PECTIN FROM COFFEE PULP: EXTRACTION, CHARACTERIZATION, AND BIOACTIVITIES

KUNAKORN DEESOPHON

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Author: Kunakorn Deesophon

Examination Committee:

Associate Professor Hongyu Chen, Ph. D.	Chairperson
Assistant Professor Sunita Chamyuang, Ph. D.	Member
Assistant Professor Natsaran Saichana, Ph. D.	Member
Yin Quan Tang, Ph. D.	Member
Amorn Owatworakit, Ph. D.	Member

Advisors:

.....Advisor

(Assistant Professor Sunita Chamyuang, Ph. D.)

.....Co-Advisor

(Assistant Professor Natsaran Saichana, Ph. D.)

.....Co-Advisor

(Yin Quan Tang, Ph. D.)

Dean:

.....

(Professor Surat Laphookhieo, Ph. D.)

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Kunakorn Deesophon

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Author	Kunakorn Deesophon
Degree	Master of Science (Biological Science)
Advisor	Assistant Professor Sunita Chamyuang, Ph. D.
Co-Advisor	Assistant Professor Natsaran Saichana, Ph. D. Yin Quan Tang, Ph. D.

ABSTRACT

Coffee pulp, a significant by-product of coffee processing in Thailand, represents a promising source of value-added compounds. This study investigates the physicochemical characteristics and bioactive properties, specifically the prebiotic and anticancer potential, of pectin derived from coffee pulp (CP). Pectin was isolated using a double extraction method and subsequently modified enzymatically (SFD). Comprehensive characterization involved thermogravimetric analysis (TGA) for purity, Fourier-transform infrared spectroscopy (FTIR) for the degree of esterification (%DE), gel permeation chromatography (GPC) for molecular weight, and high-performance anion-exchange chromatography (HPAEC-PAD) for sugar composition and structure. Bioactivity assessments included the DPPH assay for antioxidant potential, *in vitro* growth studies with *Pediococcus* sp. (PE), *Lactococcus lactis* (LL), and *Lactobacillus acidophilus* (LA) to determine Prebiotic Index and Activity Scores, and the CCK-8 assay for cytotoxicity against SW480 colon cancer and CCD 841 CoN normal colon cells, with apoptosis confirmed via Annexin V-FITC/PI staining. Results were benchmarked against commercial prebiotics (inulin), pectins (HMP, L20, L40), and 5-Fluorouracil (5-FU).

The extraction yielded 7.99% low methoxy pectin (LMP) with 4.76% DE, 68.05% purity, a weight-average molecular weight (M_w) of 68.6 KDa, and a linear structure (CP). Enzymatic modification yielded SFD, which retained LMP characteristics (0.00% DE) but exhibited increased branching and a significantly

reduced Mw of 1.10 KDa. SF demonstrated the highest antioxidant potential (IC_{50} of 101.33 µg/mL), surpassing commercial pectins which failed to reach 50% inhibition. Furthermore, CP significantly promoted the growth of all tested lactic acid bacteria, showing higher prebiotic index (PE: 0.95, LL: 3.88, LA: 3.73) and activity scores (PE: 0.25, LL: 0.40, LA: 0.72) than commercial pectins. Notably, only SFD exhibited significant anticancer activity, selectively inhibiting SW480 cell proliferation (IC_{50} of 3.40 mg/mL) more effectively than normal CCD 841 CoN cells (IC_{50} of 11.13 mg/mL), while unmodified CP showed no effect. SFD induced late-stage apoptosis in 19.80 ± 7.51% of SW480 cells, compared to 10.68 ± 1.82% in controls and 25.97 ± 3.51% with 5-FU.

These findings highlight that coffee pulp pectin is a promising natural prebiotic for food applications and gut health support. Moreover, its enzymatically modified form (MCPP) shows potential as a selective anticancer agent, warranting further investigation for therapeutic applications.

Keywords: Anticancer Properties, Apoptosis Induction, Cell Proliferation, Coffee Pulp, Enzymatic Modification, Pectin, Pectin Extraction, Prebiotics, Prebiotic Index, Prebiotic Activity Score

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ABBREVIATIONS AND SYMBOLS

5-FU	5-fluorouracil
AG	Arabinogalactan
AGS	Human gastric adenocarcinoma cell line
BMI	Body mass index
CCD 841 CoN	Normal human colon tissue
CCK-8	Cell counting kit-8
CFU	Colony forming units
CP	Coffee pulp pectin
CRC	Colorectal cancer
Da, kDa	Dalton, kilodalton
DE, %DE	Degree of esterification
DI	Deionized water
DMEM	Dulbecco's modified Eagle's medium
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	*Escherichia coli*
EMEM	Eagle's minimum essential medium
FBS	Fetal bovine serum
FTIR	Fourier-transform infrared spectroscopy
GalA	D-galacturonic acid
GPC	Gel permeation chromatograph
HG	Homogalacturonan
HMP	High methoxy pectin, High methoxy apple pectin
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
HT-29	Human colon cancer cell lines
IC_{50}	Half-maximal Inhibitory Concentration
IL-10	Interleukin 10
In	Inulin
L20	Low methoxy citrus pectin

ABBREVIATIONS AND SYMBOLS

L40	Low methoxy apple pectin
Lac	Lactose, positive control
LC	*Lacticaseibacillus casei*
LCP	Low molecular weight citrus pectin
LL	*Lactococcus lactis*
LMP	Low methoxy pectin
LP	*Lactiplantibacillus plantarum*
M_n	Number average molecular weight
MRS	De man, rogosa, sharpe
M_w	Weight average molecular weight
Neg	Negative control
NF-κB	Nuclear factor kappa B
PA	Prebiotic activity score
PBMC	Human peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PDI	Polydispersity index
PE	*Pediococcus* sp.
PI	Prebiotic index
POS	Pectin oligosaccharides
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
SF	The soluble fraction of coffee pulp pectin
SFD	Digested soluble fraction of coffee pulp pectin
SW480	Human colon adenocarcinoma cell lines
TGA	Thermogravimetric analysis
XG	Xylogalacturonan
α	Alpha

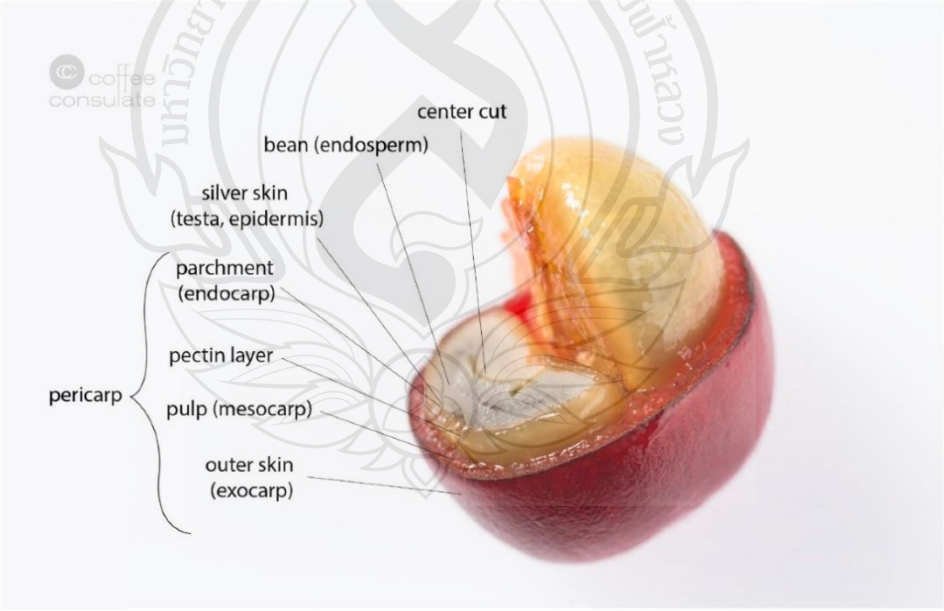
CHAPTER 1

INTRODUCTION

According to data from Office of Agricultural Economics of Thailand, Thailand is a country ranked 28th among the coffee producers produced 24,000 tons of coffee cherries in 2022. Chiang rai province is the highest coffee arabica producer in Thailand with a total production of 9,259 tons of coffee arabica in 2022 and expected to increase in upcoming years due to the high coffee demand around the world. On the other hand, this increasing trend can also increase the waste from the coffee process which caused serious environmental problems for people of nearby area due to its byproduct (Padmapriya et al., 2013). When the cherry undergoes coffee processing, only the beans are used to produce the coffee while all the other parts of cherry (Figure 1.1) are left as byproduct. One of the important byproducts is coffee pulp because the weight of pulp is almost half of the cherry weight (Reichembach & de Oliveira Petkowicz, 2020). Coffee pulp waste is typically utilized as fertilizer to reduce waste. However, large amounts of waste can have a huge impact on the environment by emitting a strong odor and deteriorate the soil and water reservoir nearby due to its acidity of the pulp. Thanks to the trend of turning agricultural waste into valuable resources through supportive government policies, technological advancements, and increased environmental awareness. These agriculture byproducts are rich in useful ingredients like carbohydrates, proteins, fibers, polyphenols, and other health-promoting compounds. According to Capanoglu et al. (2022), a huge variety of products can be made from nutritional supplements and antioxidants to biodegradable materials, natural enzymes, bio-based plastics, health peptides, organic acids, sugars, natural dyes, and even corrosion protectants. And modern extraction methods also play a key role in efficiently recovering and preserving these beneficial compounds, making them suitable for use in food, healthcare, and wellness industries (Bala et al., 2023). In a coffee pulp, however, it contains a diverse range of bioactive compounds that can be utilized in food supplements and cosmetic formulations, including lignin, hemicellulose, cellulose, lipids, caffeine, tannins, pectin, and phenolic antioxidants. (Ameca et al., 2018;

Aristizábal-Marulanda et al., 2017). One of the methods to reduce coffee pulp waste and turn this waste into value is by extracting pectin from this coffee pulp waste.

Pectin can be found generally in the higher plants including coffee cherries, pectin is a polysaccharide (dietary fiber) with a backbone of α (1,4) linked D-galacturonic acid (GalA)(Minzanova et al., 2018). Nowadays, pectin is widely used in food industrial as emulsifier, colloidal stabilizer, texturizer, and thickening and gelling agent that is popular in jams, fruit juices, desserts, and dairy products (Freitas et al., 2021; Jindal et al., 2013). Additionally, pectin is also used for packaging, fresh and cut fruits and vegetables coatings, and as microencapsulating agents (Mellinas et al., 2020). In the medical industry, Pectin serves as an effective material for encapsulating bioactive compounds, owing to its significant role in interacting with hydrophobic molecules, such as fluoroquinolone antibiotics. This interaction enhances drug incorporation into the matrix while facilitating a controlled release profile (Cacicedo et al., 2018). Furthermore, pectin-based films as the hydrocolloid bandage for wound dressing applications (Sabando et al., 2020). Recently, pectin also used as dietary supplement to improve intestinal health (Wen et al., 2022; Wikiera et al., 2014).



Source Klingel et al. (2020)

Figure 1.1 Cross section of a coffee cherry with its different layers

Colorectal cancer (CRC) is the third most diagnosed type of cancer in male and female, and it is also the second cause of death related to cancer next below lung cancer that caused the deaths globally, with an estimated 935,000 deaths in 2020 (Aprile et al., 2021). CRC is the third most prevalent cancer in Thailand, contributing to approximately 11% of the total cancer burden. Annually, around 10,000 new cases of CRC are diagnosed. (Lohsiriwat et al., 2020). Normally, CRC was diagnosed with people older than 50 years but in the present-day patients who diagnosed with CRC become younger due to many factors such as eating habits and their lifestyle, smoking, heavy alcohol consumption, red or processed meat consumption, intestinal inflammatory disease, polyps, and genetic factors (Granados-Romero et al., 2017; Sung et al., 2021). The symptoms of CRC were varied such as nausea, malaise, anorexia, vomiting, weight loss, abdominal pain, abdominal distension, alteration of chronic bowel habits, and changes in bowel movements. Furthermore, CRC patients who found cancer at distal areas of colon tend to cause rectal bleeding more than the patient who found cancer at proximal area of colon (Granados-Romero et al., 2017). So, people these days are more likely to be interested in their eating habits due to the healthy trend of the world. Thus, pectin that is one of the functional foods shown their bioactivity of immunoregulatory, hypoglycemic effect, antioxidant activity, anti-inflammatory, and antitumor activity (Delphi & Sepehri, 2016; Ho et al., 2016; Kungel et al., 2018; Lee et al., 2018; Wu et al., 2017). As previously describe pectin as a food additive may play an important role in preventing or reducing the possibility of colon cancer due to its antitumor activity. While previous studies haven't explored the potential of coffee pulp pectin toward prebiotic property and its anticancer activity as coffee pulp was not traditionally main source for pectin extraction. This study aims to upscale the extraction process of coffee pulp pectin extraction from laboratory to pilot scale by using double extraction method using citric acid and sodium hydroxide. Moreover, pectin from the double extraction process will be test its ability to be prebiotic on *Pediococcus sp., Lactococcus lactis, Lacticaseibacillus casei,* and *Lactiplantibacillus plantarum* and its anticancer activity on SW480 cell line.

1.1 Objectives

1.1.1 To upscale the extraction process of coffee pulp pectin by using double extraction method.

1.1.2. To modify the coffee pulp pectin using enzymatic modification.

1.1.3. To investigate prebiotic and anti-colorectal cancer activities of the coffee pulp pectin.

1.2 Scope of Research

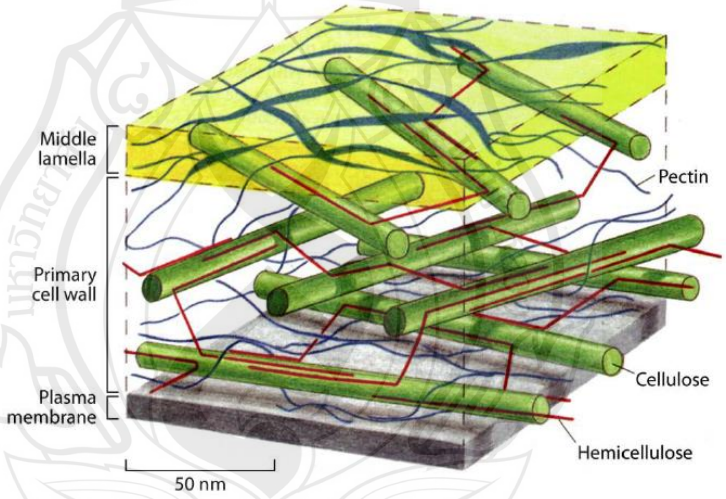
The purpose of the present work is to study the character, prebiotic property and anti-colorectal cancer effect of coffee pulp pectin extract by using double extraction method. Furthermore, to determine the prebiotic potential, then test prebiotic properties with lactic acid bacteria (*Pediococcus sp., Lactococcus lactis, Lacticaseibacillus casei,* and *Lactiplantibacillus plantarum*) to grow in a medium containing coffee pulp pectin and compare with pectin commercial. Finally, to confirm the potential to inhibit colorectal cancer, the Human colon adenocarcinoma cell lines (SW480) and normal human colon tissue (CCD 841 CoN) were confirmed by using CCK-8 assay and cell apoptosis test.

CHAPTER 2

LITERATURE REVIEW

2.1 Pectin Structure

Pectin is a complex heteropolysaccharide that acts as cementing material to hold cellulose network found in higher plant cell wall as showed in Figure 2.1. Core of this polysaccharides are made of α (1,4) linked D-galacturonic acid (GalA) (Minzanova et al., 2018). Pectin can be divided into three main types based on their structure (Figure 2.2); homogalacturonan (HG), rhamnogalacturonan I (RG-I), and substituted galacturonans such as rhamnogalacturonan II (RG-II).



