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BIOLOGICAL ACTIVITIES OF POLYSACCHARIDE EXTRACTED FROM RHIZOME OF WILD TUMERIC *Curcuma aromatica* Salisb.

Miss Ploypat Niyomploy

A Thesis Submitted in Partial Fulfillment of the Requirements
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พลอยพัฒณ์ นิยมพลอย : ฤทธิ์ทางชีวภาพของสารพอลิแซ็กคาไรด์ที่สกัดได้จากเหง้าของว่านคันทมาลา *Curcuma aromatica* Salisb. (BIOLOGICAL ACTIVITIES OF POLYSACCHARIDE EXTRACTED FROM RHIZOME OF WILD TUMERIC (*Curcuma aromatica* Salisb)
อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. พลกฤษณ์ แสงวณิช,
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งานวิจัยนี้ได้ทำการสกัดส่วนสกัดหยาบพอลิแซ็กคาร์ไรด์ของว่านคันทมาลาด้วยน้ำร้อนแล้วตกตะกอนด้วยเอทานอล จากนั้นนำส่วนสกัดหยาบพอลิแซ็กคาร์ไรด์ไปแยกโดยใช้เทคนิคโครมาโทกราฟีแบบแลกเปลี่ยนประจุโดยใช้ DEAE เซลลูโลส และโครมาโทกราฟีแบบเจลฟิลเทรชันโครมาโทกราฟี พอลิแซ็กคาร์ไรด์ที่ได้ คือ P11, P21 และ P22 พอลิแซ็กคาร์ไรด์หลักคือ P11 และ P21 นำไปศึกษาลักษณะและฤทธิ์ของการเพิ่มจำนวนเซลล์ ส่วน P22 จะศึกษาเฉพาะฤทธิ์ในการเพิ่มจำนวนของเซลล์สร้างเส้นใยที่เพาะเลี้ยงจากเนื้อเยื่อเหงือกเนื่องจากมีปริมาณน้อย จากการศึกษาลักษณะของพอลิแซ็กคาร์ไรด์หลักทั้งสองชนิดพบว่า จากผลของ HPLC มีไซโลสซึ่งเป็นน้ำตาลโมเลกุลเดี่ยวเพียงชนิดเดียวที่เป็นองค์ประกอบในโมเลกุล จากผลของ GPC พบว่า P11 และ P21 มีมวลโมเลกุลเท่ากับ 496,171 และ 157,665 ดาลตัน ตามลำดับ จากนั้นจึงนำพอลิแซ็กคาร์ไรด์ดังกล่าวมาทดสอบฤทธิ์การเพิ่มจำนวนเซลล์โดยวิธีการวิเคราะห์แบบ MTT และตามด้วยการวิเคราะห์ทางสถิติแบบ one-way ANOVA ผลการทดลองพบว่าส่วนสกัดหยาบพอลิแซ็กคาร์ไรด์ P11 และ P22 มีความสามารถในการเพิ่มจำนวนเซลล์เมื่อเทียบกับกลุ่มควบคุมดังนี้ 43.93 ± 17.18, 30.09 ± 13.66 และ 40.98 ± 10.92% ตามลำดับ สำหรับ P21 ที่ความเข้มข้นเดียวกันพบว่าสามารถยับยั้งการเจริญเติบโตของเซลล์ได้ 92.16 ± 3.49% การทดลองทั้งหมดได้ทดสอบทางสถิติแบบ one-way ANOVA พบว่ามีความแตกต่างกับกลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($p<0.05$)

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต.....พลอยพัฒณ์ นิยมพลอย.....
ปีการศึกษา.....2551..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PLOYPAT NIYOMPLOY : BIOLOGICAL ACTIVITIES OF POLYSACCHARIDE EXTRACTED FROM RHIZOME OF WILD TUMERIC *Curcuma aromatica* Salisb. ADVISOR: ASSOC. PROF. POLKIT SANGVANICH, Ph.D., CO-ADVISOR: ASSOC. PROF. PASUTHA THUNYAKITPISAL, DDS, Ph.D., 73 pp.

The hot water-soluble crude polysaccharide was extracted from *Curcuma aromatica via* boiling extraction and precipitated with ethanol. Then, the crude polysaccharide extract was fractionated by ion exchange chromatography on DEAE-cellulose column, and subsequently purified by gel filtration chromatography on Superdex G-200 column chromatography, giving three polysaccharide fractions called P11, P21 and P22. The major polysaccharide fraction P11 and P21 which both were characterized and measured cell proliferation. For P22 was only enough to study its cell proliferation because of its limited amount. Then they were studied for monosaccharide composition *via* acid hydrolysis method and high performance liquid chromatography (HPLC). The molecular weight and functional group were determined by gel permeable chromatography (GPC) and fourier transformation infrared (FT-IR), respectively. P11 and P21 are only composed of the xylose. Their molecular weight average of P11 and P21 were 469,171 and 157,665 Da, respectively. Cell proliferation assay were performed using MTT technique and statistic analysis was performed by one-way ANOVA. Crude polysaccharide, P11 and P22 at the concentration 100 μg/ml showed ability of cell proliferation. The results were shown as percentage of increasing compared to control which are 43.93 ± 17.18, 30.09 ± 13.66 and $40.98 \pm 10.92\%$ for crude polysaccharide, P11 and P22 respectively. While P21 at the same concentration showed its ability to inhibit the cell growth. The result indicated that it can inhibit the cell up to $92.16 \pm 3.49\%$. The one-way ANOVA indicated that all experimental results were significant ($p<0.05$).

Field of Study.....Biotechnology.....Student's Signature.....Ploypat Niyomploy.....
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Co-Advisor's Signature.....

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LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µg/µl	microgram per microliter
^{0}C	Degree Celsius
cm	centimeters
Conc	Concentrate
Da	Dalton
DEAE	Diethylaminoethyl
DMEM	Delbecco Modified Eagle's Medium
DMSO	Dimethylsulfoxide
ELSD	Evaporative light scattering detector
g	gram
GPC	Gel permeable chromatography
h	hour
HPLC	High performance liquid chromatography
mg	milligram
mg/ml	milligram per milliliter
min	minute
mm	millimeter
mM	milimolar
M	Molar
MTT	(3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
nm	nanometer
rpm	round per minute
sec	second
SPSS	Statistical Package for the Social Science
TFA	trifluoroacetic acid
THF	tetrahydrofuran
wt/v	weight by volume
wt/wt	weight by weight

CHAPTER I

INTRODUCTION

Carbohydrate is an important biomolecule which is the composition of organism. It can be divided into three main types which are monosaccharide, oligosaccharide and polysaccharide. After the method for carbohydrate analysis has been found, it leads to understand the role of the carbohydrate in many biological processes especially in biological recognition, cell-cell interaction and receptor-binding and response.

Important and biologically active carbohydrates are usually having high molecular weight such polysaccharides. Most of polysaccharides can be isolated from plant, animal and microorganism and have biological activities such as antitumor, anticancer, antioxidant and immunostimulating. According to the biological activities of polysaccharide, the scientists are interested in studying polysaccharides especially in studying biological activities of purified polysaccharides which are extracted from plant [1,2].

From previous publications indicated that plant polysaccharides have many biological activities such as polysaccharides from *Dioscorea nipponica* Makino (Dioscoreaceae) and *Gynostemma pentaphyllum* Makino (cucurbitaceae) have antioxidant activities [3,4]. In addition polysaccharides from *Alpinia galangal* (Zingiberaceae), root of *Aconitum carmichaeli* (Ranunculaceae) and stem of *Dendrobium huoshanense* (Orchidaceae) have immunostimulating activities [5,6,7]. Polysaccharides are extracted from pollen of *Brassica napus* (Brassicaceae) and *Phoenix dactylifera* L. (Arecaceae) have anticancer and antitumor activities respectively [8,9].

Curcuma aromatica Salisb one of pretty ginger belonging to the genus Curcuma (Zingiberaceae) is a wild plant of the forests of western Ghats and Bengal in India [10]. From past researches, there have been reported about substances that are a component of *Curcuma aromatica*, such as xanthorrhizol, 7-methanoazulene and curcumene which have antimosquito [11], zedorone has anti-feedant [12]. And it contains several major antitumor ingredients: elemicin, curcumol curdione. In addition oil extracted from *Curcuma aromatica* has been shown to exert various

medical activities such as promoting blood circulation to remove blood stasis and treating cancer [10,13]. The other substances for example curdione, neocurdione, curcumol, tetramethypyrazine and 1,2-hexadecanediol [14].

Alpinia galangal, *Curcuma zedoria* and *Curcuma xanthorrhiza* Robx. are the plants in genus Zingiberaceae which were extracted the polysaccharides that provide biological activities [5,44,45]. *Curcuma aromatica* from the same genus has not been reported on extraction and biological activities of its polysaccharides. It is interesting that polysaccharides purified from *Curcuma aromatica* may have biological activities like polysaccharides from *Alpinia galangal*, *Curcuma zedoria* and *Curcuma xanthorrhiza* and there will be many advantages in the future.

In this study, we report on the extraction, purification and characterization of the polysaccharides of *Curcuma aromatica* using DEAE-cellulose column chromatography, gel filtration Superdex-200 column chromatography, high performance liquid chromatography (HPLC) and gel permeable chromatography GPC). In addition, we also report on cell proliferation of extract polysaccharide.

Objectives:

(1) To study the cell proliferation of purified polysaccharides from *Curcuma aromatica*.

(2) To study the characterization of purified polysaccharides from *Curcuma aromatica*.

CHAPTER II

THEORETICAL AND LITERATURE REVIEW

Theoretical background

2.1. Introduction to carbohydrates

Carbohydrates comprise the most abundant group of natural products. They are prime biological substances, of which billions of tons are pronounced every year by photosynthesis by plants and microorganisms. They form the major constituents of shells of insects, crabs, and lobsters, and the supporting tissue of plants but moreover, they are presented as part of basically all cell walls, spanning from the world of microbes to mammals [15].

The general formula of carbohydrate is $C_n(H_2O)_n$ (hydrates of carbon). Later with the discovery of new compounds that did not conform to the formula, but still showed the same chemical properties, the term carbohydrate has been modified and broadened [16].

Carbohydrates include monosaccharides, oligosaccharides and polysaccharides as well as substances derived from monosaccharides by reduction of the carbonyl group (aditols), by oxidation of one or more terminal groups to form carboxylic acids, or by replacement of one or more hydroxyl groups by hydrogen (deoxy derivatives), an amino function, a thiol group, or similar heteroatomic groups. Carbohydrates exist in a large constitutional as well as stereochemical variety, as they are built up from monosaccharides of various kinds, forming branched or linear oligomers, as well as polysaccharide [15]. Under this new definition, even those molecules that do not fit the general formula $C_n(H_2O)_n$ are considered to be carbohydrates [16].

2.1.1 Classification of carbohydrates

Carbohydrates can be classified into two main groups: simple carbohydrates, molecules that contain only carbohydrates in their structure, and complex

carbohydrates (also called carbohydrate-containing molecules), molecules in which carbohydrates appear covalently bound to lipids, proteins and etc.

2.1.1.1 Simple carbohydrates

Based on their molecular sizes, simple carbohydrates can be divided into three major groups: monosaccharides and monosaccharide derivatives, oligosaccharides, and polysaccharides. The term saccharide means sugarlink. Sugar is still being used to include the lower members of carbohydrates like monosaccharides and oligosaccharides, which are usually sweet and water soluble. Monosaccharides and monosaccharide derivatives are the lower member of carbohydrates, which can not be degraded by hydrolysis to smaller carbohydrate molecule. They constitute the building blocks of oligosaccharides and polysaccharides, which are polymers of monosaccharides and their derivatives, joined by acetal-type linkages. By definition, oligosaccharides contain between 2 and 10 monosaccharide units, and polysaccharides contain more than 10 units. Both, oligo and polysaccharides can be hydrolyzed to their corresponding monosaccharides and monosaccharides derivatives [16].

(1) Monosaccharides

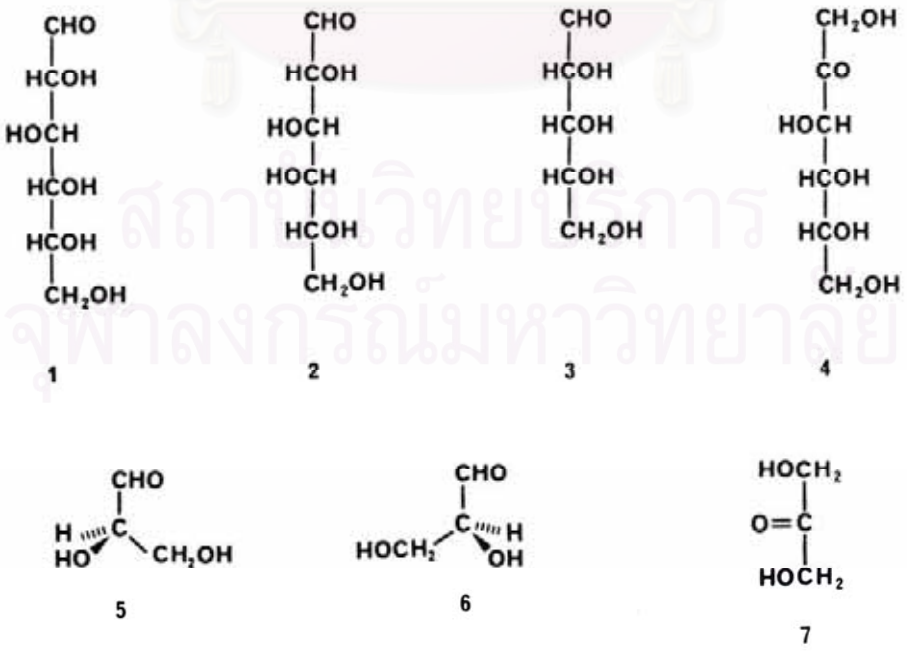
The monosaccharides are the polyhydroxy aldehydes and ketones. These compounds also are known as aldoses (the aldehydes) and ketoses (the ketones). Common aldoses (Fig 2.1) include D-glucose [1], D-galactose [2], and D-ribose [3] while D-fructose [4] is the most common ketose. The smallest compounds which clearly qualify as monosaccharides are the two enantiomers of glyceraldehyde [5] and [6] and 1,3-dihydroxyacetone [7]. In principal, monosaccharides could have quite long carbon atom chains but, in practice, those with more than seven or eight carbon atoms are rarely encountered.

Monosaccharides are further categorized according to the number of carbon atoms in the central chain. For example, a monosaccharide with a central chain of six carbon atoms is a hexose. If this compound is also an aldehyde (e.g., D-glucose [1] and D-galactose [2], then it is an aldohexose. In a like manner, aldotriose, aldotetrose and aldopentose refer to aldoses with three, four, and five carbon atoms, respectively, in the central chain. Thus, D-ribose [3] is an aldopentose.

A set of names also exits for ketoses. Ketoses with three, four, five and six carbon atoms in the central chain are ketotrioses, ketotetroses, ketopentoses, and ketohexoses, respectively. An alternate terminology frequently used for indicating the presence of the ketone group is with the “ulose” ending. This approach gives rise to the names triulose, tetrulose, pentulose, and hexulose for the ketones with three, four, five and six carbon atoms, respectively. D-frutose [4], therefore, can be described as a ketohexose or a hexulose.

(2) Oligosaccharides

Oligosaccharides are compounds formed when small numbers (2-10) of monosaccharides are coupled together. The coupling is a specific type;that is, an oxygen atom must be part of an acetal or ketal group. Examples of these type of linkages are shown (Fig 2.1) in compounds [8] and [9]. The number of monosaccharide units present in an oligosaccharide usually is indicated by a prefix such as di, tri, tetra, or penta (for two, three, four, and five units, respectively). Thus, sucrose [8] is a disaccharide while raffinose [9] is a trisaccharide. The term “sugar” often is used to refer to any member of the group of mono- and oligosaccharides [17].



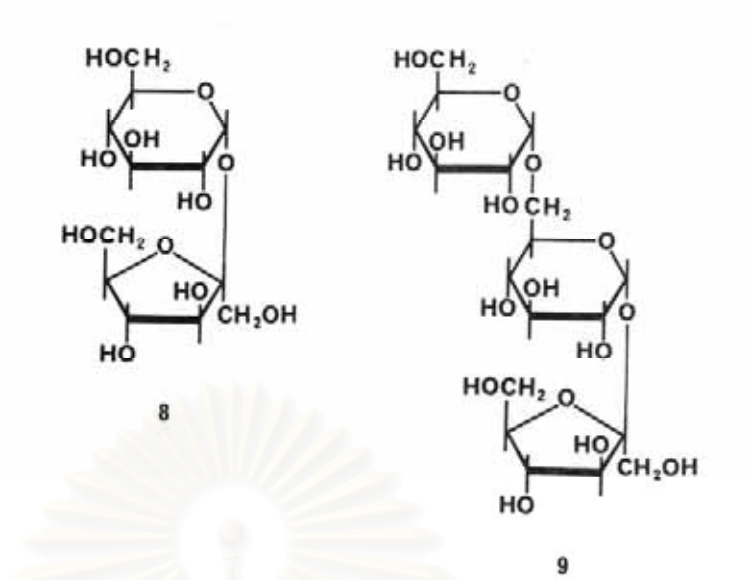


Fig 2.1 The aldoses (1-7) and ketoses (8-9)

(3) Polysaccharides

Polysaccharides (also called glycans) are natural polymers composed of more than 10 glycosidically linked monosaccharides [18]. This number is somewhat arbitary. Carbohydrates containing 5 to 15 monosaccharide residues rarely occur in nature. A few natural polysaccharides contain 80 to 100 residues. Some others, like cellulose, exceed this number, having an average of 3000 residues of D-glucose. Different molecules of a particular polysaccharide will differ in the number of residues, so rather than an exact molecular weight one refers to a distribution of molecular weights or an average molecular weight. Polysaccharides occur in almost all living organism. They serve as structural materials (e.g., cellulose) and food storage compounds (e.g., glycogen). They also confer immunological specificity (e.g., polysaccharides of bacterial capsules).

