SYNTHESIS AND BIOACTIVITIES OF PECTIN SULFATE

By

MISS Kornsima CHAYANSUPAP

A Thesis Submitted in Partial Fulfillment of the Requirements

for Doctor of Philosophy MICROBIOLOGY

Department of MICROBIOLOGY

Graduate School, Silpakorn University

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MISS KORNSIMA CHAYANSUPAP : SYNTHESIS AND BIOACTIVITIES OF PECTIN SULFATE THESIS ADVISOR : WARANYOO PULSAWAT, Ph.D.

Pectin, a heteropolysaccharide made up of three major components; homogalacturonan (HG), rhamno-galacturonan type I (RG-I) and rhamnogalacturonan type II (RG-II), is derived from cell wall of higher terrestrial plants. The pectin was submitted to chemical modification specifically sulfation and anticoagulant activity of the sulfated pectin was investigated. Successful replacement of hydroxyl groups of pectin with sulfate groups was confirmed by degree of sulfation (DS) and Fourier fransform infrared spectroscopy (FTIR) spectra of absorption bands at 830 (S=O) and 1242 (C-O-S) cm^{-1}.

Sulfated pectin (PecS) and hydrolyzed sulfated pectin (PecHS) demonstrated significantly prolonging activated partial thrombosis time (APTT) of 179 and 63.5 seconds, respectively, at a concen- tration of 50 µg ml^{-1}. The PecS elevated prothrombin time (PT) to 54 seconds at a concentration of 250 µg ml^{-1} compared to native pectin that had no significant anticoagulant effect. The result indicated the inhibition of intrinsic pathway according to the prolonged APTT. The extrinsic coagulation pathways could be inhibited with PecS at high concentrations according to an increase of prolonged PT. Both Pec and PecS at concentration of 2000 mg/ml exhibited no effected of L929 cell line after 48 h of the cell cultivation and only after 24 h exposed to Pec and PecS. However, when extending of cultivation to 72 h, it was found that the L929 cell line increased more % survival compared to the cell line exposed to Pec. On the contrary, when Pec and PecS at 2000 mg/ml were exposed cancer cell line, HeLa and HepG2, Pec and PecS contributed no sign to cease or caused cell death of HeLa, but effected to cell extention and morphology change of cells.

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Kornsima CHAYANSUPAP

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Pectin is a complex mixture of polysaccharides that makes up about one third of the cell wall dry substance of higher plants (Sundar Raj et al. 2012). Their structures comprise approximately 70% of galacturonic acid, and all the pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 position (Mohnen 2008). Pectin is wildly used in food industrial as jellifying, thickening, water binder and stabilizing agent, where it is used in the production of jams, jellies as well as a stabilizer in fruit juices and milk drink, confectionery articles, baked and dairy products or in the nonfood industry, such as in cosmetics and pharmaceutics (Bae et al. 2009), pectin has been investigated as a matrix tablets for colon specific drug delivery system. The preparation of pectin/chitosan polyelectrolyte complex scaffolds for possible bone tissue engineering applications. The using of pectin hydrogel for drug delivery, artificial skin and soft contact lenses (Mishra, Banthia, and Majeed 2012). In addition, pectin has been shown diverse of biological activities that include immunomodulating, antidote, antioxidant, apoptosis induction against colon cancer cells, gastric emptying delay, and cholesterol lowering effect in blood (Bae et al. 2009; Vityazev et al. 2010).

Pectin have no sulfate groups in their composition. When introduced of sulfate groups into pectin, the physiological activity of pectin has been changed and exhibited some bioactivities, such as anticoagulant activity, antioxidant activity and antimicrobial activity (Bae et al. 2009; Vityazev et al. 2010; Maas et al. 2012).

Sulfation methods for synthesis of pectin sulfate were influenced on several sulfated polysaccharide properties, such as degree of substitution, position of hydroxyl group substitution with sulfate group, molecular size and of products. Currently, sulfating agent were prepared by many ways, such as sulfuric acid, chlorosulfonic acid, sulfuryl chloride, sulfur trioxide and sulfamic acid. Some organic solvents had been used as reaction medium, such as pyridine, dimethyl sulfoxide and formamide (Fan, Jiang, et al. 2011).

Heparin has been using for anticoagulant activity therapy for more than 50 years. Heparin is a highly sulfated glycosaminoglycan and has the highest negative charge density of any know biological molecules consisting of glucuronic acid and 6-O-sufate, N-sulfate glucosamine. Mode of action of heparin, heparin binds to enzyme inhibitor: antithrombin III, causing a conformation change that result in its activation though an increase in the flexibility of its reactive site loop. The activation of antithrombin then inactive thrombin and other protease involved in blood clotting cascade, mostly for activated factor X. But there is disadvantage for using heparin because, heparin was isolated from animal materials may be contaminated with prions inducing BSE (bovine spongiform encephalopathy) (Fan, Wang, et al. 2011). This reason strongly motivated the necessity to find new anticoagulant to replace heparin.

In this study pectin sulfate was prepared by difference of sulfating agent including of trisulfonated sodium amine (Fan, Jiang, et al. 2011), sulfur trioxide pyridine (J. Yang et al. 2003) and chlorosulfonic acid (Maas et al. 2012) after depolymerization with hydrogen peroxide and/or hydrolysis with hydrochloric acid. Fourier transform infrared spectrometry (FT-IR) and $^{13C\text{-}NMR}$ spectra were used to confirm the introduction of sulfate group into pectin molecules. The anticoagulant activity was measured by activated partial thromboplastin time (APTT) and prothrombin time (PT). Minimal inhibitory concentration (MIC) was used to investigate the antimicrobial activity (Mahon, Lehman, and Manuselis 2014). Nephelometry method was used to determine the degree of substitution of pectin sulfate (Dodgson and Price 1962). Antioxidant activity was measured by superoxide anion scavenging activity assay, hydroxyl radical scavenging ability assay and reducing power (Y. Yang et al. 2011). And finally, anticancer activity and cytotoxicity of pectin and its derivatives were examined using MTT assay. In addition, determination of effect on biochemical assays when substituted pectin sulfate for common anticoagulant agent using in test tube.

1.2 Research objectives

1.2.1 To synthesize pectin sulfate with bioactivities.

1.2.2 To characterize the structure of pectin and its derivatives.

1.2.3 To investigate bioactivities of pectin sulfate such as anticoagulant activity, antimicrobial activity, anticancer activity and cytotoxicity.

1.3 Hypotheses

1.3.1 The hydroxyl group of pectin can be replaced by sulfate group under the suitable conditions.

1.3.2 The structure of pectin and the position of sulfate group that replace into pectin can be detected by ^{1}H-NMR, ^{13}C-NMR and Fourier transform infrared spectroscopy (FT-IR).

1.3.3 Molecular size, the number and position of sulfate group of pectin sulfate can be influencing on bioactivities of pectin sulfate such as anticoagulant activity, antimicrobial activity, anticancer activity and cytotoxicity.

1.4 Scope of the study

In this study, pectin was depolymerized with hydrogen peroxide and/or acid hydrolysis to obtain low molecular weight pectin. And then sulfation with sulfating agent under suitable conditions. After that, the structure of pectin and its derivatives were characterized by ^{1}H-NMR, ^{13}C-NMR and FT-IR. The molecular weight was determined by polyacrylamide gel electrophoresis. The degree of substitution was measured by barium sulfate nephelometry method. Finally, testing for bioactivities of pectin and its derivative were carried out by anticoagulation, antimicrobial, anticancer and cytotoxicity assay. In addition, *in vitro* biochemical assays using sulfated pectin replacing common anticoagulant agent are carried out.

CHAPTER 2

LITERATURE REVIEW

2.1 Literature review

Polysaccharide sulfates

Sulfated polysaccharides are ubiquitously in various natural biomaterials such as composition of animals, plants, and microorganisms (Yu et al. 2018). The structures of which contain a varying number of sulfate groups covalently bonded to the sugar backbone. They frequently exist on the cell walls of certain seaweeds. These polysaccharide sulfates of marine origin include agarans and carrageenans in red algae, fucans in brown algae, and ulvans in green algae (Korva et al. 2016). It is suggested that their polyanions are involved in expressing various biological functions. In addition to functional properties, individual sulfated polysaccharides exhibit various pharmacological activities, such as anticoagulant, antithrombotic, antioxidant, antimicrobial, anticancer, anti-inflammation, etc (Mestechkina and Shcherbukhin 2010).

Heparin as the first anticoagulant, was discovered in 1916 at Johns Hopkins Medical School, Baltimore, USA, by a second-year medical student, Jay McLean, who was working under the physiologist William Henry Howell. The heparin was in the main interest as the substances controlling blood clotting with the thought there was a balance between a clotting inhibitor (termed antithrombin) and a procoagulant (termed thromboplastin) (Wardrop and Keeling 2008).

The mast cells of several animal tissues produce this natural sulfated linear glycosaminoglycan. The polysaccharide chains of heparin molecule are composed of an alternating α-D-glucosamine and uronic acid residues linked via α-(1$\rightarrow$4) bonds. The majority of glucosamine residues are deacetylated at nitrogen and sulfated at amino and hydroxy groups (N-sulfo- and 6-O-sulfo); sulfo groups can be also located at position C-3. The units of uronic acid are the resi dues of 2-O-sulfo-α-L-iduronic acid and the residues of β-D-glucuronic acid epimeric at C-5 ((Mestechkina and Shcherbukhin 2010).

Due to these various biological activity properties, various synthetic polysaccharide sulfates has gain interest for synthesis along with the utilization of natural compounds such as

starch, cellulose, dextran, pullulan, curdlan, chitin, chitosan, alginate and including pectin (Korva et al. 2016). There were also a number of sulfating agent and synthesis procedures for preparation polysaccharide sulfate from natural biopolymers as shown in Table 1.

Table 1. Preparation of sulfated natural polysaccharides (modified from Suwannee Tongmalee 2015)

polysaccharide sulfates	**sulfating agent**	**degree of substitution (DS)**	**references**
starch sulfates	Na_2SO_3, $NaNO_2$	2.14	(Cui et al. 2007)
cellulose sulfates	DMF-SO_3	1.8	(Touzinsky and Gordon 1979)
dextran sulfates	HSO_3Cl, pyridine	1.9	(Walton 1952)
pullulan sulfates	SO_3- pyridine, DMF	0.17 – 1.99	(Alban, Schauerte, and Franz 2002)
curdlan sulfates	SO_3- pyridine, LiCl/Me2SO	1.7 - 2.2	(Koumoto et al. 2004)
chitin sulfates	DMF-SO_3	2.0	(Jayakumar et al. 2007)
chitosan sulfate	DMF-SO_3	0.11-0.35	(Jayakumar et al. 2007)
alginate sulfates	Na_2SO_3, $NaNO_2$	1.87	(Fan, Jiang, et al. 2011)
pectin sulfate	$N(SO_3\ Na)_3$	0.12	(Fan, Gao, et al. 2012)

The most widely used substances are chlorosulfonic acid is an effective method for adding sulfate. Sulfation of polysaccharides can performed using sulfuric acid, chlorosulfonic acid, monomethyl sulfate, sulfotrioxide, sulfuryl chloride, or sulfamic acid in the presence of formamide, DMSO, trimethylamine, or pyridine. Employing chlorosulfonic acid was believed to be better than

the other two sulfating agents which were pyridine sulfotrioxide and pyridine monomethyl sulfate according to degree of sulfation, degree of destruction, and yield of the polysaccharide sulfate derivatives. Nevertheless, hydrolytic degradation of the polysaccharide and environmental pollution were prone to occur with using the chlorosulfonic acid as sulfating reagent (Chen et al. 2015).

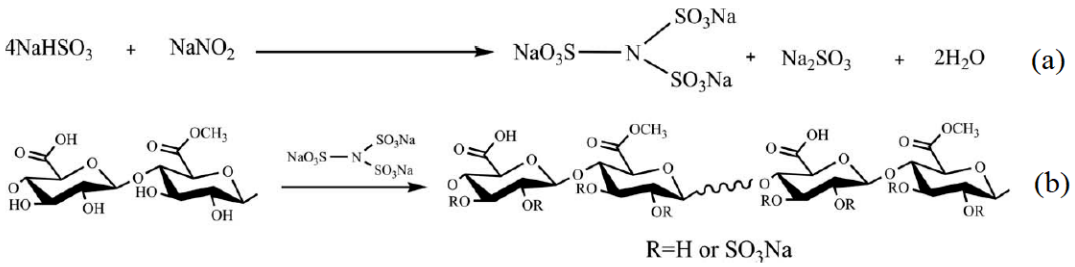

Figure 1. Synthesis reactions of the (a) sulfating reagent and (b) pectin sulfate (Fan, Gao, et al. 2012)

Currently, sulfur trioxide complex reagents are the most widely employed method for the sulfation of a polysaccharide which use sulfur trioxide–amine complexes (Figure 1) in a highly polar organic solvent such as dimethylformamide (DMF) or dimethylacetamide (DMA) (Figure 2). The advantages of sulfur trioxide complexes include their relatively stability, ease of handling, and compatibility with highly polar solvents promoting in the solubility of polysaccharides. Traditionally, sulfation of polysaccharides is attained using sulfuric acid and chlorosulfonic acid (Figure 3) to yield an active sulfur trioxide reagent.

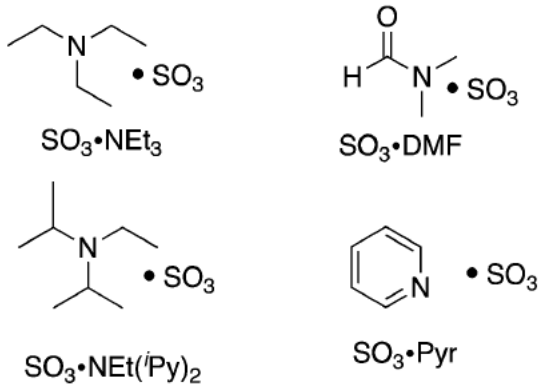

Figure 2. Sulfur trioxide complexes with amines (Caputo, Straub, and Grinstaff 2019).

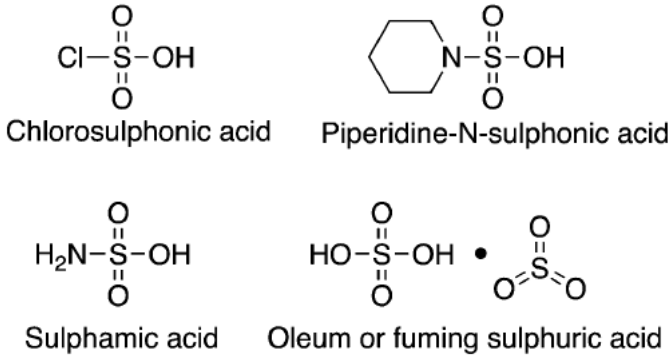

Figure 3. Structures of different sulfation reagents (Caputo, Straub, and Grinstaff 2019).