Source Scheller and Ulvskov (2010)

Figure 2.1 Simplified model of the primary cell wall

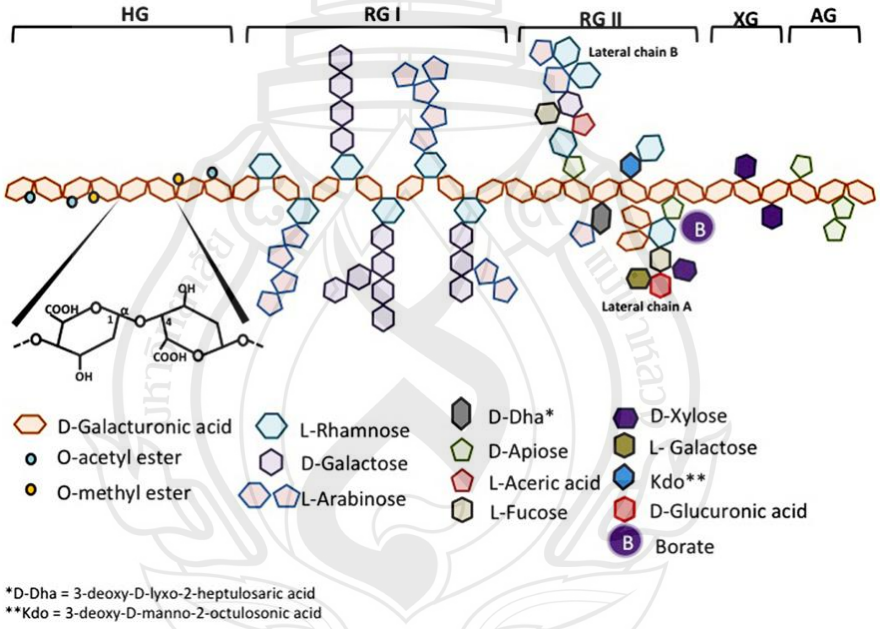
2.1.1 Homogalacturonan

A smooth region of pectin, known as homogalacturonan (HG), consists of a linear homopolymer composed of α-(1,4)-linked d-galacturonic acid (GalA) residues. These residues may undergo methylation and acetylation, influencing the polymer's functional properties. This most abundant region comprises around 65% of total pectin (Mohnen, 2008). At C-6 of some carboxyl group will be methyl esterified and partly O-

acetylated at C-2 or C-3 (O'NEILL et al., 1990). The distribution of methyl esterification and non-esterified on GalA residues are main factors for functional characteristics of pectin (LMP and HMP) (Willats et al., 2006).

2.1.2 Rhamnogalacturonan I

A hairy partly of pectin which comprise around 20–35% of pectin has more complicated structure than HG. RG-I is comprised of repeated L-rhamnose and galacturonic acid disaccharides [-α-D-GalA-1,2-α-L-Rha-1-4-] $_n$, which can also be acetylated at C-2 or C-3. Some of L-rhamnose was substituted at C-4 by different neutral sugars such as L-arabinose and L-galactose (Mohnen, 2008). The side chains include α -1,5- arabinan with 2- and 3- linked arabinose or arabinan branching, β -1,4-galactans (Nakamura et al., 2002).



Source Leclere et al. (2013)

Figure 2.2 Pectin structure. AG, arabinogalactan; HG, homogalacturonan; RG, rhamnogalacturonan; XG, xylogalacturonan

2.1.3 Rhamnogalacturonan II

A hairy part of pectin which comprises around 10% of the total pectin and even more complex. This part of pectin mainly functions on plant cell wall structure for plant growth and development (Mohnen, 2008). RG-II is a highly complex pectic polysaccharide composed of thirteen distinct sugars, including rhamnose, fucose,

xylose, galactose, and apiose or aceric acid. Its structure features twenty-one unique glycosidic linkages, organized around a backbone consisting of nine galacturonic acid (GalA) residues, which may undergo partial methyl esterification (ranging from none to three per residue) (Ishii & Kaneko, 1998).

2.2 Pectin Type

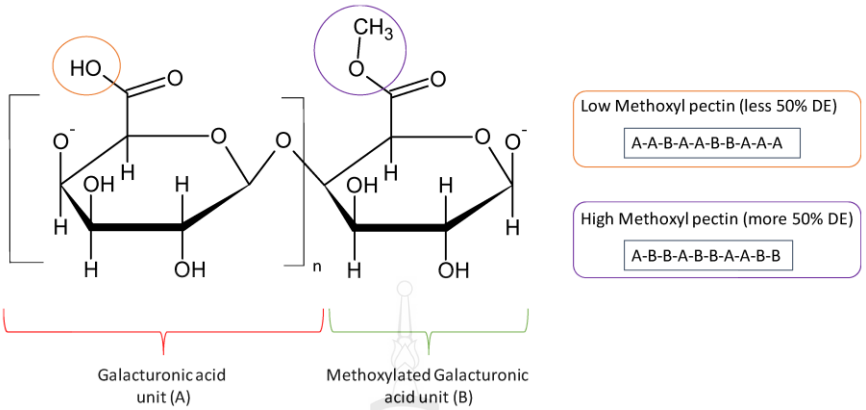
The classification of pectin types is primarily determined by the degree of esterification (DE), which reflects the number of methoxy groups substituting the carboxylic acid (COOH) moiety on galacturonic acid residues (Figure 2.3). The DE plays a crucial role in influencing pectin's gelation mechanism, processing parameters, and functional properties (Noreen et al., 2017).

2.2.1 High Methoxyl Pectin

High Methoxy Pectin (HMP) is a type of pectin characterized by a degree of esterification (DE) greater than 50%. This type of pectin could form gels at low pH values (<4.0) with a presence of high concentrations of soluble solids, such as high sugar concentrations, which is attributed to the presence of hydrogen bonding and hydrophobic interactions among the pectin chains (Morris et al., 2000; Noreen et al., 2017).

2.2.2 Low Methoxyl Pectin

Low Methoxy Pectin (LMP) is a type of pectin characterized by a DE lower than 50%. LMP could form gels under conditions of low sugar concentrations, but with the presence of acidity and calcium ions. This type of pectin is typically obtained through the process of de-esterification of HMP (Morris et al., 2000; Noreen et al., 2017). LMP can be produced using various modifying agents, including alkaline substances such as sodium hydroxide or ammonia, enzymatic treatments with pectin methyl esterase, and concentrated acids. These agents facilitate structural modifications by adjusting the degree of esterification, thereby influencing the functional and gelation properties of LMP in various applications (Fishman et al., 2015).



Source Mellinas et al. (2020)

Figure 2.3 Structure of low and high methoxyl pectin

2.3 Pectin Extraction

The main source of commercial pectin is derived from the peels of citrus fruits because of their advantageous characteristics and high extraction yield. The extraction process is typically carried out through hydrothermal extraction under acidic conditions, utilizing high temperatures (75-95 °C) and prolonged extraction times (60-300 minutes) (Mellinas et al., 2020). Nowadays, many alternative sources from agricultural waste have been widely studied as the source of pectin.

Reichembach and de Oliveira Petkowicz (2020) extracted pectin from dried coffee pulp waste. The first step involved boiling dried pulp in 80% ethanol, followed by extraction with 0.1M nitric acid under reflux conditions. This resulted in a yield of 14.6% pectin, which was determined to be HMP. Maneerat et al. (2017) extracted pectin from dried banana peel waste using two different conditions. The first condition utilized 0.05 M hydrochloric acid at pH 1.5, while the second employed deionized water at pH 6. Both conditions were conducted at a temperature of 90 °C for 120 minutes. This resulted in yields of 11% and 4.8% pectin, which were determined to be HMP and LMP, respectively. Muñoz-Almagro et al. (2019) extracted pectin from dried cocoa pod husk waste. The cocoa pod husk was extracted using 4% citric acid at pH 3 and a temperature of 95 °C for 95 minutes. This resulted in a yield of 8% pectin, which was determined to LMP. Hasem et al. (2019) extracted pectin from dried powder of durian rinds. The extraction process utilized a hydrochloric acid aqueous solution at a pH of 2.5 and a

temperature of 85°C for 60 minutes. This resulted in a yield of 73.67% pectin. Kazemi et al. (2019) employed a Box Behnken design to extract eggplant peel pectin. The extraction process utilized distilled water that was acidified with citric acid to pH 2.5, and a temperature of 90 °C for 90 minutes. This resulted in a yield of 26.1% pectin, which was determined to be HMP. In this experiment a double extraction method will be used to extract pectin from coffee pulp to obtain LMP.

2.4 Prebiotic Activity of Pectin

Prebiotics are non-digestible sugars or compounds that probiotic can use as an energy source to provide a benefit to the host, while probiotic is living microorganisms that use prebiotic to provide a benefit such as the improvement of intestinal digestion, immune system, cardiometabolic health, prevent of cancer risk, increase intestinal barrier, reducing cholesterol, and produce vitamin B to the host (Sánchez et al., 2017). One of the traditional probiotics products in daily life is yogurt that contains living *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Hamann & Marth, 1984).

Chung et al. (2017) have demonstrated the ability of secretion the pectate lyase to utilize pectin as an energy source for *Lachnospira eligens* and *Faecalibacterium prausnitzii* which is the phylum of Firmicutes that is one of the 20 most abundant bacteria in the human gut microbiome, they also found that these two bacteria can promote the production of the anti-inflammatory IL-10 in human peripheral blood mononuclear cell (PBMC) stimulation which is *in vitro* experiment. Liang et al. (2020) show the correlation of CRC patients with decreasing an abundance of *F. prausnitzii*. Thus, *F. prausnitzii* was classified as one of fostering next-generation probiotics in the human gut as butyrate-producing bacteria with an ability to use prebiotic such as pectin and its derivative to produce butyrate which can prevent the risk of colorectal cancer (Kumari et al., 2021). The butyrate compounds can induce apoptosis and reduce proliferation in colorectal cancer cell lines (Williams et al., 2003). Butyrate also has a positive relation to the body mass index (BMI) of 2-year-old children and the abundance of *Faecalibacterium* (Nandy et al., 2022).

The characteristics of native pectin such as its degree of esterification (%DE), the ratio of homogalacturonan (HG) to rhamnogalacturonan (RG), and its molecular weight (M_w) can vary depending on the plant source and extraction method used. In contrast, modified pectin depends on desire proposed through modification methods such as enzymatic modification, pH modification, and heat treatment. These modifications typically simplify the pectin structure, reduce its molecular size and %DE, and generate smaller fragments known as pectin oligosaccharides (POS). Native pectin is well recognized for its ability to support beneficial gut bacteria, encouraging the growth of species such as *Faecalibacterium prausnitzii*, *Lachnospira eligens*, *Bacteroides*, *Ruminococcaceae*, and *Bifidobacterium* spp., often leading to slower, more sustained digestion (Pascale et al., 2022). On the other hand, modified pectin with their simpler structures tend to expand their prebiotic effects. However, this broader activity may come at the cost of reduced specificity for certain microbial populations and often results in faster, less sustained fermentation compared to their native counterparts (Wang et al., 2024).

2.5 Anticancer Activity of Pectin

Numerous studies have demonstrated the potential of both nonmodified and modified pectin as an anticancer agent. Pectin exerts its anti-cancer effects through multiple mechanisms, including binding to proteins involved in tumor cell adhesion, promoting apoptosis in tumor cells, inhibiting galectin-3, a key regulator that shields cancer cells from programmed cell death, inhibition of cancer cell migration, and slowing down of cancer progression and/or aggregation (Ding & Cui, 2020).

Pectin derived from *Codonopsis pilosula* and its selenized form has been shown to trigger apoptosis in cancer cells by enhancing the expression of caspase-3, a key marker enzyme in the apoptotic pathway and Bax, a pro-apoptotic protein that facilitates mitochondrial outer membrane permeabilization in A549 lung cancer cells that associated with the intrinsic pathway of apoptosis (Chen et al., 2015). Similarly, ultrasound modified pectin extracted from sweet potato induces apoptosis-like cell death in HT-29 colon cancer cells. This effect is mediated by caspase-3 activation,

accompanied by DNA fragmentation, distinct morphological changes consistent with programmed cell death, and cell cycle arrest at the G2/M phase (Ogutu et al., 2018).

The anticancer mechanism toward galectin-3 was the interaction of pectin at the RG-I region. Galectin-3 is found highly expressed in colon cancer cells and can be found at cytoplasm, cell surfaces, and extracellular, its C-terminal carbohydrate recognition domain can bind to β-galactoside glycans on pectin's RG-I, and its N-terminal domain is used for oligomerization (self-association). This binding leads to inhibition of their ability to promote tumor growth, metastasis, cell adhesion, and angiogenesis (Ornelas et al., 2022). Toll-Like Receptor 4 is another mechanism associated with the HG domain of pectin, this receptor located on cell surface that responds for inflammatory signaling cascades (NF-κB), tumor cell survival, proliferation, migration, and invasion when biding to lipopolysaccharide of bacteria or HMGB1 released from stressed or dying cells, which can be blocked by the binding of this receptor to HG domain (Li et al., 2017).

Wang et al. (2016) studied the effect of chemoprevention of low molecular weight citrus pectin (LCP) on gastrointestinal cancer cells (AGS) and SW480 colorectal cancer cells. The result showed a decrease level of Galectin-3 expression that cancer cells used for metastasis in both cancer cell lines at 5 mg/mL, also at 5 mg/mL of LCP the AGS and SW480 can decrease cell proliferation around 24% and 28%, respectively. The study also showed that LCP at 10 mg/mL induced apoptosis in both cancer cell lines in xenograft tissues. Cheng et al. (2013) demonstrated the effect of RG-I rich potato that can inhibit proliferation of HT-29 cancer cells by reducing cyclin B1 and cyclin-dependent kinase 1 on mRNA expression level, leading to interruption of G2/M cell cycle. Maxwell et al. (2016) demonstrated that native sugar beet pulp pectin has small impact on anti-proliferative activity towards HT-29 colon cancer cell. While alkali treatment pectin that increased the ratio of RG-I (contain neutral sugar sidechains) to HG at 1 mg/mL can reduce proliferation by 20.7% and inducing apoptosis without an effect on the cell cycle of HT-29 (Table 2.1). In present day there was no study about anticancer activity of coffee pulp pectin. So, this study was aimed to investigate this ability of coffee pulp pectin.

Table 2.1 Anticancer activity of pectin

Pectin	Anticancer activity	Reference
Low molecular weight citrus pectin	At 5 mg/mL pectin on gastrointestinal cancer cells (AGS) and SW480 colorectal cancer cells • Decrease level of Galectin-3 expression (cancer cells used for metastasis) • Decrease cell proliferation	Wang et al. (2016)
RG-I rich potato pectin	At 5 mg/mL pectin on HT-29 colon cancer cells • Decrease cell proliferation • Reducing cyclin B1 and cyclin-dependent kinase 1 on mRNA expression level • Interrupting of G2/M cell cycle	Cheng et al. (2013)
Alkali treatment sugar beet pulp pectin	At 1 mg/mL pectin on HT-29 colon cancer cells • Decrease cell proliferation • Inducing apoptosis without an effect on the cell cycle	Maxwell et al. (2016)

CHAPTER 3

MATERIALS AND METHODS

3.1 Pectin Extraction

Pectin was extracted from coffee pulp using a modified version of the method outlined by Chamyuang et al. (2021). Ten kilograms of ground and dried coffee pulp was soaked in 40 liters of distilled water and incubated at room temperature for 24 hours. The solution was then filtered to remove the pulp. The pulp was then subjected to acid extraction by being soaked in 60 liters of 0.1 N citric acid and incubated at 92°C for 2 hours in a tank. After incubation, the citric acid was filtered out and retained for pectin precipitation. The coffee pulp from this step was then used in an alkaline extraction by being soaked in 60 liters of 0.5% sodium hydroxide and incubated at room temperature for 1 hour. The sodium hydroxide was filtered out. Both the acid and alkaline filtrates were mixed and adjusted to a pH of 5 using sodium hydroxide. Pectin was then precipitated using 95% ethanol food grade in a ratio of 1:1. The pectin was dried in a tray dryer at 60°C for 24 hours.

3.2 Pectin Modification

The soluble fraction (SF) of coffee pulp pectin was prepared by dissolving 1% (w/v) coffee pulp in distilled water, followed by centrifugation at 8,000 RPM for 90 minutes. The supernatant was collected and subjected to freeze-drying. Pectin digestion was carried out using pectinase derived from *Aspergillus niger* (Sigma-Aldrich). With some modification from Khatri et al. (2015), a 4% (w/v) SF solution was prepared in deionized water, and the pH was adjusted to 4.0 using 0.1 M acetic acid. This solution was mixed with a 0.1 M acetate buffer (pH 4.0) in a 1:1 ratio to yield a 2% (w/v) SF solution in acetate buffer. Pectinase enzyme was then added at a concentration of 0.25 U/mL, and the mixture was incubated at room temperature on a rocking shaker for 30

minutes. The enzymatic reaction was terminated by heating the mixture at 95°C for 10 minutes to inactivate the enzyme, yielding digested soluble fraction of coffee pulp pectin (SFD). The condition used in enzymatic modification was based on pre-study to optimize the reaction time from 0-60 minutes and pectinase concentration from 0-2 U/mL based on 2% SF concentration.

3.3 Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) was recorded following Lessa et al. (2018) using a Mettler Toledo TGA/DSC 3+ HT/1600/1033 (Switzerland) in a dry N_2(g) atmosphere at a flow rate of 20 mL/min. Experiments were performed from 25 ºC to 800 ºC with a heating rate of 10 ºC/min.

3.4 Fourier-transform Infrared Spectroscopy (FTIR)

The main functional groups of pectin were determined using Fourier-transform infrared spectroscopy (FTIR) with Spectrum GX and a DTGS detector (PerkinElmer), following the method outlined by Sato et al. (2011). One milligram of pectin was combined with 100 mg of potassium bromide and ground into a fine powder. The powder was pressed into a pellet using a hydraulic press and placed on an FTIR holder. The absorbance spectrum was measured under the following conditions: a wavenumber range of 4000 - 400 cm^{-1}, 16 scans, and a resolution of 4.

