8.805±0.042
		48	7.576±0.083	7.348±0.110
		72	7.209±0.044	7.125±0.035
	White turmeric	0	4.924±0.017	5.015±0.021
		12	7.149±0.083	7.409±0.109
		24	8.845±0.042	8.813±0.099
		48	7.348±0.110	7.616±0.117
		72	7.125±0.141	6.835±0.012
L. plantarum	Galanga	0	5.130±0.122	4.919±0.127
		12	8.296±0.110	8.873±0.058
		24	9.060±0.093	8.915±0.064
		48	7.556±0.035	7.025±0.131
		72	7.519±0.102	6.653±0.045
	White turmeric	0	4.961±0.034	5.003±0.043
		12	8.563±0.035	8.733±0.125
		24	8.796±0.045	9.049±0.074
		48	8.394±0.056	7.841±0.132
		72	8.068±0.055	7.883±0.109
B. bifidum	Galanga	0	5.122±0.002	5.008±0.124
		12	6.472±0.127	7.484±0.043
		24	8.785±0.152	8.895±0.004
		48	8.567±0.050	8.826±0.110
		72	7.745±0.105	8.078±0.065

Table 15. Growth of probiotic used water extract from galanga and ethanolic extract from white turmeric as carbon sources in minimal medium (cont.)

Probiotic	Extract	Time (h)	Viable count (Log CFU/ml)	
			E. coli	*S. aureus*
B. bifidum	White turmeric	0	4.934±0.124	5.102±0.070
		12	7.484±0.043	7.510±0.105
		24	8.895±0.004	8.609±0.004
		48	8.826±0.110	8.473±0.176
		72	8.678±0.065	8.206±0.042

Table 16. Growth of pathogen used water extract from galanga and ethanolic extract from white turmeric as carbon sources in minimal medium

Pathogen	Extract	Time (h)	Viable count (Log CFU/ml)		
			L. acidophilus	*L. plantarum*	*B. bifidum*
E. coli	Galanga	0	5.184±0.159	5.179±0.164	5.134±0.133
		12	7.996±0.006	8.943±0.063	8.704±0.281
		24	5.927±0.013	4.521±0.043	8.916±0.018
		48	4.670±0.016	3.252±0.049	7.754±0.064
		72	3.342±0.028	2.327±0.101	6.570±0.040
	White turmeric	0	5.035±0.153	5.110±0.103	4.986±0.068
		12	8.255±0.030	8.985±0.012	8.873±0.066
		24	5.972±0.003	3.942±0.032	9.013±0.057
		48	4.713±0.022	2.922±0.026	8.260±0.106
		72	3.672±0.129	1.339±0.153	8.059±0.273
S. aureus	Galanga	0	5.015±0.153	5.082±0.081	5.017±0.018
		12	8.255±0.030	8.654±0.122	8.182±0.137
		24	5.503±0.003	2.278±0.119	8.431±0.019
		48	4.713±0.022	0	7.148±0.152
		72	3.672±0.129	0	6.667±0.033

Table 16. Growth of pathogen used water extract from galanga and ethanolic extract from white turmeric as carbon sources in minimal medium (cont.)

Pathogen	Extract	Time	Viable count (Log CFU/ml)		
		(h)	*L. acidophilus*	*L. plantarum*	*B. bifidum*
S. aureus	White turmeric	0	5.024±0.068	5.041±0.075	5.023±0.175
		12	8.310±0.037	8.552±0.044	8.638±0.155
		24	5.772±0.173	4.988±0.120	9.004±0.024
		48	4.756±0.008	2.781±0.091	7.515±0.110
		72	3.545±0.127	1.424±0.204	6.819±0.028

Table 17. Inhibition of in *vitro* growth of pathogens by *L. acidophilus* in the presence of water extract from galanga, ethanolic extract from white turmeric and glucose at 24, 48 and 72 h after inoculation.