2.1.1.2 Complex carbohydrates

Other molecules contain carbohydrates as a minor or major constituent. These molecules are called complex carbohydrate such as carbohydrate antibiotics, nucleic acids, glycoproteins, proteoglycans, peptidoglycans, lipopolysaccharides, and glycolipids [16].

2.1.2 Biological roles of carbohydrates

Carbohydrates comprise the most abundant group of compounds found in natural sources; they are present both in plants and animals. They perform a wide range of functions and exits in more diverse forms than any other group of natural products [16].

In the past, carbohydrates were considered to be solely of use for energy storage, and as skeletal components. However, this view was challenged in 1963 when a protein was isolated from *Canavalia ensiformis* that demonstrated ability to bind to carbohydrates on erythrocytes. In 1982 the first animal carbohydrate binding protein was identified and this sparked interest into the wider roles of carbohydrates and carbohydrate binding proteins within biological systems. These carbohydrate binding proteins are termed lectins and it is now known that they are found in varying densities on all cell-surface membranes. They interact specifically with oligosaccharides and glycoconjugates (such as glycolipids and glycoproteins) on the surrounding cells via hydrogen bonding, metal coordination, van der waals forces, and hydrophobic interactions. It is believed that favourable interactions occur between the hydroxyl groups of the carbohydrates and the amino acid functionalities of the proteins, to aid molecular recognition processes. These interactions are relatively weak, but they are so numerous that specific interactions occur. Selectivity is believed to be further increased through additional binding of the carbohydrate to the lectin's subsites.

The study of carbohydrates within biological systems has illustrated that they are involved in a number of fundamental biological functions such as cell-cell recognition and cell-external agent interactions. These interactions can initiate beneficial biological events, such as fertilization, cell growth and differentiation (for example during embryogenesis) and immune responses, as well as detrimental disease processes, such as inflammation, viral and bacterial infections, and cancer metastasis (*vide infra*). Therefore the view that carbohydrates are of limited importance within biological systems has been challenged and renewed interest into the science 'Glycobiology' has emerged [19].

From this field of science, knowledge and understanding of the role of carbohydrates on biological processes increased and led to the concept and interest in developing medicines from carbohydrates, especially in the treatment of immune

system diseases such as infections or even cancer. Carbohydrates that are of biological importance or have bioactivity usually have high molecular weight, i.e. they are a type of polysaccharide known as glycans. Glycans may be attached to proteins to form a larger-molecule substance called glycoproteins or proteoglycans, or be attached to lipids to form glycolipids. These types of substance may be generally referred to as glycoconjugates or complex carbohydrates due to their complex chemical structure. Glycobiology is the beginning of the drug development from carbohydrate especially immunotherapy for example infection or cancer. Carbohydrates which are very important in biological activity and have a high molecular weight are polysaccharides. Polysaccharides or glycans maybe are included with protein that we call “glycoprotein” or “proteoglycan”. They are also included with lipid that we call “glycolipid” and these polysaccharides are called “glycoconjugates” or “complex carbohydrates” because they have a complex structure [1,2].

2.2 Polysaccharides

It is estimated that approximately $4x10^{11}$ tons of carbohydrates are biosynthesized each year on earth by plants and photosynthesizing bacteria. The majority of these carbohydrates are produced as polysaccharides. Polysaccharides are macromolecules consisting of a large number of monosaccharide residues. They are sometimes also called glycans. For polysaccharides which contain a substantial proportion of amino sugar residues the term glycosaminoglycan is a common one. Polysaccharides which consist of only one kind of monosaccharide are called homopolysaccharides (homoglycans); when they are built up of two or more different monomeric units they are named heteropolysaccharides (heteroglycans). In the latter type, the monosaccharide units are usually linked to each other in a definite pattern, rather than randomly. Certain sequences of monomeric building blocks are often found to be regularly repeated as so called repeating units. Homo- as well as heteropolysaccharides can be linear or branched. The size of a polysaccharide varies between approximately 16,000 and 16,000,000 daltons (Da). Polysaccharides exist in an enormous structural diversity as they are produced by a geat variety of species, including microbes, algae, plants and animals. Among these are fructans, xanthans, fucans, bacterial gel polysaccharides and are obtained from red-purple seaweeds. The most well-known polysaccharides are starch, glycogen, cellulose and chitin.

2.2.1 Classification of polysaccharides

According to their structural features, polysaccharides can be classified in two major classes: (1) homopolysaccharides, which are constituted by one type of monosaccharide and (2) heteropolysaccharides, which are made up of more than one type of monosaccharide. They can be subdivided further into linear or branched. According to their origin they can be classified as plant, animal, bacterial, fungal or algal polysaccharides. Table 2.1 and 2.2 list the major groups of polysaccharides of relatively widespread occurrence. Table I lists homoglycans, polysaccharides with a single sugar constituent and table II lists heteroglycans with two different sugar residues in the main chain, either alternating or less regular sequence [18].

2.2.2 Other plant polysaccharides

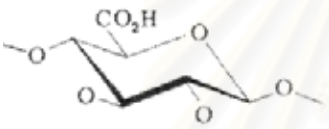
Most of the polysaccharides described here are heteroglycans, which are divided into groups according to the nature of the sugar units constituting the main chains: substituted D-glucans, D-galactans, D-galacturonans, and D-xylans. Associated plant polysaccharides are the D-fructofuranans and β-D-glucans containing mixed linkages. In view of the rapidly growing appreciation that proteins bound to carbohydrates form essential components of primary cell wall and are isolated from plant tissues of various kinds. The table 2.3 shows major of plant polysaccharide types.

Table 2.1 Major Sources of Plant Polysaccharide Types

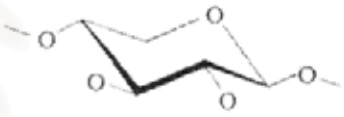
Plant polysaccharide type	Source
Glucomannoglycan	Coniferous woods, monocotyledonous plant bulbs
Galactomannan	Leguminous seeds
Arabinogalactans	Coniferous woods, leguminous stem exudates
Galacturonan	Apple and citrus fruits, soybeans
Xylan	Cereal grains, hardwoods

Representative species from less than one-half of the 90 orders of angiosperm have been investigated with respect to polysaccharide composition and the study of polysaccharide from gymnosperms has been equally selective. However, it is now

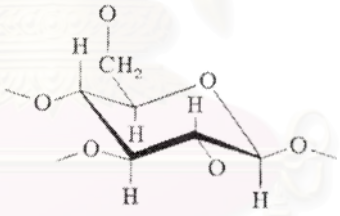
apparent that in molecular terms there is no clear discontinuity in polysaccharide structure between partially esterified D-galacturonans and highly substituted polymer with frequent insertions of L-rhamnopyranosyl residues in the main chain or between linear D-xylans and their branched glycosyl derivatives. Mannans and glucomannoglycans merge and both carry pendant galactosyl groups in proportions that vary greatly. In the same way, there are glucans that extend from cellulose to highly branched, soluble compounds in which xylosyl, galactosyl, and fucosly units are incorporated. Structure formulas for the common monosaccharide constituents of plant polysaccharides are shown in fig 2.2. Oxygen atoms that are drawn with bonds attached represent the most common position of glycosidic linkages in the molecular chains, for example, O-1 and O-4 of the α-D-Gal*p*A units.



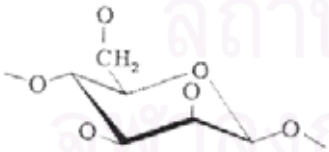
β-D-Glc *ρ*A



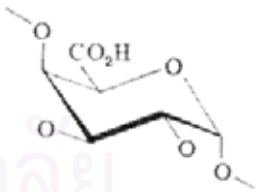
β-D-Xyl *ρ*



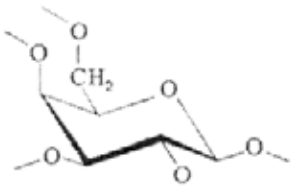
β-D-Glc *ρ*



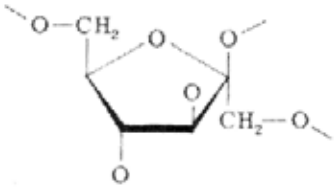
α-D-gal *ρ*A



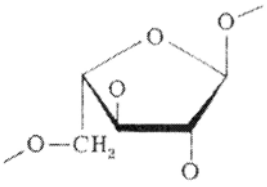
β-D-Man *ρ*



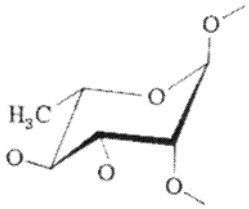
β-D-gal *ρ*



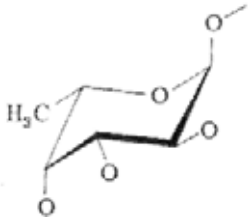
β-D-Fru *f*



α-L-Ara *f*



α-L-rha *ρ*



α-L-Fuc *ρ*

Fig 2.2 Common constituent monosaccharides; preferred modes of linkage through oxygen atoms indicated by bonds

2.3 Ion exchange chromatography

Ion chromatography is considered to be an indispensable tool in a modern analytical laboratory. Complex mixtures of anions or cations can usually be separated and quantitative amounts of the individual ions measured in a relatively short time. Higher concentrations of sample ions may require some dilution of the sample before introduction into the ion-chromatographic instrument. However, ion chromatography is also a superb way to determine ions present at concentrations down to at least the low part per billion (μg/l) ranges. Ion exchange chromatography separates compounds according to the nature and degree of their ionic charge. The net surface charge of samples varies according to the surrounding pH. When above its isoelectric point (pI) a sample will bind to an anion exchange, when below its pI a sample will bind to a cation exchanger. The column to be used is selected according to its type and strength of charge. Ion exchange chromatography has been divided into 2 types, anion exchange chromatography and cation exchange chromatography.

To release the samples in the order of binding tenacity, conditions are then altered so that bound substances are eluted differentially. This elution is usually performed by changes in pH or increases in salt concentration. At low salt concentration (low ionic strengths), all components will be tightly held on top of the column. When the ionic strength of the mobile phase is increased, the salt ions

compete with the adsorbed sample ions for the bonded charges on the column. As a result, some of the sample components will be partially desorbed and start moving through the column. If the salt concentration is higher, the resulting ionic strength causes a larger number of the sample components to be desorbed, and the speed of the movement down the column increases. The samples come off the column matrix when the ionic strength of the buffer neutralizes their charge. The least charged molecules come off first and the most highly charged come off last.

Anion Exchange Chromatography (AEC)

The surface charge of the solutes which bind will be net negative, thus to get binding of a specific protein one should be above the pI of that protein. Commonly used anion exchange resins are Q- resin, a Quaternary amine; and DEAE resin, DiEthylAminoEthane (Fig 2.3).

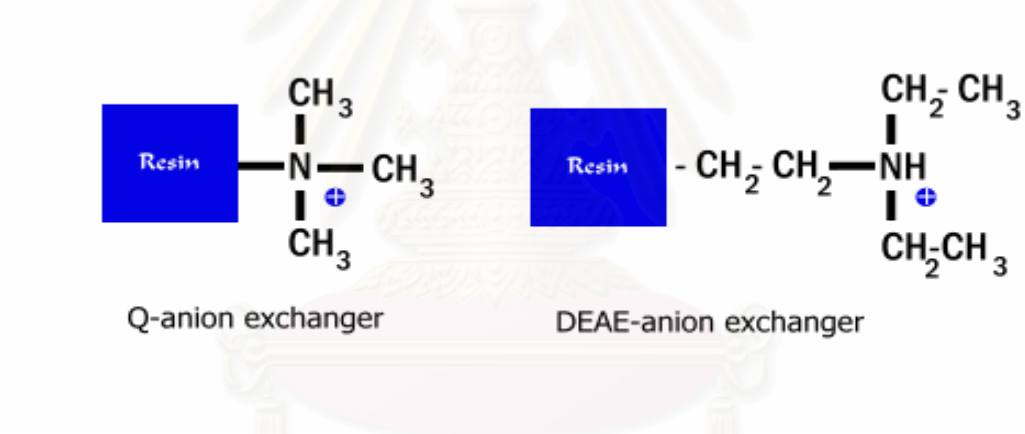


Fig 2.3 Q-resin and DEAE resin as anion exchange resin

AEC is often used as a primary chromatography step due to its high capacity. AEC is performed using buffers at pH's between 7 and 10 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1M NaCl. The salt in the solution competes for binding to the immobilized matrix and releases the protein from its bound state at a given concentration.

Cation Exchange Chromatography (CEC)

The surface charge of the solutes which bind will be net positive. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions (Fig 2.4).

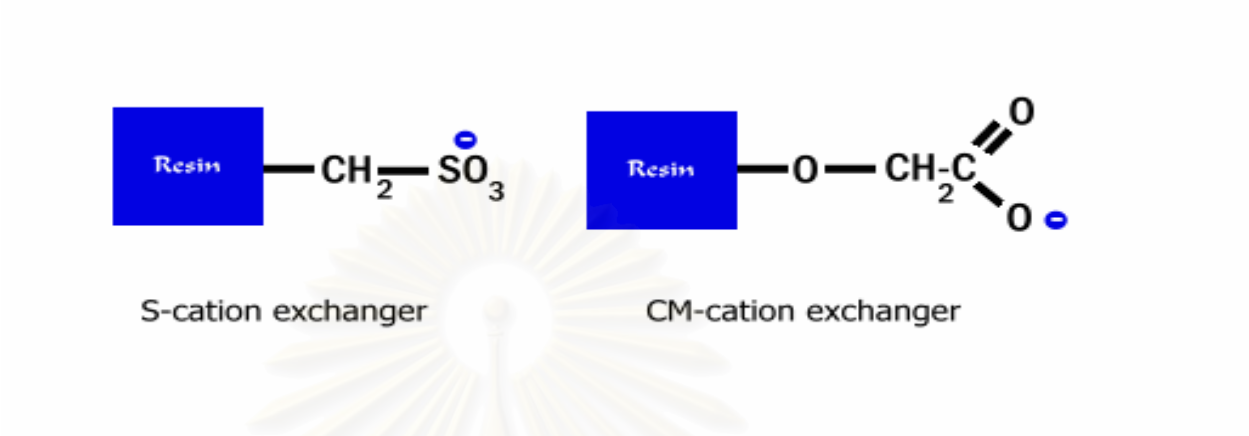


Fig 2.4 S-resin and CM-resin as cation exchange resin

CEC is less commonly used compared to AEC. Typically, CEC is performed using buffers at pH between 4 and 7 and running a gradient from a solution containing just this buffer to a solution containing this buffer with 1M NaCl [22].

2.4 Size exclusion chromatography

Size exclusion chromatography has also been called gel permeation or gel filtration chromatography. The name gel permeation chromatography is used when an organic solvent is used as a mobile phaseis. Size exclusion chromatography a powerful technique that is particularly applicable to high-molecular-weight species.

Packings for size-exclusion chromatography consist of small (~ 10 μm) silica or polymer particles containing a network of uniform pores into which solute and solvent molecules can diffuse. The first have the advantages of greater rigidity, which leads to easier packing; greater stability, which permits the use of a wider range of solvents including water; more rapid equilibration with new solvents; and stability at higher temperatures. The disadvantages of silica-based particles include their tendency to retain solutes by adsorption and their potention for catalyzing the degradation of solute molecules.

Most early size-exclusion chromatography was carried out on cross-linked styrenedivinylbenzene copolymers. The pore size of these polymers is controlled by the extent of cross-linking and hence the relative amount of divinylbenzene present during manufacture. As a consequence, polymeric packing having several different average pore sizes are marketed. Originally, styrene-divinylbenzene gels were hydrophobic and thus could only be used with nonaqeous mobile phases. Now, however, hydrophilic gels are available making possible the use of aqueous solvents for the separation of large, water-soluble molecules such as sugars. These hydrophilic gels are sulfonated divinylbenzenes or polyacrylamides.

The supports for size exclusion chromatography are beads which contain holes of given sizes. The size of the pores in the matrix determines the rate at which samples of various sizes diffuse into the beads and some samples are completely excluded. Larger molecules, which can't penetrate the pores, move around the beads and migrate through the spaces which separate the beads faster than the smaller molecules, which may penetrate the pores. The column is then eluted with just buffer and the samples come out in order with the largest first.