Pectin polysaccharide

Pectin is an excellent carbohydrate polymer derived from mainly natural resources and it is the structural component of plant cell wall (Mishra, Banthia, and Majeed 2012). The highest concentrations of pectin are found in the middle lamella of cell wall, with a gradual decrease as one passes through the primary wall toward the plasma membrane (Sundar Raj et al. 2012), has functions in plant growth, morphology, development, and plant defense. Pectin is a family of galacturonic acid-rich polysaccharides including homogalacturonan (Figure 4. A), rhamnogalacturonan I (Figure 4. B), and the substituted galacturonans rhamnogalacturonan II (RG-II) (Figure 4. C), and xylogalacturonan (XGA) (Mohnen 2008). The universally used jellifying, thickening, water binder and stabilizing agent pectin which nowadays is an indispensable

component of a great variety of products in either the food industry, where it is used in the production of jams, jellies as well as a stabilizer in fruit juices and milk drink, confectionery articles, baked and dairy products or in the nonfood industry, such as in cosmetics and pharmaceutics. Furthermore, pectin is shown to have diverse biological activities that include immunomodulating, antidote, antioxidant, apoptosis induction against colon cancer cells, gastric emptying delay, and cholesterol lowering effect in blood (Bae et al. 2009; Vityazev et al. 2010). It has been reported to help reduce blood cholesterol in a wide variety of subjects and experimental conditions as comprehensively reviewed and consumption of at least 6 g/day of pectin is necessary to have a significant effect in cholesterol reduction. Amounts less than 6 g/day of pectin are not effective. Pectin reduces rate of digestion by immobilizing food components in the intestine. This results in less absorption of food. The thickness of the pectin layer influences the absorption by prohibiting contact between the intestinal enzyme and the food, thus reducing the latter's availability. Due to its large water binding capacity, pectin gives a feeling of satiety, thus reducing food consumption (Sundar Raj et al. 2012).

Pectin is soluble in pure water. Monovalent cation salts of pectinic and pectic acids are usually soluble in water; di- and trivalent cations salts are weakly soluble or insoluble. Dry powdered pectin, when added to water, has a tendency to hydrate very rapidly, forming clumps. These clumps consist of semidry packets of pectin contained in an envelope of highly hydrated outer coating. Clump formation can be prevented by dry mixing pectin powder with water-soluble carrier material or by the use of pectin having improved dispersibility through special treatment during manufacturing. The most important use of pectin is based on its ability to form gels. The particular structure of pectin imposes some specific constraints. Low methoxyl pectin (< 50% esterification) form thermoreversible gel in present of calcium ion at low pH (3-4.5) whereas high methoxyl pectin rapidly form thermally irreversible gel in the present of sufficient sugars such as sucrose at low pH (< 3.5), the lower the methoxyl content, the slower the set. The degree of esterification can be reduced using commercial pectin methylesterase, leading to a higher viscosity and firmer gelling in the present of calcium ion (Sundar Raj et al. 2012).

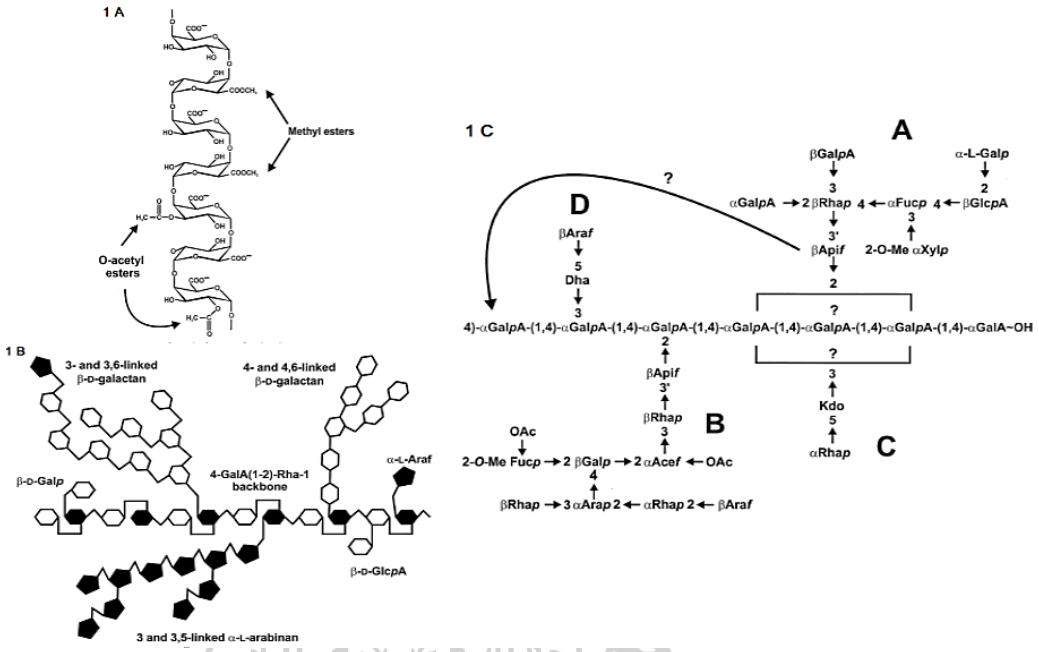

Figure 4. The structure of pectin (Ridley, O'Neill, and Mohnen 2001)

[A] In Figure 4 is the primary structure of homogalacturonan. Homogalacturonan is a linear polymer of 1-4 linked α-D-GalpA residues. Some of the carboxylates of the GalpA residues are esterified with methanol. The GalpA residues may also be esterified with acetic acid at C2 and C3. [B] In Figure 4 is the major structural features of rhamnogalacturonan I. The backbone is composed of the disaccharide repeating unit [4- α-D-GalpA-(2)-α-L-Rhap-(1)]. Branched and linear oligosaccharides composed predominantly of a-L-Araf and b-d-Galp residues are linked to C4 of some of the Rhap residues. Some of the Rhap residue may also be O-acetylated at C2 and/or C3. [C] In Figure 4 is the primary structure of rhamnogalacturonan II. The backbone of RG-II is composed of at least seven 1-4 linked α-D-GalpA residues. Four structurally different oligosaccharide side chains (A–D) are linked to the RG-II backbone.

Pectin has no sulfate groups in their composition. The effect of introduction of sulfate groups into pectin macromolecules on the physiological activity and anticoagulant properties of pectin has been examined. In 2009, Bae *et al.* (Bae et al. 2009) reported citrus pectin with chemical modification, specifically sulfation (DS 1.5). The pectin derivative was exhibited antimicrobial

effects against *Bacillus cereus* and *Vibrio fischeri* and the anticoagulant activity, which increased in a concentration dependent manner. These results demonstrated that the incorporation of sulfate groups into the pectin structure appeared to play an important role in improving its biological activities.

In 2010, Vityazev et al. (Vityazev et al. 2010) was prepared pectin sulfate from *Bergenia crassifolia* L, *Lemna minor* L and galacturonan as a backbone of pectins. Chlorosulfonic acid proved to be the optimal reagent for sulfation of both galacturonan and derivative pectins, and their anticoagulant activities were depended on the quantity of sulfate groups in the pectin macromolecules.

And in 2012, Maas et al. (Maas et al. 2012) investigated citrus pectin which was chemically sulfated. The results demonstrated that the antimicrobial effect of citrus pectin was enhanced by introducing sulfate groups into the pectin structure. In addition, the sulfated pectin exhibited anticoagulant activity. They inhibited thrombin by a mechanism depending on antithrombin and heparin cofactor II. Their hemorrhagic potential was also similar to that of heparin.

Hemostasis

Hemostasis is derived from a Greek word, which means stopping of the circulation of blood (Ogedegbe 2002). Hemostasis is a fundamental defense mechanism of all vertebrates and involves two complementary processes composing of the formation of blood clot or thrombus to prevent blood loss from a damaged tissues, vessels or organs and the process of thrombus dissolution or fibrinolysis, once endothelial repair has occurred (Gentry 2004). A breakdown in the vascular system is rapidly repaired to maintain blood flow and the integrity of the vasculature. The vascular system prevents bleeding through vessel contraction, diversion of blood flow from damaged vessels, initiation of contact activation of platelets with aggregation, and contact activation of the coagulation system (Ogedegbe 2002).

There are 3 mechanisms that works together to stop the flow of blood.

1) Vasoconstriction
2) Primary hemostasis (platelet plug formation)
3) Secondary hemostasis (blood coagulation)

1) Vasoconstriction: Vasoconstriction of a damaged blood vessel slows the flow of blood and thus helps to limit blood loss. This process is mediated by local controls, vasoconstrictors such as thromboxane are released at the site of the injury and systemic control, epinephrine released by the adrenal glands stimulates general vasoconstriction.

2) Primary hemostasis or platelet plug formation is recruited to the site of injury, where they become a major component of the developing thrombus; blood coagulation, initiated by tissue factor, to generate of thrombin and fibrin. These events occur concomitantly and under normal conditions, regulatory mechanisms contain thrombus formation temporally and spatially (Furie and Furie 2008).

Platelets, also called thrombocytes, are small cellular fragments that lack a nucleus that can vary in size up to 3 μm in diameter. They arise from the megakaryocyte in the bone marrow and circulate in blood at a platelet count that ranges from 200,000 to 400,000/ μL (Lefkowitz 2008). When a blood vessel is damaged, the blood is exposed to collagen fibers in the basement membrane of the vessel. Platelets are activated by collagen (Furie and Furie 2008). The endothelial cells secrete von Willebrand factor (vWF). vWF is secreted from the endothelial cell both into the plasma and abluminally into the subendothelial matrix. It is a large multimeric protein that acts as the intercellular glue binding platelets to one another and also to the subendothelial matrix at an injury site, which is needed for platelet adhesion to exposed subendothelial collagen in the arterioles (Lefkowitz 2008). In addition, the endothelial cells produce a variety of other adhesion molecules, which include P-selectin, intercellular adhesion molecules (ICAMs), and platelet endothelial cell adhesion molecules (PECAMs) (Ogedegbe 2002). Activated platelets release chemicals such as ADP, and thromboxane that cause the aggregation of more platelets to the site of injury. Platelet aggregation results in the formation of a platelet plug which acts to stem the flow of blood from the

broken vessel. It is essential that platelets become activated only at the site of a broken vessel. Otherwise activated platelets would form plugs and induce clots in inappropriate places (Figure 5). Healthy vessels secrete an enzyme called prostacyclin that functions to inhibit platelet activation and aggregation.

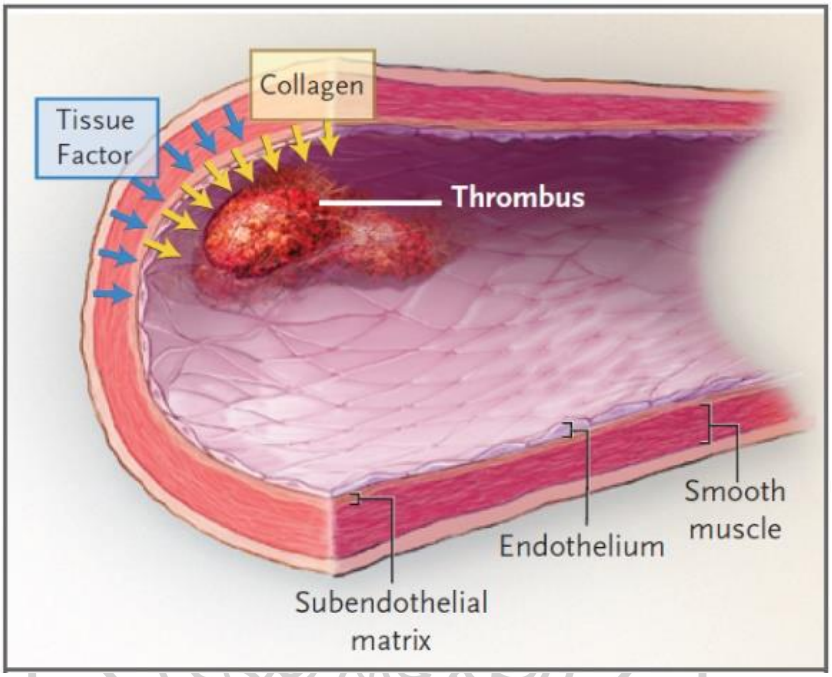

Figure 5. Response to Vascular Injury (Furie and Furie 2008).

Collagen and tissue factor associated with the vessel wall provide a hemostatic barrier to maintain the high-pressure circulatory system. Collagen (yellow arrows), located in the subendothelial matrix beneath the endothelium, is not exposed to flowing blood under normal conditions. Tissue factor (blue arrows), located in the medial (smooth muscle) and adventitial layers of the vessel wall, and comes in contact with flowing blood when the vessel is disrupted or punctured. Both collagen and thrombin initiate thrombus formation. Collagen is a first line of defense, and tissue factor a second line of defense (Furie and Furie 2008).

3) Secondary hemostasis or blood coagulation are divided into intrinsic, extrinsic, and common pathway (Figure 6). The intrinsic pathway was thought to be initiated by activation of contact factors and involves the subsequent activation of kallikrein, factor XII, factor XI, and factor IX, leading to factor X activation. The extrinsic pathway is initiated by tissue factor (TF) expression upon tissue injury and after complete formation of TF with factor VII also results in activation of factor X. In the common pathway, activated factor X activates prothrombin ultimately leading to fibrin formation (Schoenmakers, Reitsma, and Spek 2005). Some of the coagulation factors such as fibrinogen and prothrombin are referred to their common names, whereas others such as factors VIII and XI are referred to their Roman numeral nomenclatures. Activation of a factor is indicated by the addition of low case "a" next to the Roman numeral in the coagulation cascade such as factor VIIa, factor Xa and factor XIIa. Some of the common names were derived from the original patients in whom symptoms leading to the determination of the factor deficiency were found. Examples are the Christmas factor and Hageman factor (Table 1) (Ogedegbe 2002).

Table 2. Coagulation factors (Ogedegbe 2002).

Factor	Common Name	Function	Pathway Participation
Factor I	Fibrinogen	Thrombin substrate, polymerizes to form fibrin	Common
Factor II	Prothrombin	Serine protease	Common
Factor III	Tissue factor	Cofactor	
Factor IV	Ionic calcium	Mineral	
Factor V	Labile factor	Cofactor	Common
Factor VII	Stable factor	Serine protease	Extrinsic
Factor VIII	Antihemophiliac factor		
von Willebrand factor (vWF)	Cofactor	Intrinsic	
Factor IX	Christmas factor	Serine protease	Intrinsic
Factor X	Stuart Prower factor	Serine protease	Common
Factor XI	Plasma thromboplastin antecedent (PTA)	Serine protease	Intrinsic
Factor XII	Hageman factor	Serine protease	Intrinsic
Factor XIII	Fibrin stabilizing factor	Transglutaminase	Common
Prekallikrein	Fletcher factor	Serine protease	Intrinsic
High molecular weight kininogen	Fitzgerald factor, HMWK	Cofactor	Intrinsic
Platelet factor 3	Phospholipids, PF3	Assembly molecule	

The intrinsic pathway is begun with formation of the primary complex on **collagen** by **high molecular weight kininogen** (HMWK), **prekallikrein**, and Factor X. **Prekallikrein** is converted to **kallikrein** and Factor XII becomes Factor XIIa. Factor XIIa converts Factor XI into Factor XIa. Factor XIa activates Factor IX, which with its co-factor Factor VIIIa form the **tenase** complex, which activates Factor X to Factor Xa (Figure 6).