Extracts	Incubation time	Pathogens (Log CFU/ml)	
	(h)	*E. coli*	*S. aureus*
Galanga	24	-3.280	-3.564
	48	-3.836	-3.148
	72	-4.873	-3.513
White turmeric	24	-3.117	-3.323
	48	-3.583	-2.955
	72	-4.430	-3.618
Glucose	24	-1.788	-2.125
	48	-2.983	-2.114
	72	-3.466	-3.185

- denotes a decrease of pathogens.

Table 18. Inhibition of in *vitro* growth of pathogens by *L. plantarum* in the presence of water extract from galanga, ethanolic extract from white turmeric and glucose at 24, 48 and 72 h after inoculation.

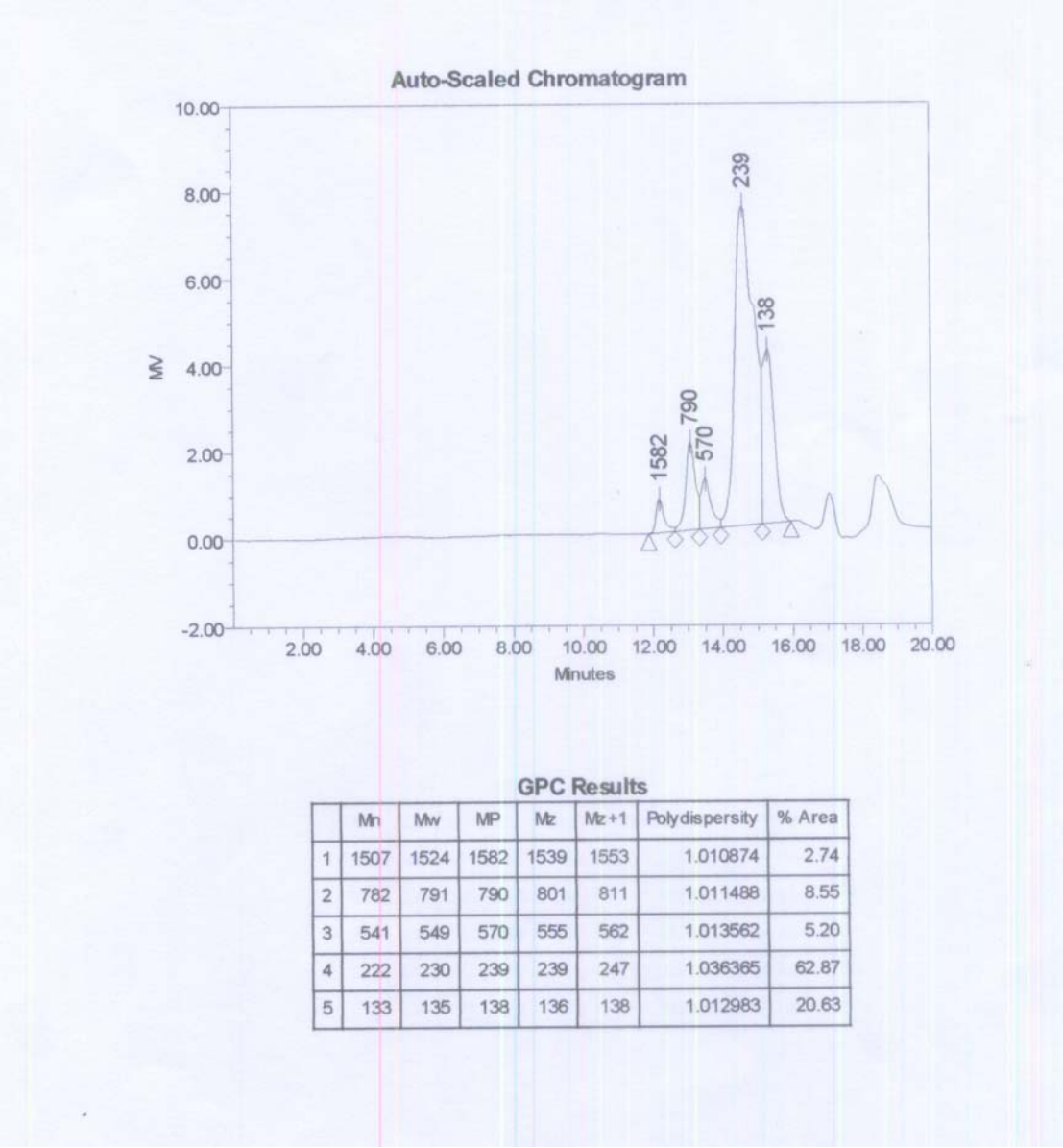
Extracts	Incubation time (h)	Pathogens (Log CFU/ml)	
		E. coli	*S. aureus*
Galanga	24	-4.583	-6.878
	48	-5.153	-7.902
	72	-5.827	-7.218
White turmeric	24	-5.080	-4.067
	48	-5.418	-5.114
	72	-6.923	-5.733
Glucose	24	-2.520	-3.813
	48	-3.426	-4.984
	72	-5.043	-5.087