The degree of esterification (%DE) was calculated utilizing OriginPro 2025 software, adhering to the methodology established by Chatjigakis et al. (1998). This analysis employed Equation (1) to quantify the spectral peak areas at 1740 cm^{-1}, corresponding to esterified carboxyl groups, and at 1600 cm^{-1}, associated with free carboxyl groups.

$$\%\,DE = \left(\frac{A\ 1740}{A\ 1740 + A\ 1600}\right) \times 100 \qquad (1)$$

Where A 1740 is area of peak at 1740 cm^{-1} and A 1600 is area of peak at 1600 cm^{-1}

3.5 Pectin Sugars Determination

The hydrolysis procedure was conducted according to Wang et al. (2021). Initially, 5 mg of pectin was hydrolyzed in 12 mL of 4 M trifluoroacetic acid at 105°C for 6 hours. Following hydrolysis, 4 mL of the digested sample was transferred to a pear-shaped flask for evaporation and subsequently wash again with methanol at 50°C three times. The monosaccharide composition of pectin was analyzed using High-Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). The analysis utilized the Thermo Scientific Dionex ICS-6000 system (Thermo Fisher Scientific Inc., USA) equipped with a Dionex CarboPac PA1 column (4 × 250 mm). Prior to analysis, the sample was dissolved in ultrapure water, filtered through a nylon membrane with a pore size of 0.22 μm, and stored in the autosampler chamber at 4°C. An injection volume of 25 μL was used, with ultrapure water and 16 mM sodium hydroxide as eluents. A monosaccharide standard was employed to generate a calibration curve for quantifying monosaccharide concentrations. Galacturonic acid content was quantified using a modified colorimetric method based on Lefsih et al. (2018) and Mestres et al. (2022), employing 3-phenylphenol or m-hydroxybiphenyl as reagents. Pectin samples were prepared by diluting a 1 mg/mL stock solution in deionized water tenfold. Subsequently, 400 μL of the diluted pectin solution was transferred into a test tube and placed in a cold bath. To this, 2.4 mL of 96% sulfuric acid containing 0.1% NaCl was added, followed by immediate vortexing for 10 seconds. The reaction mixture was then incubated in a 95°C water bath for 10 minutes, with shaking at the 5 and 9 minute marks. After heating, the samples were rapidly cooled in an ice bath. Next, 40 μL of a 0.15% 3-phenylphenol solution was added, vortexed briefly, and 200 μL of each sample was transferred to a 96-well plate. Absorbance measurements were recorded at 520 nm. Calibration curve was prepared using D-(+)-Galacturonic acid monohydrate at concentrations of 8, 16, 32, and 64 μg/mL.

3.6 Gel Permeation Chromatograph Analysis

The average molecular weight (M_w) of pectin samples was estimated using gel permeation chromatograph (GPC), a technique modified from Dangi and Yadav (2020). The molecular weight was determined using gel permeation chromatography technique with Shimadzu, Nexera Series consisting of column set; TSKgel guardcolumnPWXL, TSKgel G5000PWXL (Exclusion limit $2.5X10^6$), TSKgel G4000PWXL (Exclusion limit $1X10^6$), TSKgel G3000PWXL (Exclusion limit $2X10^5$), and TSKgel G2500PWXL (Exclusion limit $5X10^3$) connected to a refractive index detector. The sample (3 mg/mL) was dissolved in 0.1 M sodium nitrate. A nylon 66 membrane with a pore size of 0.45 μm was used to filter the sample (3 mg/mL) after it had been dissolved in 0.1 M sodium nitrate and eluted at a rate of 0.6 mL/min at 40 °C prior to injection. Pullulan standards with known molecular weights ranging from M_w 180-805,000 Da were used to calibrate the GPC. To determine the molecular weight of pectin, a linear regression equation derived from pullulan standards employed in conventional calibration was utilized.

3.7 Antioxidant Activity

The antioxidant activity of pectin was assessed relative to ascorbic acid as a reference, employing a modified version of the method outlined by Wathoni et al. (2019). Pectin solutions were prepared at concentrations of 1000, 500, 250, 135, 62.5, 31.25, and 15.625 μg/mL in deionized (DI) water, while ascorbic acid solutions (positive control) were prepared at concentrations of 32, 16, 8, 4, 2, 1, 0.5, and 0.25 μg/mL in DI water. These solutions were subsequently mixed with 0.2 mM DPPH in ethanol at a ratio of 2:1 (sample to DPPH) and incubated at room temperature in the absence of light for 30 minutes. The absorbance of the samples was measured at 517 nm using a UV-visible spectrophotometer, with ethanol serving as the blank. The IC_{50} values were determined using the IC_{50} Calculator provided by AAT Bioquest, Inc.

3.8 Probiotic Growth Stimulation

The microbial strains *Pediococcus sp., Lactococcus lactis, Lacticaseibacillus casei, Lactiplantibacillus plantarum*, and *Escherichia coli* were inoculated at a 1% (v/v) concentration into prebiotic media and incubated at 37°C with 200 rpm agitation for 36 hours. The growth of the microbial strains was monitored every four hours, and the number of colony forming units (CFU) was determined by counting colonies on MRS (DE MAN, ROGOSA, SHARPE) plates for the first three strains and nutrient agar plates for *E. coli*. The plates were incubated at 37°C for 24 - 48 hours.

3.9 Prebiotic Index

The prebiotic index (PI) was determined by calculating the ratio of probiotic growth in the presence of a prebiotic compared to probiotic growth in a control carbohydrate (lactose) using the following equation (2)

$$P_I = \frac{CFU\ of\ probiotic\ in\ prebiotic\ carbohydrate}{CFU\ of\ probiotic\ in\ control\ carbohydrate} \qquad (2)$$

The prebiotic effectiveness of a carbohydrate was evaluated by determining its prebiotic index (PI) value. A PI value close to 1 suggests low effectiveness of the evaluated carbohydrate, whereas a value higher than 1 indicates a positive impact on probiotic growth (Figueroa-Gonzalez et al., 2019).

3.10 Prebiotic Activity Score

Prebiotic activity score (PA) was determined according to Figueroa-Gonzalez et al. (2019) following equation (3).

$$P_A = \frac{(LogP_{24} - LogP_0)prebiotic}{(LogP_{24} - LogP_0)lactose} - \frac{(LogE_{24} - LogE_0)prebiotic}{(LogE_{24} - LogE_0)lactose} \quad (3)$$

Where PA is a prebiotic activity score, LogP represents the log-transformed colony-forming units (CFU/mL) of probiotic bacteria measured at 24 hours (P24) and 0 hours (P0) when cultured with the prebiotic and lactose, LogE represents the log-transformed CFU/mL of *E. coli* at 24 hours (E24) and 0 hours (E0) under the same conditions.

A high prebiotic activity score (PA) indicates favorable growth of probiotic bacteria when compared to lactose. Conversely, *E. coli* should show a slower growth rate when cultivated on prebiotics in comparison to lactose. As a result, the pectin prebiotic activity score can be determined using equation 4 in relation to a specific probiotic strain.

3.11 Cell Culture

The human colorectal cancer cell line (SW480) and the human colorectal cell line (CCD 841 CoN; ATCC CRL-1790) were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's modified Eagle's medium (DMEM) for SW 480 and Eagle's minimum essential medium (EMEM) for CCD 841 CoN both supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA) and 1% penicillin/streptomycin (P/S) (10,000 units/mL) at 37 °C with 5% CO_2 in a humidified atmosphere.

3.12 Cell Proliferation Assay

The effects of pectin on SW480 cell proliferation were evaluated using the SuperKine™ Maximum Sensitivity Cell Counting Kit-8 (CCK-8) by following manufacturer protocol, SW480 and CCD 841 CoN cells were seeded at a concentration of 5 x 10^3 and 1.25 x 10^4 cells/well, respectively in a 96-well plate for 24 hours. A series of pectin concentrations (1, 2, 3, 4, and 5 mg/mL) were prepared by diluting the pectin solution with complete media (DMEM or EMEM + 10% FBS + 1% P/S). The cells

were then treated with the various pectin concentrations and incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 for 48 hours. Following the incubation period, the media in each well were discarded, and 100 µL of 10% CCK-8 solution in complete media was added to each well and incubated at 37 °C for 1.5 hours. The absorbance was measured at 450 nm using a microplate reader. The cell proliferation rate was calculated using equation (4).

$$Cell\ proliferation\ (\%) = 100 - [(\frac{Abs\ of\ control\ sample - Abs\ of\ tested\ sample}{Absorbance\ of\ control\ sample})x100] \quad (4)$$

3.13 Annexin V-FITC/PI Flow Cytometric Analysis

To observe the apoptosis inducing effects of pectin on SW480, Annexin V–FITC Apoptosis Detection Kit (Elabscience, E-CK-A211) was used according to the manufacturer's protocol and Cheng et al. (2013). SW480 cells were seeded at 2.5×10^5 cells per well in 6-well plates and incubated for 24 hours before replacing the medium with varied pectin concentrations for an additional 48-hour incubation. The cells and media were harvested via trypsinization, washed twice with PBS, and resuspended in 100 µL binding buffer. Samples were stained with 2.5 µL Annexin V-FITC and 2.5 µL PI, incubated on ice in the dark condition for 20 minutes, and adding 400 µL of Annexin V binding buffer. Cells were analyzed using a FACScan flow cytometer, with apoptosis quantified via CytExpert software.

3.14 Statistical Analysis

Experimental and statistical analysis were calculated using SPSS (Version 20.0). One-way analysis of variance (One-way ANOVA) was used for chemical properties and prebiotic activity score. For the pectin extraction and cell proliferation part, factorial designs were used with univariate analysis and were compared by Duncan's Multiple Range Test (DMRT). Data were presented as mean ± standard error, and a significant difference was analyzed at the 0.05 level by one-way ANOVA. The

pectin extraction and cell proliferation part were analyzed using factorial designs with univariate analysis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Pectin Extraction

Pectin was extracted from coffee pulp (CP), a by-product of the coffee production process, using a double extraction method involving 0.1 N citric acid and 0.5% w/v sodium hydroxide solution. This method achieved a yield of 7.99%. The combination of acid and basic, which acid extraction will hydrolyze the complex structure to release the pectin from plant cell wall (Yi et al., 2024) and basic extraction facilitated the saponification of ester groups during the basic extraction phase, resulting in low methoxy pectin (LMP) with a degree of esterification of 4.76%. By contrast, acid extraction alone produced high methoxy pectin (HMP) (Chamyuang et al., 2021).

For comparison, Hasanah et al. (2019) extracted pectin from coffee pulp using citric acid at 85°C for 125 minutes, yielding HMP with a 7.8% yield. Reichembach & de Oliveira Petkowicz (2020) utilized 0.1 M nitric acid under reflux conditions for 30 minutes, following a pretreatment with ethanol boiling, and obtained HMP with a 14.6% yield. While the double extraction method yields lower pectin content compared to Reichembach and de Oliveira Petkowicz (2020), It offers advantages in terms of scalability, as it requires only heating and stirring instead of reflux conditions. Additionally, it is safer due to the use of weak organic citric acid, commonly employed in the food industry, rather than inorganic nitric acid that can contribute to equipment degradation through corrosion and posing environmental risks due to their potential to generate hazardous waste and emissions (Pérez-Martínez et al., 2013). This method also produces LMP, which forms a gel without sugar, beneficial for low-sugar product formulations.

4.2 Thermo Gravimetric Analysis (TGA)

The purity of pectin based on the burning point was determined by using TGA at heating rate of 10 ºC per minute from 25 ºC to 1,200 ºC under nitrogen atmosphere. The thermogram from 25−90°C represents the wight loss of moisture that was absorbed in the sample and range from between 90−250°C shows polysaccharide pyrolytic decomposition (Wang et al., 2016). Purity of pectin was reported in Table 4.1. CP, SF, and SFD show lower purity around 5-10% compared to the commercial pectin (HMP, L20, and L40) due to the unknown impure which does not bastion after 580 ºC. Since alcohol-based precipitation presents a limitation, it can lead to the unwanted retention of acid-soluble ash within the extract, thereby reducing pectin purity (Guo et al., 2015). To enhance purification, additional purification steps can be implemented in the extraction process. These may include membrane-based separation, which isolates pectin by molecular size through selective membranes such as microfiltration and ultrafiltration, complemented by diafiltration. Alternatively, cationic materials can be employed to selectively bind anionic pectin, either through protein-based precipitation utilizing sodium caseinate and bovine serum albumin or metal-based separation involving aluminum sulfate and iron (III) chloride (Baghdadi et al., 2023).

Table 4.1 Weight percentage of pectin samples

Sample	Percentage of weight (%)			Normalize (%)	
	Moisture	Pectin	Unknown impure	Pectin	Unknown impure
HMP	11.87	61.27	26.86	73.14	26.86
L20	11.34	61.34	27.32	72.68	27.32
L40	11.32	60.96	27.72	72.28	27.72
CP	13.81	54.24	31.95	68.05	31.95
SF	14.98	49.68	35.34	64.66	35.34
SFD	12.99	51.78	35.23	64.77	35.23

4.3 Fourier-transform Infrared Spectroscopy (FTIR)

The structural characteristics and chemical composition of pectin were analyzed using Fourier Transform Infrared (FTIR) spectroscopy. The absorption band within the range of 3400–3200 cm^{-1} corresponds to O–H stretching vibrations, while the band near 2900 cm^{-1} is indicative of C–H stretching vibrations. The peak observed at 1740 cm^{-1} (not found in SFD) is attributed to C=O stretching vibrations of esterified carboxyl groups, whereas the peak at 1600 cm^{-1} (at 1590 cm^{-1} in L40 and 1580 cm^{-1} in SFD) signifies COO^- antisymmetric stretching vibrations of free carboxylate groups. These free carboxylate groups are instrumental in calculating the degree of esterification of pectin (Sato et al., 2011). Amidated pectin exhibits additional distinct bands, notably around 1650 cm^{-1} (only at 1670 cm^{-1} in L40) corresponding to the amide I peak, associated with C=O stretching vibrations, and near 1550 cm^{-1} for the amide II peak, linked to N–H bending and C–N stretching vibrations (Mishra et al., 2008). The bands at 1450 cm^{-1} and 1377 cm^{-1} represent CH_2 and CH_3 bending modes, respectively. Furthermore, the peak at 1410 cm^{-1} is associated with COO^- symmetric stretching vibrations of carboxylate groups, while the peak at 1240 cm^{-1} relates to C–O stretching vibrations within the side chain (Kozioł et al., 2022). In the region spanning 1160 to 990 cm^{-1}, multiple bands correspond to glycosidic linkages, including C–C, C–O–C, and C–OH bonds. Within the "fingerprint" region, absorption below 920 cm^{-1} is predominantly attributed to vibrations of C–O–C bridges, characteristic of polysaccharides (Lessa et al., 2018).

FTIR analysis revealed that CP and SF exhibited similar chromatogram (Figure 4.1), reflecting their shared composition. In contrast, SFD demonstrated the disappearance of the band at 1740 cm^{-1}, indicative of hydrolyzed ester bonds. This phenomenon may be attributed to impurities in the pectinase enzyme (Poly-(1,4-α-D-galacturonide) glycanohydrolase), potentially containing pectin methylesterase activity. Additionally, the shift in peak from 1600 cm^{-1} to 1580 cm^{-1} suggests the formation of carboxylate ions during enzymatic digestion, alongside molecular structural changes from polymer to oligomer, as supported by Zhang et al. (2010). Their study utilized pectinase derived from *Aspergillus niger* to digest polygalacturonic acid, yielding

oligogalacturonic acid. Furthermore, the FTIR spectrum revealed a reduction in glycosidic linkage bands within the range of 1000–1200 cm^{-1}, consistent with cleavage of glycosidic bonds during enzymatic degradation.

The degree of esterification (%DE) was calculated as the ratio of the area of esterified carboxyl groups (at 1740 cm^{-1}) to the total area of carboxyl groups (sum of the areas at 1740 cm^{-1} and 1600 cm^{-1}) (Table 4.3). High levels of sugar promote the gelation of high methoxy pectin (HMP) for HMP sample at low pH values. Conversely, low methoxy pectin (LMP), such as L20, L40, CP, SF, and SFD, forms gels in the presence of calcium ions and acidic conditions. This property makes LMP advantageous for the formulation of low-sugar products.