The extrinsic pathway is initiated by tissue factor and factor VIIa activate factor X, forming factor Xa. Factor Xa is then abled to activate prothrombin to form thrombin. Thrombin is converted fibrinogen to fibrin. Fibrin is formed a mesh that, in concert with the platelets, plugs the break in the vessel wall. The fibrin mesh is further stabilized by factor XIII, which sews up the clot (much like forming an intricate network of cross-stitched strands of fibrin), the mechanism was shown in Figure 6. (Gentry 2004).

The common pathway is a step that thrombin activated fibrin to form fibrin clot formation (Figure 6).

Regulation of Hemostasis

Hemostasis is exquisitely regulated for two major reasons. First, hemostasis must be restricted to the local site of vascular injury. That is, activation of platelets and coagulation factors in the plasma should occur only at the site of endothelial damage, tissue factor expression, and procoagulant phospholipid exposure. Second, the size of the primary and secondary hemostatic plugs must be restricted so that the vascular lumen remains patent. After vascular injury, intact endothelium in the immediate vicinity of the injury becomes “activated.” This activated endothelium presents a set of procoagulant factors that promote hemostasis at the site of injury. In contrast, the anticoagulant factors are generally secreted by the endothelium and are soluble in the blood. Thus, the activated endothelium maintains a balance of procoagulant and anticoagulant factors to limit hemostasis to the site of vascular injury. After vascular injury, the endothelium surrounding the injured area participates in five separate mechanisms that limit the initiation and propagation of the hemostatic process to the immediate vicinity of the injury. These mechanisms

involve prostacyclin (PGI_2), antithrombin III, proteins C and S, tissue factor pathway inhibitor (TFPI), and tissue-type plasminogen activator (t-PA) (Weitz 2001; Lefkowitz 2008; Tanaka, Key, and Levy 2009).

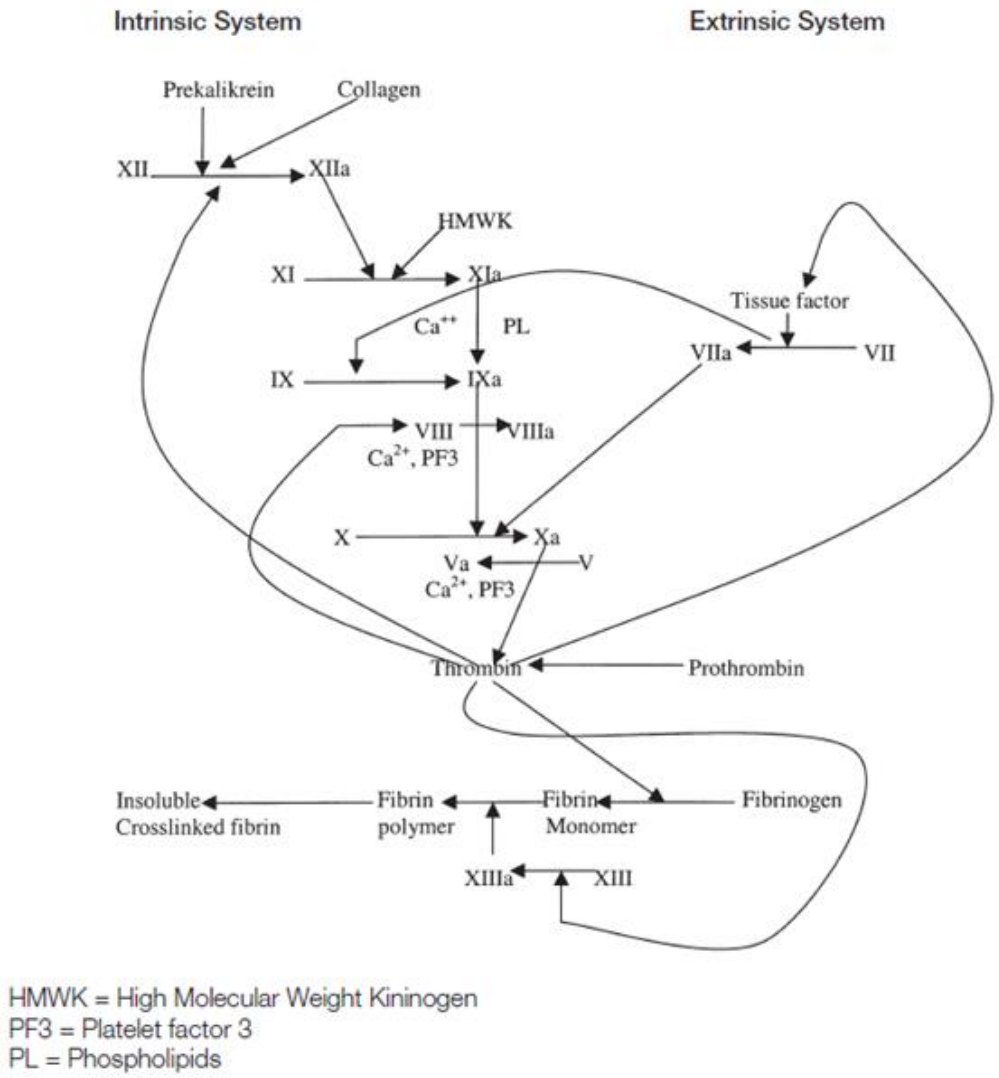

Figure 6. Coagulation cascade (Ogedegbe 2002).

Anticoagulants

Anticoagulant medicines reduce the ability of the blood clot. This is necessary if the blood clots too much, as blood clots can block blood vessels and lead to conditions such as a stroke or a heart attack.

Vitamin K antagonist

Vitamin K antagonist have been the mainstay of long-term oral anticoagulation since their discovery in the early part of the 20th century. Coumarin derivatives are currently the sole drugs available for oral anticoagulation therapy (warfarin in North America and phenprocoumon in Europe) that, inhibit gamma-carboxylation of serine protease zymogens, including prothrombin, Factors VII, IX, X, protein C, and protein S. The gamma-carboxylated domain (called the Gla-domain) is critical for enzymatic functions of vitamin K-dependent proteins because this protein domain binds to calcium ions on negatively charged phospholipid surfaces. Coumarins thus reduce the enzymatic activation of serine proteases, but they do not directly antagonize thrombin activity in contrast to heparin-AT complex. Both procoagulant (prothrombin, Factors VII, IX, X) and anticoagulant (protein C and S) proteins are affected, but the net clinical effect of vitamin K antagonists is anticoagulation because thrombin generation is suppressed by nonfunctional prothrombin and Factor X. (Tanaka, Key, and Levy 2009; Liem and DeLoughery 2011).

Heparin, low molecular weight heparin and fondaparinux

Unfractionated heparin and low molecular weight heparin are foundation for prevention and treatment of venous thrombosis and are widely used in combination with antiplatelet drugs, such as aspirin and glycoprotein IIb/IIIa antagonists, and thrombolytic agents in patients with acute coronary ischemic syndromes. Low molecular weight heparin is gradually replacing heparin for treatment of patients with venous thrombosis and is rapidly establishing a niche for itself as a treatment of unstable angina (Weitz 2001).

The mechanism of heparin, the heparin-antithrombin complex inactivates a number of coagulation enzymes, including thrombin (factor IIa) and factors Xa, IXa, XIa, and XIIa, thrombin and factor Xa are the most responsive to inhibition (Jack Hirsh et al. 2001).

Directed Thrombin inhibitors

Direct thrombin inhibitors are a new class of anticoagulants that bind directly to thrombin and block its interaction with its substrates (Di Nisio, Middeldorp, and Büller 2005) that, have

potential advantages over heparin. Whereas thrombin bound to fibrin or fibrin degradation products is relatively protected from inactivation by heparin bound thrombin is readily inhibited by direct thrombin inhibitors. Direct thrombin inhibitors produce a more predictable anticoagulant response than heparin because, unlike heparin, they do not bind to plasma proteins. Likewise, direct thrombin inhibitors are not neutralized by platelet factor 4, a highly cationic, heparin binding protein released from activated platelet (Weitz 2001).

Table 3. Example of drug, polysaccharides and polysaccharide sulfates using for anticoagulation application ((Suwannee Tongmalee 2015)

Type of active compound	**Name**	**APTT (s)**	**Mechanism in anticoagulation**	**reference**
drug	heparin	500 (14 µg/mL)	antithrombin III (AT), Xa, thrombin (IIa)	(Arlov 2012)
	hirudin	-	antithrombin III	(SCHMITZ, ROTHE, and DODT 1991)
	wafarin	-	II, VII, IX, X	(J Hirsh et al. 2003)
polysaccharide	fucoidans	238 (28 µg/mL)	VIII, IX, XI and XII	(J. Wang et al. 2010)
	carrageenan	86.4 (-)	Xa	(Liang et al. 2014)
	fucan	250 (100 µg/mL)	thrombin (IIa)	(Dore et al. 2013)
Polysaccharide sulfate	alginate sulfates	170 (75 µg/mL)	Xa, thrombin (IIa)	(Fan, Jiang, et al. 2011)
	chitosan sulfates	160 (75 µg/mL)	Xa, thrombin (IIa)	(Fan, Wu, et al. 2012)
	cellulose sulfates	200.8 (22 µg/mL)	Xa, thrombin (IIa), AT-III	(Z. M. Wang et al. 2007)

Cell culture

Cell culture is cultured under conditions in which those cells cannot rearrange themselves into tissues, which will lose the original properties and functions of the tissue. Cell line can be divided into 3 types as following.

1) Primary cell line refers to cells that are isolated from living things or organs of living organisms directly. They retain properties such as shape and chromosomes similar to origin organs. Fibroblast cells and epithelial cells are examples of primary cell line. Disadvantages of this cell line is unable to prolong cultivation and the cells often die after subcultivation.
2) Diploid cell line refers to cells originated from the primary cell line, such as MRC-5 (Medical Research Council 5). Human embryonic lung is a cell with high sensitivity to isolation. This cell line is suitable for vaccination preparation. The disadvantage of this cell line is ability to subculture for 50-100 times, then the cell will die.
3) Continuous cell line refers to cells endlessly divide, therefore subcultivation can be performed for hundreds of times. The cell originates from the transformation of the diploid cell line to cancer cells. The disadvantage of this cell line resulted from frequent mutation of normal cells causing cells to have altered properties and also resulted in the decrease of sensitivity of the isolation. Examples of the continuous cell line are Vero cell and HeLa cell.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Methods

3.1.1 Materials and equipment

1. Erlenmeyer flask 1000, 125, 100 mL (SCHOTT, Germany)
2. Beaker (SCHOTT, Germany)
3. Pasture pipette (Hirschmann Laborgerate)
4. Test tube (Pyrex, USA)
5. Round bottom 250 mL (Pyrex, USA)
6. Balance (Sartorius)
7. Shaking incubator (Gallenkamp)
8. Incubator (Gallenkamp)
9. Vortex mixer (Vision Scientific)
10. Airflow controller (Wiwatsan)
11. Laminar airflow (Ehret)
12. Hot air oven (Memmert)
13. Centrifuge (Hettich Zentrifugen Micro 22R, Germany)
14. Centrifuge tube (Extragenr, USA)
15. Autoclave (Hirayama, Japan)
16. Water bath (Fisher Scientific, UK)
17. Microscope (Nikon, Japan)
18. Hemacytometer (Neubauer Improved Bright-line, Canada)
19. 96 well plate (Corning Incorporated, USA)
20. Micropipette (Discovery+, Poland)
21. Micropipette Tip (Extragene, USA)
22. Tissue culture flask (Corning, USA)
23. Cryotube (Nunc, Denmark)
24. CO_2 Incubator (New Brunswick Scientific, USA)

25. Gel electrophoresis (Biorad, Horizon)
26. Fourier transform infrared spectroscopy (FT-IR) (Spectrum 100, Perkin Elmer)

3.1.2 Chemicals

1. Acetic acid ($C_2H_4O_2$; RCI Labscan)
2. Acetone
3. Alcian Blue 8GX (AMRESCO)
4. Ammonium persulphate ($(NH_4)_2S_2O_8$; OmniPur)
5. APTT reagent (C.K. PREST)
6. Barium chloride ($BaCl_2$; SIGMA)
7. Boric acid (H_3BO_3; RANKEM)
8. Bromphenol blue (AMRESCO)
9. chlorosulfunic acid ($ClSO_3H$; Merck)
10. Dimethyl sulfoxide (DMSO; Merck)
11. Ethanol (C_2H_5OH; Merck)
12. Fetal bovine serum (FBS, Gibco)
13. Formamide ($HCONH_2$; Fluka AG)
14. Gelatin (Fluka)
15. Glucose ($C_6H_{12}O_6$; UNIVAR)
16. Hydrochloric acid (HCl; BAKER)
17. Hydrogen peroxide (H_2O_2; UNIVAR)
18. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, Germany)
19. N, N, N′, N′-Tetramethylethylenediamine ($(CH_3)_2NCH_2CH_2N(CH_3)_2$; SIGMA)
20. Polyacrylamide (Plusone)
21. Potassium sulphate (K_2SO_4; UNIVAR)
22. PT reagent (NEOPLASTINE Cl PLUS)
23. Sodium hydroxide (NaOH; ALPHA, INDIA)
24. Sucrose ($C_{12}H_{22}O_{11}$; UNIVAR)
25. Trichloroacetic acid ($C_2HCl_3O_2$; AnalaR NARMAPUR)

26. Tris ($C_4H_{11}NO_3$; Ameresco)

3.1.3 Chemicals for cell cultures

1. D-MEM (Gibco, USA)
2. Fetal Bovine serum (Gibco, USA)
3. Trypsin/EDTA (Gibco, USA)

3.1.4 Bacteria

1. *Escherichia coli* (TISTR 073 190717)
2. *Serratia marcescens* (TISTR 1354)
3. *Salmonella* sp.
4. *Pseudomonas aeruginosa* (TISTR 1540 150717)
5. *Staphylococcus aureus* (TISTR 2329 18717)
6. *Bacillus cereus* (TISTR 2372 180717)

3.2 Methods

3.2.1 Preparation of pectin sulfate

[1.] Sulfation of pectin with trisulfonated sodium amine ($N(SO_3Na)_3$) (The method was applied from Fan, Jiang, et al. 2011).

Pectin sulfate were performed following this step: in first step, trisulfonated sodium amine was adjusted to suitable pH using sodium hydroxide. Then, 0.1 g pectin was added to the sulfating agent under magnetic stirring for the certain time that sulfation was occurred at the appropriate temperature. After that, the reaction solution was dialyzed for 72 h with distilled water and precipitated with 3 times 95% ethanol overnight. Next, centrifugation at 8000 rpm for 5 min to collect the precipitate. Finally, the pectin sulfate was dried at 37˚C for 2 days.