- denotes a decrease of pathogens.

Table 19. Inhibition of in *vitro* growth of pathogens by *B. bifidum* in the presence of water extract from galanga, ethanolic extract from white turmeric and glucose at 24, 48 and 72 h after inoculation.

Extracts	Incubation time (h)	Pathogens (Log CFU/ml)	
		E. coli	*S. aureus*
Galanga	24	-0.212	-0.683
	48	-0.621	-0.722
	72	-1.337	-0.492
White turmeric	24	-0.096	-0.157
	48	-0.147	-0.223
	72	-0.210	-0.317
Glucose	24	-0.040	-0.102
	48	-0.088	-0.149
	72	-0.176	-0.219

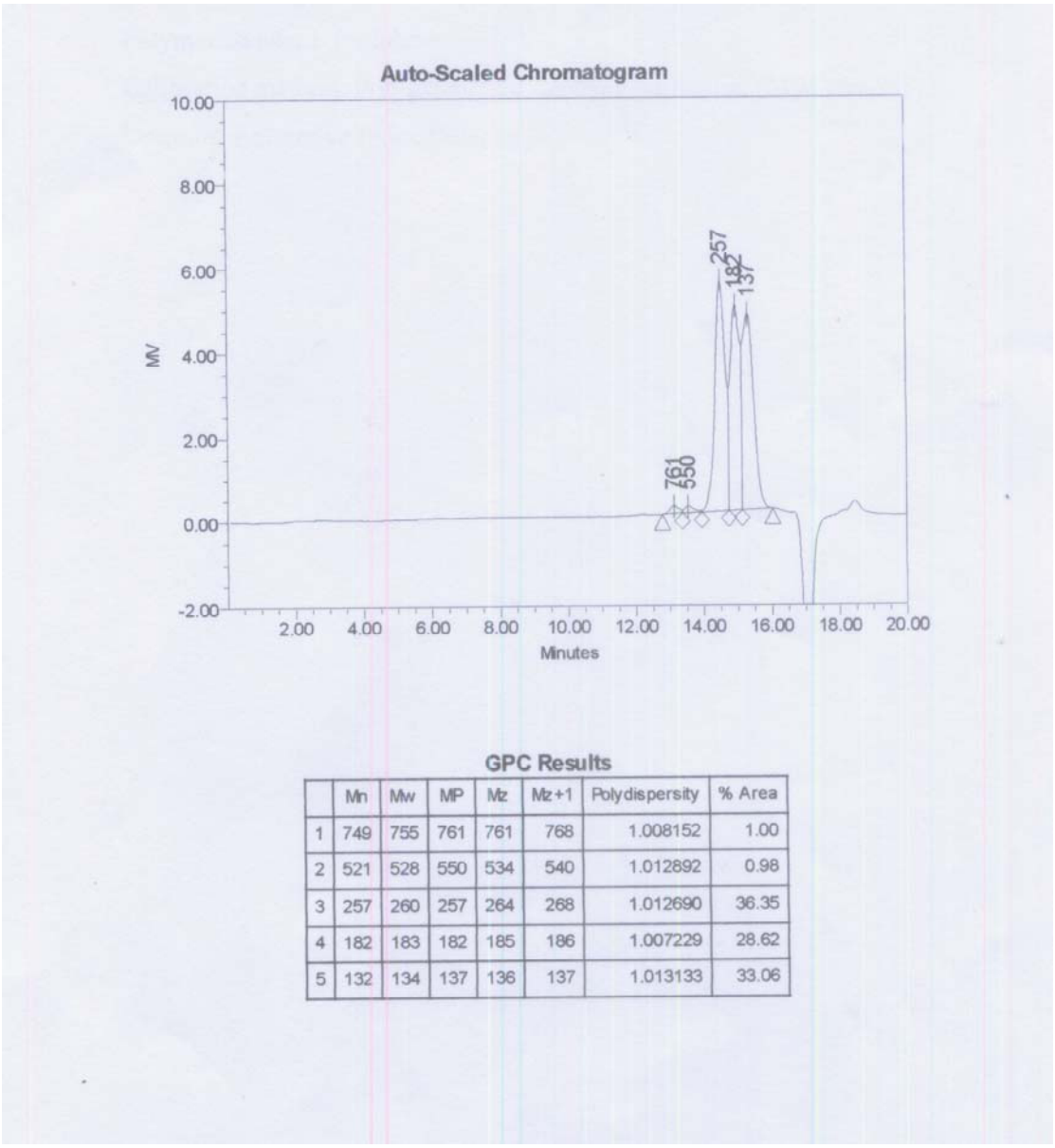
- denotes a decrease of pathogens.