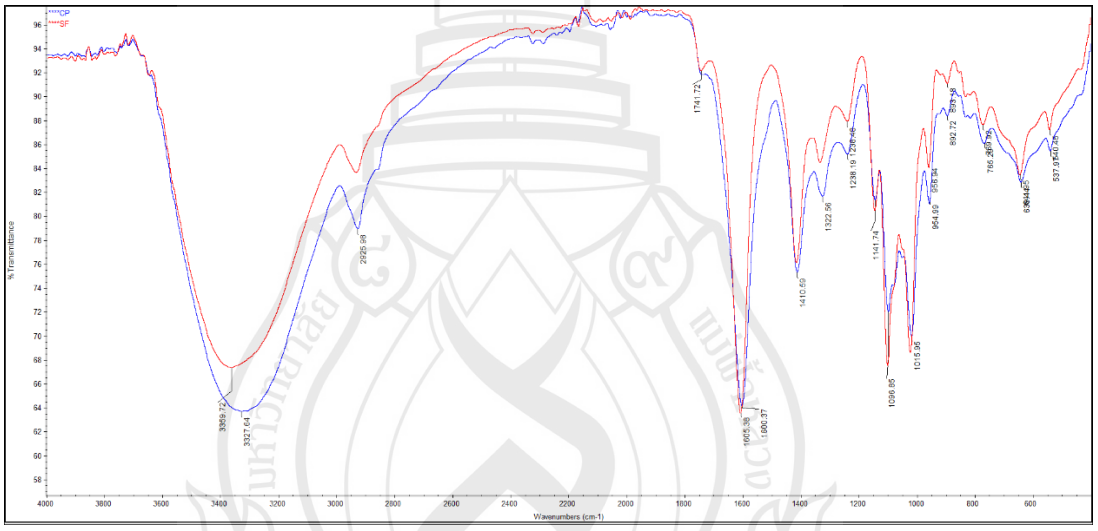


Figure 4.1 FTIR spectra of coffee pulp pectin (CP) and soluble fraction of coffee pulp pectin (SF)

Table 4.2 FTIR absorption bands and functional group assignments

Wavenumber (cm^{-1})	**Assignment**	**Note**
3400–3200	O–H stretching vibrations	-
2970-2900	C–H stretching vibrations	-
1745-1735	C=O stretching vibrations	Not found in SFD
1650	Amide I C=O stretching vibrations	Only L40 at 1670 cm^{-1}
1600	COO^- antisymmetric stretching vibrations	At 1590 cm^{-1} in L40 and 1580 cm^{-1} in SFD
1550	Amide II N–H bending and C–N stretching vibrations	-
1450	CH_2 bending	-
1410	COO^- symmetric stretching vibrations	-
1377	CH_3 bending	-
1240	C–O stretching vibrations within the side chain	-
1160-990	C–C, C–O–C, and C–OH bonds	-
>920	C–O–C bridges	Fingerprint region

Table 4.3 Degree of esterification of pectin samples

Sample	% DE
HMP	55.62
L20	16.77
L40	30.48
CP	4.76
SF	7.59
SFD	0

Note % DE is degree of esterification

4.4 Sugar Composition

After hydrolyzing pectin samples with trifluoroacetic acid were analyst their sugar content includes rhamnose, arabinose, galactose, and glucose using HPAEC-PAD. While The sugar composition in commercial pectin samples shown higher rhamnose and galactose but lower arabinose compared to pectin derived from coffee pulp (CP, SF, and SFD) (Table 4.4). Structural characterization of pectin can be assessed through branching indices that quantify its linearity and side-chain complexity. A higher R1 value suggests a predominantly linear pectin structure with minimal branching, whereas a lower R2 value similarly indicates a highly linear configuration with reduced side-chain extensions. In contrast, R3 provides insight into the overall size of the branching side chains, with increased values correlating to more extensive branching. Additionally, R4 specifically evaluates the branching side-chain size while excluding arabinose contributions (Denman & Morris, 2015).

CP exhibited significantly highest R1 value of 31.90 ± 1.41 with the lowest R2 value of 0.01 ± 0.00, indicating a predominantly linear structure with minimal residual side-chain polysaccharides. as evidenced by the elevated HG/RG-I ratio. This ratio quantifies the relative abundance of HG domains per RG-I domain, derived from the calculated of HG% and RG-I%. The highest HG/RG-I ratio observed in CP got the highest value of 27.36 ± 1.58 further confirms its linear structural characteristics. According to Denman and Morris (2015), increasing the processing temperature

enhances the linearity of pectin by promoting the degradation of RG-I regions and associated sugar side chains. The coffee pulp pectin, extracted at 92°C. Additionally, the drying method appears to influence pectin linearity, as evidenced by differences in R1 values between samples processed via different techniques. CP subjected to ethanol precipitation followed by hot air drying exhibited a higher R1 value compared to SF, which supernatant of CP was freeze-dried after centrifugation, resulting in a lower R1 value of 28.31 ± 1.35. In this study, hot air drying was selected over freeze-drying primarily due to the limited capacity and high operational costs associated with freeze dryers at the pilot scale. Additionally, hot air drying is more suitable for upscale production, offering lower maintenance requirements and reduced operational costs.

Furthermore, HG/RG-I ratio This suggests that thermal exposure during drying may contribute to increased linearity of pectin. Moreover, enzymatic digestion with pectinase resulted in increased branching of SFD, as evidenced by the reduction in its R1 value to 24.6 ± 0.78 and a decrease in the HG/RG-I ratio to 21.25 ± 0.93. This indicated that enzymatic modification by pectinase induced structural alterations by increasing the RG-I of pectin.

Galacturonic acid content was quantified using 3-phenylphenol or m-hydroxybiphenyl under the influence of concentrated sulfuric acid. During this process, uronic acids, including galacturonic acid, undergo hydrolysis, generating furan-based derivatives containing carboxyl (-COOH) functional groups. These reaction products subsequently interact with 3-phenylphenol, producing pink-colored complexes whose intensity correlates with uronic acid concentration, allowing for precise quantitative analysis (Zhao et al., 2024). The galacturonic acid content in pectin samples varied across different extraction sources and methods. Variability in galacturonic acid levels is influenced by plant origin and extraction parameters such as temperature, pH, and solvent composition. Pérez-Martínez et al. (2013) reported that pectin extracted from cladode flour exhibited a decline in galacturonic acid content with increasing temperature and pH.

Drying methods also appear to affect GalA content and pectin linearity. The freeze-dried supernatant of SF demonstrated a higher galacturonic acid content 67.88 ± 1.60% than the coffee pulp pectin itself CP, suggesting that centrifugal separation and freeze-drying may contribute to GalA preservation. Reichembach and

de Oliveira Petkowicz (2020) reported a 79.50% GalA from *Coffea arabica* L. pulp extracted using nitric acid reflux. In contrast, Li et al. (2023) found a 45.01% GalA in pectin extracted from *Coffea arabica* L. husks via cellulase extraction at 45°C. Post extraction modifications significantly impact the GalA content of pectin, as observed in SFD, where enzymatic digestion with pectinase (polygalacturonase) reduced GalA to 42.27 ± 0.55%. This aligns with findings by Funami et al. (2011), demonstrating that polygalacturonase digestion leads to a decline in GalA levels. Conversely, protease treatment does not alter GalA content, whereas a combination of arabinanase and galactanase enhances GalA levels by selectively degrading neutral sugars, potentially exposing additional galacturonic acid residues.

Table 4.4 Percentage sugar composition of pectin samples

Sample	Rhamnose (%)	Arabinose (%)	Galactose (%)	Glucose (%)	GalA (%)	R1	R2	R3	R4	HG (%)	RG-I (%)	HG/RG-I
HMP	0.84 ± 0.00 [a]	0.60 ± 0.00 [f]	2.98 ± 0.03 [a]	3.58 ± 0.03 [c]	65.61 ± 0.15 [b]	14.84 ± 0.09 [f]	0.01 ± 0.00 [a]	4.26 ± 0.04 [cd]	3.55 ± 0.04 [a]	64.77 ± 0.15 [b]	5.26 ± 0.03 [a]	12.31 ± 0.06 [e]
L20	0.42 ± 0.00 [c]	0.26 ± 0.03 [e]	1.20 ± 0.00 [c]	17.00 ± 0.15 [b]	49.9 ± 0.49 [e]	26.54 ± 0.23 [c]	0.01 ± 0.00 [c]	3.48 ± 0.08 [e]	2.86 ± 0.00 [b]	49.48 ± 0.49 [e]	2.3 ± 0.03 [d]	21.51 ± 0.12 [c]
L40	0.68 ± 0.03 [b]	0.50 ± 0.03 [d]	2.08 ± 0.03 [b]	18.46 ± 0.37 [a]	60.71 ± 1.12 [c]	18.63 ± 0.29 [e]	0.01 ± 0.00 [b]	3.8 ± 0.12 [de]	3.06 ± 0.13 [b]	60.03 ± 1.08 [c]	3.94 ± 0.12 [b]	15.24 ± 0.27 [d]
CP	0.28 ± 0.03 [d]	0.92 ± 0.03 [b]	0.54 ± 0.00 [f]	0.84 ± 0.00 [d]	55.45 ± 0.69 [d]	31.90 ± 1.41 [a]	0.01 ± 0.00 [e]	5.27 ± 0.64 [ab]	1.90 ± 0.26 [d]	55.17 ± 0.73 [d]	2.02 ± 0.09 [e]	27.36 ± 1.58 [a]
SF	0.42 ± 0.00 [c]	1.16 ± 0.03 [a]	0.82 ± 0.03 [d]	0.64 ± 0.03 [de]	67.88 ± 1.60 [a]	28.31 ± 1.35 [b]	0.01 ± 0.00 [d]	4.71 ± 0.14 [bc]	1.95 ± 0.08 [d]	67.46 ± 1.60 [a]	2.82 ± 0.06 [c]	23.94 ± 1.06 [b]
SFD	0.26 ± 0.03 [d]	0.84 ± 0.00 [c]	0.62 ± 0.03 [e]	0.46 ± 0.03 [e]	42.27 ± 0.55 [f]	24.6 ± 0.78 [d]	0.01 ± 0.00 [d]	5.67 ± 0.58 [a]	2.40 ± 0.17 [c]	42.01 ± 0.52 [f]	1.98 ± 0.10 [e]	21.25 ± 0.93 [c]

Note Values are mean ± S.D of triplicates. Sugar ratios of pectin are also shown as R_1 = GalA/(Rha + Ara + Gal); R_2 = Rha/GalA; R_3 = (Ara + Gal)/Rha; R_4 = Gal/Rha; HG = GalA – Rha; RG-I = 2Rha + Ara + Gal. Each sugar and ratio are analyzed independently from other sugar and ratio. Letter a-f use to classify significantly different group based on percentage sugar or ratio, ranking from highest (a) to lowest (f) value at $p < 0.05$

4.5 Gel Permeation Chromatography

The molecular weight distribution of pectin samples was shown by the GPC chromatogram (Table 4.5) including the weight average molecular weight (M_w), number average molecular weight (M_n), and polydispersity index (PDI) that measures the molecular weight distribution of the polymers. SFD was found to be significantly reduced in size as a result of enzymatic digestion for 30 minutes as showed a shift to the right in retention time compared to SF, suggesting the formation of lower molecular weight fragments. While the broad peak of PDI obtained for non-modify pectin suggested the complexity of the polymer molecular structure with wide dispersion of molecular weights, the intense peaks of PDI recorded for enzymatic modification pectin or SFD showed the homogeneity of pectin molecules in terms of molecular weight (Dangi & Yadav, 2020; Hua et al., 2015). Smaller oligosaccharides and a higher proportion of monosaccharides produced during hydrolysis could result in a decrease in molecular weight (Ho et al., 2017) due to the pectinase enzyme, specifically Poly-(1,4-α-D-galacturonide) glycanohydrolase called Endo-Polygalacturonase that hydrolyzes the α-1,4 glycosidic bonds or the backbone of the pectin which result a six galacturonic acid with M_w around 1.10×10^3 for 0.25 U/mL at 30 minutes hydrolysis time. Pectin with lower molecular weight (M_w) called pectin oligosaccharides shows improved solubility due to its shorter polymer chains that facilitate greater interaction with water molecules. In contrast, the complex structure of high M_w pectin limits its ability to dissolve. As enzymatic modification can lower M_w, optimizes solubility for applications like beverages (Son et al., 2024). Pectin oligosaccharides show improve bioactivity by exposing more fermentable galacturonic acid chains and arabinose residues, accelerating microbial metabolism and short chain fatty acid production (Pascale et al., 2022). Also enhancing galectin-3 binding leading to suppress tumor growth, metastasis, and induce apoptosis (Wang et al., 2016).

Table 4.5 Molecular weight of pectin samples

Sample	Peak	Area (%)	M_w (Da)	M_n (Da)	PDI (M_w /M_n)
HMP	1	100	$6.11 \times 10^5 \pm 0.01$	$1.40 \times 10^5 \pm 0.01$	4.37 ± 0.05
L20	1	100	$5.06 \times 10^5 \pm 0.02$	$1.86 \times 10^5 \pm 0.15$	2.73 ± 0.22
L40	1	100	$2.54 \times 10^5 \pm 0.01$	$6.87 \times 10^4 \pm 0.14$	3.69 ± 0.06
CP	1	90.69	$6.86 \times 10^4 \pm 0.10$	$3.45 \times 10^4 \pm 0.07$	1.99 ± 0.06
	2	9.31	$1.64 \times 10^3 \pm 0.03$	$1.37 \times 10^3 \pm 0.06$	1.20 ± 0.03
SF	1	82.35	$1.12 \times 10^5 \pm 0.02$	$3.76 \times 10^4 \pm 0.08$	3.00 ± 0.10
	2	5.71	$1.33 \times 10^3 \pm 0.04$	$1.13 \times 10^3 \pm 0.03$	1.18 ± 0.00
	3	6.80	31.00 ± 1.00	29.00 ± 0.00	1.06 ± 0.00
	4	2.55	8.00 ± 1.00	7.00 ± 0.00	1.10 ± 0.01
	5	2.59	1.00 ± 0.00	1.00 ± 0.00	1.23 ± 0.02
SFD	1	3.64	$6.30 \times 10^4 \pm 0.16$	$4.77 \times 10^4 \pm 0.19$	1.32 ± 0.04
	2	9.34	$3.82 \times 10^3 \pm 0.05$	$3.10 \times 10^3 \pm 0.03$	1.23 ± 0.01
	3	40.06	$1.10 \times 10^3 \pm 0.01$	$1.05 \times 10^3 \pm 0.01$	1.04 ± 0.00
	4	16.08	308.00 ± 7.00	290.00 ± 8.00	1.06 ± 0.00
	5	30.88	32.00 ± 1.00	28.00 ± 1.00	1.13 ± 0.04

Note Values are mean ± S.D of triplicates. M_w is weight average molecular weight, M_n is number average molecular weight, PDI is polydispersity index.

4.6 Antioxidant Activity

The antioxidant potential of coffee pulp pectin was evaluated through the DPPH radical scavenging assay. This method relies on the ability of antioxidant compounds to neutralize DPPH free radicals, resulting in a visible color transition of the sample from purple to yellow, attributable to the formation of DPPH-H (Wathoni et al., 2019). The antioxidant activity of the pectin samples was assessed at concentrations of 1000, 500, 250, 135, 62.5, 31.25, and 15.625 µg/mL, with results compared to ascorbic acid tested at 32, 16, 8, 4, 2, 1, 0.5, and 0.25 µg/mL (Figure 4.2). The IC_{50} values for CP, SF, and SFD pectin samples were determined to be 259.28, 101.33, and 236.65 µg/mL,

respectively, whereas the IC_{50} for ascorbic acid was significantly lower at 4.19 µg/mL. Commercial pectin, including HMP, L20, and L40, did not achieve 50% inhibition at the maximum concentration of 1000 µg/mL, exhibiting scavenging activity of 36.00%, 18.07%, and 23.60%, respectively. These findings highlight the superior DPPH radical scavenging ability of coffee pulp pectin, underscoring its higher antioxidant potential relative to commercial pectin samples.

SF demonstrated a higher antioxidant capacity than CP, likely attributable to its greater purity. The presence of insoluble components in CP may interfere with the DPPH assay, requiring centrifugation to eliminate these impurities prior to analysis. This purification step reduces sample concentration, potentially affecting the accuracy of antioxidant measurements. However, CP still demonstrated a greater antioxidant potential than the coffee pulp pectin reported by Biratu et al. (2024), which had an IC_{50} of 642.3 µg/mL. In contrast, the pectin extracted from mangosteen rind displayed significantly higher antioxidant activity, with an IC_{50} of 161.93 µg/mL (Wathoni et al., 2019), highlighting the variability in antioxidant properties across different sources and extraction conditions. While SFD exhibited IC_{50} values lower than SF but comparable to CP, which contrasts with findings by Hosseini Abari et al. (2021), who reported a 50% increase in DPPH radical scavenging activity for apple pectin following enzymatic digestion with pectinase from *Streptomyces hydrogenans* YAM1, achieving an IC_{50} of 2 mg/mL, compared to native apple pectin.

Qi et al. (2022) demonstrated that, at the same molecular weight de-esterified, polygalacturonase digested *Lonicera japonica* Thunb pectin exhibited significantly enhanced antioxidant capacity at IC_{50} of 9.41 mg/mL compared to its non-de-esterified form at IC_{50} of 27.06 mg/mL. This enhancement is due to the increased availability of carboxyl (-COOH) and electrophilic functional groups after de-esterification, which increase radical scavenging activity. Furthermore, their findings also highlighted the synergistic contributions of various pectin domains to antioxidant activity, with oligogalacturonides from the HG domain demonstrating the highest effectiveness, followed by RG-II, while RG-I exhibited the weakest radical scavenging capability.