[2.] Sulfation of pectin with sulfur trioxide pyridine (SO_3.Py) (The method was applied from Yang et al. 2003)

Synthesis of pectin sulfate were performed as follows: first of all, pectin was dissolved in dimethyl sulfoxide (DMSO) at the final concentration 1.0% under magnetic stirring at suitable temperature for 30 min. Next, SO_3.Py complex was added into the solution mixture. The molar ratio of SO_3.Py complex to sugar residues and reaction time were varied. After that, the reaction was cooled at room temperature in an ice water bath and neutralized with 15% NaOH. Then, dialyzed with distilled water for 72 h and precipitated with 3 times 95% ethanol overnight. Next, centrifugation at 8000 rpm for 5 min to collect the precipitate. Finally, the pectin sulfate was dried at 37˚C for 2 days.

[3.] Sulfation of pectin with chlorosulfonic acid

The sulfation of pectin was performed following this step. At first, 0.5 g of pectin was mixed in 10 mL formamide, 10 mL pyridine. Then, certain volume of chlorosulfonic acid (in a proportion of 10 mole of chlorosulfonic acid per mole of free hydroxyl) was dropped to the mixture under magnetic stirring at 0˚C (Maas et al. 2012). The reaction was performed at a suitable temperature for certain time that prefer for reaction occur. After that, the solution was precipitated overnight with 3times of 95% ethanol and centrifuged at 8000 rpm for 5 min to collect precipitate. Then, the precipitate was resuspended in distilled water and dialyzed for 72 h. Next, centrifuged at 8000 rpm for 5 min to collect pectin sulfate. Finally, pectin sulfate was dried at 37˚C for 2 day.

3.2.2 Depolymerization of pectin

[1.] Depolymerization with hydrogen peroxide

Oxidative method involving hydrogen peroxide (Fan et al. 2011) was used to degrade pectin. First of all, 5.0 g pectin was homogenous with 200 mL distilled water at temperature that prefer to reaction occurred. Then, 30% (w/w) H_2O_2 was slowly dropped into the solution at final concentration 1.5%. After degradation for suitable time, 3 times of 95% ethanol were added to precipitate the pectin. After that, the precipitate was centrifuged at 8000 rpm for 5 min. Finally, pectin was dried at 37˚C for 2 day.

[2.] Hydrolyzation with hydrochloric acid

1.0 g pectin was hydrolyzed in 100 mL 0.1 M HCl at suitable temperature for approximately time. Then, the reaction was terminated by adding 0.1 M NaOH in an ice-water bath and precipitated with 3 times 95% ethanol overnight, the precipitate was collected by centrifuged at 8000 rpm for 5 min to collect pectin. Finally, pectin was dried at 37˚C for 2 day.

3.2.3 Chemical structure analyses of pectin sulfate

[1] Measurement of degree of substitution

This research was used the barium sulfate nephelometry method to determine the DS of pectin sulfate (Dodgson and Price 1962). 0.03 g of pectin and its derivatives were dissolved in 10 mL distilled water. Then, 0.25 mL of the test solution, 1.0 mL of distilled water, 0.625 mL glutin-barium chloride solution and 0.350 mL 8% trichloroacetic acid were added into the test tube and stirred for 1 min. After that the reaction was allowed to set for 20 min. The absorbency of barium sulfated was measured with a spectrophotometer at 360 nm. A standard curve was monitoring with different concentration of potassium sulfate instead of the test solution while the other gents in the same conditions given above (water was used as blank test). The DS of the pectin sulfate was determined by comparison with standard curve and calculated following equation (Touzinsky and Gordon 1979).

$$\mathbf{DS} = \frac{\mathbf{MwU\ [S]}}{\mathbf{3200 - MwG\ [S]}}$$

Where DS was the sulfur content (%) of pectin sulfate, Mw U was molecular weight of sugar unit, MwG was molecular weight of substituent group and [S] was concentration of sulfate.

[2] FTIR measurements

IR spectra of samples were evaluated with a Perkin Elmer precisely Spectrum 100 Fourier transform infrared spectrometer. The test specimens were prepared by the KBr-disk method.

[3] ^{13}C-NMR and ^{1}H-NMR spectra of the pectin sulfate

^{13}C-NMR and ^{1}H-NMR spectra were recorded on a NMR spectrometer at an ambient temperature. The samples were dissolved in D_2O. Tetramethylsilane (TMS) was used as internal standard.

3.2.4 Bioactivities analyses

[1] In vitro coagulation assay

Anticoagulation activity of all samples were evaluated by coagulation assay, activated partial thromboplastin time (APTT) and prothrombin time (PT) assay. These assays were performed by semi-automated coagulation analyzer. For APTT assay, 50 µl citrated normal human plasma was mixed with 50 µl sample and incubated at 37˚C for 3 min. Then, 50 µl pre-incubated C.K. Prest was added to the mixture and incubated at 37˚C for 3 min. After that, 50 µl pre-incubated 0.025 mol/L $CaCl_2$ was added and recorded clotting time. For PT assay, 50 µl citrated normal human plasma was mixed with 50 µl sample and incubated at 37˚C for 3 min. Next, 100 µl pre-incubated neoplastine reagent was added to the mixture and recorded clotting time.

[2] Antimicrobial assay

Antimicrobial activity (Minimal inhibitory concentration: MIC) of all samples were investigated by micro broth dilution method using the standard protocol (CLSI M07-A7 for bacteria) against six bacterial strains: *Escherichia coli* (TISTR 073 190717), *Serratia marcescens* (TISTR 1354), *Salmonella* sp., *Pseudomonas aeruginosa* (TISTR 1540 150717), *Staphylococcus aureus* (TISTR 2329 18717) and *Bacillus cereus* (TISTR 2372 180717). Pectin and its derivatives were serially diluted with NB containing 10% glucose and 0.5% phenol red to get the varying concentration and 100 µl of each concentration was placed in the well. The drug-free positive control is also included for comparison. Microtitre plates were incubated for 24 h at 37 °C, the MIC were calculated (Mahon, Lehman, and Manuselis 2014)

[3] Cell culture

Human keratinocyte cells and L929 cells (for cytotoxicity assay) and HeLa (human cervical adenocarcinoma epithelial cell line) cells, HepG2 (human hepatoma cell line) cells were cultured as monolayers in DMEM containing 10% bovine serum and 100 U/mL penicillin/100 µg streptomycin at 5% CO_2 in a 37°C and humidified atmosphere. Adherent cells were detached by trypsinization and then plated onto 10-cm culture dishes, 24- or 96-well plates and used for experiments at ~70–80% confluency.

Human peripheral blood mononuclear cells (PBMCs) were prepared from peripheral blood. Venous blood was collected from normal healthy volunteer by venipuncture, into a heparinized tube (10 unit of heparin per mL blood). The heparinized blood (~10 mL) was diluted at a 1:1 ratio with PBS. The diluted blood was then overlaid with 10 mL of Ficoll Hypaque (specific gravity 1.077 g/liter), and centrifuged at 400 g for 30 min at ambient temperature to separate PBMCs or buffy coat from erythrocytes. PBMCs were collected by aspiration, suspended in PBS and then centrifuged at 400 g for 10 min at 4°C. Purified PBMCs were suspended in RPMI 1640 medium containing 10% heat inactivated fetal

bovine serum (FBS), and enumerated by microscopy. The viable of cells were determined by trypan blue exclusion (Fernandez-Botran and Vetvicka 2000; Freshney 2005).

[4] Cytotoxicity

MTT assay was used to evaluate the cytotoxicity of all samples. PBMC and L929 cells were seeded into 96-well plates at a density of and 3 x 10^5 and 1 × 10^4 cells/well respectively and incubated with serum-free media in the presence of different concentrations of samples and incubated for 24 h. Then, 100 µl of 0.5 mg/mL MTT was added and incubation was continued for another 4 h (The mitochondrial succinate dehydrogenase in live cells converts MTT into visible formazan crystals during incubation). The formazan crystals were then solubilized in dimethylsulphoxide (DMSO) and the absorbance was measured at 540 nm by using an enzyme-linked immunosorbent assay (ELISA) microplate reader. Finally, relative cell cytotoxicity was calculated compared with the absorbance of the untreated control group.

[5] Anticancer assay

Anti-cancer activity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay using HeLa and HepG2 cells lines. Cell suspensions (200 µl) at a density of 1 × 10^4 cells/mL were plated in 96-well microtiter plates and incubated for 24 h. Then, pectin and its derivatives at different concentrations were added to each well and further incubated for 48 h under the same conditions. MTT solution (20 µl of 5 mg/mL in Ca^{2+}, Mg^{2+}-free PBS) was added to each well and incubated for 2 h. Then, an old medium (150 µl) containing MTT was gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a Microplate Reader at 550 nm. And the IC_{50} values were calculated.

For apoptotic cells feature observation, Cells were grown on the 6-cm culture dishes, and then they were treated with different concentrations of pectin and its derivatives for 12 or 24 h. A light microscope was used to study the morphological change of pectin and its derivatives-treated cells.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Hydrolyzation with hydrochloric acid and sulfation with chlorosulfonic acid for Pectin

Pectin at amount of 0.5 g was hydrolyzed with 50 mL of 0.3 M HCl at 100 °C for 1 h. Then, the reaction was terminated by adding 0.1 M NaOH into the reaction flask which was placing on an ice-water bath and subsequently precipitation was carried out using 3-fold volume of cold acetone and left for overnight. The precipitate was collected by centrifugation at 8000 rpm for 5 min in order to collect pectin. Finally, obtained pectin was dried at 37˚C for 2 day.

The sulfation of pectin was performed following these steps. First, 0.5 g of pectin was mixed with 10 mL of formamide and 10 mL of pyridine. Then, 1.0 mL of chlorosulfonic acid was slowly dropped to the mixture under magnetic stirring at 0˚C (Maas et al. 2012). The reaction was conducted at a certain temperature and reaction duration preferencing for reaction occurring. Later, the solution was precipitated with 3-fold volume of cold acetone and left overnight. Thereafter the precipitate was collected by centrifugation at 8000 rpm for 5 min and subsequently resuspended with distilled water and dialyzed for 72 h. Next, pectin sulfate was harvested by centrifugation at 8000 rpm for 5 min. Finally, obtained pectin sulfate was dried at 37˚C for 2 day.

Note: Lyophilization assay was used to collect the precipitated of sulfated hydrolyzed pectin.

Native pectin (Pec), pectin sulfate with chlorosulfonic acid (PecS), pectin sulfate with trisulfonate sodium amine (PecS1) and pectin sulfate with sulfur trioxide pyridine had molecular weight above 90 kDa. PecS was obtained with yield of 79.98±1.78 at degree of substitution (DS) of 1.45±0.06 as demonstrated in Table 4, Figure 7 and Figure 8. Nevertheless, the molecular weight of hydrolyzed pectin (PecH) and depolymerized pectin (PecD) were significantly decreased and less than 50 kDa with retaining its yield of 76.73±0.70 and 51.04±1.53, respectively. There were no observation of substituted sulfate in PecH and PecD similarly to Pec. Lastly, when PecH was

sulfated, the yield was slightly decreased to 60.69±1.94 with DS of 1.27 ±0.04 which was accounted for 20.90% and 12.41% decreases of yield and DS, respectively. The hydrochloric acid was employed to reduce the size of pectin because it gave higher yield than depolymerization with hydrogen peroxide. Figure 9 exhibited mechanism of sulfation reaction which hydroxyl groups of C-2 and C-3 of monosaccharide were substituted by sulfate groups. Chlorosulfonic acid gave the highest DS and coagulation activity. It demonstrated that sulfating agent had substantial effect on DS according to the result shown in table 4 (Vityazev et al. 2010).

In comparison, Fan et al. (Fan, Gao, et al. 2012) obtained pectin sulfate with 23, 20 and 16 kDa with DS values of pectin sulfates of 0.12, 0.21, and 0.37, respectively. Although the molecular weight of PecS and PecHS obtained in this research were higher, the achieved DS were also significantly higher at 3.92 and 3.43 folds compared to their pectin sulfate with highest DS. The DS was suggested to increase with molecular weight as demolished sulfate group may occurred as a result of hydrolysis or degradation of the polysaccharides. There could be resulted from other factors such as pH and temperature of sulfating conditions. The DS of PecS and PecHS were slightly less than the starch sulfates, cellulose sulfates, dextran sulfates, curdlan sulfates and chitin sulfates but significantly higher than chitosan sulfates and pectin sulfates as shown in Table 1.

Table 4. The effect of sulfation and hydrolysis on molecular weight, degree of substitution (DS) and % sulfate content of pectin derivatives.

Pectin derivatives	**Yield (%)**	**Molecular weight kDa**	**Degree of substitution (DS)**
Native pectin	100±0.00	> 90	0.01±0.01
Hydrolyzed pectin	76.73±0.70	< 50	0.00±0.00
Depolymerized pectin	51.04±1.53	< 50	0.00±0.00
Sulfated pectin with trisulfonate sodium amine	83.44±1.05	> 90	0.47±0.08
Sulfated pectin with sulfur trioxide pyridine	70.05±0.97	> 90	0.84±0.11
Sulfated pectin with chlorosulfonic acid	79.98±1.78	> 90	1.45±0.06
Sulfated hydrolyzed pectin with chlorosulfonic acid	60.69±1.94	< 50	1.27±0.04

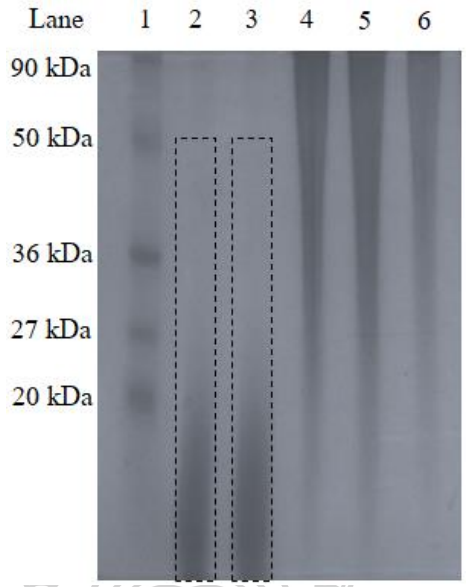

Figure 7. Estimation of molecular weight of PecD, PecH, PecS1, PecS2 and PecS by polyacrylamide gel electrophoresis (PAGE)

Note: Lane (1) marker, (2) PecD, (3) PecH, (4) PecS1, (5) PecS2 and (6) PecS

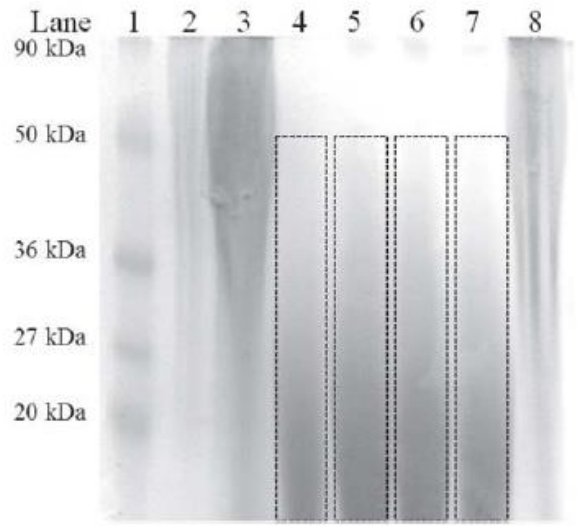

Figure 8. Estimation of molecular weight of Pec, PecH, PecS and PecSH by polyacrylamide gel electrophoresis (PAGE) (Pulsawat and Chayansupap 2014).