GPC Results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity	% Area
1	1507	1524	1582	1539	1553	1.010874	2.74
2	782	791	790	801	811	1.011488	8.55
3	541	549	570	555	562	1.013562	5.20
4	222	230	239	239	247	1.036365	62.87
5	133	135	138	136	138	1.012983	20.63

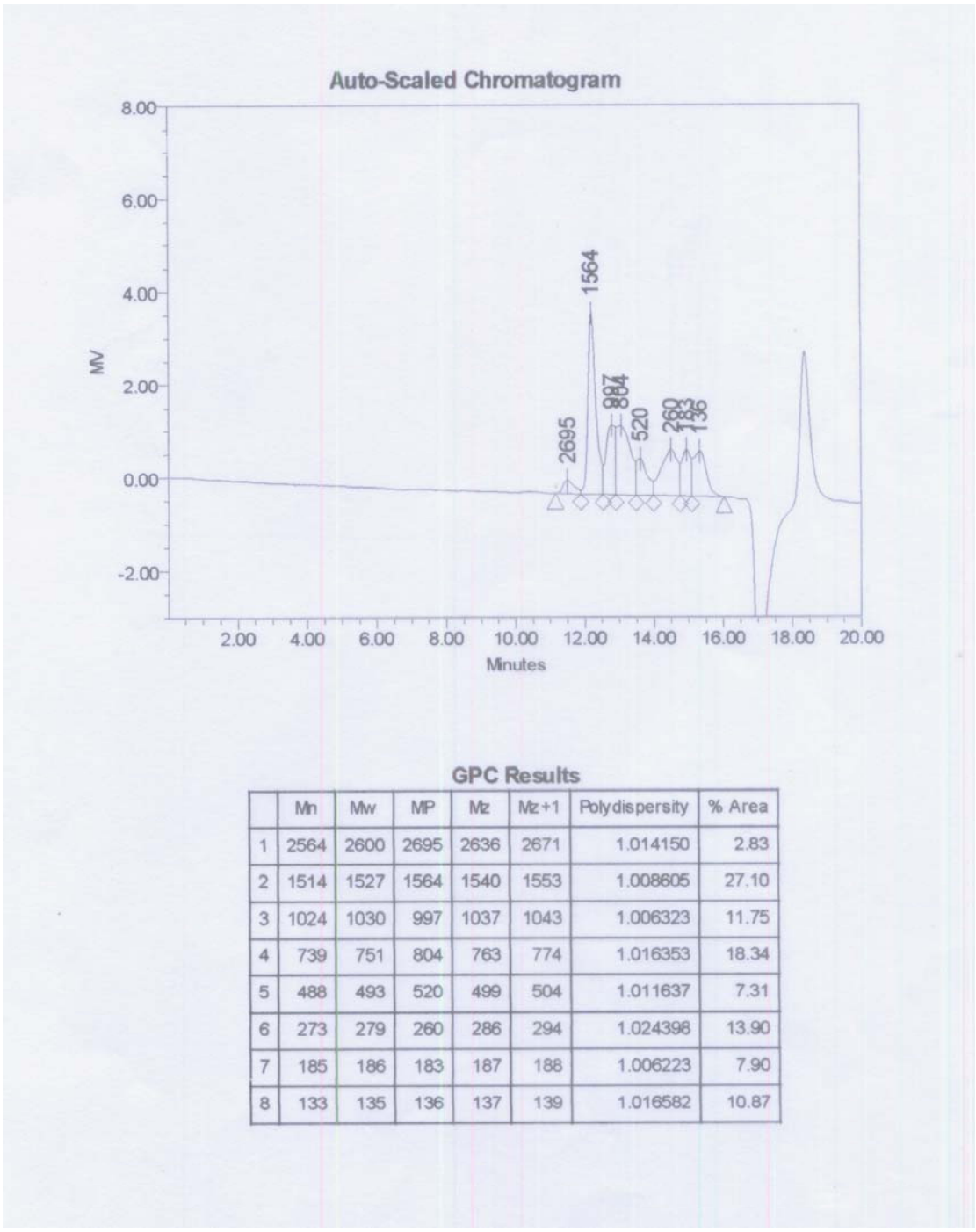
Figure 13. GPC chromatogram of water extract of galanga (sediment part)