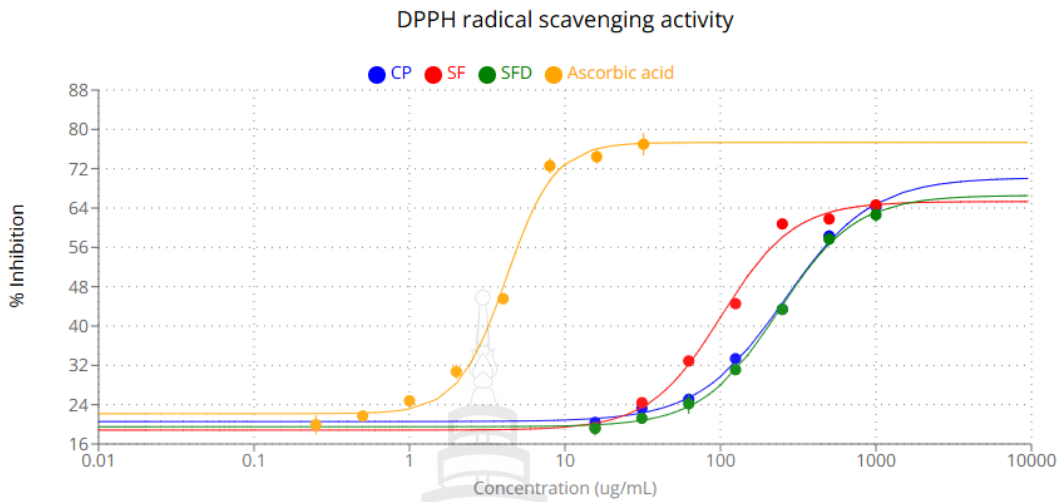


Figure 4.2 DPPH radical scavenging activity of CP (blue), SF (red), SFD (green), and ascorbic acid (yellow)

4.7 Prebiotic Activities

The relative cell growth of *Pediococcus sp.* (PE), *Lactococcus lactis* (LL), *Lacticaseibacillus casei* (LC), *Lactiplantibacillus plantarum* (LP), and *Escherichia coli* (EC) at 24 hours of growth on media containing 1% (w/v) prebiotic supplements, as described in material and method section is presented in Table 4.6. The results indicate that inulin (In), coffee pulp pectin (CP), and its soluble fraction (SF) significantly enhance the growth of all tested bacterial strains compared to lactose (Lac), the negative control (Neg), high methoxy apple pectin (HMP), low methoxy citrus pectin (L20), and the enzymatically modified soluble fraction of coffee pulp pectin (SFD). This highlights the higher prebiotic potential of unmodified coffee pulp pectin over Lac as standard references and commercial pectins. CP significantly promoted the growth of all bacterial strains, enhancing the stability of bacterial growth over time. In contrast, lactose initially increased the growth of LL to 10.65 ± 0.06 Log (CFU/mL) (Figure 4.3 B) at 8 hours but subsequently decreased by 24 hours. CP also supported the growth of all lactic acid bacteria at 24 hours, with PE reaching 8.53 0.01 Log (CFU/mL), followed by LL at 9.62 ± 0.03 Log (CFU/mL) and LC at 8.39 ± 0.09 Log (CFU/mL), and LP at 7.90 ± 0.07 Log (CFU/mL) demonstrating superior growth compared to commercial pectins (Figure 4.3). Additionally, at 24 hours lactose (except for LP), inulin and SF

significantly enhanced the growth of all lactic acid bacteria. While SFD promotes the growth of PE and LC but inhibits the growth of LL and LP. However, the commercial pectins HMP and L20 exhibited poor growth performance compared to the negative control and even inhibited growth at a significant level of $p < 0.05$, as shown in both Figure 4.3 and Table 4.6.

These results align with findings reported by Olano-Martin et al. (2002), which suggested that high-methylated pectin inhibits the growth of lactic acid bacteria, while low-methylated pectin promotes their growth after 24 hours of cultivation. Similarly, Dongowski and Lorenz (1998) concluded that low methoxy pectin supports bacterial growth more effectively than high methoxy pectin due to its longer degradation time of high methoxyl pectin. Pectins are glycosidically linked polysaccharides composed of over 65% galacturonic acids and neutral sugars, including rhamnose, arabinose, glucose, and xylose, which constitute a significant component of plant cell walls and could act as the prebiotic substant. The specific composition can vary depending on the source; for instance, apple, lemon and coffee pulp exhibit different contents of neutral sugars (Kravtchenko et al., 1992).

4.8 Prebiotic Index

The Prebiotic Index (PI) was calculated for various carbohydrate and prebiotic sources as data showed in Table 4.7. The highest PI value was observed for LP with SF, yielding a score of 66.46 ± 14.08. Only CP exhibited index close to or greater than 1 for all bacterial strains, indicating that CP positively influenced probiotic growth. Additionally, In and SF exhibited close to or more than 1 indices for PE, LC, and LP but except for LL. While SFD showed positively influenced just for PE and LC. In contrast, HMP and L20 demonstrated no efficacy in promoting probiotic bacterial growth.

When examining the homogeneity of all pectin samples as described in Table 4.5, coffee pulp derived pectins (CP, SF, and SFD) were displayed non-homogeneity, likely due to the presence of sugar side chains in the coffee pulp (Arpi et al., 2021). Interestingly, while commercial pectins such as HMP and L20 contained higher levels

of rhamnose, galactose, and glucose (Table 4.4), they failed to enhance the prebiotic indices of PE, LL, and LC. This highlights the importance of molecular weight (M_w) in modulating biological activity. Commercial pectins exhibited relatively high M_w values (610.62 kDa for HMP and 506.84 kDa for L20), which may have limited their prebiotic performance. In contrast, the lower M_w pectins particularly CP (68.60 kDa) successfully supported the growth of all tested lactic acid bacteria. However, SFD with lower M_w of 1.10 kDa got positively influenced on prebiotic indices of PE and LC but not for LL and LP. This result consistent with findings by Ho et al. (2017), extremely low molecular weight pectin fragments may not further improve growth, suggesting an optimal range of M_w for prebiotic efficacy.

Furthermore, CP did not undergo purification step like commercial pectins. This characteristic may benefit CP by enhancing the prebiotic index for all lactic acid bacteria tested. Conversely, the commercial pectins, which exhibited homogeneity, did not promote the growth of lactic acid bacteria. It is important to note that the non-homogeneity of CP warrants further investigation into other sugar determination. Hydrolysis of the pulp can yield fermentable sugars such as xylose, arabinose, fructose, glucose, sucrose, and maltose, which may serve as valuable food sources for probiotic bacteria (Heeger et al., 2017). The diverse sugar composition in each prebiotic sample likely plays a crucial role in determining the prebiotic index.

4.9 Prebiotic Activity Score

The potential of a prebiotic substrate to enhance the growth of probiotic bacteria is quantified by the Prebiotic Activity Score (PA), which compares the growth of probiotics supplemented with prebiotics to that of other organisms, such as *E. coli* (EC), also supplemented with the same prebiotics. A higher score indicates greater prebiotic activity (Zhang et al., 2018). In this study, SFD demonstrated the highest PA for PE, potentially due to its inhibitory effect on EC, similar to the behavior observed with HMP. However, SFD showed limited bioactivity to promote the growth of LL and LP. In contrast, CP with no activity to inhibit EC but also exhibited positive PA values across three tested lactic acid bacteria, excluding LP, with significant differences

observed at $p < 0.05$ as data displayed in Figure 4.4. Interestingly, L20 had the lowest activity scores for all tested strains, even though it shared LMP characteristics with CP, indicating a growth inhibition effect on probiotics but promoted the growth of EC. HMP shown low activity score for PE and LP likewise, the pectin with high M_w improved the survivability though low pH condition even poorly promote the growth of these two probiotics (Larsen et al., 2018). Meanwhile, In as commercial prebiotic only exhibited positive PA value for PE and LC as a result that it also highly promoted EC growth.

One of the factors that affect prebiotic activity score is metabolic diversity or the presence of specific enzyme hydrolysis and transport systems for specific prebiotic such as β-galactosidase which affect the growth of lactic acid bacteria (Zhang et al., 2018). Like *Pediococcus* sp. and *L. lactis* that can utilize galactose, arabinose, and fructose which are normally found in coffee pulp (Kleerebezem et al., 2020; Todorov et al., 2022). Or *L. acidophilus* that can produce many glycosyl hydrolases α- and β-glucosidases, β-galactosidases, and α-L-fucosidases that can digest oligosaccharides (Chamberlain et al., 2022).

Table 4.6 Relative cell growth at 0 h to 24 h, reported as Log CFU/mL, for bacterial cultures grown in different treatments

Bacteria	Relative Log of growth prebiotic supplements (CFU/mL)							
	Lac	**In**	**Neg**	**HMP**	**L20**	**CP**	**SF**	**SFD**
PE	1.22±0.07^{b}	1.46±0.03^{a}	0.26±0.05^{d}	0.02±0.07^{e}	-0.26±0.10^{f}	1.25±0.04^{b}	0.66±0.07^{c}	1.19±0.12^{b}
LL	1.67±0.03^{b}	1.00±0.16^{c}	0.51±0.02^{d}	-1.13±0.01^{h}	-0.03±0.03^{f}	1.98±0.04^{a}	0.34±0.06^{e}	-0.83±0.10^{g}
LC	0.56±0.11^{c}	1.01±0.14^{b}	0.39±0.18^{c}	-0.45±0.34^{d}	-1.42±0.19^{e}	1.11±0.11^{a}	0.97±0.19^{ab}	0.56±0.07^{b}
LP	-4.42±0.15^{f}	0.04±0.14^{c}	-0.94±0.10^{d}	-1.96±0.14^{e}	-0.80±0.11^{d}	0.74±0.16^{b}	1.08±0.04^{a}	-
EC	2.75±0.03^{a}	2.62±0.06^{b}	1.73±0.01^{d}	-0.38±0.02^{h}	1.62±0.09^{e}	2.14±0.02^{c}	1.47±0.05^{f}	-0.09±0.12^{g}

Note Values are mean ± S.D of triplicates. PE, LL, LC, LP, and EC: refers to *Pediococcus* sp., *L. lactis*, *L. casei*, *L. Plantarum*, and *E. coli*, respectively. Each bacterial strain is analyzed independently from other strain and letter a-h is used to classify significantly different group based on relative growth, ranking from highest (a) to lowest (h) index at $p < 0.05$

Table 4.7 Prebiotic index at 24 h for bacterial cultures grown in different treatments

Bacteria	Prebiotic index						
	In	**Neg**	**HMP**	**L20**	**CP**	**SF**	**SFD**
PE	1.14±0.34^{b}	0.08±0.01^{c}	0.05±0.01^{c}	0.03±0.01^{c}	0.95±0.11^{b}	1.71±0.24^{b}	5.44±1.12^{a}
LL	0.30±0.07^{b}	0.09±0.00^{b}	0.00±0.00^{b}	0.03±0.01^{b}	3.87±0.47^{b}	0.32±0.07^{a}	0.03±0.01^{b}
LC	2.23±0.43^{bc}	0.72±0.19^{cd}	0.08±0.06^{d}	0.01±0.00^{d}	6.27±2.48^{a}	3.25±0.81^{b}	2.53±0.87^{bc}
LP	5.77±2.12^{c}	0.87±0.42^{c}	6.19±0.68^{c}	0.73±0.34^{c}	28.78±11.61^{b}	66.46±14.08^{a}	0.03±0.01^{c}

Note Values are mean ± S.D of triplicates. PE, LL, LC, and LP: refers to *Pediococcus* sp., *L. lactis*, *L. casei*, and *L. plantarum* respectively. Each bacterial strain is analyzed independently from other strain and letter a-c is used to classify significantly different group based on prebiotic index, ranking from highest (a) to lowest (c) index at $p < 0.05$

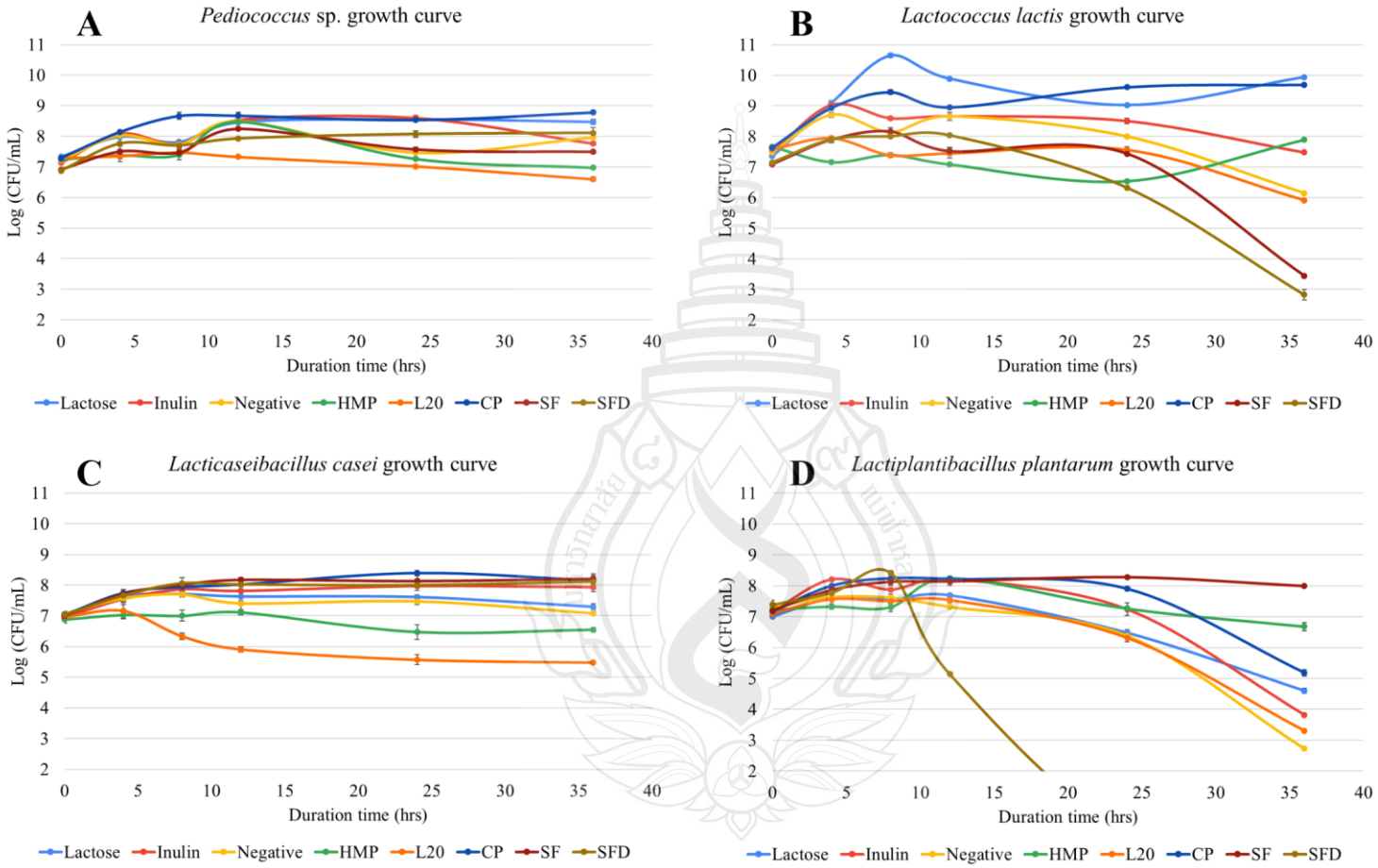


Figure 4.3 Growth of *Pediococcus* sp. (A), *L. lactis* (B) and *L. casei* (C) and *L. plantrrum* (D), cultured with different types of carbon sources