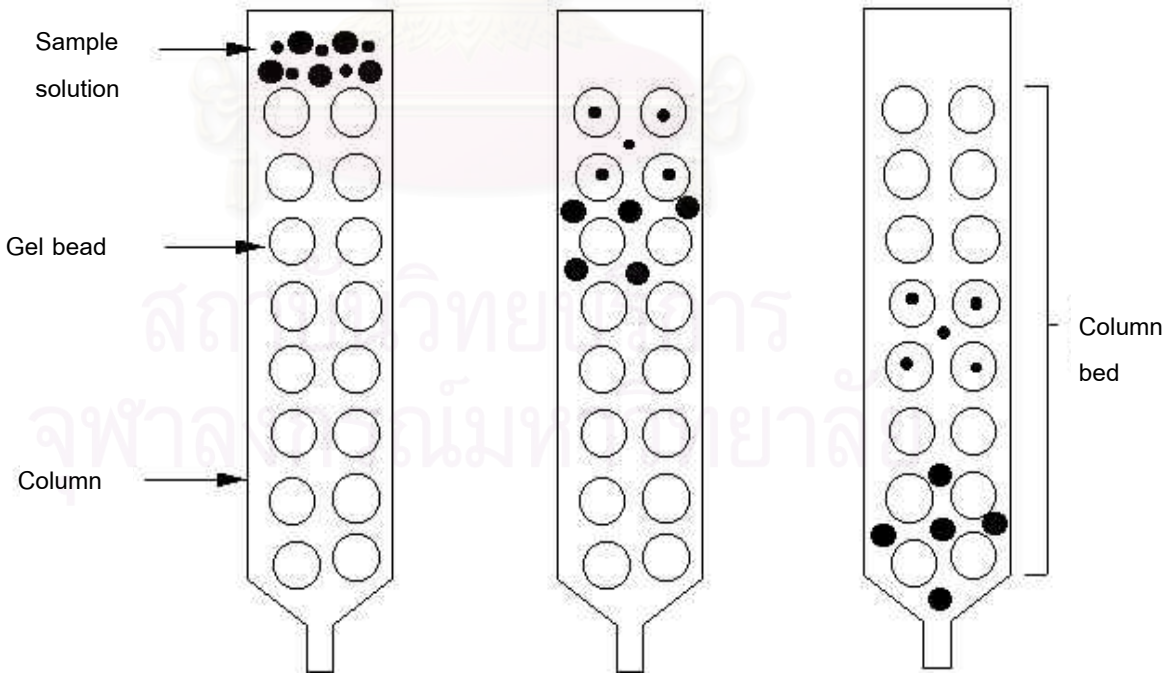


Fig 2.5 Gel filtration chromatography

2.5 High performance liquid chromatography

High performance liquid chromatography (HPLC) in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector as show in fig 2.6 [23].

HPLC is primarily an analytical separation technique, used to detect and quantitate analytes of interest in more or less complex mixtures and matrices. However, it is also used to isolate and purify compounds [24].

The basic operating principle of HPLC is to force the analyte through a column of the stationary phase (usually a tube packed with small spherical particles with a certain surface chemistry) by pumping a liquid (mobile phase) at high pressure through the column. The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses the length of the column. The amount of retardation depends on the nature of the analyte, stationary phase and mobile phase composition. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time and is considered a reasonably unique identifying characteristic of a given analyte. The use of pressure increases the linear velocity (speed) giving the components less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. Common solvents used include any miscible combinations of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the separation of the analyte components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent [25].

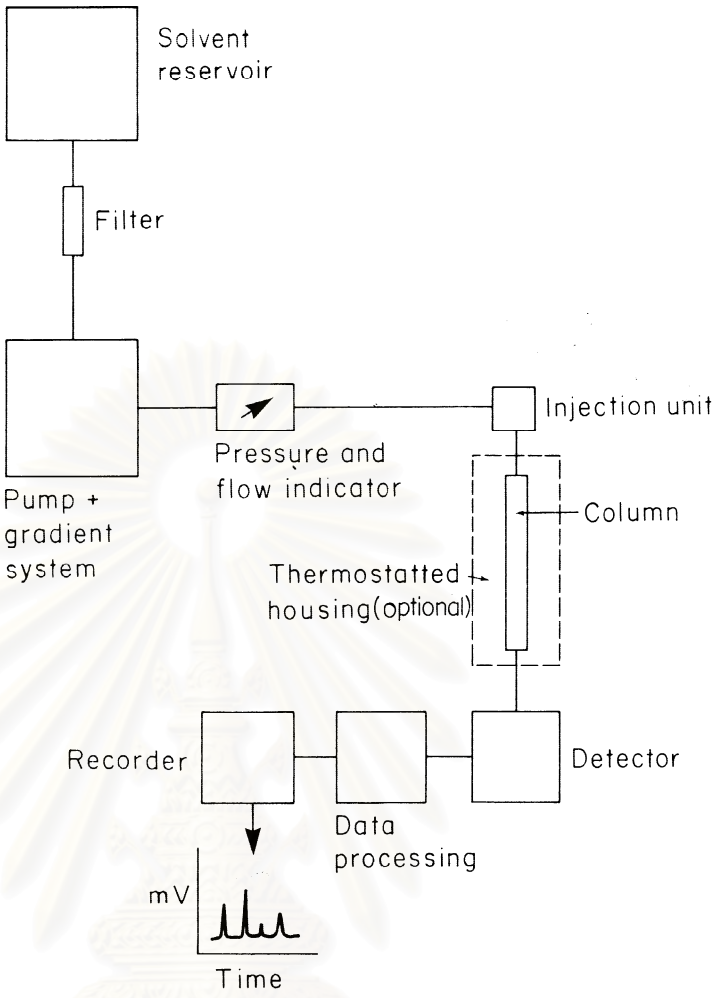


Fig 2.6 The HPLC instrument consists of an injection, a pump, a column and a detector

2.6 IR spectroscopy

Infra spectroscopy (IR spectroscopy) is the subset of spectroscopy that deals with the infrared region of the electromagnetic spectrum. It can be used to identify organic compounds or investigate sample.

Infrared spectroscopy exploits the fact that molecules have specific frequencies at which they rotate or vibrate corresponding to discrete energy livels (vibrational modes). These resonant frequencies are determined by the shape of the molecular potential energy surfaces, the masses of the atoms and, by the associated vibronic coupling. In order for a vibrational mode in a molecule to be IR active, it must be associated with changes in the permanent dipole. In particular, in the Born-Oppenheimer and harmonic approximations, i.e. when the molecular Hamiltonian

corresponding to the electronic ground state can be approximated by a harmonic oscillator in the neighborhood of the equilibrium molecular geometry, the resonant frequencies are determined by the normal modes corresponding to the molecular electronic ground state potential energy surface. Nevertheless, the resonant frequencies can be in a first approach related to the strength of the bond, and the mass of the atoms at either end of it. Thus, the frequency of the vibrations can be associated with a particular bond type. Simple diatomic molecules have only one bond, which may stretch. More complex molecules have many bonds, and vibrations can be conjugated, leading to infrared absorptions at characteristic frequencies that may be related to chemical groups. For example, the atoms in a CH_2 group, commonly found in organic compounds can vibrate in six different ways: symmetrical and antisymmetrical stretching, scissoring, rocking, wagging and twisting.

A beam of infrared light is produced and split into two separate beams. One is passed through the sample, the other passed through a reference which is often the substance the sample is dissolved in. Examination of the transmitted light reveals how much energy was absorbed at each wavelength. This can be done with a monochromatic beam, which changes in wavelength over time, or by using a fourier transform instrument to measure all wavelengths at once. From this, a transmittance or absorbance spectrum can be produced, showing at which IR wavelengths the sample absorbs. Analysis of these absorption characteristics reveals details about the molecular structure of the sample. The sample that is measured can be gaseous sample, liquid sample or solid sample (Fig 2.7) [26].

IR spectroscopy finds widespread application to qualitative and quantitative analyses. Its single most important use has been for the identification of organic compounds whose mid-infrared spectra are generally complex and provide numerous maxima and minima that are useful for comparison purpose. Indeed, in most instances, the mid-infrared spectrum of an organic compound provides a unique fingerprint, which is readily distinguished from the absorption patterns of all other compounds; only optical isomers absorb in exactly the same way. In addition to its application as a qualitative analytical tool, infrared measurements are finding increasing use for quantitative analysis as well. Here, the high selectivity of the method often makes possible the quantitative estimation of analysis in a complex mixture with little or no prior separation steps. The most important analyses of this type have been of atmospheric pollutants from industrial processes. Another

important use of infrared absorption spectroscopy is as a detector for gas chromatography, where its power for identifying compounds is coupled with the remarkable ability of gas chromatography to separate the components of complex mixtures. This application has been fostered by the development of high-speed fourier transform spectrometers [21].

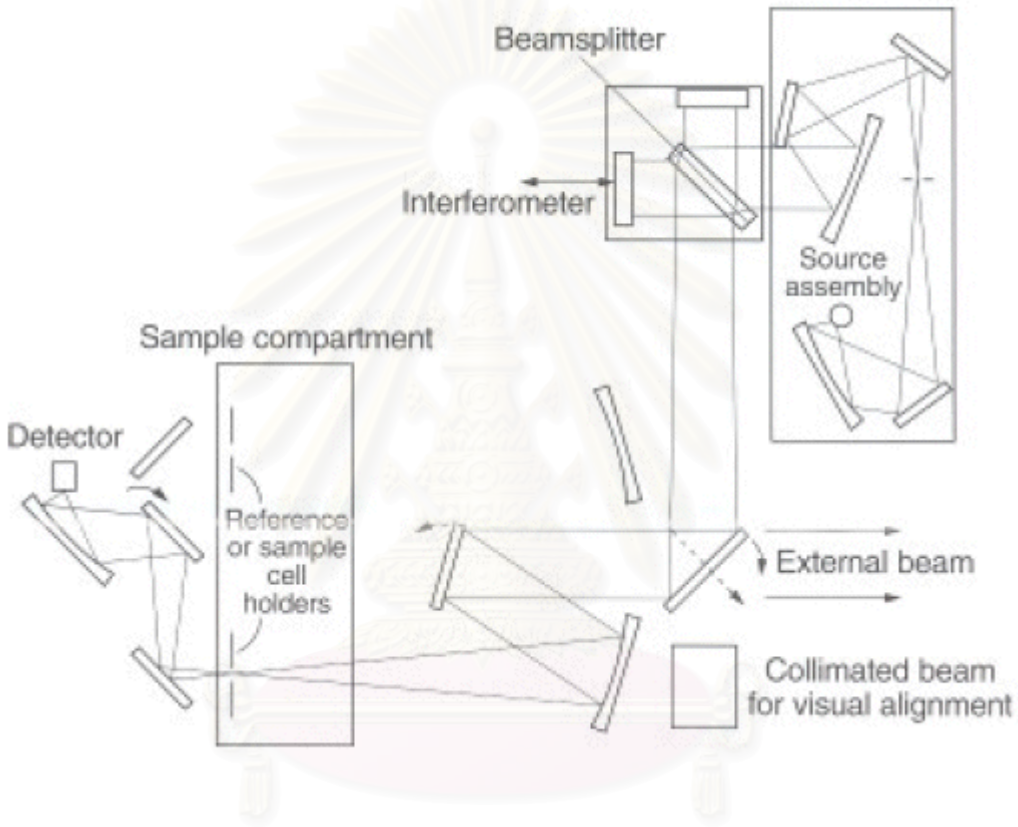


Fig 2.7 The principle of IR spectroscopy

2.7 MTT assay

MTT (3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, first described by Mosman in 1983, is the measurement of mitochondrial activity for calculate the number of cell that are dividing in the culture (cell proliferation). The tetrazolium rings of the yellow MTT are splited by mitochondrial reductase enzyme from viable cell and then they are become to be purple formazan which is impermeable to cell membranes. A solubilization solution, usually using dimethylsulfoxide, is added to dissolve the purple formazan product into a colored

solution. The number of surviving cells is calculated by measure the absorbance of the colored solution. The absorbance of the colored solution is measured by spectrophotometer (between 500-600 nm depend on the solubilization solution). The number of surviving cells is directly proportional to the level of the formazan product created [27].

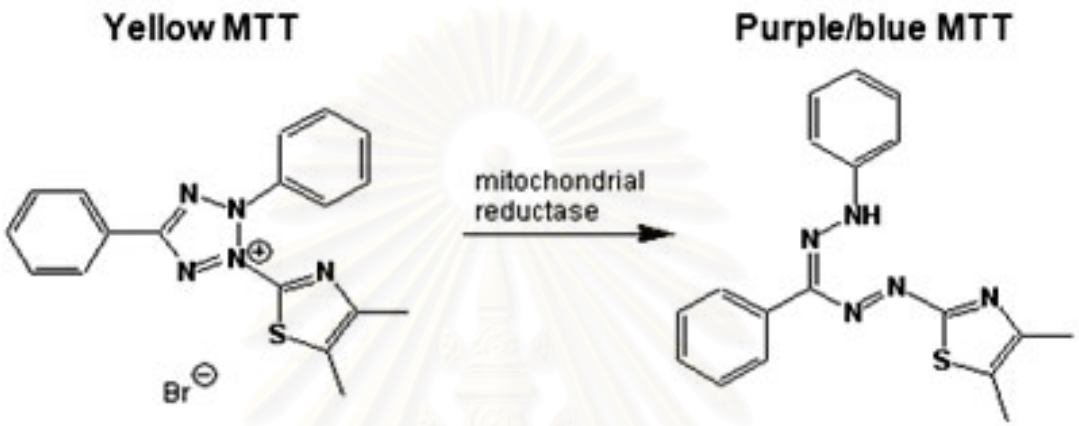


Fig 2.8 The principle of MTT assay. The tetrazolium rings of the yellow MTT are splited by mitochondria reductase enzyme to be purple formazan

2.8 Colorimetric method for carbohydrate analysis

The phenol - sulfuric acid method is an example of a colorimetric method that is widely used to determine the total concentration of carbohydrates present [28]. The method detects virtually all classes of carbohydrates, including mono-, di-, oligo- and polysaccharides. Although the method detects almost all carbohydrates varies, Thus, unless a sample is known to contain only one carbohydrate, the results must be expressed arbitrarily in terms of one carbohydrate. In this method, the concentrated sulfuric acid break down any polysaccharides, oligosaccharides, and disaccharides to monosaccharides. Pentose (5 carbon compounds) they are dehydrated to furfural, and hexose (6 carbon compounds) to hydroxymethyl furfural. These compounds then react with phenol to produce a yellow-gold color. For products that are very high in xylose (a pentose), such as wheat bran or corn, xylose should be used to construct the standard curve for the assay, and measure the absorption at 480 nm. For products that are high in hexose sugars, glucose is commonly used to create the standard curve, and

the absorption is measured at 490 nm. The color for this reaction is stable for several hours, and the accuracy of the method is within ±2% under proper conditions [29].

2.9 Human gingival fibroblast cells

Gingiva is a part of the oral mucosa and covers the alveolar process and the neck. Gingiva can be divided into 2 parts, namely the free gingiva and the attached gingiva. Microanatomy of the gingiva can be divided into 2 parts: the epithelium and the connective tissue. The free gingiva consists of the oral epithelium which spreads on the outer portion or in the visible part of the oral cavity, and the oral sulcular epithelium which is attached to the enamel, and the junctional epithelium which attaches to the surface. There is a layer of connective tissue beneath the oral epithelium and the oral sulcular epithelium with the junction between the epitheliums and the connective tissue having a jagged shape. The part where the connective tissue extends into the epithelial tissue is called the connective tissue papilla, while the part where the epithelial tissue extends into the connective tissue is called the epithelial ridges or the rete pegs. In the junctional epithelium of the healthy gingiva, no connective tissue papilla and rete pegs are found. The junctional epithelium has a different structure from the oral epithelium and the oral sulcular epithelium.

The connective tissue, considered as the main component of the gingiva, consists mainly of collagen fibers (approximately 60% of the total volume of all connective tissues), fibroblasts (approximately 5%), blood vessels, nerves and matrix (approximately 35%). Many other types of cells are also components of the connective tissue of the gingiva such as mast cells, macrophages, neutrophilic granulocytes, lymphocytes, and plasma cells. Fibroblasts are the most common type of cell in the connective tissue (65% of all cells) and play a role in the production of many types of fibers and matrices of the connective tissue which involve both in wound healing responses and in normal metabolic precesses. Fibroblasts may be spindle-shaped or stellate with an oval-shaped nucleus. In the cytoplasm of fibroblasts, there are rough endoplasmic reticulums with a high number of ribosomes, golgi complexes can generally be found, and mitochondria are large and numerous. Furthermore, there are many small to no filaments in the cell and in the cell membrane there are many large-sized vesicles. There are many types of connective tissues that are made of fibroblasts such as collagen fiber, reticulin fiber, oxytalan

fiber, and elastic fiber. A large amount of collagen fiber can be found in the gingival connective tissue, where the fiber is inside the connective
tissue in bundles [30,31].

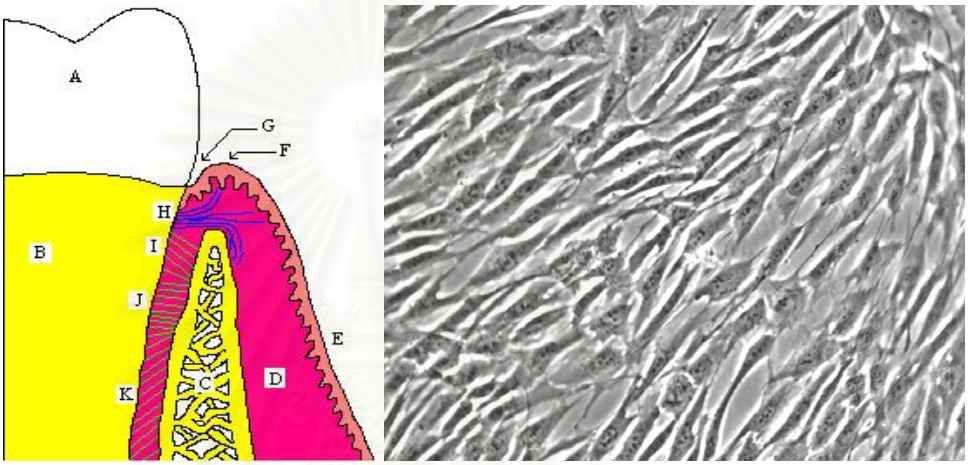


Fig 2.9 The left picture, A diagram of the periodontium. A, crown of the tooth, covered by enamel B, root of the tooth, covered by cementum c, aleolar bone D, subepithelial connective tissue E, oral epithelium F, free gingival margin G, gingival sulcus H, principal gingival fibers I, alveolar crest fibers of the PDL J, horizontal fibers of the PDL K, oblique fibers of the PDL. And the right picture is the gingival fibroblast cells.

2.10 Zingiberaceae

Zingiberaceae, or the ginger family, is one of the largest families of flowering plants with 1,300 species classified into around 52 genera. This is usually divided into three sub-families: Siphonochiloideae, Tamijioideae, Alpinioideae and Zingiberoideae.