Note: Lane (1) marker, (2) Pec, (3 and 8) PecS, (4) PecH, (5) PecH with size exclusion and (6 and 7) PecHS. Lane (4-7) shown in dash line box present the modified pectin with molecular weight lower than 50 kDa.

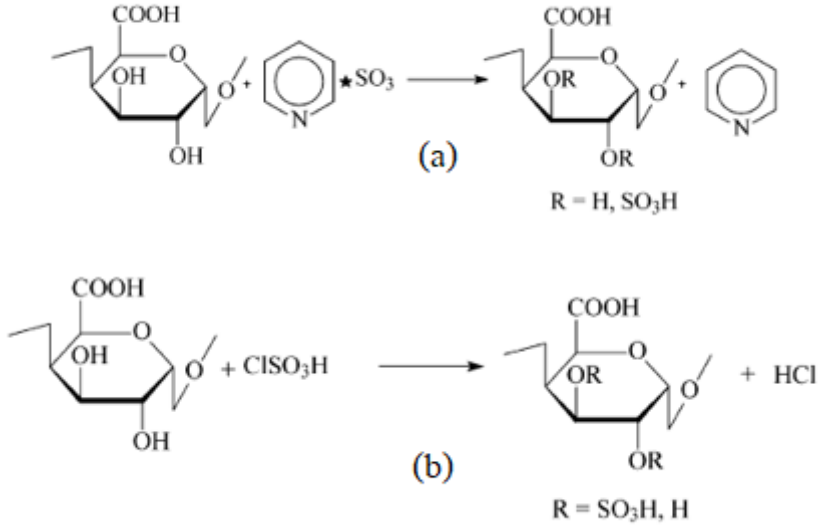

Figure 9. Synthesis reactions of pectin sulfate with (a) sulfur trioxide pyridine and (b) chlorosulfonic acid (Vityazev et al. 2010).

4.2 Structural analyses of Pectin sulfate

4.2.1 FTIR measurements

FTIR spectra of samples were evaluated with a Perkin Elmer precisely Spectrum 100 Fourier transform infrared spectrometer. The test specimens were prepared by the KBr-disk method.

Sulfated pectin (PecS) was synthesized with DS of 1.5. Figure 10 illustrated the FTIR spectra of pectin and PecS. FTIR spectra showed two peaks from PecS, the peak at 830 cm^{-1} was indicating a symmetrical C-O-S vibration associated to a C-O-SO_3 group and the other at 1243 cm^{-1} describing an asymmetrical S=O stretching vibration. While, peaks at 826 and 1251 cm^{-1} from PecHS displayed in Figure 11 (Fan, Gao, et al. 2012). However, these two peaks were absent in FTIR spectra of Pec and PecH, which demonstrated that the pectin sulfates were synthesized successfully. The signal at 3390, 2941, 1746 and 1676 cm^{-1} of pectin denoteed the –OH, –CH, C=O of ester and C=O of acid stretching of galacturonic acid, respectively.

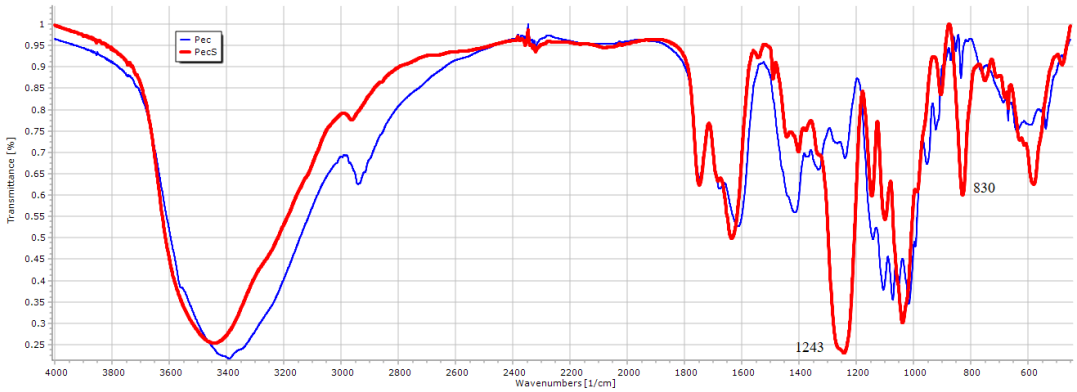

Figure 10. FTIR Spectra of pectin (Pec) and pectin sulfates (PecS).

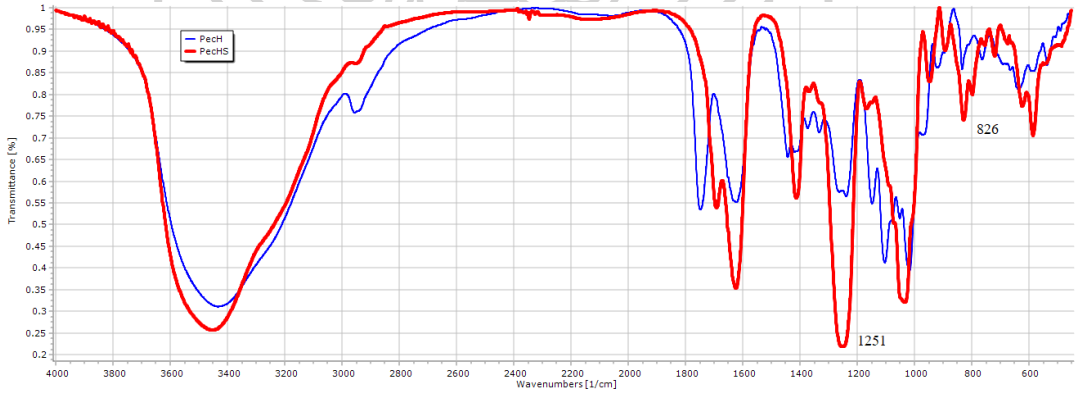

Figure 11. FTIR Spectra of pectin (PecH) and pectin sulfates (PecHS).

4.2.2 ^{13}C-NMR and ^{1}H-NMR analysis of pectin and pectin sulfate

Figure 16 and 17 demonstrated the ^{13}C-NMR spectra of the PecS. The very weak signal at 175.28 and 100.80 ppm were assigned to the C-6 of COOH groups and C-1 of a-D-GalA (Zhang et al. 2015). The signals at 67.90, 73.26, 77.65 and 52.84 ppm were assigned to the C-2, C-3, C-5, and O-CH_3 of pectin sulfate compared to 67.76, 70.48, 77.66, 71.82 and 52.88 ppm which were assigned C-2 to C-5 and O-CH_3 spectra of native pectin, respectively (Table 5). As for ^{1}H spectra of native pectin and PecS were illustrated in Table 5 and Figure 18. There were significantly ^{13}C and ^{1}H spectra shift at C-3 and C-5 which moved the spectra to the high field. This was correspondent to the suggestion of Vityazev et al. (Vityazev et al. 2010) that the presence of sulfate group at the C-3 atom could shift the signal of atom C-5 to a higher field. This can led to suggestion of the postulation that the sulfate group majorly replace at C-3.

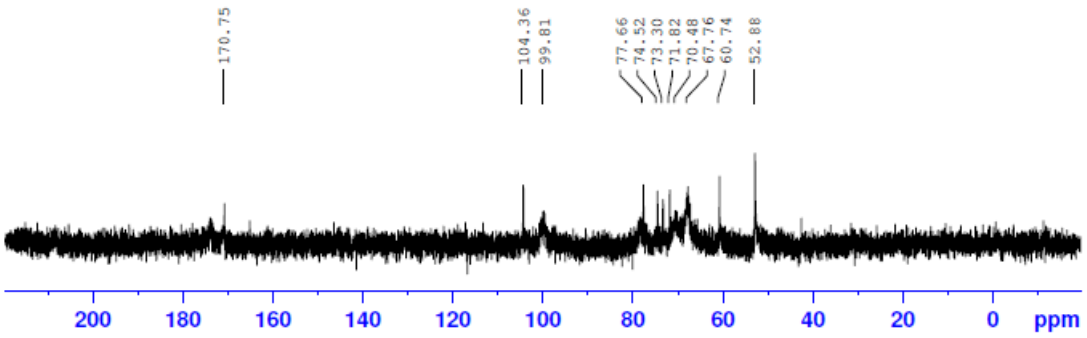

Figure 12. ^{13}C-NMR of pectin (Pec) showing spectra at 220 – 0 ppm

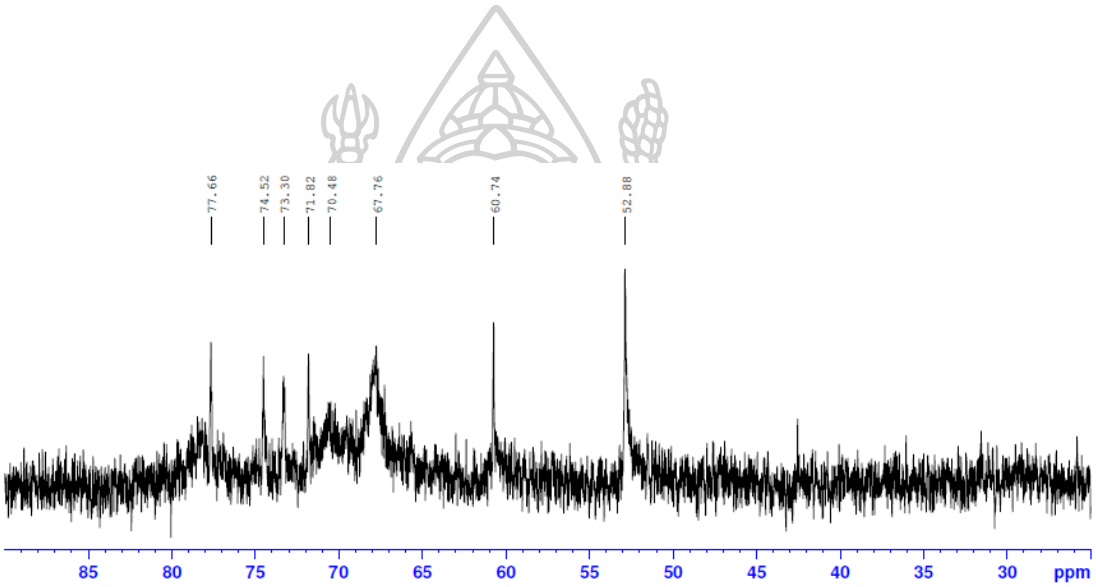

Figure 13. ^{13}C-NMR of pectin (Pec) showing spectra at 90 – 0 ppm

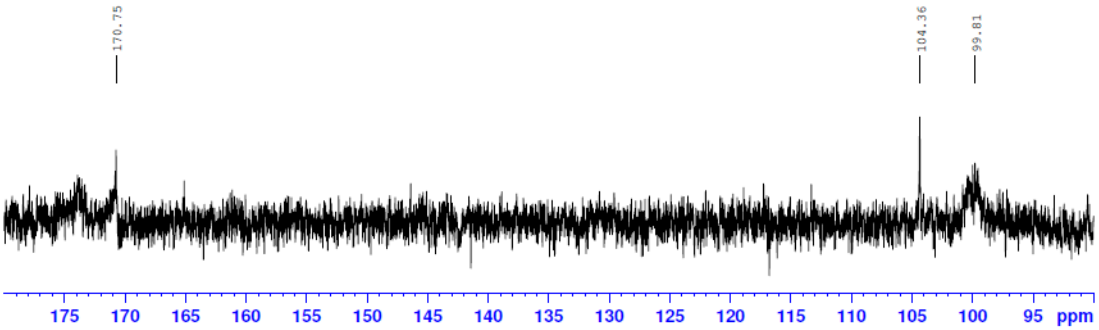

Figure 14. ^{13}C-NMR of pectin sulfate (Pec) showing spectra at 180 – 90 ppm

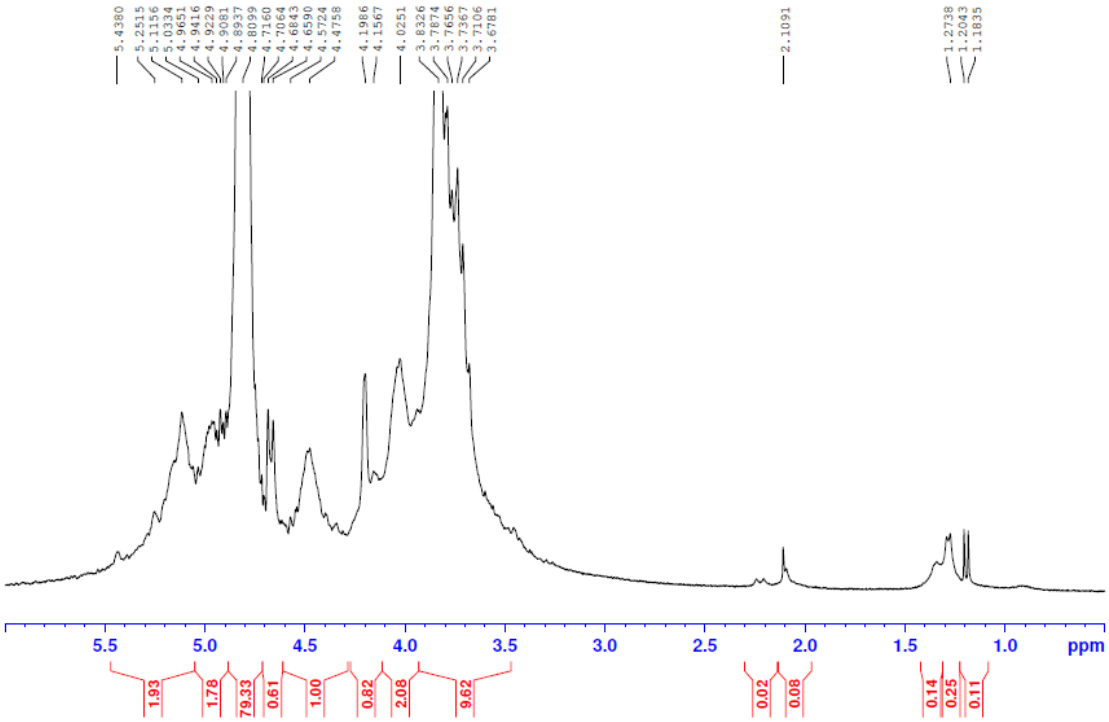

Figure 15. ^{1}H-NMR of pectin (Pec)