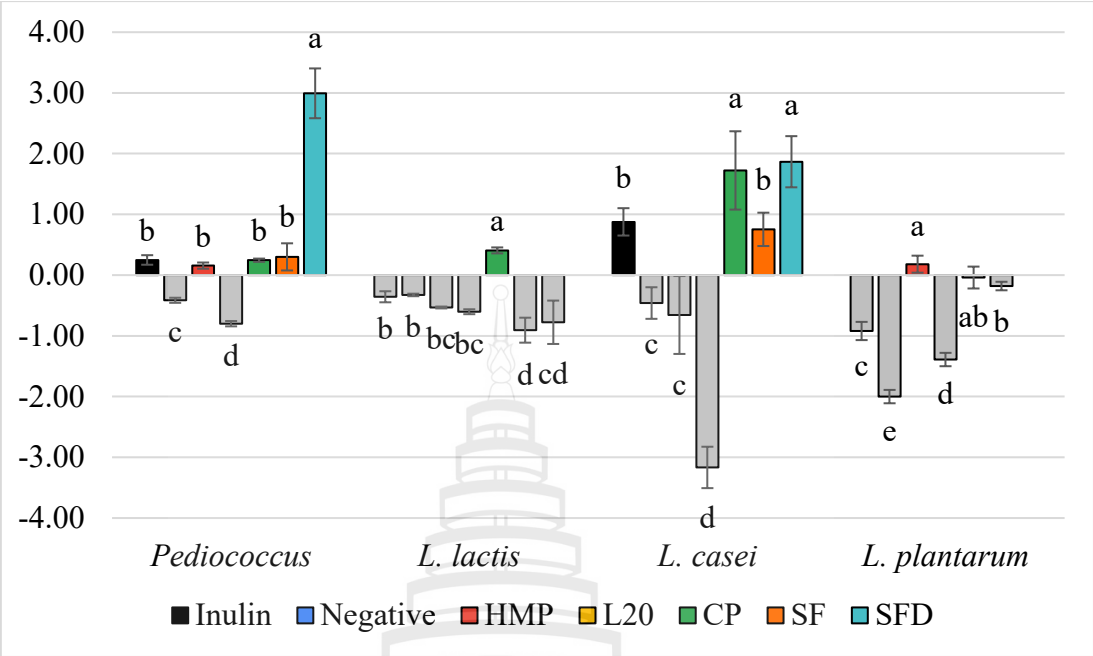


Figure 4.4 Prebiotic activity score in different samples. Each bacterial strain is analyzed independently from other strain and letter a-e use to classify significantly different group based on activity scores, ranking from highest (a) to lowest (e) activity scores at $p < 0.05$

4.10 Anti-colorectal Cancer Activity

The antiproliferative effects of pectin on SW480 colon cancer cells and CCD 841 CoN normal colon cells were assessed using the CCK-8 assay, which employs the tetrazolium salt WST-8. This compound is enzymatically reduced by dehydrogenases in viable cells, producing a water-soluble formazan dye, with its concentration directly correlating to cell viability. Following 48 hours of treatment at 1, 2, 3, 4, and 5 mg/mL, in Figure 4.5 nonmodified pectin exhibited no significant inhibition of SW480 proliferation, except for L40 at 3 mg/mL and SF at 5 mg/mL, which still maintained over 80% cell viability. In contrast, modified pectin (SFD) demonstrated significant inhibition at 2–5 mg/mL, with an IC_{50} value of 3.40 mg/mL, indicating enhanced cytotoxic effects on SW480 cells. 5-FU exhibited IC_{50} of 0.047 mg/mL (359.99 µM), demonstrating its cytotoxic potency. Structurally, 5-FU is a heterocyclic aromatic compound analogous to pyrimidine bases in DNA and RNA, with a fluorine substitution

at the C-5 position of uracil. This structural modification enables its incorporation into nucleic acids, disrupting cellular processes and inducing cytotoxic effects, ultimately leading to cell death (Qi et al., 2022; Zhang et al., 2008). Moreover, 5-FU exhibited an IC_{50} of 0.052 mg/mL (399.15 µM) toward CCD 841 CoN (Figure 4.6), suggesting lower sensitivity in CCD 841 CoN compared to SW480. This trend aligns with differences in doubling time, as SW480 cells have a higher proliferative rate than CCD 841 CoN, making them more susceptible to treatment-induced cytotoxicity.

SFD exhibited an IC_{50} of 11.13 mg/mL toward CCD 841 CoN (Figure 4.6), nearly three times higher than its IC_{50} for SW480 (3.40 mg/mL), indicating lower sensitivity in normal colon cells. Despite this, SW480 maintained over 80% cell proliferation in response to SFD at comparable concentrations, reinforcing the role of low molecular weight (M_w) pectin in cancer cell inhibition. SFD's M_w was approximately 62 times lower than CP, alongside a reduced in R1 and HG/RG-I ratio with more branching due to enzymatic digestion, emphasizing the importance of structural modifications in enhancing antiproliferative effects of pectin. Likewise, Wang et al. (2016) reported that low-molecular-weight citrus pectin at 5.0 mg/mL for 24 hours reduced the proliferation of AGS gastric cancer cells to 76% and SW480 colon cancer cells to 72%, highlighting its potential antiproliferative effects in different cancer cell lines. In addition, Cheng et al. (2011) demonstrated that HG-rich ginseng pectin exhibited antiproliferative effects on HT-29 colon cancer cells, reducing cell viability to 68% at 5 mg/mL after 72 hours of incubation. This finding suggests that HG domains may contribute to pectin's bioactivity in inhibiting cancer cell proliferation. On the other hand, Maxwell et al. (2016) observed that citrus pectin with a molecular weight nearly three times lower had no significant effect on the proliferation of HT-29. In contrast, the soluble fraction of alkali-treated sugar beet pectin, enriched in RG-I domains, reduced cell proliferation to 79.3% at 1.0 mg/mL after 48 hours of incubation, suggesting that RG-I may contribute to pectin's antiproliferative activity. Similarly, Cheng et al. (2013) reported that RG-I-enriched pectin from potato at 5 mg/mL decreased HT-29 proliferation to 55% after 72 hours of incubation. The observed reduction in the HG/RG-I ratio and lower M_w in SFD may be linked to interaction with galectin-3, a protein known to specifically bind to the RG-I region that contains galactose side chains. This binding potentially interferes with the role of galectin-3 in

promoting tumor progression, including processes such as cell adhesion, metastasis, angiogenesis, and tumor growth, and promote apoptotic activity (Ornelas et al., 2022), reinforcing the hypothesis that RG-I is one of the key domains involved in pectin's anticancer properties.

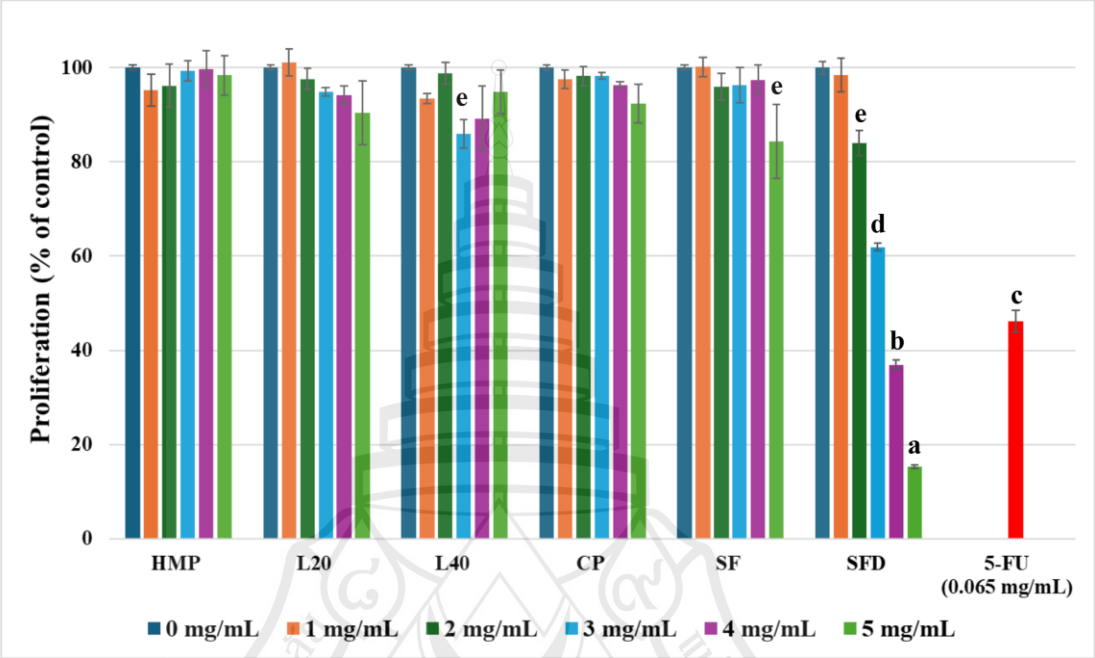


Figure 4.5 Effect of Pectin and 5-FU (500 µM, 0.065 mg/mL) on cell proliferation of SW480 cells after 48 hours of incubation by CCK-8. The letters a-e indicate significant differences from control, ranking from highest (a) to lowest (e) antiproliferative effects at $p < 0.05$

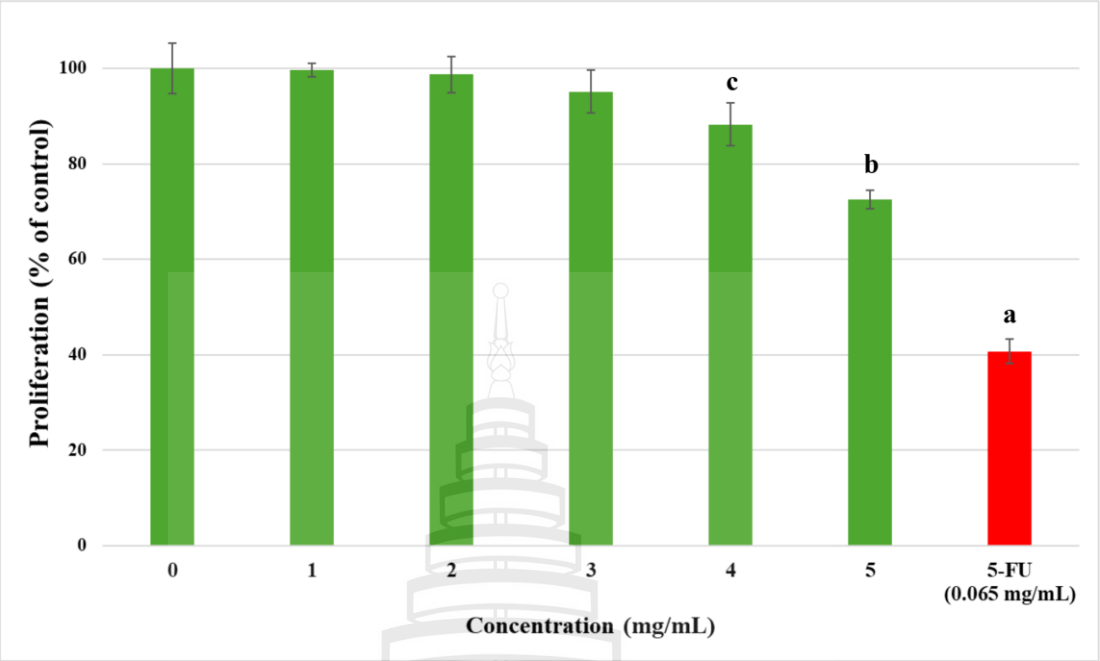


Figure 4.6 Effect of SFD and 5-FU (500 µM, 0.065 mg/mL) on cell proliferation of CCD 841 CoN cells after 48 hours of incubation by CCK-8. The letters a-c indicate significant differences from control, ranking from highest (a) to lowest (c) antiproliferative effects at $p < 0.05$

4.11 Effects of Pectin on SW480 Cells Apoptosis

The apoptosis-inducing effects of pectin were evaluated in SW480 cells using the Annexin V-FITC/PI staining assay. Annexin V binds to phosphatidylserine (PS), a phospholipid typically localized to the inner leaflet of the plasma membrane. During apoptosis, PS translocates to the outer leaflet, allowing Annexin V-FITC to bind and fluoresce, marking early apoptotic cells. Propidium Iodide (PI), a membrane-impermeable dye, penetrates cells with compromised membrane integrity, identifying late apoptotic or necrotic cells. Flow cytometry enables the quantification of these populations, distinguishing Annexin V-positive/PI-negative cells as early apoptotic and Annexin V-positive/PI-positive cells as late apoptotic or necrotic.

HMP, L20, L40, CP, and SF pectin were excluded from the experimental samples due to their lack of antiproliferative effects on SW480 cells. Their limited bioactivity suggests structural or compositional differences that do not contribute to significant cancer cell inhibition. Following IC_{50} determination from CCK-8 proliferation tests, 3.40 mg/mL of SFD and 0.05 mg/mL (360 μM) of 5-FU were selected for comparison against untreated (complete medium) and vehicle control (0.1 M acetate buffer, pH 7). Untreated SW480 cells maintained 86.81 ± 2.23% viability, with 6.35 ± 1.77% late apoptotic cells, while the vehicle control showed 84.82 ± 1.05% viability and 10.68 ± 1.82% late apoptotic cells, with no significant difference between them. In contrast, SFD (3.40 mg/mL) significantly reduced viability to 71.61 ± 7.50%, increasing late apoptosis to 19.80 ± 7.51%, while 5-FU at 0.05 mg/mL (360 μM) further lowered viability to 67.54 ± 4.56%, with 25.97 ± 3.51% late apoptotic cells (Figure 4.7, Table 4.8), both showing significant differences compared to controls. SFD increased late apoptosis by 9.12% relative to vehicle control. No significant variations were observed in early apoptotic cell populations across treatments. Along with Wang et al. (2016) who reported the suppression of Bcl-xL and Survivin which is anti-apoptotic proteins after treating AGS and SW480 cells with 10 mg/mL low molecular weight citrus pectin, also using TUNEL analysis to confirm apoptosis induction in xenograft tissues. RG-I-enriched alkali-treated sugar beet pectin from Maxwell et al. (2016) significantly reduced cell viability while markedly increasing both early and late

apoptotic cell populations, highlighting its potential anticancer properties. While Cheng et al. (2013) reported that RG-I-enriched pectin from potato at 5 mg/mL did not induce significant apoptosis on HT-29 cells but induced significant G2/M cell cycle arrest. Previous studies have provided strong evidence of pectin's involvement in the intrinsic apoptosis pathway. Chen et al. (2015) demonstrated that pectin enhances the expression of pro-apoptotic markers caspase-3 and Bax which associated to mitochondrial outer membrane permeabilization in A549 lung cancer cells. In addition, Wang et al. (2016) reported a downregulation of the anti-apoptotic proteins Bcl-xL and Survivin in AGS and SW480 cancer cell lines. Together, these findings strongly support the role of pectin in promoting apoptosis through intrinsic pathways.

The structural composition of pectin, particularly its HG and RG-I domains, plays a critical role in its antiproliferative and apoptosis-inducing properties. HG-rich pectin is exposing reactive carboxyl groups, which contribute to cancer cell suppression. RG-I-enriched pectin, with its complex side chains also associated with antiproliferative effects. Additionally, low molecular weight pectin has been shown to significantly reduce cancer cell proliferation and induce apoptosis, reinforcing the importance of its fragmentation for enhanced bioactivity. In contrast, the degree of esterification (%DE) appears to have no direct impact on cell proliferation.

The limitations of the CCK-8 and Annexin V-FITC/PI staining assays revealed discrepancies in assessing SW480 cell viability following 3.4 mg/mL SFD treatment. Based on CCK-8 analysis, proliferation was approximately 50%, whereas Annexin V-FITC/PI staining indicated 71% live cells, suggesting methodological differences in viability assessment. CCK-8 quantifies metabolic activity via mitochondrial dehydrogenase, while Annexin V-FITC/PI staining evaluates plasma membrane integrity, implying that mechanisms beyond apoptosis may be influencing cell viability. The observed 9.12% increase in late apoptosis compared to the vehicle control points to additional pathways, such as mitoptosis, which can occur through inner or outer mitochondrial membrane degradation (Lyamzaev et al., 2020). Furthermore, the inhibition of Galectin-3, a key regulator of cell adhesion, migration, immune evasion, and angiogenesis, may also contribute to reduced cancer cell survival, as its suppression enhances apoptotic susceptibility (Fortuna-Costa et al., 2014). Additionally, cell cycle arrest, an important determinant of cancer cell viability, warrants further exploration.

Cheng et al. (2013) previously reported that pectin induces G2/M arrest, suggesting a potential mechanism beyond apoptosis that contributes to its antiproliferative effects.