Members of the family are small to large herbaceous plants with distichous leaves with basal sheaths that overlap to form a pseudostem. The plants are either self-supporting or epiphytic. Flowers are hermaphroditic, usually strongly zygomorphic, in determinate cymose inflorescences, and subtended by conspicuous, spirally arranged bracts. The perianth is comprised of 2 whorls, a fused tubular calyx, and a tubular corolla with one lobe larger than the other two. Flowers typically have

two of their stamenoids (sterile stamens) fused to form a petaloid lip, and have only one fertile stamen. The ovary is inferior and topped by two nectaries, the stigma is funnel-shaped [32,33].

2.11 *Curcuma aromatica* Salisb

2.11.1 General background

Curcuma aromatica (Kanthamala) is in family Zingiberaceae. It is a wild plant, cultivated throughout Inia mainly in Bengal and Kerala (Travancore). There are medicinal plants which are in the same family with it for example *Curcuma longa* (Kaminchan), *Curcuma zedoria* (Kaminaoi), *Alpinia galangal* (Kha) and etc.

Kanthamala is suitable for cultivation in mid-hills under sub-humid and sub-temperate climatic conditions. It can be grown in locations situated around and above 1300 m altitude. The location may be sunny or partially under shade. And Kanthamala prefers clayey loam soils rich in organic matter (humus) content with adequate moisture. The pH of the soil may be slightly acidic to neutral [10,34].

Kingdom	Plantae
Subkingdom	Tracheobionta
Division	Magnoliophyta
Class	Liliopsida
Subclass	Zingiberaidae
Order	Zingiberales
Family	Zingiberaceae
Subfamily	Zingiberoideae
Genus	Curcuma L
Species	*Curcuma aromatica* Salisb

Its synonyms, **Sanskrit**: Vana- haridra; **Hindi**: Jangli Haldi; **English**: Cochin turmeric, Yellow zedoary; **French**: safran des Indes; **Gujrati**: kapurkachali, Vanahaladara; **Bengal**: banhaludi; **Tamil**: Kasturi- manjal; **Telgu**: Kasturi- manjai; **Bombay**: Ambehaldi; **Malayalam**: Anakuva, Kattumannar [35].

2.11.2 Morphology description

It is erect, perennial herb. Rhizomes are large, tuberous, yellow or orange-read inside and aromatic in taste. Leaves are large, green, oblong- lanceolate/

oblong elliptic, with acuminate apex, 38-60 x 10-20 cm size, often variegated above, pubescent beneath, base deltoid with long petioles. Rootstock large, of palmately branched, sessile annulated biennial tubers. Flowering stem appears with or before the leafing stem, as thick as the forefinger and sheathed. Flowers are fragrant, shorter than the bracts, in spikes 15-30 cm long; flowering bracts 3.8-5 cm long, ovate, recurved, cymbiform, rounded at the tip, pale green, connate below forming pouches for the flower, bracts of the coma 5-7.5 cm long, more or less tinged with red or pink. Calyx 8 mm long, irregular with 3-lobed, corolla tube 2.5 cm long with upper half like funnel-shaped, lobes pale rose-coloured, the lateral lobes oblong, the dorsal longer, ovate, concave, arching over the anthers. Lip yellow, obovate, deflexed, subentire or obscurely 3-lobed. lateral staminodes oblong, obtuse and as long as corolla-lobes [36].

Fig 2.10 *Curcuma aromatica* Salisb flowers, leaves and rhizomes

2.11.3 Chemical constituents

Analysis of rhizomes in *C. aromatica* gave the % values : Ether extracted 12.06, alcoholic extract 1.14, water extract 6.50, moisture content 13.33, albuminoids 30.63, starch 23.46, crude fiber 8.40, ash value 4.46 [36].

2.11.4 Medicinal applications

The rhizomes are the important herb which is used as medicinal plant in therapy. The rhizomes are used to cure in leucoderma and diseases of blood. In India, the rhizomes are applied as tonic, carminative, antidote to snack bite and astringent. In the Konkan, the rhizomes are utilized to stop the eruption of exanthematous fever. In addition, they are used in scabies and for the eruption os smallpox and removing stillborn baby from womb. Futhermore, the rhizomes are also applied for fratured bones and healing wounds, The paste made of rhizomes and milk is utilized for stomachache and blood dysentery. And the paste of rhizomes with benzoin is generally a domestic remedy in headache. Juice of *C. aromatica* is given for curing indigestion, dysentery and rheumatism. Moreover, the rhizomes are applied as an component of body deodorants. *C. aromatica* also is utilized as a color indicator in the apparatus used for purification of tap water [37].

2.12 Literature review

Carbohydrates are essential components of all living orginisms and are the most abundant class of biological molecules. Carbohydrates include monosaccharide, oligosaccharides and polysaccharides. Polysaccharides exist in an enormous structure diversity as they are produced by a variety of species such as microbes, algae, animals and especially in plants. Current applications used many kinds of natural polysaccharide and their derivatives in the pharmaceutical applications. For plant polysacchrides, published data indicate that they have many biological activities such as antioxidant activity, antitumor activity, anticancer activity, immunity activity and cell proliferation.

For literatures of antioxidant activity were presented as follow, in 2007 Li *et al.* used boiling water for extract the polysaccharide from *Lycium barbarum* fruit. *L. barbarum* polysaccharide showed notable inhibitory activity in the β-carotene-linoleat model system, exhibited a moderate concentration-dependent inhibition of the $DPPH^{\cdot}$ radical, showed significant reducing power, superoxide scavenging ability, inhibition of mice erythrocyte hemolysis mediated by peroxyl free radicals and also ferrousion chelating potency [38]. In the same year, Wang and Luo reported on the different fractions of polysaccharide purified from *Gynostemma pentaphyllum* Makino (GMA, GMB and GMC) were obtained from fractionation by DEAE-Sepharose CL-6B column and purification by Sephadex G-100 column chromatography. GMC had the

higher superoxide radical scavenging and hibitory effects on self-oxidation of 1,2,3-phentriol [4].

In 2008, Luo used boiling water to extract the crude polysaccharide from *Dioscorea nipponica* Makino. Crude polysaccharide contained predominantly water extractable polysaccharide (DMB) which was purified by DEAE-Sepharose CL-6B and Sephadex G-100 column chromatography. Futhermore DMB exhibited a higher activity at scavenging superoxide radical than vitamin C [3]. In the same year, Chen *et al.* also reported that the water-soluble polysaccharide was extracted from the fruiting bodies of *Ganoderma artum* and isolated by gel-filtration chromatography. The results indicated that the polysaccharide showed strong $DPPH^{\cdot}$ radical and superoxide anion radical scavenging activity [39].

In 2009, Capek *et al.* reported that crude polysaccharides were isolated from the aerial parts of sage (*Salvia officinalis* L.) by extraction with water (A), hot ammonium oxalate (B) and dimethyl sulfoxide (C). Six ion-exchange fractions from A were examined for $DPPH^{\cdot}$ and hydroxyl radical scavenging activities. The results showed that the radical scavenging abilities of the most active crude polysaccharides A, B and C on $DPPH^{\cdot}$ radical were found in the range 80-90% while the most active purified fraction A_3-A_6 achieved 75-92% [40]. In the same year, Xu *et.al* reported that the purified polysaccharide from fern *Pteridium aquilium* was obtained by fraction using DEAE-Sepharose fast-Flow column chromatography and purification using sephacyl S-400 HR column chromatography. The purified polysaccharide was demonstrated to have strong reducing power (FRAP value : 827.6 μmol/l), moderate scavenging activities against $DPPH^{\cdot}$ radical (83.1%) and superoxide radicals (60.5%) and moderate inhibitory power for self-oxidation of 1,2,3-phentriol (52.4%) [41].

In 2005, Ishurd et al. also reported the polysaccharide from Libyan dates (*Phoenix dactylifera* L.) had a anticancer activity on the allogenic solid Sarcoma-180 in mice [9] Futhermore, The antitumor activity of the pollen polysaccharide from *Brassica napus* L was reported by Yang *et al*. in 2007 [8].

For the published data about immunity activity were showed as follow, In 2006 Zhao *et al.* reported that the water-soluble polysaccharide was isolated from fuzi, the root of *Aconitum carmichaeli* Debx. by hot-water extraction and follow by anion exchange and gel filtratiion chromatography. The polysaccharide showed potent stimulating effects on marine lymphocyte proliferation induced by concanavalin A or lipopolysaccharide both in vitro and in vivo as well as on splenocyte antibody

production [6]. And in 2009 Yu *et al.* also reported that *Aloe vera* polysaccharide was effective in enhancing innate immunity in oral ulcer animals [42].

Furthermore cell proliferation from plant polysaccharides were showed as the follow, in 1994 Tong et al reported on that the effects of the four plant polysaccharides i.e., pachyman polysaccharides (PPS), Acanthopanax senticosus polysaccharides (ASPS), polysaccharides of tremella fuciformis (TF) and lentinan on cell proliferation in mice sarcoma (ascitic type) S180 and human chronic myelogenous leukemia K562 cells were studied with MTT assay. It was found That TF and lentinan had no effect on both cell line, but PPS and ASPS could obviously inhibit the proliferation of them [43]. In addition, in 2008 Yuan et al reported that the crude polysaccharide from Ligusticum chuanxiong Hort. was obtained by extraction and ethanol precipitation. The major polysaccharides LCA, LCB and LCC were isolated and purified from crude polysaccharide. The results showed that all purified polysaccharide exhibited cytotoxicity to human liver carcinoma cell line and LCB has the higest cytotoxicity activity among them [44]. In 2009, Lu et al reported that polysaccharide from Patrinia herterophylla Bunge (PHB-P) might act as an inhibitor of Hela cell line proliferation in vitro and might be a potential, natural apoptosis-inducing anticancer agent [45].

Moreover the publications also have been reported on cell proliferation in human gingival fibroblast cell as the following. In 2000 Chayavivattanavonk reported that the capsaicin at the different concentration 0.002, 0.003, 0.006, 0.010, 0.020 and 0.030% were tested in human gingival fibroblast cells by using MTT assay. The results showed the concentration 0.002% of capsaicin is the lowest concentration which toxic to human gingival fibroblast cells [46]. In 2001, Yu et al reported on cell proliferation of arecolin. They found that arecolin is cytotoxic to human gingival fibroblast cells at the concentration higher than 50 μg/ml [47]. Finally in 2008 Issa et al reported that the metal ions such as Cd^{2+}, Hg^{2+}, MeHg, Au^{3+}, Cu^{2+} at concentrations of 14, 20.2, 12.1, 40, 46 and 92 mg respectively can inhibit only 50% of mitochondrial activity in human gingival fibroblast cells. In the other hand [48], in 2000 Jettanacheawchankit et al. reported that acemannan extracted from Aloe vera at the different concentrations 200, 400, 800 and 1,000 μg/ml significantly induce cell proliferation ($p < 0.05$) of human gingival fibroblast cells [49].

Zingiberaceae family also has the researches about the biological activities from their polysaccharides. In 2000, KI *et al.* reported on the antitumor activity of the partially purified polysaccharide from *Curcuma zedoria*. The antitumor activity was studied in mice transplanted with sarcoma 180 cells. The results showed that the polysaccharide fraction CZ-1-III from *C. zedoria* at dose of 6.25 mg/kg showed 50% inhibition in solid tumor growth which were inhibited. These results strongly suggested that the polysaccharide fraction CZ-1-III from *C. zedoria* decrease tumor size of mouse. In the year later, they also reported on the effected of polysaccharide from rhizomes of *C. zedoria* on macrophage function. The results indicated that the polysaccharide fraction CZ-1-III had macrophage-stimulating activity and the possibility of being used as a biological response modifier [50].

In 2003, Bendjeddou *et al*. reported that hot water polysaccharide from *Alpinia galangal* was tested for its immunostimulating activity in mice. The results indicated that the polysaccharide showed a marked stimulating effect on the reticulo-endothelial system (RES) and increased the number of peritoneal exudate cells (PEC), and spleen cells of mice [5]. Moreover, the immunostimulating activity of crude polysaccharide extract isolated from *Curcuma xanthorrhiza* Roxb. was reported by Kim *et al*. in 2007. The polysaccharide from *C. xanthorrhiza* stimulated the phagocytic activity of raw 264.7 cells and also increased the release of NO, H_2O_2, INF-α and PGE_2 [51].

In this research, *Curcuma aromatica* was choosen to study because of this plant is also in Zingiberaceae family. The biological activities were presented as follow, in 1990 Santhanam *et al*. an ointment of white soft paraffin containing 1% of powder *C. aromatica* rhizomes was used to wound on laboratory rabbits [52]. In 1997, Kim *et al*. found that the methanolic and aqueous extracts from *C. aromatica* had ability antioxidant effect using Fenton's reagent/ethyl linoleate system [53]. For the researches about anti-inflammatory activity, in 1998 Li reported that the volatile oil derived from *C. aromatica* showed significant anti-inflammatory activity on the paw of mice [54]. The curcumin was found in *C. aromatica* by Masuda *et al*. in 2001. They also presented the mechanism of curcumin as antioxidant on polyunsaturated lipids [55]. In 2005, Choochote *et al*. reported on crude rhizome extracts and volatile oil of *C. aromatica* had anti-mosquito potential [11].

For the antitumor activity, in 2000 Wan *et al*. used *C. aromatica* oil to inhibit two tumor types. The inhibitory effects of *C. aromatica* oil on proliferation

immunohistochemical staining of proliferation cell nuclear antigen (PCNA). The two tumor inhibitory rates of *C. aromatica* oil in two tumor types were 50% and 51% [56]. In the same year Wu *et al*. found the mechanism of inhibition of curcumin on proliferation of HL-60 cells [57]. In addition, in 2001 Busquet *et al*. found that the systematic administration of curcumin to rats bearing the highly cachectic Yoshida AH-130 ascites hepatoma resulted in an important inhibition of tumor growth (31% total cell number) [58]. Futhermore, in 2006 Thippeswamy *et al*. reported that inhibition of tumor angiogenesis and/or activation extract from the dried rhizome of *C. aromatica*, had potent antiangiogenic and pro-apoptotic activity in Ehrlich ascites tumor [10].

In present, the research also has never been reported about extraction and cell proliferation of polysaccharide from *C. aromatica*. Lead to the idea of this research which expects to found bioactive polysaccharide as the polysaccharides isolated from other plant in Zingiberaceae.

CHAPTER III

EXPERIMENTAL

3.1 Material

3.1.1 Plant material

Fresh rhizomes of *Curcuma aromatica* were purchased from the Chatujak Market in March 2007 (Bangkok, Thailand). A voucher specimen (BK60395) is deposited at the Bangkok Herbarium (BK) of the Plant Variety Protection Division, Department of Agriculture, Thailand by Kongkanda Chayamarit (Collector). Fresh rhizomes were sliced and dried at 50°C overnight in the oven. The dried rhizomes were grinded and then purified by chromatography technique in the next step.

3.1.2 Human gingival fibroblast cells

Human gingival fibroblast cells were obtained from faculty of Dental medicine of Chulalongkron University, Thailand. Human gingival fibroblast cells were grown in cell culture dishes (100 x 20 mm) and preserved by using DMEM (Delbecco Modified Eagle's Medium) which were composed of 10% FBS (Fetal Bovine Serum), antibiotics and L-glutamined. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The media were changed every 2 days. For subcultivation cells were separated from culture plates with trypsin solution for 45 sec. Cell proliferation assay (MTT assay) was determined by using fifth-passage of human gingival fibroblast cells [53].

3.1.3 Chemicals and reagents

Antibiotic-antimycotic: Invitrogen, U.S.A

Butanol: Fluka, Germany

Chloroform: Merck Ag Darmstadt, Germany

Dimethyl sulfoxide: Fisher Scientific, UK

Ethanol 95%: Liquor Distillery Organization, Thailand

Fetal Bovin serum: Thermo scientific, U.S.A

Fructose: Amersham pharmacia Biotech, U.S.A

Galactose: Amersham phar macia Biotech, U.S.A

Gel filtration resin, superdex200: Amersham pharmacia Biotech, U.S.A

Glucose: Analytical UNIVAR reagent, U.S.A

Ion exchange resin, DEAE-cellulose: Amersham pharmacia Biotech, U.S.A

L-glutamine: Amersham pharmacia Biotech, U.S.A

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide): Molecular Probes, U.S.A

N-Acetyl D-glucosamine: Amersham pharmacia Biotech, U.S.A

Phenol: Carlo Erba Reagents, Italy

Ribose: Amersham pharmacia Biotech, U.S.A

Sodium bicarbonate: Carlo Erba Reagents, Italy

Sodium chloride: Carlo Erba Reagents, Italy

Sodium hydroxide: Carlo Erba Reagents, Italy

Sulfuric acid 95-97%: Merck Ag Darmstadt, Germany

Tetrahydrofuran: Merck, Germany

Trypsin-EDTA: Gibco, Canada

3.1.4 Apparatus and Instruments

Apparatus

Culture cell dish: Coming Incorporated Corning, U.S.A

Dialysis bag: SnakeSkin Dialysis Tubing, Pierce, U.S.A

Eppendroff: Axygen Scientific, U.S.A

Micropipette: PiPetteman, Gilson, France

Pipette tips: Bioline, U.S.A

Well plate: Nunc, Denmark

Instruments

AKTA prime: Amersham Oharmacia Biotech, U.S.A

Centrifuge: KUBOTA KR-20000T, Kubota medical Appliance supply, Japan

Column XK 1.6x15cm: Amersham pharmacia biotech wikstroms, Sweden

Column XK 1.6x60cm: Amersham pharmacia biotech wikstroms, Sweden

Cooling: EYELA, Japan

Electronic Balance: METTLER AT200, METTLER TOLEDO, Switzerland

Fourier transformation infrared (FT-IR): PerkinElmer, USA)

Freeze dryer: Labconco, U.S.A

Heating oven: SAFETY LAB, Asian Chemical and Engineering Co., LTD, Thailand

High performance liquid chromatography (HPLC): Alltech, USA

Incubator: Thermo scientific, U.S.A

LichrosphereNH_2 column: Merck, Germany

Microcentrifuge: Biofuge pico Heraeus, Kendo, Germany

Microtiter plate reader: Pecan sunrise 3550 UV, TECAN, Austria

Refrigarated centrifuge: Hettich, Germany

Rotary evaporator: BUCHI Rotavapor R-200, BUCHI, Switzerland

Speed Vac: MAXI dry plus, Denmark

Sonicator: BHA-1000, Branson, U.S.A

Vortex mixture: Vortex-Genie2, Scientific Industries, U.S.A

Water bath shaking: Memmert, Germany

Waters 600E: Meadows Instrumentation, U.S.A

3.2 Methods of polysaccharides purification

3.2.1 Determination of polysaccharide concentration

Phenol sulfuric acid method was used to determines polysaccharide concentration using glucose as a polysaccharide standard to generate a standard curve. The total volume of this method is 3,550 µl by pipetting 1,000 µl of polysaccharide samples into tubes before adding 50 µl of phenol (80% wt/wt) and 2,500 µl of Conc. H_2SO_4. The polysaccharide solutions were shaked and left for 10 min at room temperature. After that the polysaccharide solutions (200 µl) were measured by using microplate reader at wavelength 490 nm for calculating the amount of polysaccharides by comparing with standard graph of glucose [29].