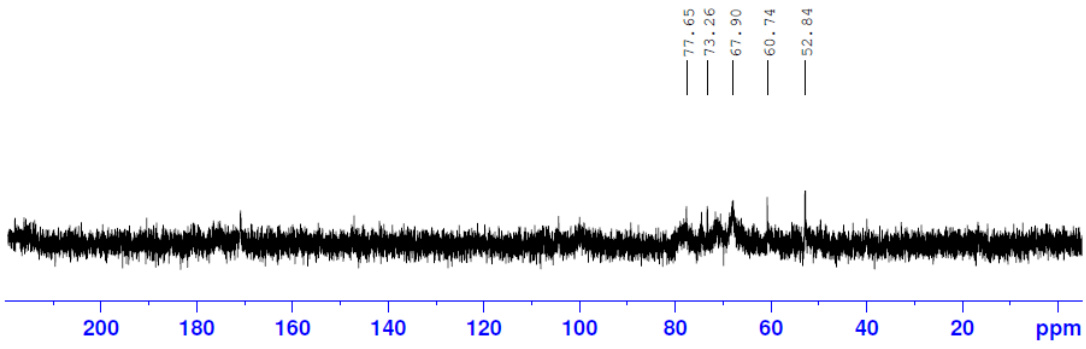

Figure 16. ^{13}C-NMR of pectin sulfate (PecS) showing spectra at 220 – 0 ppm

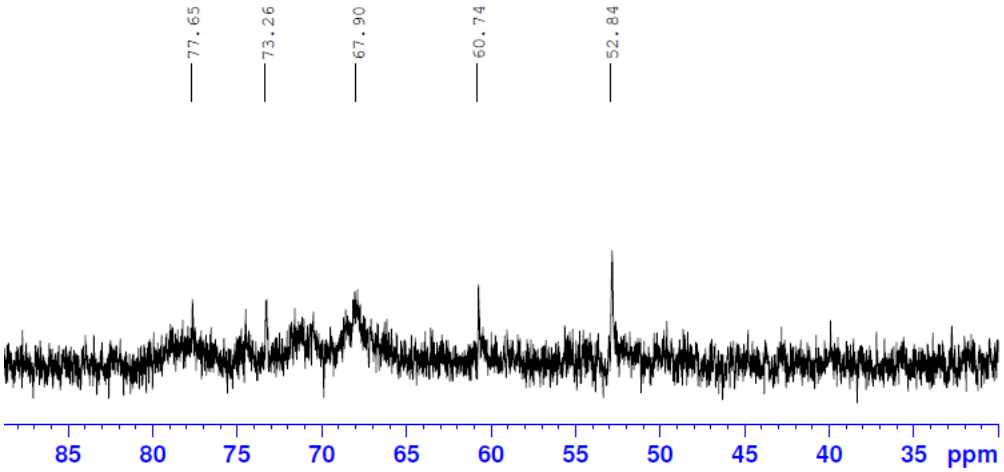

Figure 17. ^{13}C-NMR of pectin sulfate (PecS) showing spectra at 85 – 30 ppm

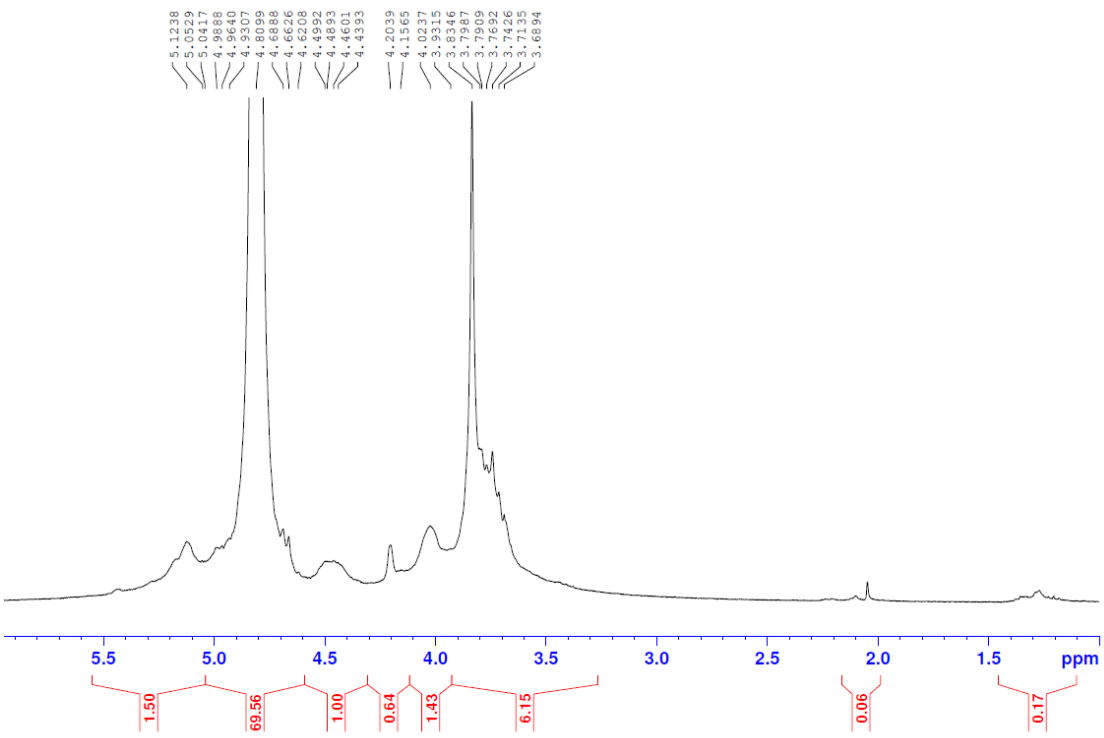

Figure 18. ^{1}H-NMR of pectin sulfate (PecS)

Table 5. The chemical shifts of signals in ^{13}C and ^{1}H NMhR spectra of pectin (Pec) and pectin sulfate (PecS).

^{1}H-NMR	H-1	H-2	H-3	H-4	H-5	H-6	O-CH_3
Pec	5.44	3.68	4.03	4.20	4.68	-	3.74
PecS	5.12	3.69	4.02	4.20	4.50	-	3.74
^{13}C-NMR	C-1	C-2	C-3*	C-4	C-5*	C-6	O-CH_3
Pec	99.81	67.76	70.48	77.66	71.82	170.75	52.88
PecS	n/a	67.90	73.26	n/a	77.65	n/a	52.84

Note: * referred to significant spectra shifted.

n/a referred to weak or no spectra signal can be depicted.

- referred to no available data.

4.3 Bioactivities assays

4.3.1 *In vitro* coagulation assay

Anticoagulation activity of all samples were evaluated by coagulation assay, activated partial thromboplastin time (APTT). These assays were performed by semi-automated coagulation analyzer. For APTT assay, 50 µl citrated normal human plasma was mixed with 50 µl of sample and incubated at 37˚C for 3 min. Then, 50 µl of pre-incubated C.K. Prest was added to the mixture and incubated at 37˚C for 3 min. After that, 50 µl of pre-incubated 0.025 mol/L of $CaCl_2$ was added and recorded clotting time.

The anticoagulant activities of all samples were investigated by partial thromboplastin time (APTT) and prothrombin time (PT) assay. The result showed that the high level of negative charge density distribution along the chain of pectin, which is resulted from the sulfate groups contributing an important role in anticoagulant activity. The higher of DS which increased the negative charge of sulfated pectin, could neutralize the positive charge of amino acid residues of the coagulation factor such as antithrombin, heparin cofactor II, protease nexin I, plasminogen I activator inhibitor, protein C inhibitor, protein Z-dependent protease inhibitor or direct inhibit the

activity or generation of coagulation factor (Fan, Jiang, et al. 2011; Vityazev et al. 2010). At the concentration range of 10-1000 µg/mL, Figure 19 and Table 6 demonstrated the prolongation time of APTT to more than 500s by PecS at concentration of 1000 µg/mL whereas there was unpresented APTT prolongation (approximately 34s of APTT time) of Pec at all examined concentration.

The determination of APTT time of Pec, PecHS and PecS was carried at the concentration range of 5-100 µg/mL, it was found that PecS and PecHS prolonged APTT from 39 s to more than 900 s and from 36 s to 174 s, respectively (Figure 20). PecS and PecHS were more effective than native Pec at the same concentration, while PT of PecS and PecHS were increased with an increase of concentration in the normal range of PT. The result presented in Figure 21. The APTT assay determined the activity in the intrinsic pathway and PT assay determined the activity in the extrinsic pathway. Sulfated polysaccharides inhibited the activity of thrombin and factor Xa in the intrinsic pathway, therefor sulfated pectin could prolong APTT (Fan, Jiang, et al. 2011).

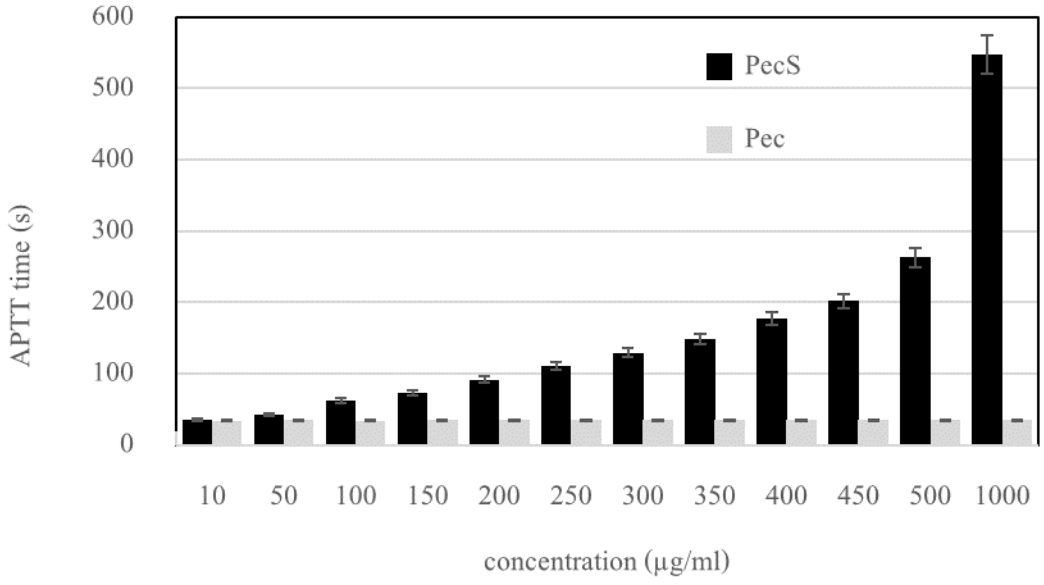

Figure 19. Anticoagulant activities of Pec and PecS with respect to APPT (10-1000 μg/mL)

Table 6. Anticoagulation activity of pectin and sulfated pectin on APTT assay.

Pectin derivatives	Concentration (µg/mL)	APTT time (s)
Pectin	10	34.13 ± 0.25
	50	34.27 ± 0.40
	100	34.13 ± 0.81
	150	34.77 ± 0.61
	200	34.90 ± 0.62
	250	34.87 ± 0.25
	300	35.70 ± 0.36
	350	35.03 ± 0.32
	400	34.50 ± 1.06
	450	34.67 ± 0.32
	500	35.53 ± 0.29
	1000	34.93 ± 0.06
Pectin sulfate	10	35.67 ± 0.71
	50	42.10 ± 0.26
	100	62.50 ± 2.52
	150	73.20 ± 0.87
	200	91.60 ± 0.79
	250	109.90 ± 2.62
	300	128.90 ± 1.90
	350	148.53 ± 0.83
	400	176.80 ± 6.52
	450	201.97 ± 5.42
	500	263.10 ± 3.18
	1000	547.50 ± 2.72

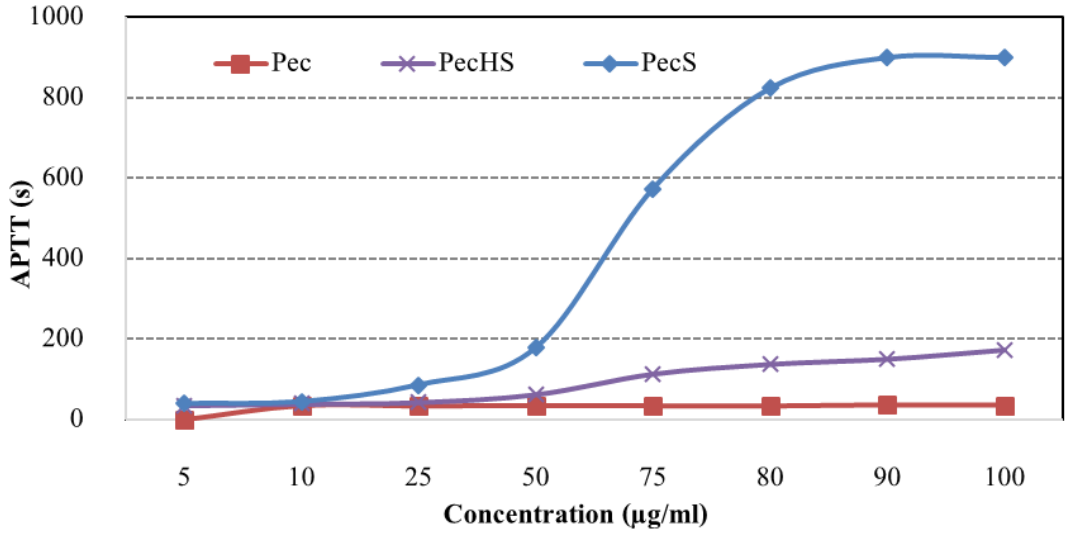

Figure 20. APTT time of Pec, PecS and PecHS

(Normal APTT 22-38s, heparin APTT 174 s at 5 µg/mL).

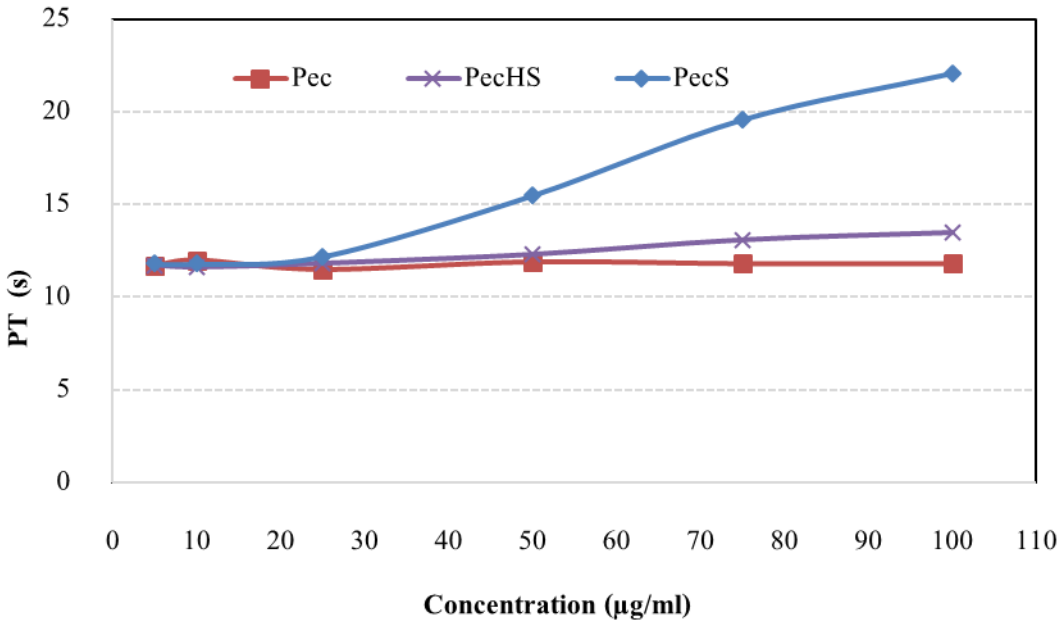

Figure 21. PT time of Pec, PecS and PecHS (Normal PT 10-14s).