GPC Results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity	% Area
1	749	755	761	761	768	1.008152	1.00
2	521	528	550	534	540	1.012892	0.98
3	257	260	257	264	268	1.012690	36.35
4	182	183	182	185	186	1.007229	28.62
5	132	134	137	136	137	1.013133	33.06

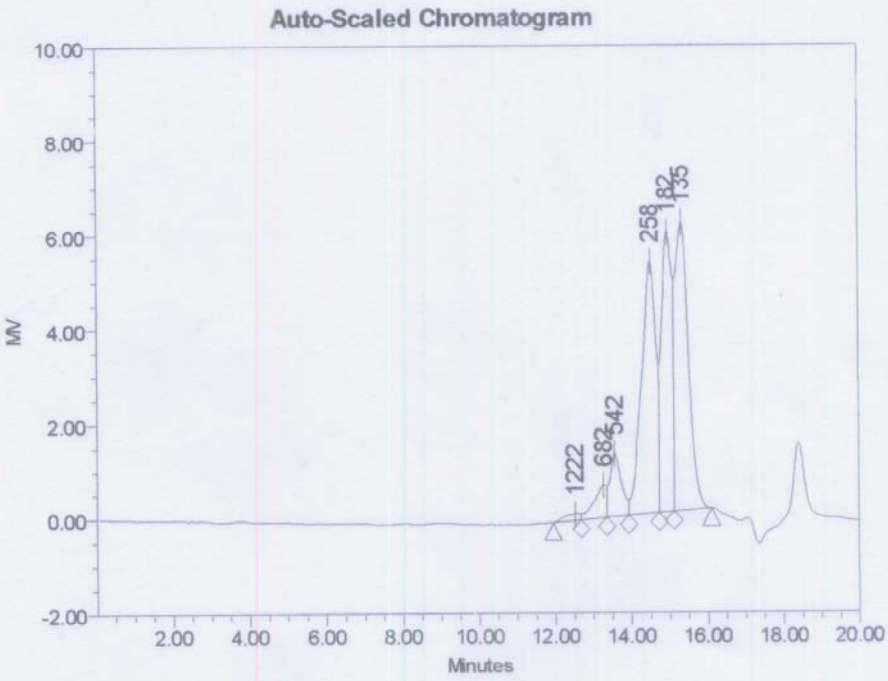
Figure 14. GPC chromatogram of water extract of galanga (supernatant part)