3.2.2 Extraction and precipitation of crude polysaccharide

Crude polysaccharide was extracted and precipitated by a modified method of Bendjeddou (2003) and Chi (2006). The 600 g of dried rhizomes powder of *Curcuma aromatica* were dissolved in distilled water at 1 g per 20 ml. The suspension

was stirred on the water bath at 95ºC for 3 h. Then allowed it to be cooled before kept overnight at 4ºC. After that, it was centrifuged for 30 min, 6,000 rpm at 20°C. The supernatant was concentrated in a rotary evaporator under pressure at 50ºC and filtrated. The filtrate was precipitated with 4 volumes of 95% ethanol and the suspension was kept overnight at 4ºC. The precipitate was assembled by centrifugation for 45 min, 8,000 rpm at 20°C. Washed with ethanol, air dried and then dissolved in distilled water. The solution was deprotenized 3 times with sevage reagent (butanol and chloroform in 1:5 ratios) for protein precipitation. The supernatant was dialyzed against distilled water for 72 h and precipitated by 4 volumes of 95% ethanol and kept overnight at 4ºC. After centrifugation for 45 min, 8,000 rpm at 20°C crude polysaccharide was washed by 95% ethanol and freeze dried for 24-36 h, giving the dried crude polysaccharide. Crude polysaccharide was calculated the amount of polysaccharide by using phenol sulfuric acid method at 490 nm [5,6].

3.2.3 Column Chromatography

3.2.3.1 Anion exchange chromatography

Crude polysaccharide was dissolved in distilled water at 1.5 mg/ml and then injected to DEAE-cellulose anion exchange column (1.6 cm x 15 cm; first with distilled water for 500 ml at 10 ml/10 min/ tube, followed gradient elution by 0.5 M NaCl for 500 ml at 10 ml/10 min/ tube) [3,4,42].

3.2.3.2 Gel filtration Chromatography

Fractions containing interesting activity were pooled and dialyzed with distilled water. The solutions were dried and then dissolved in distill water. The solutions were injected in a column of Superdex G-200 (1.6 x 60 cm) size exclusion resin equilibrated with distilled water. The flow rate was 10 ml/20 min/ tube for 1,000 ml of distilled water. The obtained polysaccharides absorption were calculated by phenol sulfuric acid method at 490 nm [3,4,42].

3.3 Cell proliferation

3.3.1 MTT assay

Cell proliferation assay of polysaccharides was determined by using modified MTT assay of Thunyakitpaisal (2004). The human gingival fibroblast cells were plated at $7x10^4$ cell/well in 500 µl of DMEM onto 24-well plastic plate and incubated at 37°C, 5% CO_2 for 24 h. The DMEM was removed from the 24-well plastic plate, added 500 µl serum free DMEM per well and then incubated at 37°C, 5% CO_2 for 24 h. The serum free DMEM was changed every 3 h, and this procedure was repeated 2 times. The polysaccharides were then mixed with the serum free DMEM at various concentrations (0.1, 1, 10 and 100 µg/ml). The polysaccharide solutions were added in 500 µg/ml per well after removing the serum-free DMEM and incubated for 24 h at 37°C, 5% CO_2. The cell proliferation was determined by MTT method. The MTT solution (7 mg/ml) was added in 400 µl to each well plastic plate and incubated for 8-10 min at 37°C, 5% CO_2. Then the MTT solution was removed from the wells. The MTT-formazan crystals in cells were dissolved by adding 800 µl of dimethylsulfoxide (DMSO) to all of the wells and then transferred to cuvettes for measure the number of living cells by spectrophotometer, using test wavelength at 570 nm [59,60].

3.3.2 Statistic analysis

All the data were analyzed by using the SPSS (Statistical Package for the Social Science) program (one-way ANOVA) and measurements of the mean ± standard deviation were determined. Significances of difference were tested by Turkey's method, the levels of significance were indicated as $p<0.05$ and signified by using star (*) [59].

3.4 Method of polysaccharides identification

3.4.1 The functional group of polysaccharides by FT-IR spectroscopy

The polysaccharides P11 and P21 (0.05 mg) were grinded with KBr powders (2-3 mg) and then pressed into pellets for determine the functional groups of polysaccharides by using Fourier transformation infrared (FT-IR) spectroscopy.

3.4.2 Determination of the molecular weight of polysaccharides by GPC

The molecular weight of P11 was determined on a Waters 600E composed of a Waters Ultrahydrosel linear 1 column and a refractive Index Detector. The purified polysaccharide P11 solution (50µl) was injected with 1 M tetrahydrofuran (THF) as a mobile phase at 1 ml/min. Polystyrene was used as a standard (MW = 6,520-188,000 Da).

The molecular weight of P21 was determined on the same instrument as P11. The purified polysaccharide P21 solution (20µl) was injected with buffer (pH 11) as a mobile phase at 0.6 ml/min. Pullulan was used as standard (MW 5,900-788,000 Da).

3.4.3 Monosaccharide composition of polysaccharide by HPLC

Polysaccharide samples (10 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 100°C for 5 h and neutralized by NaOH. The solutions were dialyzed with distilled water for 3-5 h and dried by using Speed Vac. After hydrolyzation the residues were dissolved in distilled water and injected to high performance liquid chromatography (HPLC) using LichrosphereNH_2 column, eluted with 85% Acetonitile: 15% distilled water at a flow rate 1.5 ml/min at 25°C and detected by evaporative light scattering detector (ELSD). The standards of monosaccharide using 2 mg/ml of glucose, galactose, fructose, ribose and xylose [61,62].

CHAPTER IV

RESULTS AND DISCUSSION

The polysaccharides from *C. aromatica* were analyzed by using all methods as described in the chapter III. The results will be shown and discussed in each part from this chapter, respectively.

4.1 Determination of polysaccharides yield

Dried rhizomes powder 600 g from *C. aromatica* were extracted by hot water and precipitated by 4 volumn of 95% ethanol, giving a dried crude polysaccharide (a solid brown) was 6,160 mg (1.03%). Then crude polysaccharide was purified by anion exchange chromatography on DEAE-cellulose column and gel filtration chromatography on Superdex 200 column. We got P11 = 248.46 mg (0.04%), P21 = 111.26 mg (0.019%) and P22 = 0.65 mg (0.0001%) as show in table 4.1. The amount of polysaccharides was calculated by phenol sulfuric acid method and glucose is used as a standard (Fig 4.1). From table 4.1, purified polysaccharide P11 has the highest amount of polysaccharide and the amount of P21 is higher than P22. From the results of each polysaccharide fraction P11 and P21 at the retention time 40-60 and 80-200 min were collected respectively for study their characterization and ability in cell proliferation. In P22 was also collected but only study its ability in cell proliferation because its amount of polysaccharide was very low quantity so it's not enough for a further study.

Table 4.1 The polysaccharide yield of *C. aromatica* from 600 g dried rhizomes powder

Sample	Amount of polysaccharide (mg)	Polysaccharide yield (%)
Crude polysaccharide	6,160	1.03
P11	248.46	0.04
P21	111.26	0.019
P22	0.65	0.0001

4.2 Characterization of crude polysaccharide by column chromatography

4.2.1 Anion exchange chromatography

Crude polysaccharide from *C. aromatica* was purified by anion exchange chromatography. DEAE-cellulose was used as ion exchange resin. From the purification result, DEAE-cellulose can separate this crude polysaccharide (Fig 4.1). This crude polysaccharide was loaded on the DEAE-cellulose column; the chromatogram presented an unabsorbed fraction (P1) and a large absorbed fraction (P2) as shown in fig 4.1. The unabsorbed fraction (P1) and absorbed fraction (P2) were eluted by distilled water and 0.5 M NaCl, respectively.

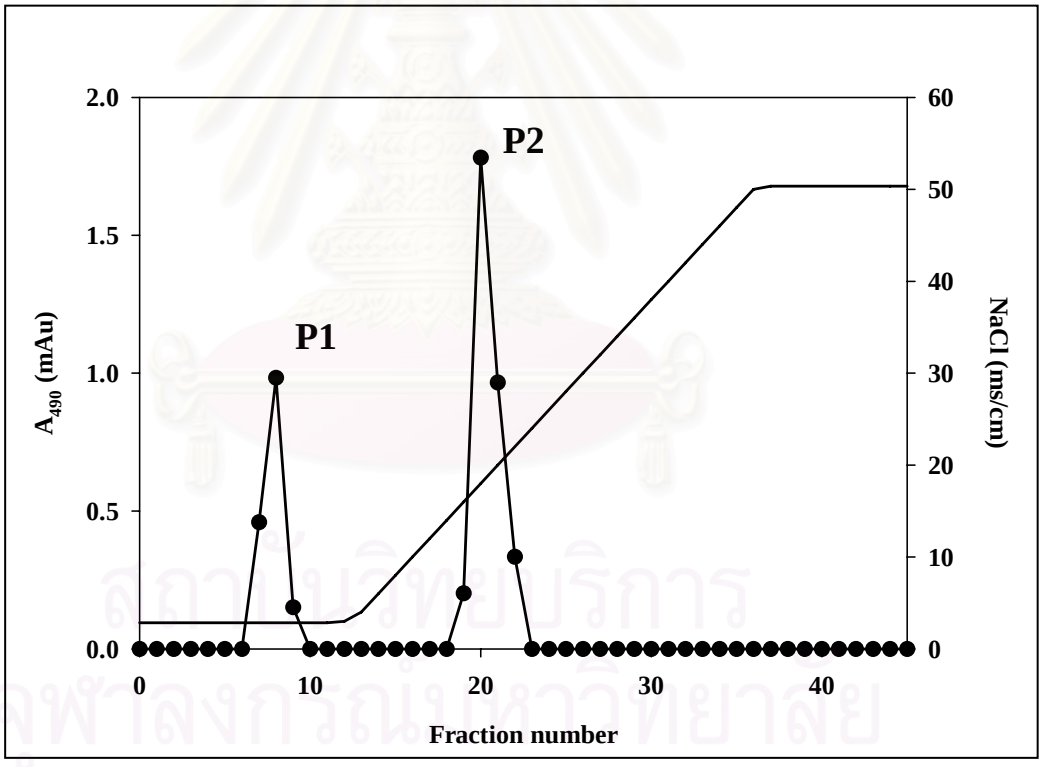


Fig 4.1 Anion exchange chromatography using DEAE-cellulose resin of crude polysaccharide

4.2.2 Gel filtration chromatography

Crude polysaccharide was purified by using DMEM-cellulose anion exchange column and yields a neutral polysaccharide (P1) and an acidic polysaccharide (P2). The next step was purification by using gel filtration chromatography technique. Two types of gel filtration resin, Superdex G-75 and Superdex G-200 were used to purify P1 and P2. Both of polysaccharides were eluted by using distilled water.

From this result, Superdex G-75 and Superdex G-200 can not separate P1 because chromatogram showed only single peaks. And these peaks were eluted at the beginning of the fraction (Fig 1D and 2D in appendixD). From this result, P1 should be very large polysaccharide which composes of many types of polysaccharide. On the other hand, P2 could be separated by using Superdex G-200, giving P21 and P22 (Fig 4.2).

From the result in table 4.1 and chromatogram of polysaccharides (Fig 4.2) indicated that P11 and P21 were a major polysaccharide and P22 was a minor polysaccharide. Both of major polysaccharides (P11 and P21) were collected for characterization and testing on cell proliferation of human gingival fibroblast cells. For a minor polysaccharide (P22) was collected and enough for only testing on cell proliferation.

4.3 Identification of purified polysaccharides

After we obtained two major purified polysaccharides (P11 and P21) from purification by using DEAE-cellulose anion exchange chromatography and gel filtration chromatography Superdex G-200 column. The next step was identification which composed of finding their functional group by using FT-IR spectroscopy, molecular weight by using Gel Permeable Chromatography (GPC) and monosaccharide composition by using acid hydrolysis method and followed by High Performance Liquid Chromatography (HPLC). The results showed as below.

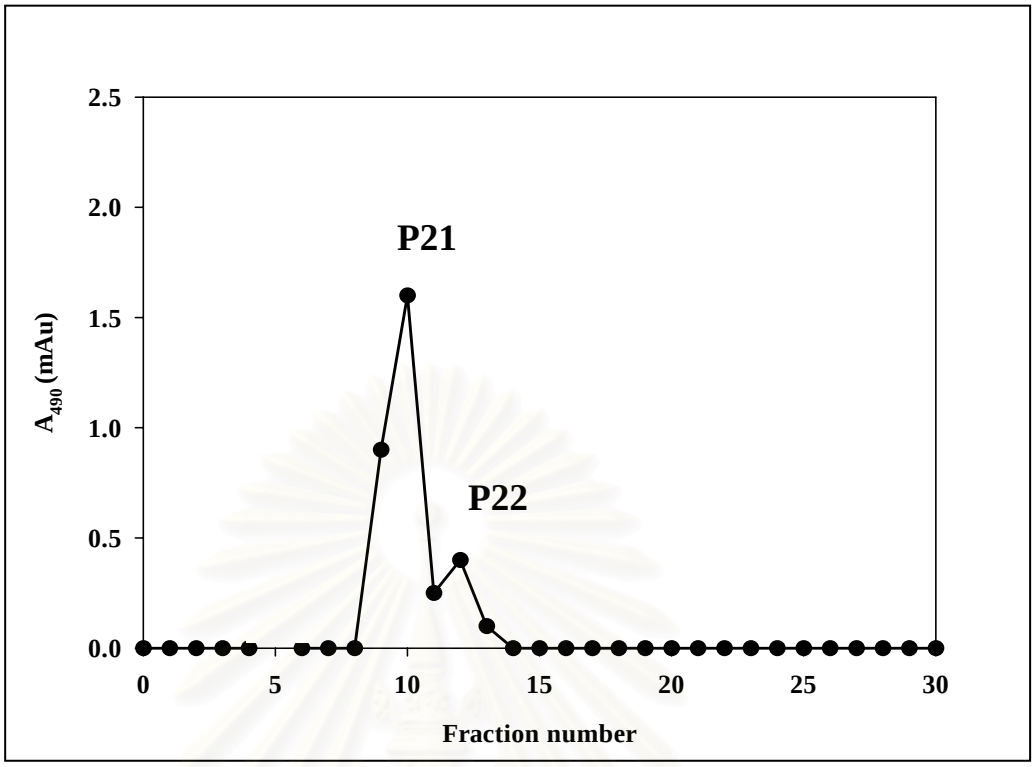


Fig 4.2 Gel filtration chromatography on Superdex G-200 column of P2

4.3.1 The functional group of polysaccharides by FT-IR spectroscopy

The results from FT-IR spectroscopy was used to confirm that P11 and P21 were polysaccharides (Fig 4.3). The intense bands between 3600-3200 cm^{-1} were O-H stretching in the constituent sugar residues of polysaccharides which both polysaccharides P11 and P21 showed their peaks at 3411.06 cm^{-1} and 3414.4 cm^{-1}. The absorbance at 2929.13 cm^{-1} and 2929.50 cm^{-1} of P11 and P21 represented C-H stretching in sugar ring and both of polysaccharides also had residual water bands around 1640-1644 cm^{-1}. Bands between 1200 up to 1460 cm^{-1} were C-H which peaks of P11 and P21 presented at 1427.99 cm^{-1} and 1365.40 cm^{-1} respectively.

The band at 1000-1160 cm^{-1} related to C-O bond. The bands from 1000 cm^{-1} to 1125 cm^{-1} were form of xylan and at 1035 cm^{-1} to 1111 cm^{-1} indicated C-O-C and O-H in pyran structure. Both polysaccharides showed those functional groups because they have peaks at 1078.76 cm^{-1} (P11) and 1091.70 cm^{-1} (P21). Furthermore, P11 also had other peaks at 1154.84 cm^{-1} and 1022.93 cm^{-1} which indicated C-O bond and form of xylan.

In addition, the O-H deformation vibration and 730 up to 900 cm^{-1} were C-H deformation vibration which showed only in P11 at 860.06 cm^{-1}.

4.3.2 The molecular weight of polysaccharides by GPC

The results from gel permeable chromatography indicated that P11 and P21 had average molecular weight of 469,171 and 157,665 Da, respectively (Fig 4.4).