4.3.2 Antimicrobial assay

MIC (Minimal inhibitory concentration) of all samples were investigated using the standard protocol against six bacterial strains: Escherichia coli (TISTR 073 190717), Serratia marcescens (TISTR 1354), Salmonella sp., Pseudomonas aeruginosa (TISTR 1540 150717), Staphylococcus aureus (TISTR 2329 18717) and Bacillus cereus (TISTR 2372 180717). Pectin and its derivatives were dissolved with NB containing 10% glucose and 0.5% phenol red to get 10,240 µg/mL. Then, 190 µl of NB was added at the first well, 100 µl at 2-11 and 95 µl at the last. After that 10 µl of sample was dropped in first well, serial dilution to get the varying concentration between 0.5-512 µg/mL. Finally, 5 µl of bacteria (10^6 cells/mL) was added in each well and incubated for 24 h at 37 °C, the MIC were calculated.

Figure 22 exhibited Pec, PecS, PecH and PecHS were not inhibited all of bacteria in this test, suggest that pectin was polysaccharide that find in nature can be a food sources of bacteria.

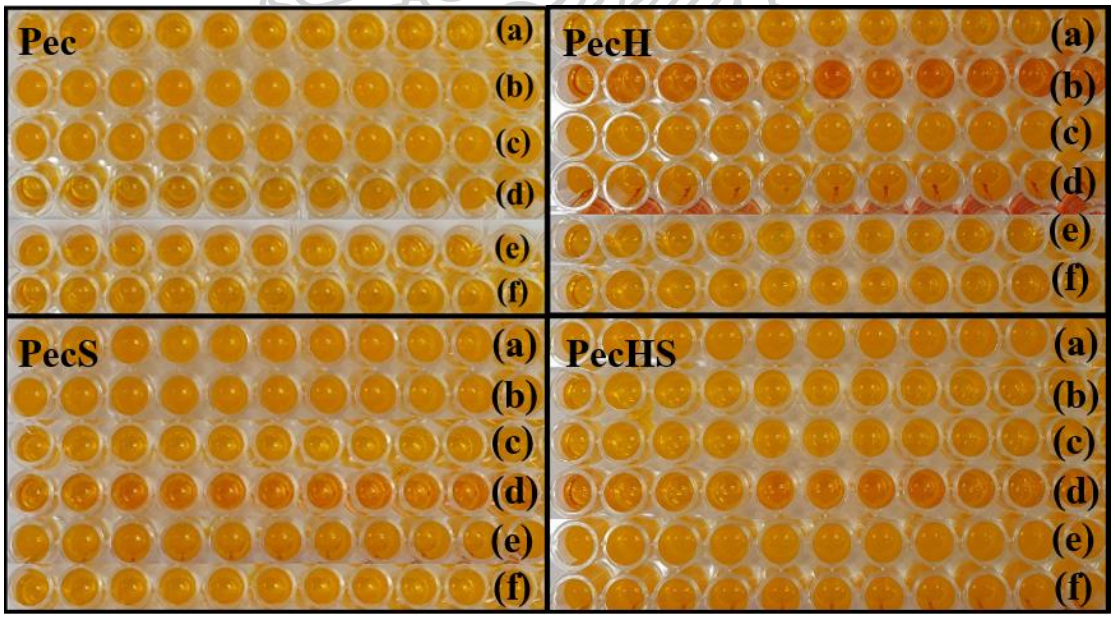

Figure 22. Antimicrobial activity (Minimal inhibitory concentration: MIC) of Pec, PecS, PecH and PecHS investigated by micro broth dilution method using the standard protocol at difference concentration. (a) *E. coli*, (b) *Salmonella* sp., (c) *S. marcescens*, (d) *P. aeruginosa*, (e) *S. aureus* and (f) *B. cereus*

4.3.3 Cytotoxicity assay (L929 cells, MK cells, PBMC, HeLa cells and HepG2 cells)

Cytotoxicity tests for cell culture, including L929 (Mouse connective tissue fibroblast cells), MK (Monkey kidney cells), PBMC (Peripheral)HeLa (Human cervical adenocarcinoma cell line) and HepG2 (human hepatoma cell line) of PecS and PecHS by MTT assay method. This method examines producing of mitochondria dehydrogenase and cofactor resulting in capability in reducing 3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) into crystal purple formazan. When applying MTT solution to examined cell culture, the mitochondria of normal cell will reduce MTT to crystal purple formazan. On the contrary, if the culture cell is malfunction or die, cell will express clear color. DMSO was used to dissolve formazan and the obtained solution was then determined absorbance at 540 nm using spectrophotometer. The absorbance value is correspondent to alive culture cell or inverted to cell death.

Both Pec and PecS were not cause of cell death in all of cells in this study. the half maximal inhibitory concentration (IC_{50}) of Pec for L929, PBMC, MK, HeLa and HepG2 cells were 324,927 µg/mL, 232,925 µg/mL, 16,427 µg/mL, 13,884 µg/mL and 8,389 µg/mL, respectively and IC_{50} of PecS were 925,770 µg/mL, 29,062 µg/mL, 26,746 µg/mL, 10,223 µg/mL and 8,245 µg/mL, respectively. The high of IC_{50} clearly demonstrated that Pec and PecS were not toxic to both of normal cells and cancer cells, the result presented in Figure 23-27.

Pec and PecS concentration of 2000 µg/mL exhibited no effect of L929 cell line after 48 h of the cell cultivation and only after 24 h exposed to Pec and PecS (Figure 28 (c) and 28 (d)). However, when extending of cultivation to 72 h, it was found that the L929 cell line increased more % survival (Figure 28 (e) and 28 (f)). It is worth notice that the cells started proliferation markedly with increased cultivation time after exposed to Pec and PecS. This suggested that Pec and PecS may have a role to play in the process of wound healing. The wound healing process involves a series of cellular events like cell migration, proliferation, and remodeling (Navya and Khora 2017).

On the contrary, when Pec and PecS at 2000 µg/mL were exposed cancer cell line, HeLa, Pec and PecS contributed no sign to cease or caused cell death of HeLa, but effected to cell extension and the changing the morphology of cells as illustrate in Figure 29 (c) and 29 (d). It is

also believed that the anticancer activity of polysaccharide is partly associated with its immunological enhancement effect (Shaoping Nie, Steve Cui 2017).

The similar manner was found with HepG2 that Pec and PecS posed cell extention and contributed cell changing of shape in Figure 30 (d) and 30 (h).

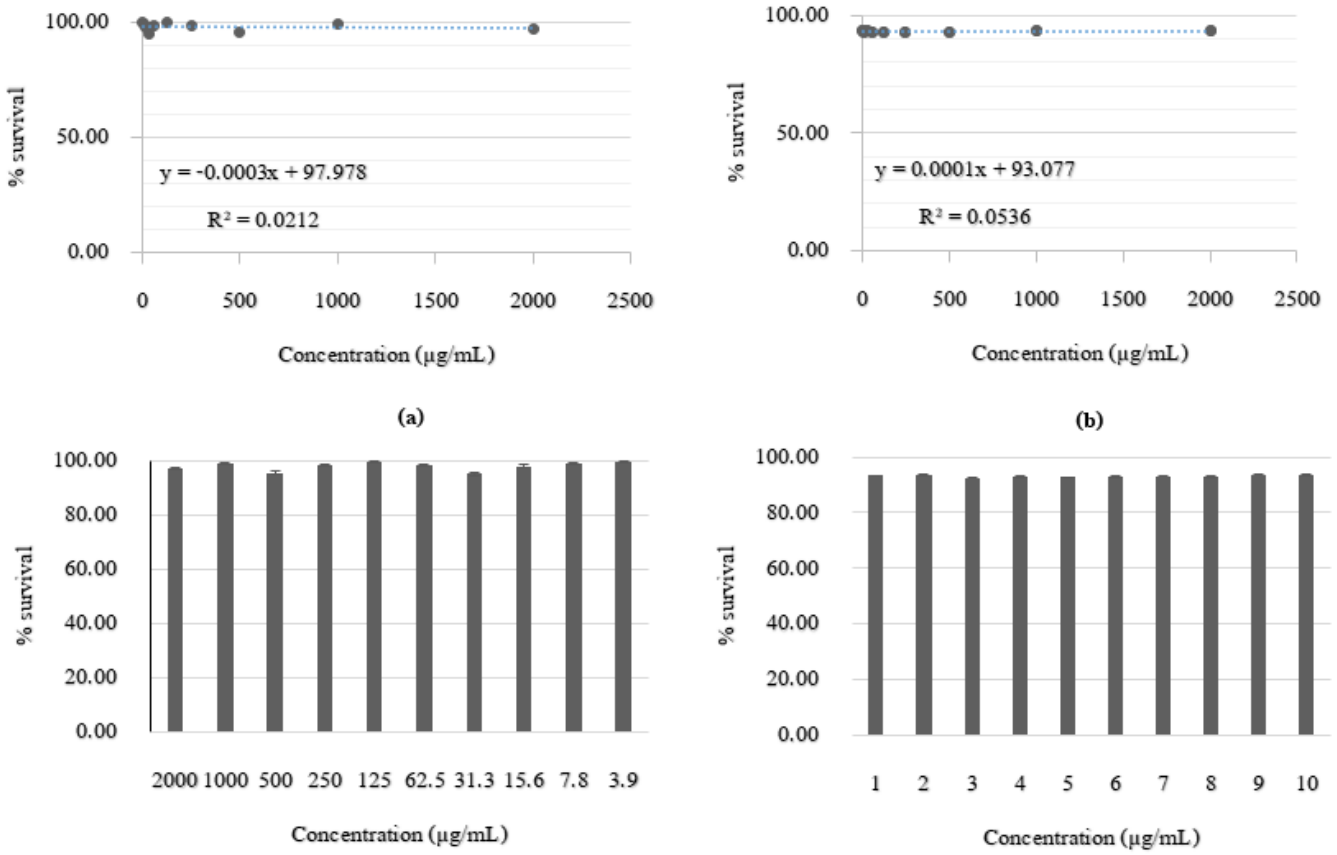

Figure 23. Relationship between the concentration of samples and % survival of L929 cells.

(a) Pec and (b) PecS

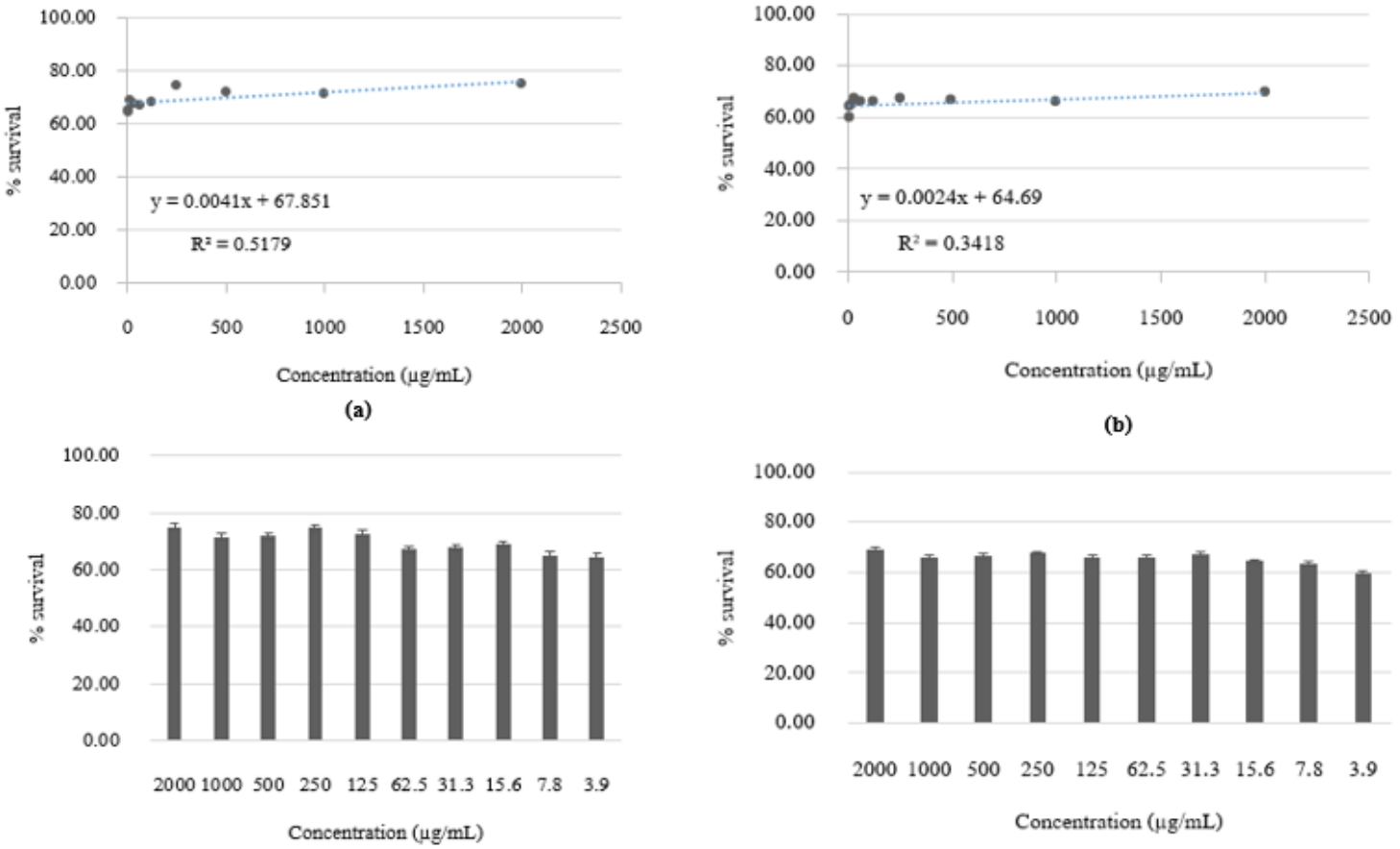

Figure 24. Relationship between the concentration of samples and % survival of MK cells.