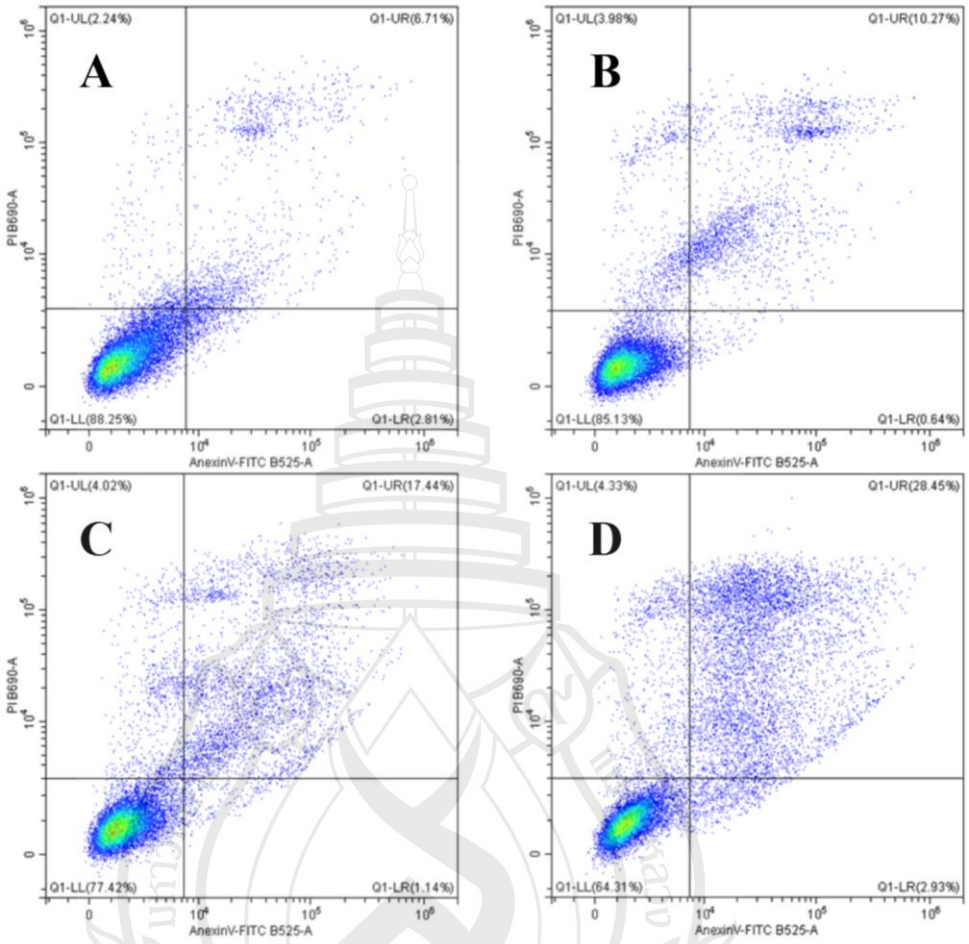


Figure 4.7 Effects of Untreated (A), Vehicle control (B), SFD at 3.4 mg/mL (C), and 5-FU at 0.05 mg/mL (360 μM) (D) on SW480 cells apoptosis after 48 hours of incubation by Annexin V-FITC/PI. The X and Y axes reflect log Annexin V-FITC and PI fluorescence, respectively.

Table 4.8 Percentage of SW 480 apoptosis cells using flow cytometry

Treatment	Live cells (%)	Early apoptotic cells (%)	Late apoptotic cells (%)	Total cell death (%)
Untreated	86.81 ± 2.23	1.20 ± 1.39	5.64 ± 2.94	13.19 ± 2.23
V-control	84.82 ± 1.05	1.11 ± 0.50	3.40 ± 1.17	15.19 ± 1.05
SFD	71.61 ± 7.50 [ab]	1.23 ± 0.28	19.80 ± 7.51 [a]	28.40 ± 7.49 [ab]
5-FU	67.54 ± 4.56 [ab]	2.78 ± 0.22	25.97 ± 3.51 [ab]	32.49 ± 4.56 [ab]

Note Values are mean ± S.D of triplicates. The superscript indicates significant differences from Untreated (a) and V-control (b) at $p < 0.05$ in each type of cell death/alive.

CHAPTER 5

CONCLUSION

Coffee pulp, a byproduct of the coffee processing industry, can pose significant environmental challenges when improperly disposed of. However, utilizing coffee pulp as a source for pectin extraction presents a promising solution to manage this waste effectively. The double extraction method yielded 7.99% pectin, characterized as low methoxy pectin (LMP), capable of forming gels in the presence of ions such as Ca^{2+}, making it suitable for low-sugar industrial products, with a purity of 68% which is 5% lower than commercial pectin (HMP, L20, and L40). After enzymatic modification with pectinase, SFD displayed significantly lower molecular weight 62 and 101 times lower than CP and SF, respectively, while maintaining LMP characteristics. Compared to commercial pectin, CP, SF, and SFD contained lower concentrations of rhamnose and galactose but higher arabinose, indicating larger yet less frequent branching structures. Additionally, while CP and SF were HG-domain rich, SFD exhibited a greater presence of RG-I domains, suggesting that enzymatic modification enhances, suggesting that enzymatic modification increases branching of pectin. Furthermore, pectin derived from coffee pulp (CP, SF, and SFD) demonstrated antioxidant activity by achieving 50% radical scavenging efficiency whereas commercial pectin failed to reach this threshold. Prebiotic properties, as indicated by both the Prebiotic Index and Prebiotic Activity Score, comparable to those of inulin, a well-established commercial prebiotic. In contrast, other commercial pectins did not exhibit significant prebiotic properties, except for HMP's activity score related to *Pediococcus* sp. and *Lactobacillus acidophilus*. SFD demonstrated dose dependent anti-colorectal cancer activity on SW 480 colon cancer cells while non-modify pectin can't show anti proliferation lower than 80%, also exhibited a higher IC_{50} for CCD 841 CoN normal colon cells, suggesting lower sensitivity toward normal cells. Along with its apoptosis inducing effects on SW480 cells that significantly reduces viability and increased late apoptosis comparable to vehicle control. The bioactivity of pectin is closely linked to its structural composition, particularly the HG and RG-I domains, more importantly the

lower M_w of SFD that exhibits enhanced apoptotic potential, whereas the degree of esterification appears unrelated to cell proliferation. Future research should focus on different mechanisms beyond apoptosis that influence cancer cell viability. This exploration could enhance its commercial viability and contribute to sustainable waste management practices in the coffee industry.

Pectin extracted from coffee pulp shows promising potential for commercialization, as evidence by their biological activity that acts as antioxidant and prebiotic agent. Its LMP characteristics make it fitting for use as a food additive in low sugar product, as an ingredient in nutraceutical, or even as a dietary supplement in capsule form. However, there might be some concern due to the dark color of this pectin may interfere with the appearance of clear or light-colored products, Additionally, appropriate protective packaging is needed of its moisture sensitivity. Importantly, in *vivo* studies are necessary to prove its health claims and ensure in human applications. SFD shows potential for pharmaceutical application, with this study representing the first step in evaluating its anticancer activity. Further investigation is essential to advance its development, including clarifying the specific pathway, in vivo study, and clinical trials to confirm its safety in humans.

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APPENDIX A

THERMO GRAVIMETRIC ANALYSIS DATA

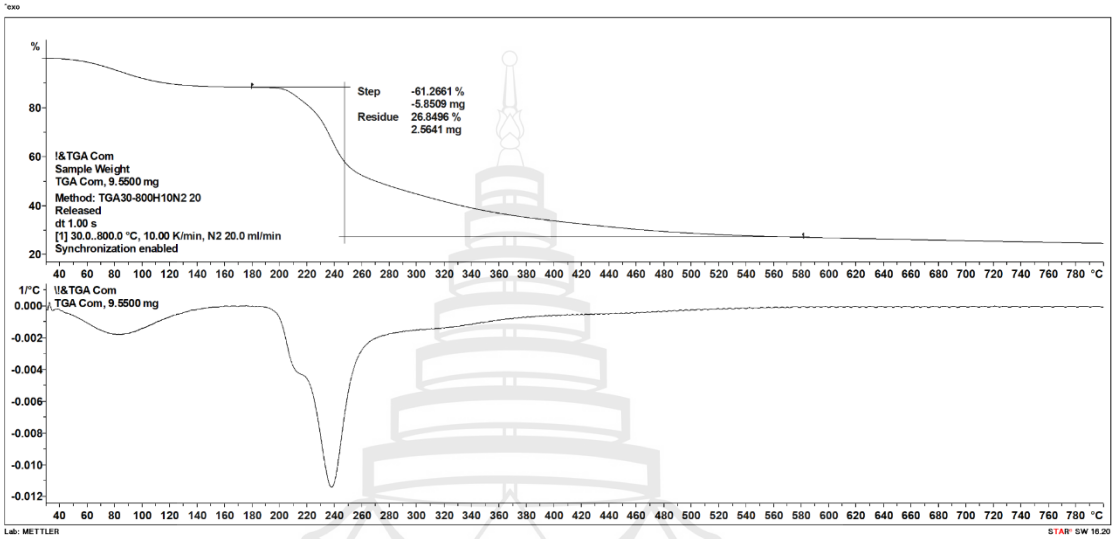

Figure A1 TGA chromatogram of high methoxy apple pectin (HMP)

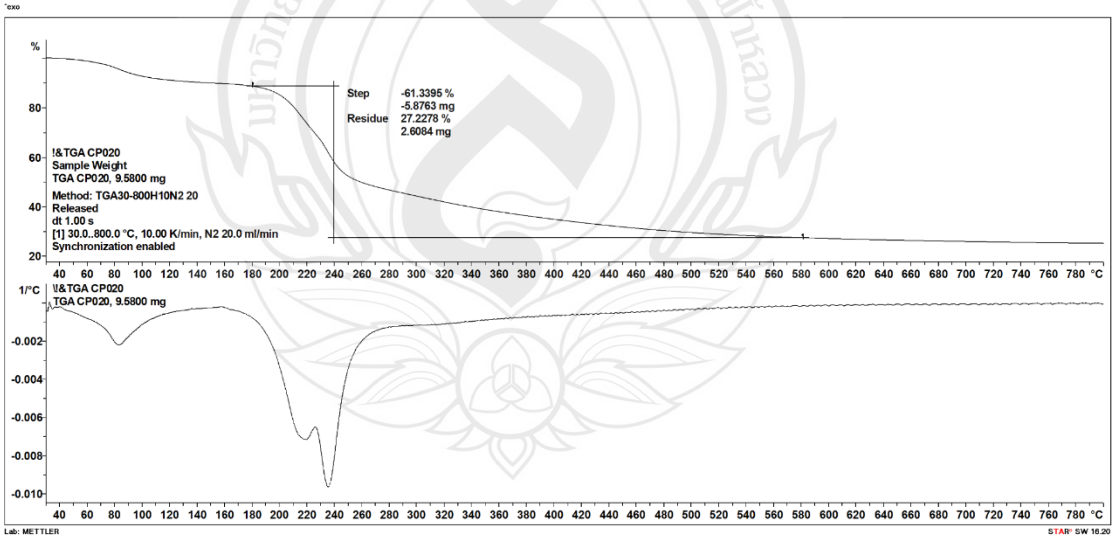

Figure A2 TGA chromatogram of low methoxy citrus pectin (L20)

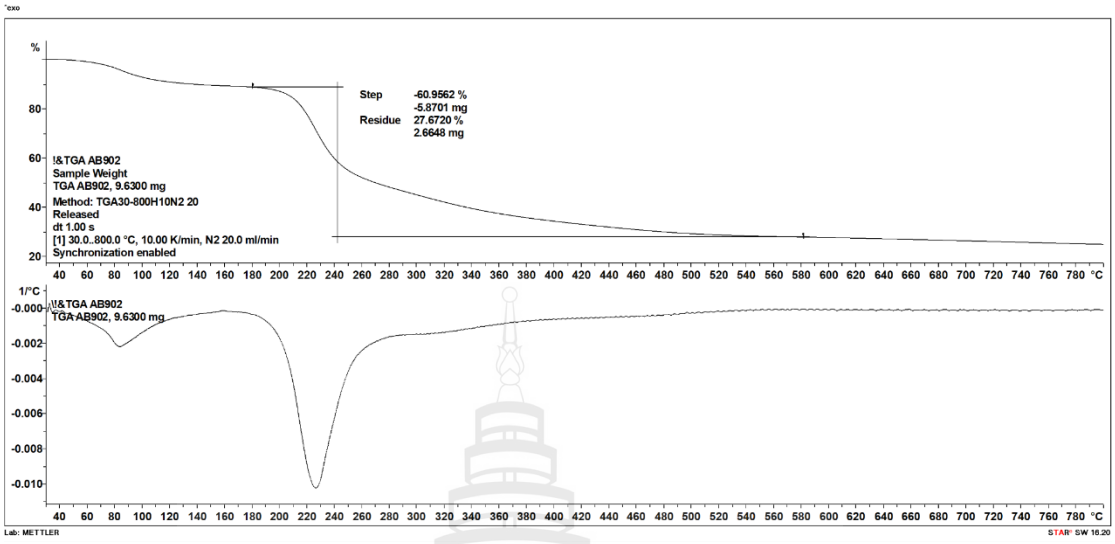

Figure A3 TGA chromatogram of low methoxy apple pectin (L40)

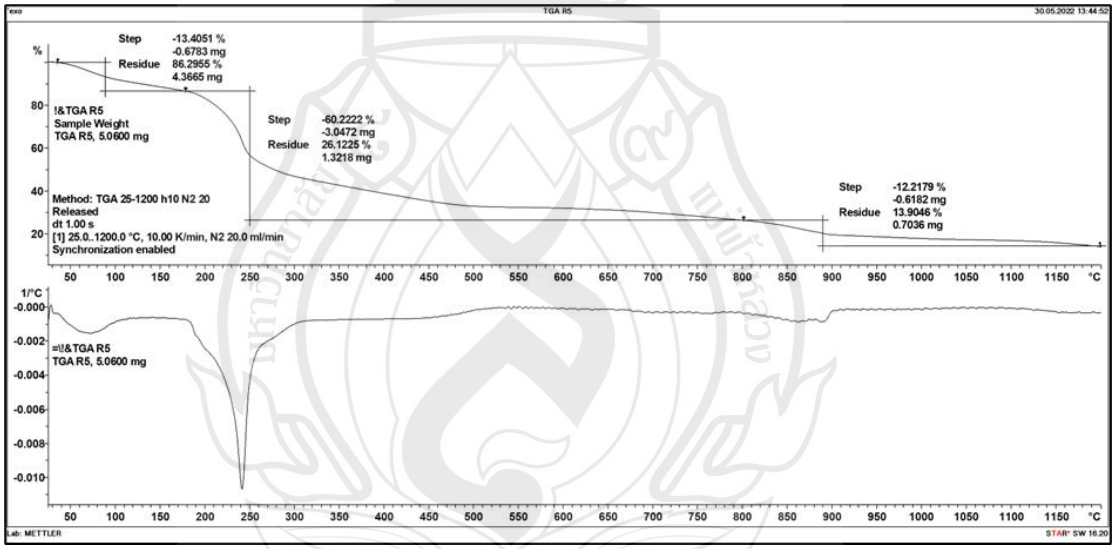

Figure A4 TGA chromatogram of coffee pulp pectin (CP)

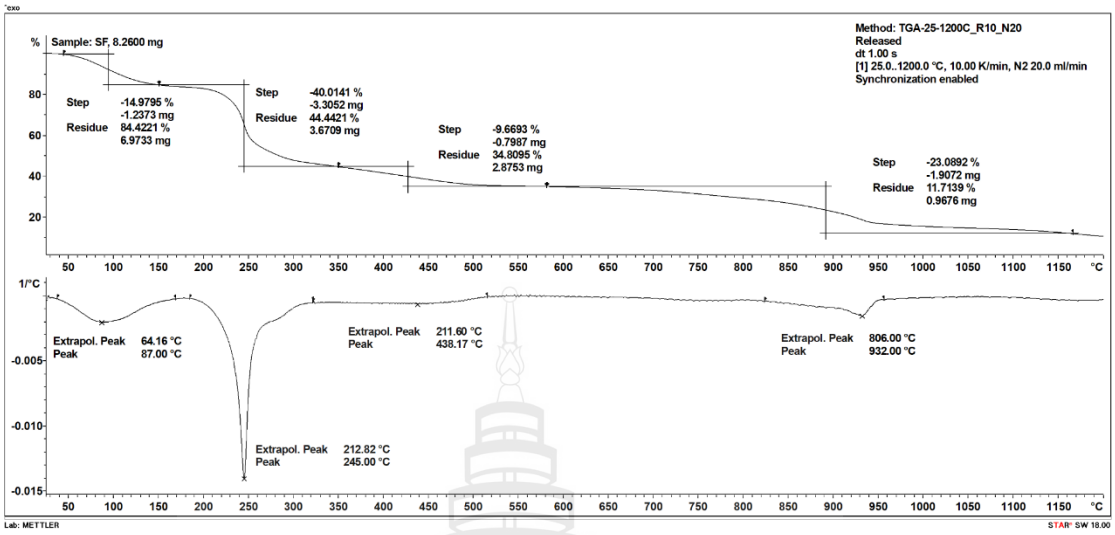

Figure A5 TGA chromatogram of soluble fraction of coffee pulp pectin (SF)