The fact that P21 was eluted by 0.5 M NaCl in DEAE-cellulose anion exchange chromatography and P21 had prominently negative charges on its molecule indicated the P21 is acidic polysaccharide. Due to P21 is acidic polysaccharide so it can be eluted by using buffer (pH = 11) from GPC.

On the other hand, P11 was eluted by using tetrahydrofuran (THF) from GPC column. From this reason, it indicated that a neutral polysaccharide which had an equal amount of positive charges and negative charges on its molecule. In addition P11 was also eluted by water in DEAE-cellulose anion exchange which confirmed that P11 is a neutral polysaccharide.

4.3.3 Monosaccharide composition of polysaccharides by HPLC

The polysaccharides composition of P11 and P21 were identified by acid hydrolysis method and followed by HPLC. The results from HPLC show that both polysaccharides P11 and P21 contained on peak at retention time of 4.650 min (Fig 4.5B) and 4.750 min (Fig 4.5C) respectively. This is the same retention time of xylose standard as shown in fig 4.5A. From this comparison, it suggested that both polysaccharides P11 and P21 are only consisted of xylose.

In addition, the results from FT-IR spectroscopy also supported this because their functional groups from FT-IR spectroscopy showed that they had a form of xylan.

4.4 Cell proliferation assay

4.4.1 The effect on cell proliferation of human gingival fibroblast cells of crude polysaccharide, P11 and P22

The different concentration of crude polysaccharide at 1, 10, 100, 1,000 and 10,000 μg/ml were added into human gingival fibroblast cells for determine its ability in cell proliferation. The results from crude polysaccharide at the concentration of 1 μg/ml showed that it can not increase the proliferation of human gingival fibroblast cells and also doesn't toxic to cells. The crude polysaccharide at the concentration of 10, 100, 1,000 and 10,000 μg/ml showed ability to increase cells as percentage of decreasing compared to control which are 25.90 ± 8.03, 43.93 ± 17.18, 19.10 ± 5.23 and 24.29 ± 3.04%, respectively (Table 1E in appendixE). The one-way ANOVA indicated that experimental results of crude polysaccharide at the concentration 10, 100, 1,000 and 10,000 μg/ml were significant ($p<0.05$) (Fig 4.7).

P11 and P22 were obtained from purified crude polysaccharide by using DEAE-cellulose anion exchange chromatography and gel filtration chromatography Superdex G-200. The different concentration of P11 and P22 at 0.1, 1, 10, 100 and 1,000 μg/ml were added into human gingival fibroblast cells for determine their ability in cell proliferation. The results showed that both P11 (Table 2E in appendixE) and P22 (Table 4E in appendixE) at concentration of 0.1 and 1 μg/ml can not increase the proliferation of cells and also don't toxic with cells. On the other hand, both P11 and P22 at the concentration of 10, 100 and 1,000 μg/ml showed ability to increase the proliferation of human gingival fibroblast cells (Fig 4.7). The results of P11 were shown as percentage of increasing compared to control which are 18.19 ± 10.57, 30.09 ± 13.66 and 18.49 ± 14.42%, respectively. The result of P22 indicated that it can increase the cell to 26.22 ± 18.67, 40.98 ± 10.92 and 25.31 ± 15.62%, respectively. The one-way ANOVA indicated that experimental results of crude polysaccharide at the concentration 10, 100, and 1,000 μg/ml were significant ($p<0.05$)(Fig 4.7).

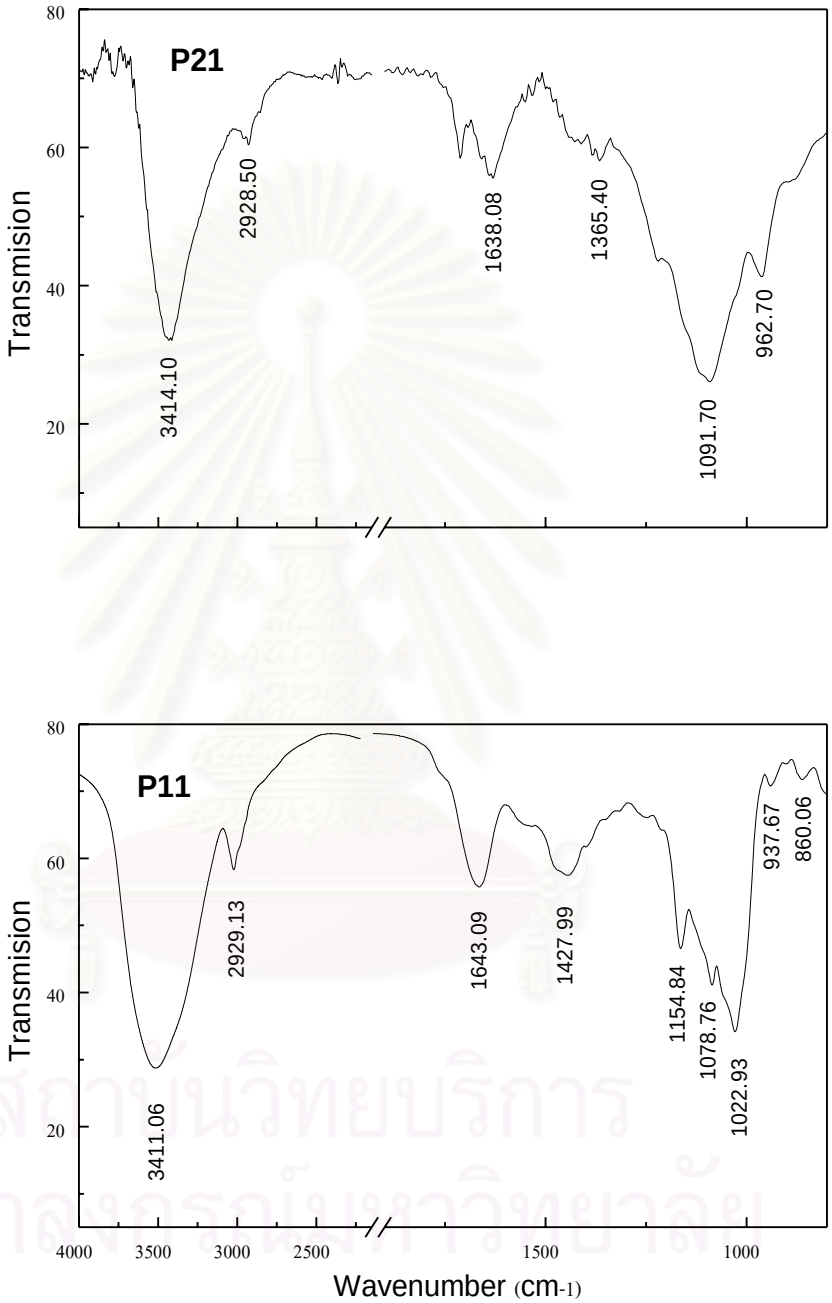


Fig 4.3 FT-IR spectroscopy of P11 and P21 from *C. aromatica* Salisb. Both polysaccharides were grinded with KBr for determination.

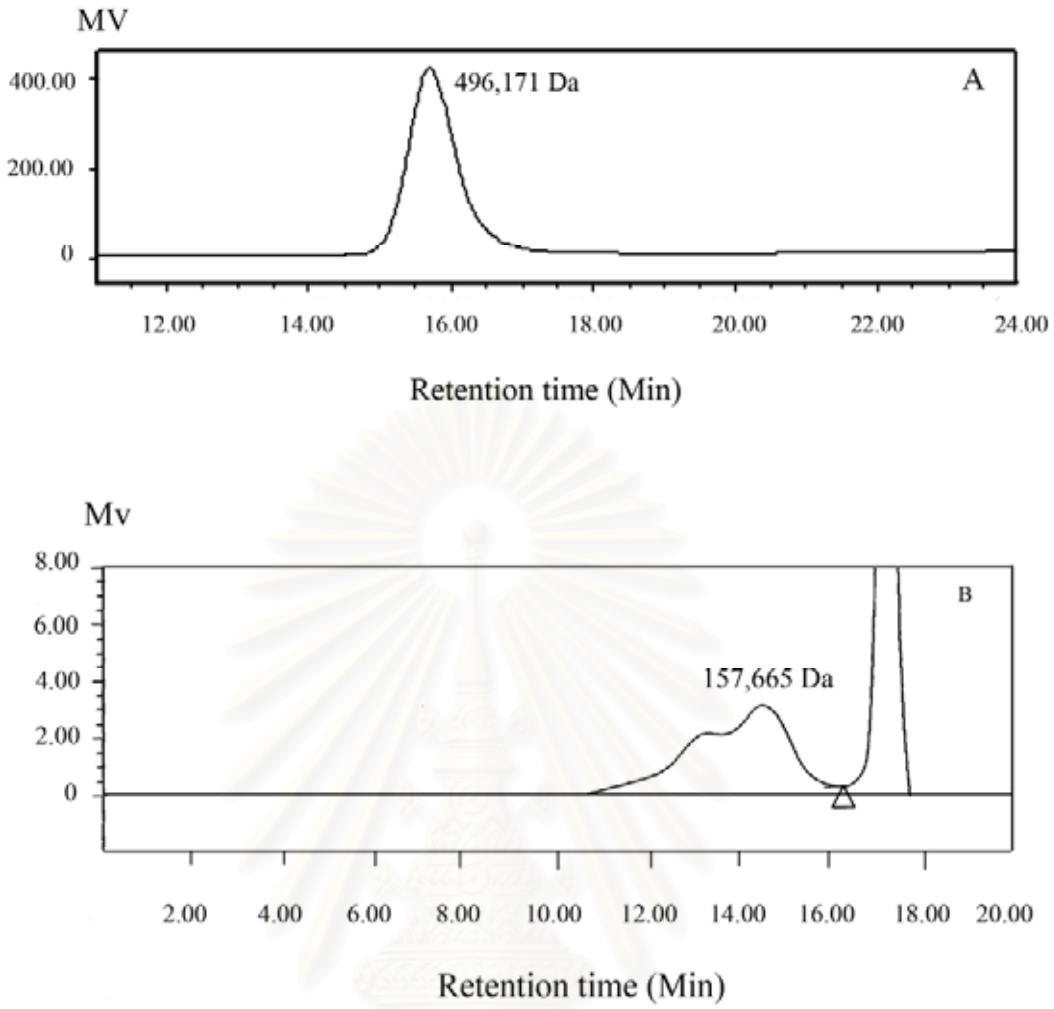


Fig 4.4 The molegular weight of P11 (A) and P21 (B) from gel permeable chromatography

Crude polysaccharide, P11 and P22 at the concentration of 100 μg/ml have the most highest to increase the number of human gingival fibroblast cells. Jettanacheawchankit (2004) reported that acemannan extracted from *Aloe vera* at the different concentrations 200, 400, 800 and 1,000 μg/ml can induce cell proliferation of human gingival fibroblast cells [49]. From the comparison, crude polysaccharide, P11 and P21 used less initial concentration than acemannan in inducing cell proliferation. The principle of MTT assay is a measure of mitochondria activity in cells or a measure of the number of surviving cells. So the results of crude polysaccharide, P11 and P22 also indicate that they can help mitochondria to work normally because human gingival fibroblast cells obtain the energy from mitochondria to use in cell proliferation [47].

4.4.2 The effect on cell proliferation of human gingival fibroblast cells of P21

The polysaccharide P21 was also obtained from purified crude polysaccharide by using DEAE-cellulose anion exchange chromatography and gel filtration chromatography Superdex G-200. The different concentration of P21 at 0.1, 1, 10 and 100 μg/ml were added into human gingival fibroblast cells for determine its ability in cell proliferation. The result from P21 (Table 3E in appendixE) at the concentration 100 μg/ml showed ability to inhibit the cell growth. The result was shown as percentage of decreasing compared to control which is $92.16 \pm 3.49\%$. The one-way ANOVA indicated that experimental results was significant ($p<0.05$). On the other hand, the other concentrations of P21 can not increase and inhibit the proliferation of human gingival fibroblast cells. It indicates that the other concentrations of P21 0.1, 1 and 10 μg/ml don't toxic to cells.

From the results above, indicated that P21 at the concentration of 100 μg/ml has ability to inhibit the number of human gingival fibroblast cells. This effect might be explained as P21 may decrease or obstruct mitochondria function [47]. Because of inefficient mitochondria function human gingival fibroblast cells lack the energy for repair and regenerated themselves. The arecolin was reported by Yu (2001) [47] that it is cytotoxic to human gingival fibroblast cells by inhibiting the mitochondrial function. The concentrations of arecolin at 50-200 μg/ml can inhibit 16-56% of mitochondrial activity while P21 at the concentrations of 100 μg/ml can inhibit cell around 90% of mitochondrial activity. From this comparison indicates that P21 is higher efficient inhibitor than arecolin. Although arecolin has a high concentration 200 μg/ml, its efficiency as inhibitor is still less than that of P21. Furthermore, P21 at concentration of 100 μg/ml can inhibit around 90% of mitochondrial function but Issa (2008) [59] reported that the metal ions such as Cd^{2+}, Hg^{2+}, MeHg, Au^{3+}, Cu^{2+} and Co^{2+} at concentrations of 14, 20.2, 12.1, 40, 46 and 92 mg respectively can inhibit only 50% of mitochondrial activity. This result suggested that P21 is more efficient than these metal ions.

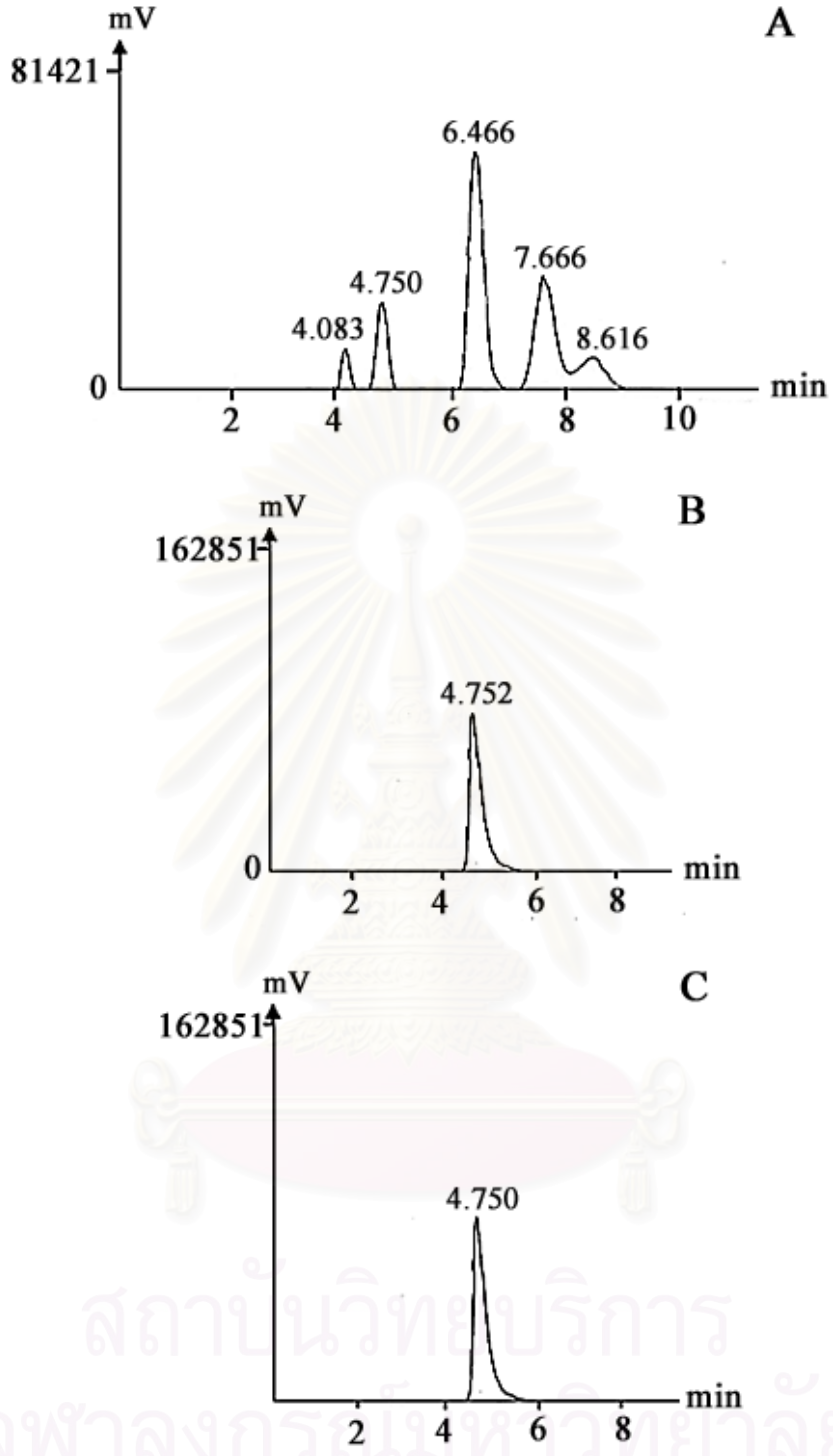


Fig 4.5 HPLC showed the monosaccharide standard (A) from left to right, ribose, xylose, fructose, glucose and galactose. The monosaccharide composition of P11 and P21 were composed of only xylose (B, C) respectively.