GPC Results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity	% Area
1	2564	2600	2695	2636	2671	1.014150	2.83
2	1514	1527	1564	1540	1553	1.008605	27.10
3	1024	1030	997	1037	1043	1.006323	11.75
4	739	751	804	763	774	1.016353	18.34
5	488	493	520	499	504	1.011637	7.31
6	273	279	260	286	294	1.024398	13.90
7	185	186	183	187	188	1.006223	7.90
8	133	135	136	137	139	1.016582	10.87

Figure 15. GPC chromatogram of ethanolic extract of white turmeric (sediment part)



GPC Results

	Mn	Mw	MP	Mz	Mz+1	Polydispersity	% Area
1	1314	1336	1222	1360	1384	1.017180	0.86
2	757	770	682	785	801	1.017730	3.42
3	521	528	542	534	541	1.012799	6.37
4	268	273	258	279	285	1.019113	31.02
5	183	185	182	186	187	1.007082	24.62
6	131	134	135	135	137	1.015832	33.70

Figure 16. GPC chromatogram of ethanolic extract of white turmeric (supernatant part)

APPENDIX B

1. Modified phenol-sulfuric method (Fox and Robyt, 1991)

The proper dilution of sample (25 µl) was added into 96-well plate and 25 µl of phenol (5 %w/v) was added. The solution was mixed by hand mildly for 30 seconds. The 96-well plate was put down on an ice cube before added 125 µl conc. H_2SO_4. The solution was mixed again for 30 seconds and wrapped with film wrap before put in the plastic zip bag. The bag was boiled at 80 °C for 30 minutes. After cooling to room temperature, the mixture was measured the absorbance at 492 nm using microplate reader comparing to calibration curve of glucose (Figure 11).

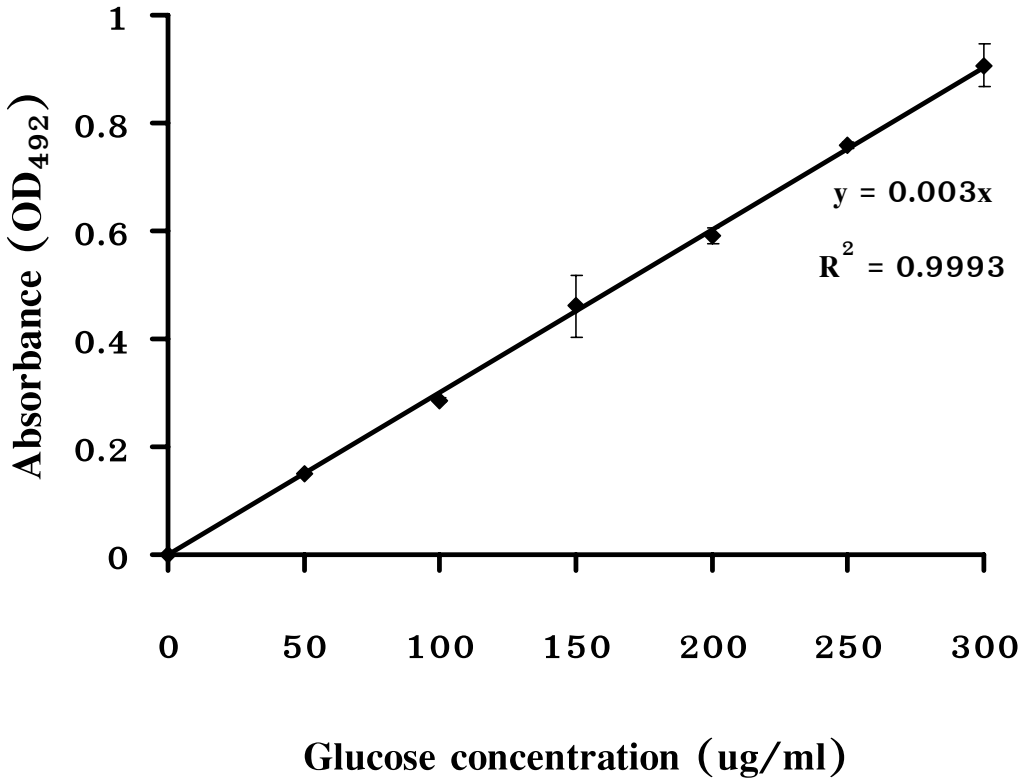


Figure 17. Calibration curve for modified phenol-sulfuric method (Fox and Robyt, 1991) for the determination of total sugar concentration express in glucose equivalent (µg/ml). Points are means±S.D. of assays done in triplicate.