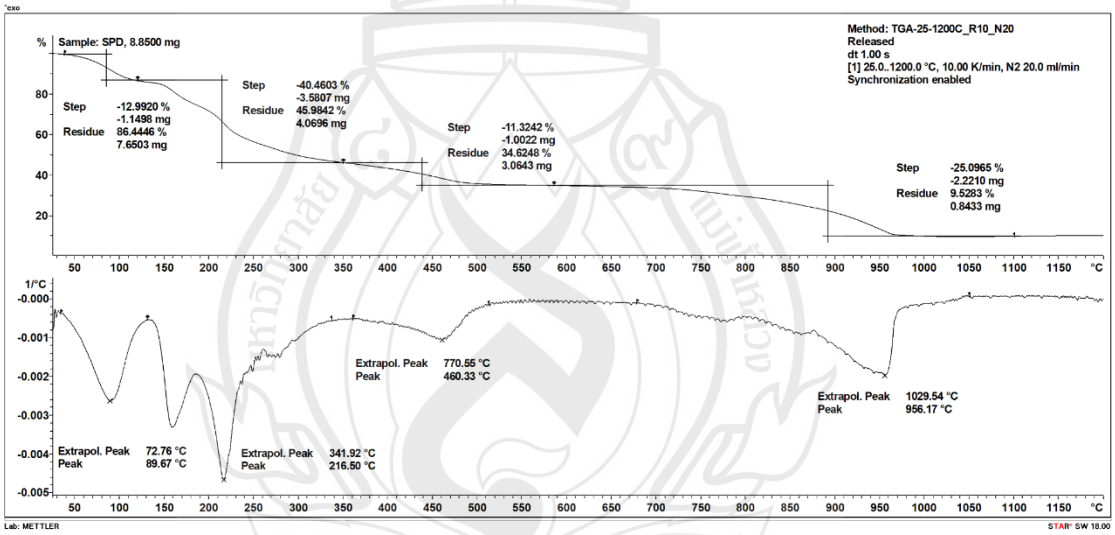

Figure A6 TGA chromatogram of digested soluble fraction of coffee pulp pectin (SFD)

APPENDIX B

FOURIER TRANSFORM INFRARED SPECTROSCOPY DATA

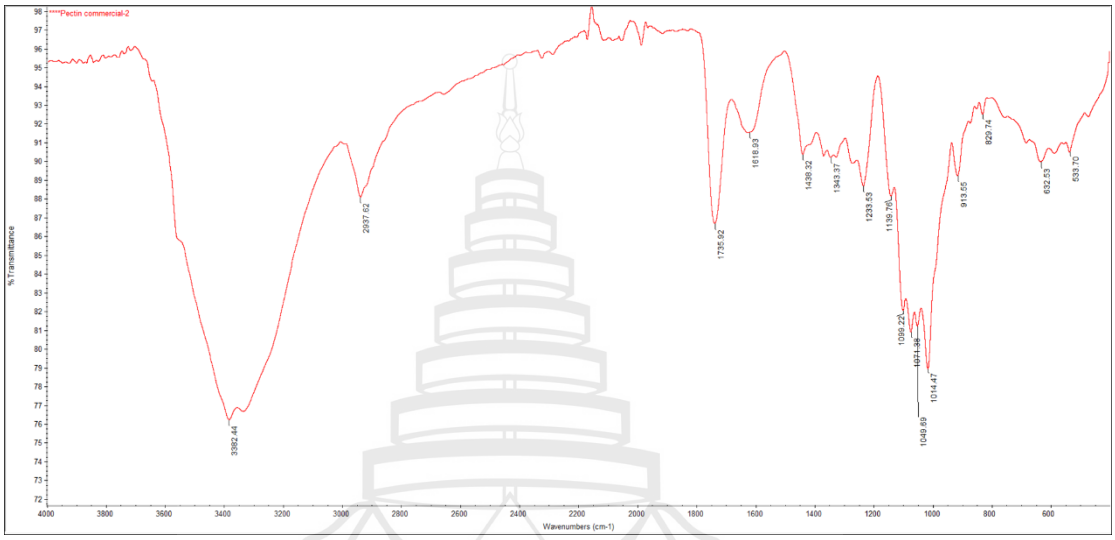

Figure B1 FTIR spectra of high methoxy apple pectin (HMP)

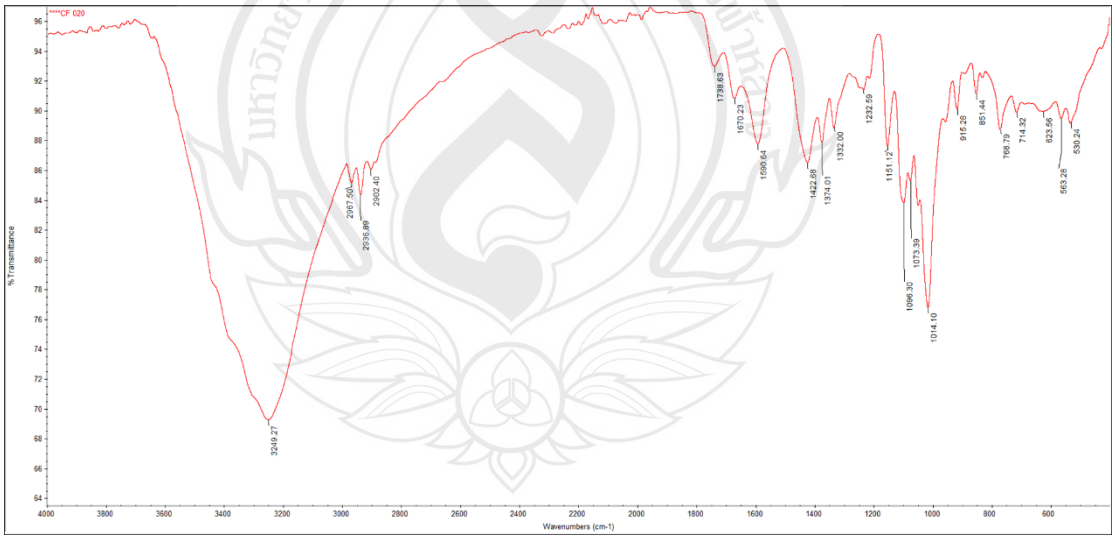

Figure B2 FTIR spectra of low methoxy citrus pectin (L20)

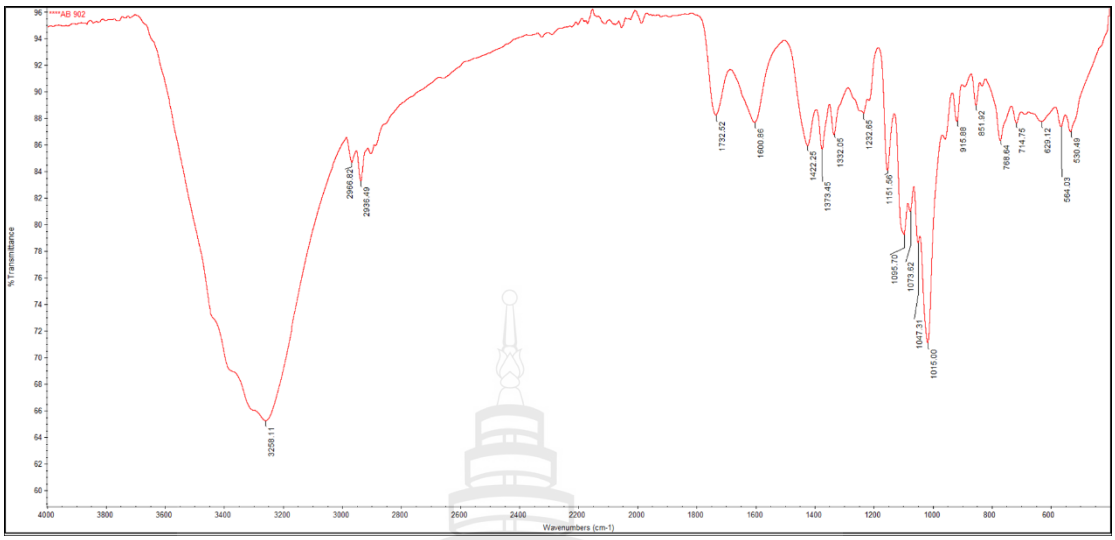

Figure B3 FTIR spectra of low methoxy apple pectin (L40)

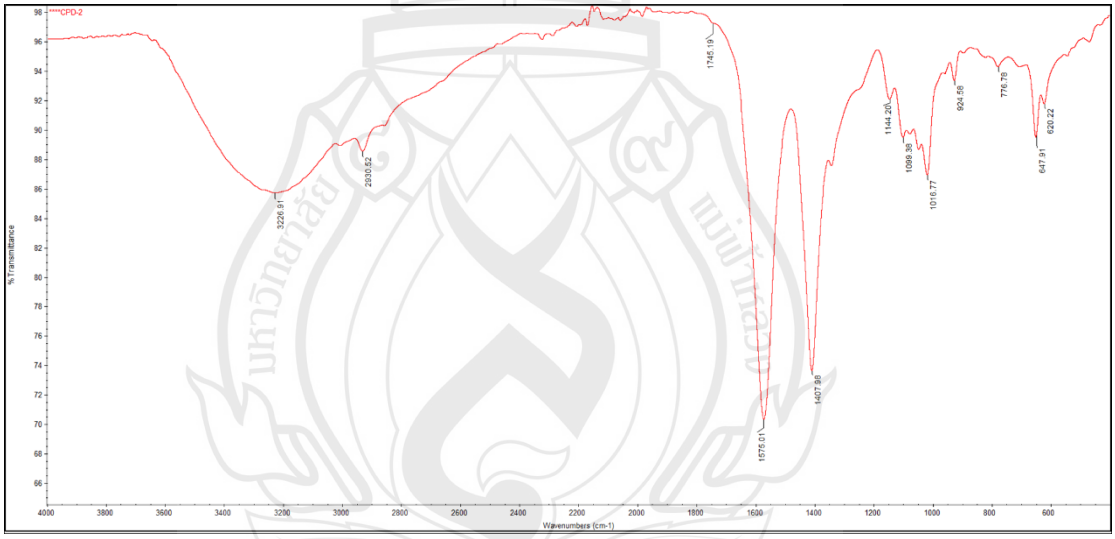

Figure B4 FTIR spectra of digested soluble fraction of coffee pulp pectin (SFD)

APPENDIX C

PREBIOTIC ACTIVITIES DATA

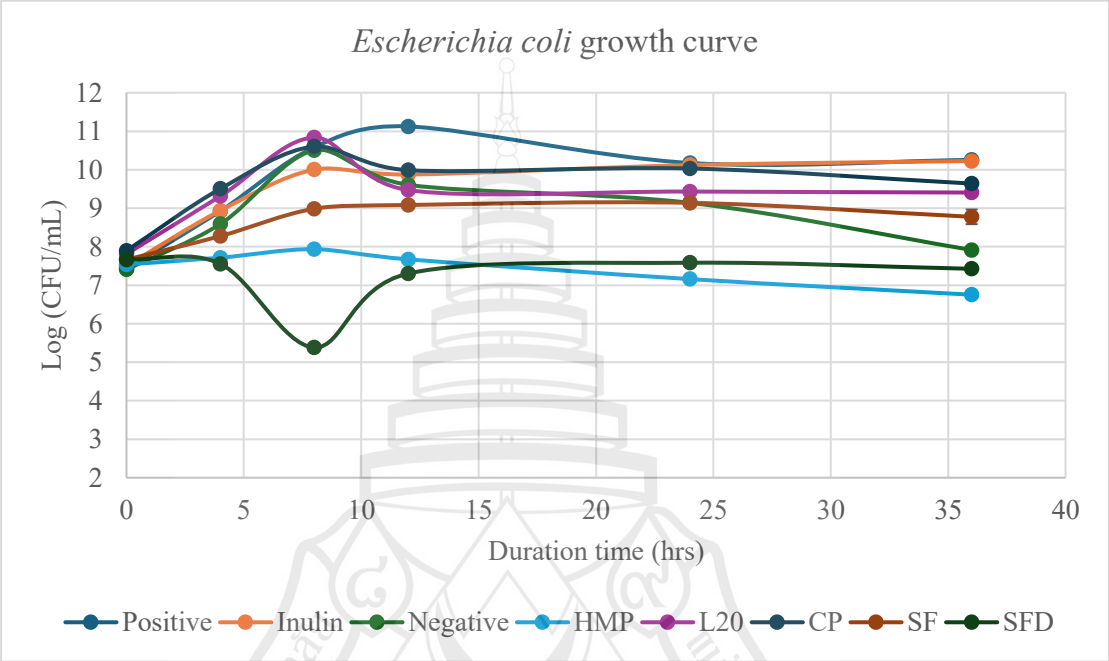

Figure C1 Growth of *Escherichia coli* cultured with different types of carbon sources

APPENDIX D

ANTI-COLORECTAL CANCER ACTIVITY DATA

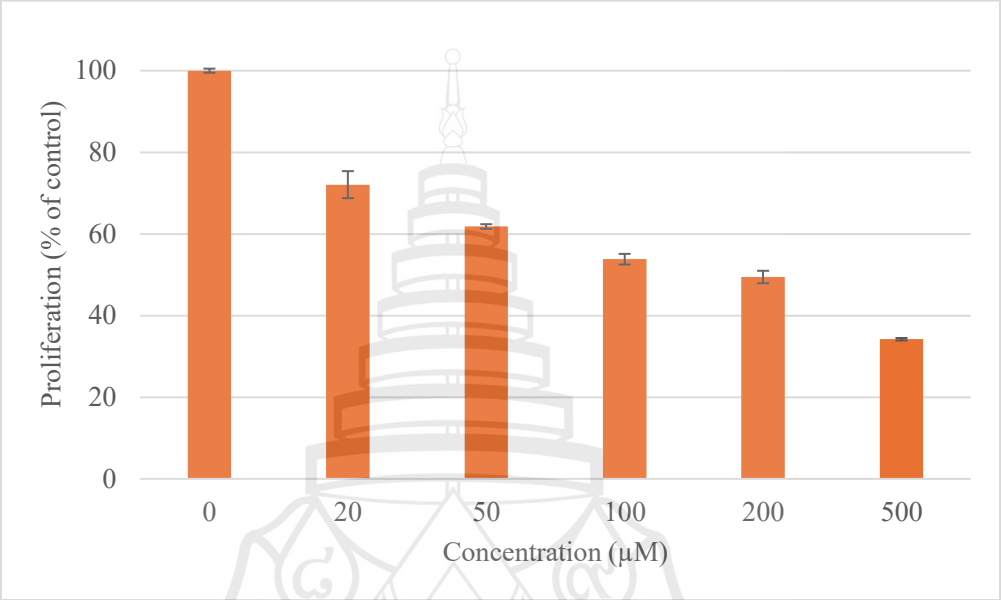

Figure D1 Effect of 5-FU on cell proliferation of SW480 cells after 48 hours of incubation by CCK-8

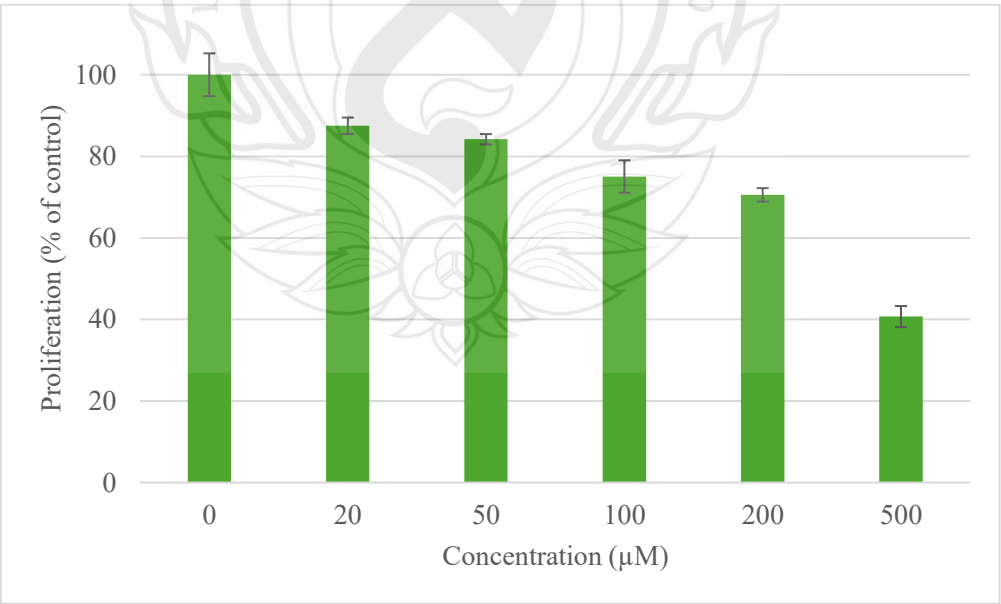

Figure D2 Effect of 5-FU on cell proliferation of CCD 841 CoN cells after 48 hours of incubation by CCK-8

CURRICULUM VITAE

NAME Kunakorn Deesophon

EDUCATIONAL BACKGROUND

2021 Bachelor of Science
Major in Biotechnology
Mae Fah Luang University, Thailand

WORK EXPERIENCE

2022-2024 Teaching Assistant, Fundamentals of Biology
Mae Fah Luang University

2021-2022 Research Assistant, Pectin production from coffee pulp for reduction of the agricultural waste during coffee processing
Mae Fah Luang University

SCHOLARSHIP

2021 Mae Fah Luang University Graduate Scholarship