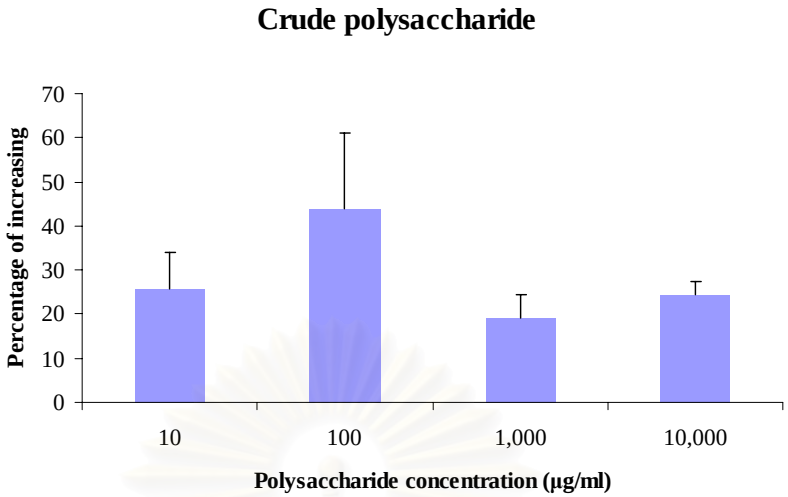


Fig 4.6 Percentage of increasing in human gingival fibroblast cells at the different concentration of crude polysaccharide compared to control (p<0.05, n=8). Control = $7x10^4$ cells

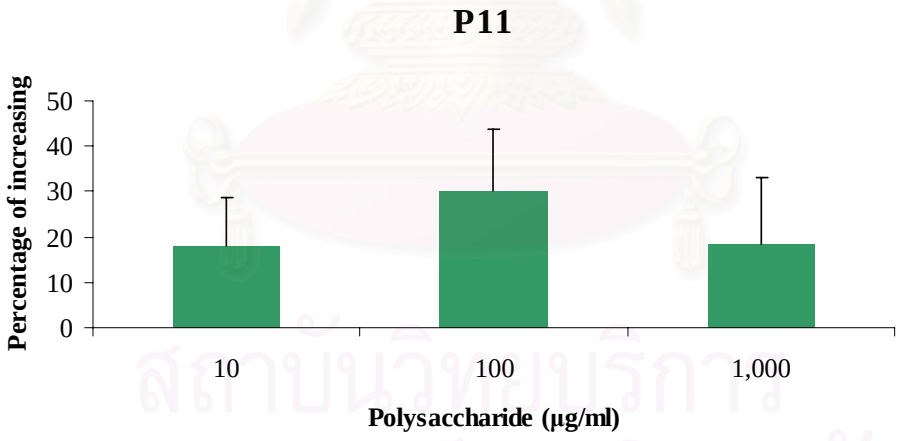


Fig 4.7 Percentage of increasing in human gingival fibroblast cells at the different concentration of P11 compared to control (p<0.05, n=8). Control = $7x10^4$ cells

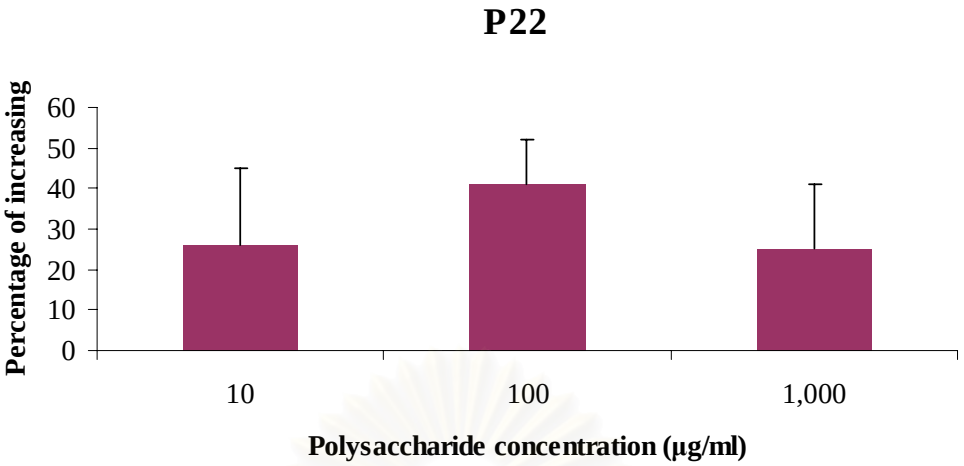


Fig 4.8 6 Percentage of increasing in human gingival fibroblast cells at the different concentration of P22 compared to control (p<0.05, n=8). Control = $7x10^4$ cells

CHAPTER V

CONCLUSION

The major polysaccharides P11 and P21 from *Curcuma aromatica* were extracted, purified, characterized and tested on cell proliferation but the minor polysaccharide P22 was only tested on cell proliferation because of its limited amount. Crude polysaccharide from *Curcuma aromatica* was extracted by hot water and then precipitated by 4 volume of 95% ethanol by a modified method [5,6]. The amount of crude polysaccharide is 6.16 g (1.03%). Then crude polysaccharide was purified in two steps by using DEAE-cellulose anion exchange chromatography and gel filtration chromatography on Superdex G-200, giving P11 = 248.46 mg (0.04%), P21 = 111.26 mg (0.019%) and P22 = 0.65 mg (0.0001%).

The major polysaccharide P11 and P21 were characterized by FT-IR, GPC and HPLC as described in Experimental section. The results from FT-IR spectroscopy indicated and confirmed that P11 and P21 are polysaccharides from their functional groups and also contain xylan structure. After that monosaccharide composition of P11 and P21 were investigated by hydrolysis method and HPLC. The results suggested that xylose is only one monosaccharide in P11 and P21. Then the molecular weight of P11 and P21 were determined by GPC. The results from GPC showed P11 and P21 are homogenous polysaccharides with weight-average molecular weight of 469,171and 157,665Da, respectively.

Crude polysaccharide and the purified polysaccharides P11, P21 and P22 were tested in proliferation of human gingival fibroblast cells by using MTT assay. The results from crude polysaccharide, P11 and P22 indicated that the concentration at 100 μg/ml have the highest ability to significantly increase the cell proliferation of human gingival fibroblast cells. In contrast P21 at the concentration 100 μg/ml can significantly decrease the cell proliferation of human gingival fibroblast.

As P11 and P22 had an ability to proliferate cells, these major polysaccharides can be used as an ingredient of medications for the treatment of gingival ulcers. On

the other hand, P21 had an ability to decrease cell proliferation, thus the polysaccharide should be tested against malignant human gingival fibroblast cell line to observe its ability to inhibit the mentioned malignant cells.

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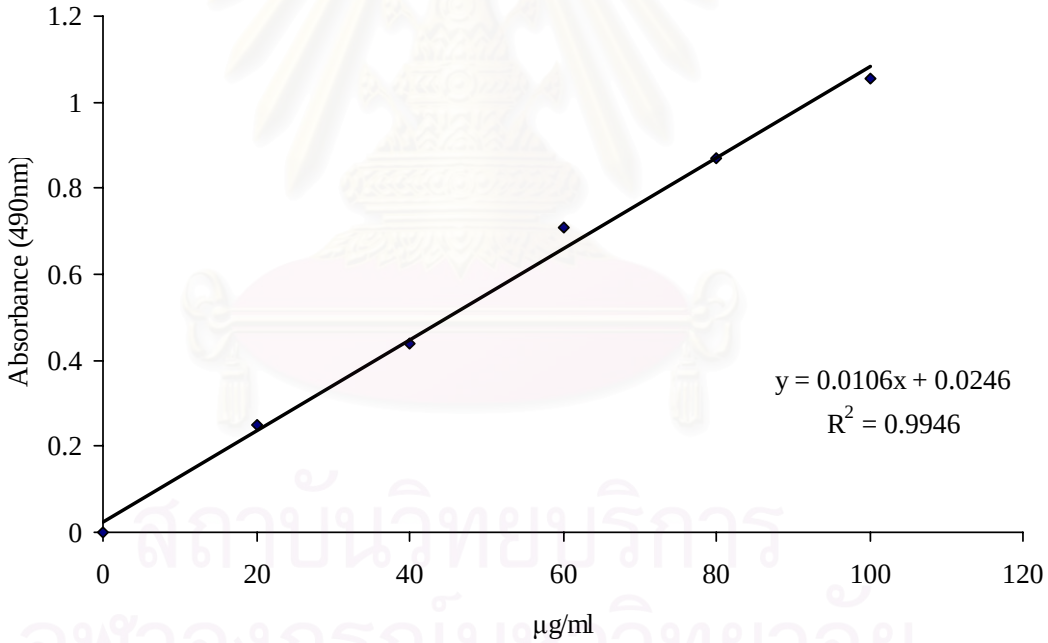
APPENDICES

Appendix A

Table 1A Solution for phenol sulfuric acid assay

Chemical	Final concentration	Amount
Phenol	10% w/v	8 g
Distilled water		to 100 ml
Sulfuric acid	100% w/v	5 ml

Fig 1A Calibration curve of polysaccharide standard

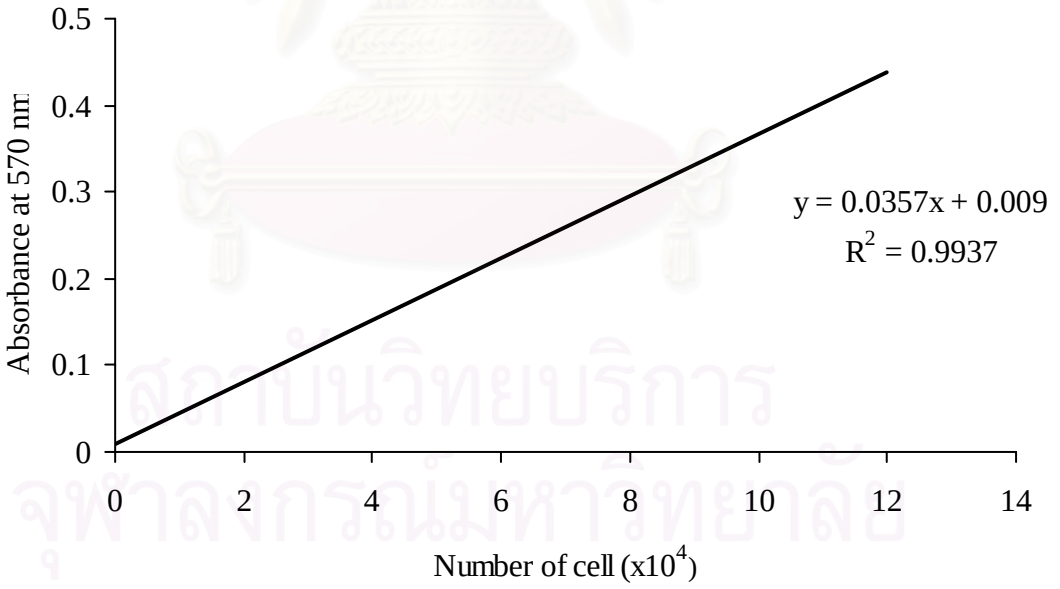


Appendix B

Table 1B Media for human gingival fibroblast cells

Chemical	Final concentration	Amount
DMEM	1 M	10 ml
Distilled water		to 1,000 ml
Fetal bovine serum	10% v/w	100 ml
L-glutamine	2mM	10 ml
Pennicillin G	100 μg/ml	
Streptomycin sulfate	100 μg/ml	mixed to 10 ml
Amphotericin B	20 μg/ml	

Fig 1B Calibration curve of number of cell standard



Appendix C

Table 1C Solution for MTT assay

Chemical	Final concentration	Amount
MTT	0.7 mg/ml	0.0112 g
Serum free DMEM		16 ml

Appendix D

Fig 1D Gel filtration chromatography Superdex G-75 column of P1

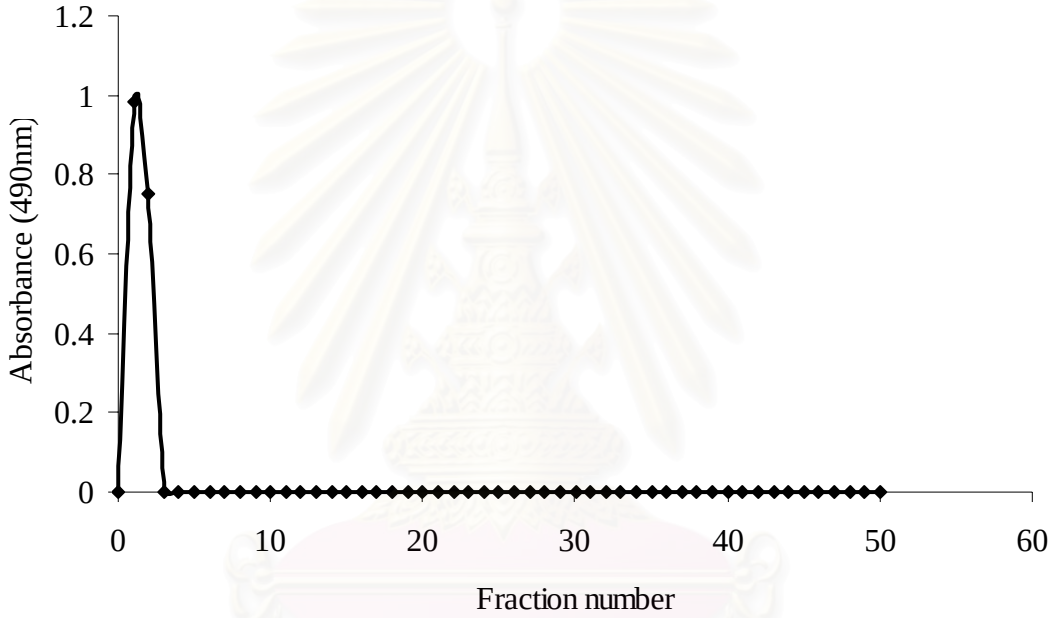


Fig 2D Gel filtration chromatography Superdex G-200 column of P1

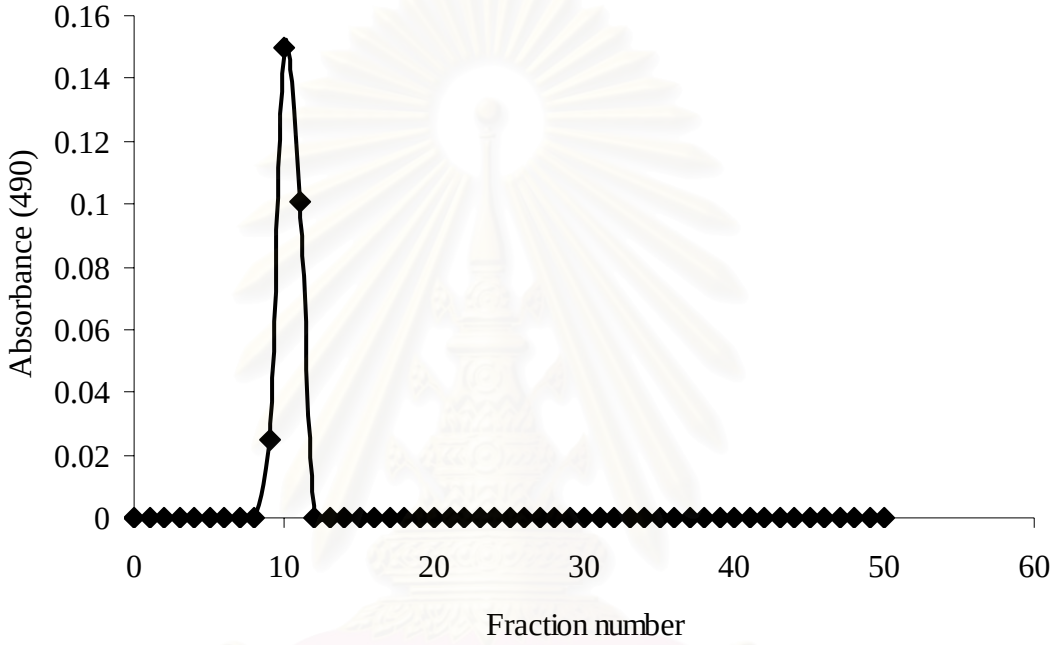


Fig 3D Gel filtration chromatography Superdex G-75 column of P2

Appendix E

Table 1E The statistic results of the effect of different concentrations from crude polysaccharide (1 = Control, 2 = 1 mg/ml, 3 = 0.1 mg/ml, 4 = 0.01 mg/ml and 5 = 0.001 mg/ml) on the proliferation of human gingival fibroblast cells

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	CONC	N	Percent	N	Percent	N	Percent
AB570	1	8	100.0%	0	.0%	8	100.0%
	2	8	100.0%	0	.0%	8	100.0%
	3	8	100.0%	0	.0%	8	100.0%
	4	8	100.0%	0	.0%	8	100.0%
	5	8	100.0%	0	.0%	8	100.0%

Descriptives

	CONC			Statistic	Std. Error
AB57	1	Mean		.24262	.006074
		95% Confidence Interval for Mean	Lower Bound	.22826	
			Upper Bound	.25699	
		5% Trimmed Mean		.24264	
		Median		.24300	
		Variance		.000	
		Std. Deviation		.017179	
		Minimum		.219	
		Maximum		.266	
		Range		.047	
		Interquartile Range		.03250	
		Skewness		-.255	.752
		Kurtosis		-1.167	1.481
	2	Mean		.28725	.004308
		95% Confidence Interval for Mean	Lower Bound	.27706	
			Upper Bound	.29744	
		5% Trimmed Mean		.28756	
		Median		.29100	
		Variance		.000	
		Std. Deviation		.012186	
		Minimum		.267	
		Maximum		.302	
		Range		.035	
		Interquartile Range		.02075	
		Skewness		-.866	.752
		Kurtosis		-.308	1.481
	3	Mean		.34525	.014192