2. Modified dinitrosalicylic acid from Miller (1959) (Robertson *et al.*, 2001)

The proper dilution of sample (100 µl) was added into 96-well plate and 100 µl of 3,5-dinitrosalicylic acid was added. The solution was mixed by hand mildly for 30 seconds and wrapped with film wrap before put in the plastic zip bag. The bag was boiled at 80 °C for 30 minutes. After cooling to room temperature, the mixture was measured the absorbance at 575 nm using microplate reader comparing to calibration curve of glucose (Figure 12).

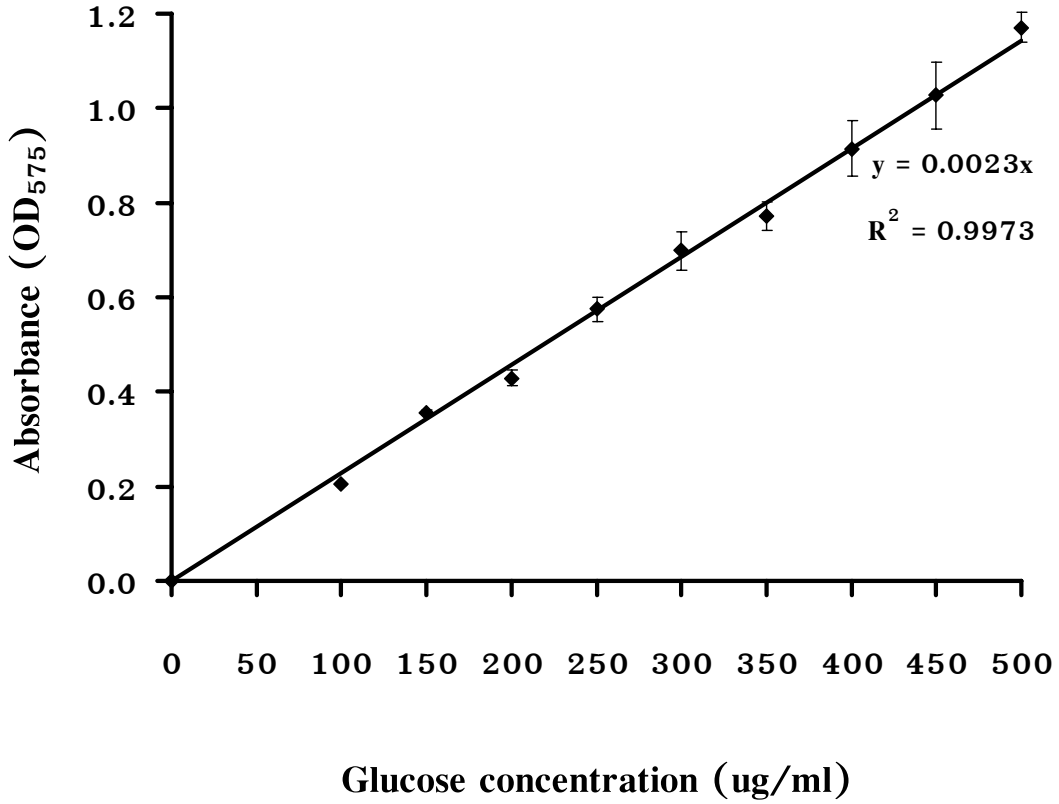


Figure 18. Calibration curve for modified dinitrosalicylic acid from Miller (1959) (Robertson *et al.*, 2001) for the determination of reducing sugar concentration express in glucose equivalent (µg/ml). Points are means±S.D. of assays done in triplicate.

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