		95% Confidence Interval for Mean	Lower Bound	.31169	
			Upper Bound	.37881	
		5% Trimmed Mean		.34278	
		Median		.33800	
		Variance		.002	
		Std. Deviation		.040142	
		Minimum		.306	
		Maximum		.429	
		Range		.123	
		Interquartile Range		.05175	
		Skewness		1.452	.752
		Kurtosis		2.202	1.481
	4	Mean		.30375	.006670
		95% Confidence Interval for Mean	Lower Bound	.28798	
			Upper Bound	.31952	
		5% Trimmed Mean		.30350	
		Median		.30000	
		Variance		.000	
		Std. Deviation		.018866	
		Minimum		.284	
		Maximum		.328	
		Range		.044	
		Interquartile Range		.03800	
		Skewness		.252	.752
		Kurtosis		-2.187	1.481
	5	Mean		.27238	.008294
		95% Confidence Interval for Mean	Lower Bound	.25276	
			Upper Bound	.29199	
		5% Trimmed Mean		.27075	
		Median		.26300	
		Variance		.001	
		Std. Deviation		.023458	
		Minimum		.250	
		Maximum		.324	
		Range		.074	
		Interquartile Range		.02375	
		Skewness		1.831	.752
		Kurtosis		3.553	1.481

Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	CONC	Statistic	df	Sig.	Statistic	df	Sig.
AB570	1	.157	8	.200(*)	.934	8	.551
	2	.242	8	.187	.888	8	.226
	3	.233	8	.200(*)	.869	8	.148
	4	.250	8	.149	.845	8	.086
	5	.280	8	.064	.795	8	.025

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Test of Homogeneity of Variance

		Levene Statistic	df1	df2	Sig.
AB570	Based on Mean	1.818	4	35	.147
	Based on Median	1.482	4	35	.229
	Based on Median and with adjusted df	1.482	4	17.474	.250
	Based on trimmed mean	1.681	4	35	.176

Multiple Comparisons

Dependent Variable: AB570
Tukey HSD

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
(I) CONC	(J) CONC				Lower Bound	Upper Bound
1	2	-.04462(*)	.012168	.007	-.07961	-.00964
	3	-.10262(*)	.012168	.000	-.13761	-.06764
	4	-.06112(*)	.012168	.000	-.09611	-.02614
	5	-.02975	.012168	.127	-.06473	.00523
2	1	.04462(*)	.012168	.007	.00964	.07961
	3	-.05800(*)	.012168	.000	-.09298	-.02302
	4	-.01650	.012168	.659	-.05148	.01848
	5	.01487	.012168	.739	-.02011	.04986
3	1	.10262(*)	.012168	.000	.06764	.13761
	2	.05800(*)	.012168	.000	.02302	.09298
	4	.04150(*)	.012168	.013	.00652	.07648
	5	.07287(*)	.012168	.000	.03789	.10786
4	1	.06112(*)	.012168	.000	.02614	.09611
	2	.01650	.012168	.659	-.01848	.05148
	3	-.04150(*)	.012168	.013	-.07648	-.00652
	5	.03137	.012168	.097	-.00361	.06636
5	1	.02975	.012168	.127	-.00523	.06473
	2	-.01487	.012168	.739	-.04986	.02011
	3	-.07287(*)	.012168	.000	-.10786	-.03789
	4	-.03137	.012168	.097	-.06636	.00361

* The mean difference is significant at the .05 level.

Table 2E The statistic results of the effect of different concentrations from polysaccharide P11 (Control = 1, 2 = 100μg/ml, 3 = 10μg/ml, 4 = 1μg/ml, 5 = 0.1μg/ml) on the proliferation of human gingival fibroblast cells

Case Processing Summary

	CONTROL	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
AB570	1	6	100.0%	0	.0%	6	100.0%
	2	6	100.0%	0	.0%	6	100.0%
	3	6	100.0%	0	.0%	6	100.0%
	4	6	100.0%	0	.0%	6	100.0%
	5	6	100.0%	0	.0%	6	100.0%

Descriptives

	CONTROL			Statistic	Std. Error
AB570	1	Mean		.29483	.002960
		95% Confidence Interval for Mean	Lower Bound	.28722	
			Upper Bound	.30244	
		5% Trimmed Mean		.29509	
		Median		.29650	
		Variance		.000	
		Std. Deviation		.007250	
		Minimum		.283	
		Maximum		.302	
		Range		.019	
		Interquartile Range		.01300	
		Skewness		-.894	.845
		Kurtosis		-.085	1.741
	2	Mean		.38083	.015945
		95% Confidence Interval for Mean	Lower Bound	.33985	
			Upper Bound	.42182	
		5% Trimmed Mean		.38143	
		Median		.39550	
		Variance		.002	
		Std. Deviation		.039056	
		Minimum		.330	
		Maximum		.421	
		Range		.091	
		Interquartile Range		.07900	
		Skewness		-.705	.845
		Kurtosis		-1.789	1.741
	3	Mean		.34683	.012335
		95% Confidence Interval for Mean	Lower Bound	.31512	
			Upper Bound	.37854	

		5% Trimmed Mean		.34643	
		Median		.34600	
		Variance		.001	
		Std. Deviation		.030215	
		Minimum		.314	
		Maximum		.387	
		Range		.073	
		Interquartile Range		.06025	
		Skewness		.167	.845
		Kurtosis		-1.956	1.741
	4	Mean		.33500	.008595
		95% Confidence Interval for Mean	Lower Bound	.31291	
			Upper Bound	.35709	
		5% Trimmed Mean		.33572	
		Median		.34000	
		Variance		.000	
		Std. Deviation		.021052	
		Minimum		.302	
		Maximum		.355	
		Range		.053	
		Interquartile Range		.03800	
		Skewness		-.749	.845
		Kurtosis		-.797	1.741
	5	Mean		.32567	.008671
		95% Confidence Interval for Mean	Lower Bound	.30338	
			Upper Bound	.34795	
		5% Trimmed Mean		.32457	
		Median		.32050	
		Variance		.000	
		Std. Deviation		.021238	
		Minimum		.306	
		Maximum		.365	
		Range		.059	
		Interquartile Range		.02975	
		Skewness		1.553	.845
		Kurtosis		2.681	1.741

Tests of Normality

	CONTROL	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
AB570	1	.176	6	.200(*)	.922	6	.522
	2	.289	6	.128	.839	6	.129
	3	.180	6	.200(*)	.919	6	.499
	4	.232	6	.200(*)	.904	6	.397
	5	.234	6	.200(*)	.865	6	.207

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Test of Homogeneity of Variance

		Levene Statistic	df1	df2	Sig.
AB570	Based on Mean	4.694	4	25	.006
	Based on Median	1.908	4	25	.140
	Based on Median and with adjusted df	1.908	4	11.791	.175
	Based on trimmed mean	4.479	4	25	.007

Multiple Comparisons

Dependent Variable: AB570
Tukey HSD

(I) CONTROL	(J) CONTROL	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.08600(*)	.015023	.000	-.13590	-.03610
	3	-.05200(*)	.015023	.038	-.10190	-.00210
	4	-.04017	.015023	.163	-.09007	.00974
	5	-.03083	.015023	.400	-.08074	.01907
2	1	.08600(*)	.015023	.000	.03610	.13590
	3	.03400	.015023	.304	-.01590	.08390
	4	.04583	.015023	.084	-.00407	.09574
	5	.05517(*)	.015023	.024	.00526	.10507
3	1	.05200(*)	.015023	.038	.00210	.10190
	2	-.03400	.015023	.304	-.08390	.01590
	4	.01183	.015023	.959	-.03807	.06174
	5	.02117	.015023	.739	-.02874	.07107
4	1	.04017	.015023	.163	-.00974	.09007
	2	-.04583	.015023	.084	-.09574	.00407
	3	-.01183	.015023	.959	-.06174	.03807
	5	.00933	.015023	.983	-.04057	.05924
5	1	.03083	.015023	.400	-.01907	.08074
	2	-.05517(*)	.015023	.024	-.10507	-.00526
	3	-.02117	.015023	.739	-.07107	.02874
	4	-.00933	.015023	.983	-.05924	.04057

* The mean difference is significant at the .05 level.

Table 3E The statistic results of the effect of different concentrations from polysaccharide P21 (Control = 1, 2 = 100μg/ml, 3 = 10μg/ml, 4 = 1μg/ml, 5 = 0.1μg/ml) on the proliferation of human gingival fibroblast cells

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	CONC	N	Percent	N	Percent	N	Percent
AB570	1	8	100.0%	0	.0%	8	100.0%
	2	8	100.0%	0	.0%	8	100.0%
	3	8	100.0%	0	.0%	8	100.0%
	4	8	100.0%	0	.0%	8	100.0%
	5	8	100.0%	0	.0%	8	100.0%

Descriptives

	CONC			Statistic	Std. Error
AB570	1	Mean		.26800	.019441
		95% Confidence Interval for Mean	Lower Bound	.22203	
			Upper Bound	.31397	
		5% Trimmed Mean		.26578	
		Median		.24300	
		Variance		.003	
		Std. Deviation		.054988	
		Minimum		.212	
		Maximum		.364	
		Range		.152	
		Interquartile Range		.09150	
		Skewness		.846	.752
		Kurtosis		-.737	1.481
	2	Mean		.02938	.003196
		95% Confidence Interval for Mean	Lower Bound	.02182	
			Upper Bound	.03693	
		5% Trimmed Mean		.02919	
		Median		.02800	
		Variance		.000	
		Std. Deviation		.009039	
		Minimum		.015	
		Maximum		.047	
		Range		.032	
		Interquartile Range		.00775	
		Skewness		.644	.752
		Kurtosis		2.297	1.481
	3	Mean		.22325	.008575
		95% Confidence	Lower Bound	.20297	

		Interval for Mean	Upper Bound	.24353	
		5% Trimmed Mean		.22472	
		Median		.22850	
		Variance		.001	
		Std. Deviation		.024253	
		Minimum		.171	
		Maximum		.249	
		Range		.078	
		Interquartile Range		.02525	
		Skewness		-1.578	.752
		Kurtosis		3.073	1.481
	4	Mean		.31713	.032538
		95% Confidence Interval for Mean	Lower Bound	.24018	
			Upper Bound	.39407	
		5% Trimmed Mean		.31686	
		Median		.30650	
		Variance		.008	
		Std. Deviation		.092032	
		Minimum		.194	
		Maximum		.445	
		Range		.251	
		Interquartile Range		.16975	
		Skewness		.239	.752
		Kurtosis		-1.431	1.481
	5	Mean		.28862	.023854
		95% Confidence Interval for Mean	Lower Bound	.23222	
			Upper Bound	.34503	
		5% Trimmed Mean		.28719	
		Median		.27000	
		Variance		.005	
		Std. Deviation		.067470	
		Minimum		.207	
		Maximum		.396	
		Range		.189	
		Interquartile Range		.11650	
		Skewness		.767	.752
		Kurtosis		-.654	1.481

Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	CONC	Statistic	df	Sig.	Statistic	df	Sig.
AB570	1	.280	8	.064	.875	8	.170
	2	.189	8	.200(*)	.927	8	.487
	3	.213	8	.200(*)	.861	8	.124
	4	.199	8	.200(*)	.932	8	.535
	5	.191	8	.200(*)	.896	8	.268

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Test of Homogeneity of Variance

		Levene Statistic	df1	df2	Sig.
AB570	Based on Mean	6.407	4	35	.001
	Based on Median	4.425	4	35	.005
	Based on Median and with adjusted df	4.425	4	27.185	.007
	Based on trimmed mean	6.232	4	35	.001

Multiple Comparisons

Dependent Variable: AB570
Tukey HSD

			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
	(I) CONC	(J) CONC				Lower Bound	Upper Bound
Tukey HSD	1	2	.23863(*)	.028910	.000	.15551	.32174
		3	.04475	.028910	.539	-.03837	.12787
		4	-.04912	.028910	.448	-.13224	.03399
		5	-.02063	.028910	.952	-.10374	.06249
	2	1	-.23863(*)	.028910	.000	-.32174	-.15551
		3	-.19388(*)	.028910	.000	-.27699	-.11076
		4	-.28775(*)	.028910	.000	-.37087	-.20463
		5	-.25925(*)	.028910	.000	-.34237	-.17613
	3	1	-.04475	.028910	.539	-.12787	.03837
		2	.19388(*)	.028910	.000	.11076	.27699
		4	-.09387(*)	.028910	.020	-.17699	-.01076
		5	-.06537	.028910	.182	-.14849	.01774
	4	1	.04912	.028910	.448	-.03399	.13224
		2	.28775(*)	.028910	.000	.20463	.37087
		3	.09387(*)	.028910	.020	.01076	.17699
		5	.02850	.028910	.860	-.05462	.11162
	5	1	.02063	.028910	.952	-.06249	.10374
		2	.25925(*)	.028910	.000	.17613	.34237
		3	.06537	.028910	.182	-.01774	.14849
		4	-.02850	.028910	.860	-.11162	.05462

* The mean difference is significant at the .05 level.

Table 4E The statistic results of the effect of different concentrations from polysaccharide P22 (Control = 1, 2 = 100μg/ml, 3 = 10μg/ml, 4 = 1μg/ml, 5 = 0.1μg/ml) on the proliferation of human gingival fibroblast cells

Case Processing Summary

		Cases					
		Valid		Missing		Total	
	CONC	N	Percent	N	Percent	N	Percent
AB570	1	8	100.0%	0	.0%	8	100.0%
	2	8	100.0%	0	.0%	8	100.0%
	3	8	100.0%	0	.0%	8	100.0%
	4	8	100.0%	0	.0%	8	100.0%
	5	8	100.0%	0	.0%	8	100.0%

Descriptives

	CONC			Statistic	Std. Error
AB570	1	Mean		.25213	.019853
		95% Confidence Interval for Mean	Lower Bound	.20518	
			Upper Bound	.29907	
		5% Trimmed Mean		.24869	
		Median		.23800	
		Variance		.003	
		Std. Deviation		.056154	
		Minimum		.203	
		Maximum		.363	
		Range		.160	
		Interquartile Range		.08625	
		Skewness		1.357	.752
		Kurtosis		1.120	1.481
	2	Mean		.35175	.009388
		95% Confidence Interval for Mean	Lower Bound	.32955	
			Upper Bound	.37395	
		5% Trimmed Mean		.35167	
		Median		.35500	
		Variance		.001	
		Std. Deviation		.026553	
		Minimum		.317	
		Maximum		.388	
		Range		.071	
		Interquartile Range		.04825	
		Skewness		-.048	.752
		Kurtosis		-1.810	1.481
	3	Mean		.31588	.016046
		95%	Lower Bound	.27793	

		Confidence Interval for Mean	Upper Bound	.35382	
		5% Trimmed Mean		.31536	
		Median		.31900	
		Variance		.002	
		Std. Deviation		.045385	
		Minimum		.263	
		Maximum		.378	
		Range		.115	
		Interquartile Range		.09350	
		Skewness		.257	.752
		Kurtosis		-1.435	1.481
	4	Mean		.26638	.008102
		95% Confidence Interval for Mean	Lower Bound	.24722	
			Upper Bound	.28553	
		5% Trimmed Mean		.26692	
		Median		.27600	
		Variance		.001	
		Std. Deviation		.022916	
		Minimum		.231	
		Maximum		.292	
		Range		.061	
		Interquartile Range		.04200	
		Skewness		-.593	.752
		Kurtosis		-1.496	1.481
	5	Mean		.27700	.012362
		95% Confidence Interval for Mean	Lower Bound	.24777	
			Upper Bound	.30623	
		5% Trimmed Mean		.27644	
		Median		.26950	
		Variance		.001	
		Std. Deviation		.034965	
		Minimum		.236	
		Maximum		.328	
		Range		.092	
		Interquartile Range		.07025	
		Skewness		.598	.752
		Kurtosis		-1.035	1.481

Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	CONC	Statistic	df	Sig.	Statistic	df	Sig.
AB570	1	.286	8	.052	.837	8	.070
	2	.230	8	.200(*)	.919	8	.423
	3	.197	8	.200(*)	.897	8	.270
	4	.272	8	.084	.885	8	.210
	5	.204	8	.200(*)	.901	8	.292

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Test of Homogeneity of Variance

		Levene Statistic	df1	df2	Sig.
AB570	Based on Mean	1.544	4	35	.211
	Based on Median	.968	4	35	.437
	Based on Median and with adjusted df	.968	4	19.304	.447
	Based on trimmed mean	1.269	4	35	.301

Multiple Comparisons

Dependent Variable: AB570
Tukey HSD

			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
	(I) CONC	(J) CONC				Lower Bound	Upper Bound
Tukey HSD	1	2	-.09963(*)	.019578	.000	-.15591	-.04334
		3	-.06375(*)	.019578	.020	-.12004	-.00746
		4	-.01425	.019578	.949	-.07054	.04204
		5	-.02488	.019578	.710	-.08116	.03141
	2	1	.09963(*)	.019578	.000	.04334	.15591
		3	.03588	.019578	.372	-.02041	.09216
		4	.08538(*)	.019578	.001	.02909	.14166
		5	.07475(*)	.019578	.004	.01846	.13104
	3	1	.06375(*)	.019578	.020	.00746	.12004
		2	-.03588	.019578	.372	-.09216	.02041
		4	.04950	.019578	.107	-.00679	.10579
		5	.03887	.019578	.294	-.01741	.09516
	4	1	.01425	.019578	.949	-.04204	.07054
		2	-.08538(*)	.019578	.001	-.14166	-.02909
		3	-.04950	.019578	.107	-.10579	.00679
		5	-.01063	.019578	.982	-.06691	.04566
	5	1	.02488	.019578	.710	-.03141	.08116
		2	-.07475(*)	.019578	.004	-.13104	-.01846
		3	-.03887	.019578	.294	-.09516	.01741
		4	.01063	.019578	.982	-.04566	.06691

* The mean difference is significant at the .05 level.

BIOGRAPHY

Miss Ploypat Niyomploy was born on December 5, 1983 in Kanchanaburi, Thailand. She graduated a Bachelor Degree of Science with the second class honour, from Department of Zoology, Faculty of Science, Kasetsart University in 2006. She was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2006.