(a) Pec and (b) PecS

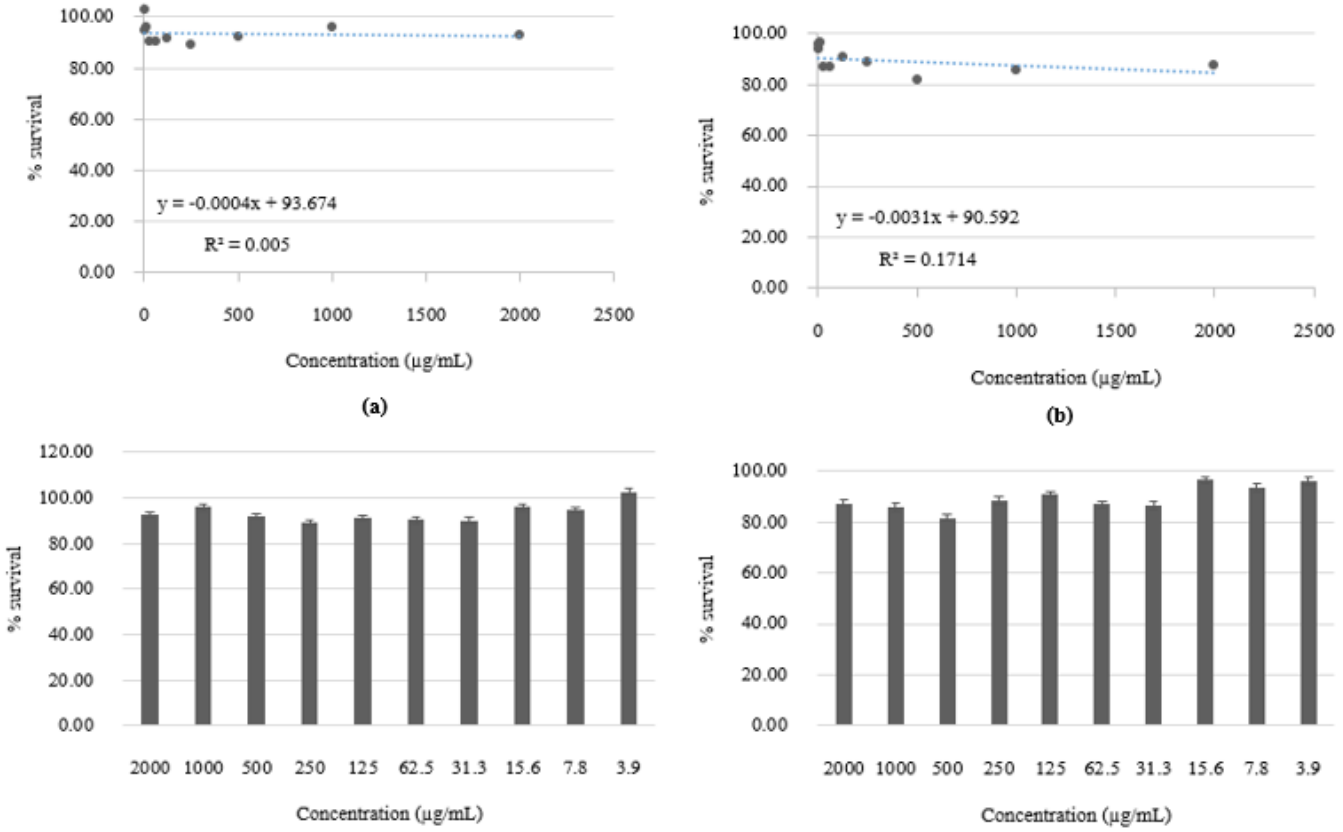

Figure 25. Relationship between the concentration of samples and % survival of PBMC cells.

(a) Pec and (b) PecS

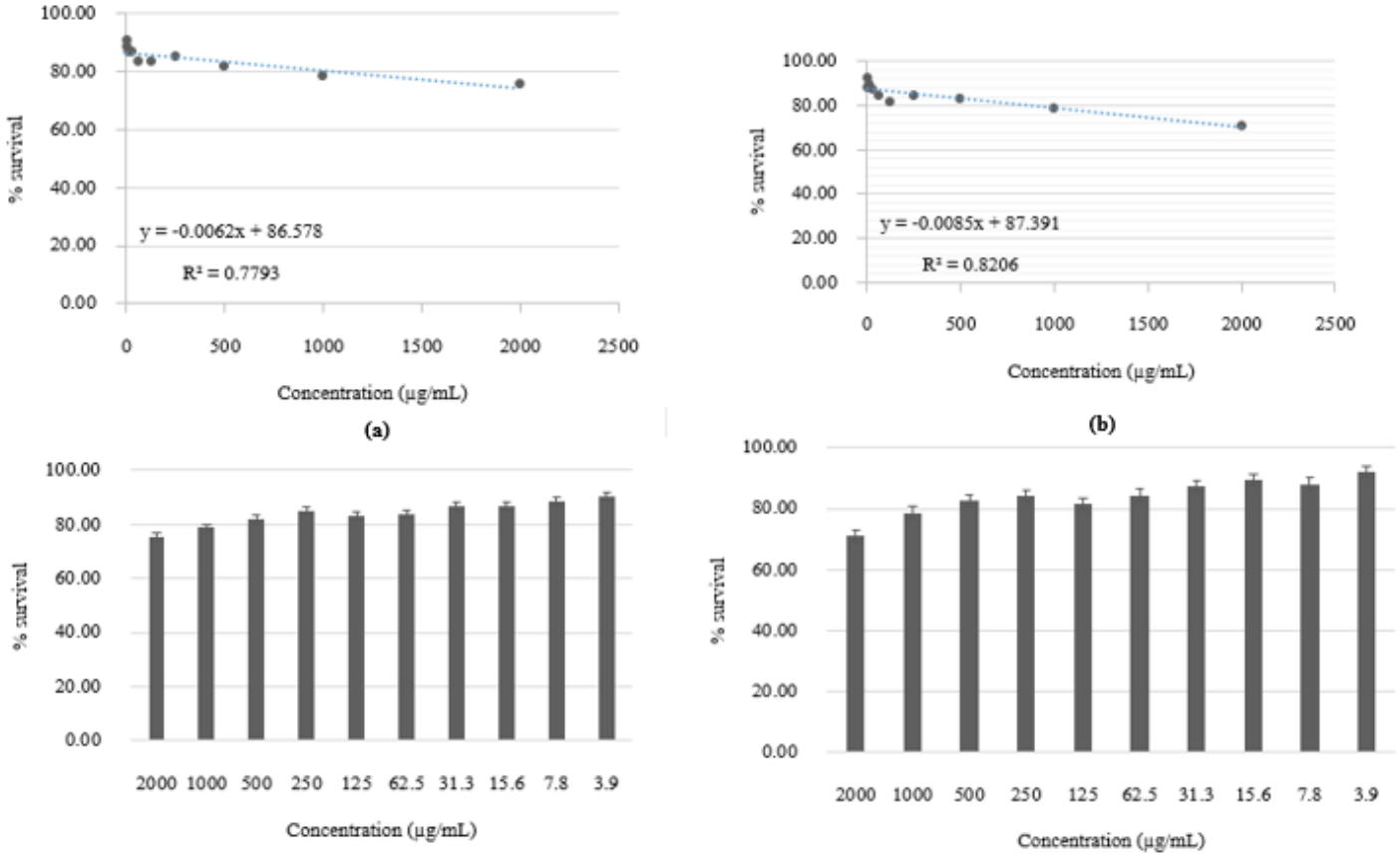

Figure 26.Relationship between the concentration of samples and % survival of HeLa cells.

(a) Pec and (b) PecS

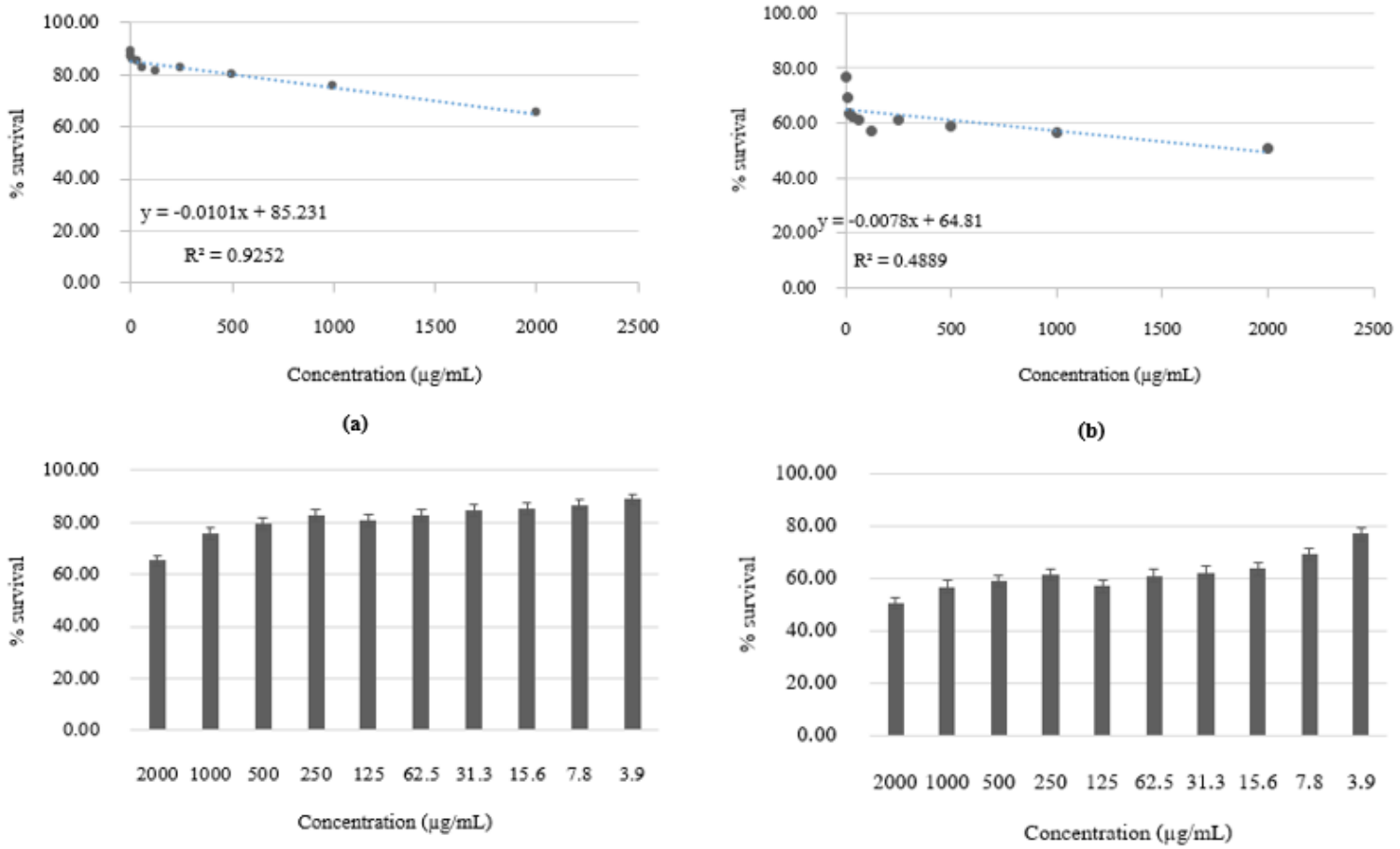

Figure 27. Relationship between the concentration of samples and % survival of HepG2 cells.

(a) Pec and (b) PecS

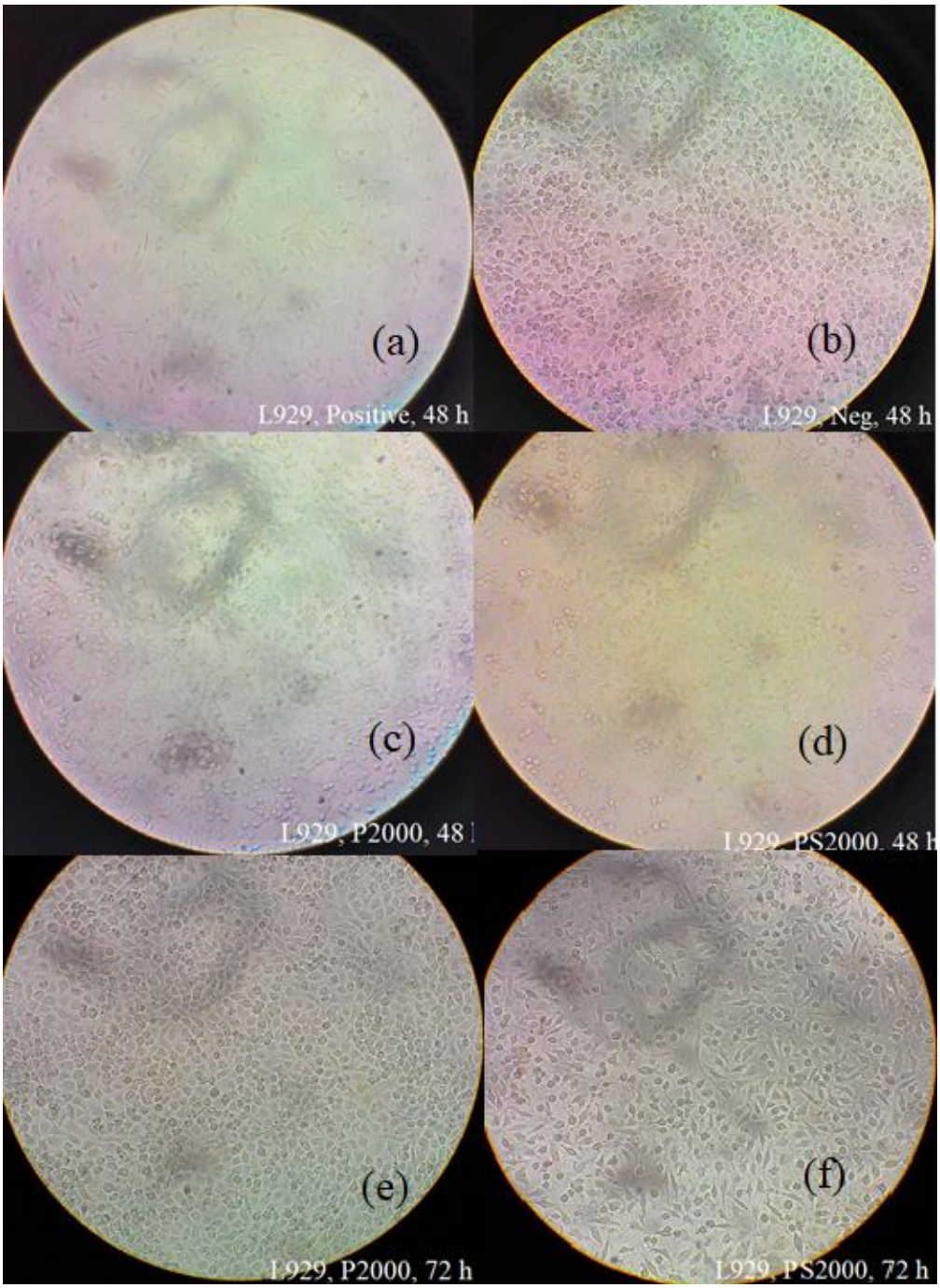

Figure 28. Effect of Pec and PecS at concentration 2000 µg/mL on Cell line L929 after cultivation for 48-72 h. (a) Positive control, (b) Negative control, (c) Pec at conc. 2000 µg/mL at 48 h, (d) PecS at conc. 2000 µg/mL at 48 h., (e) Pec at conc. 2000 µg/mL at 72 h, and (f) PecS at conc. 2000 µg/mL at 72 h.

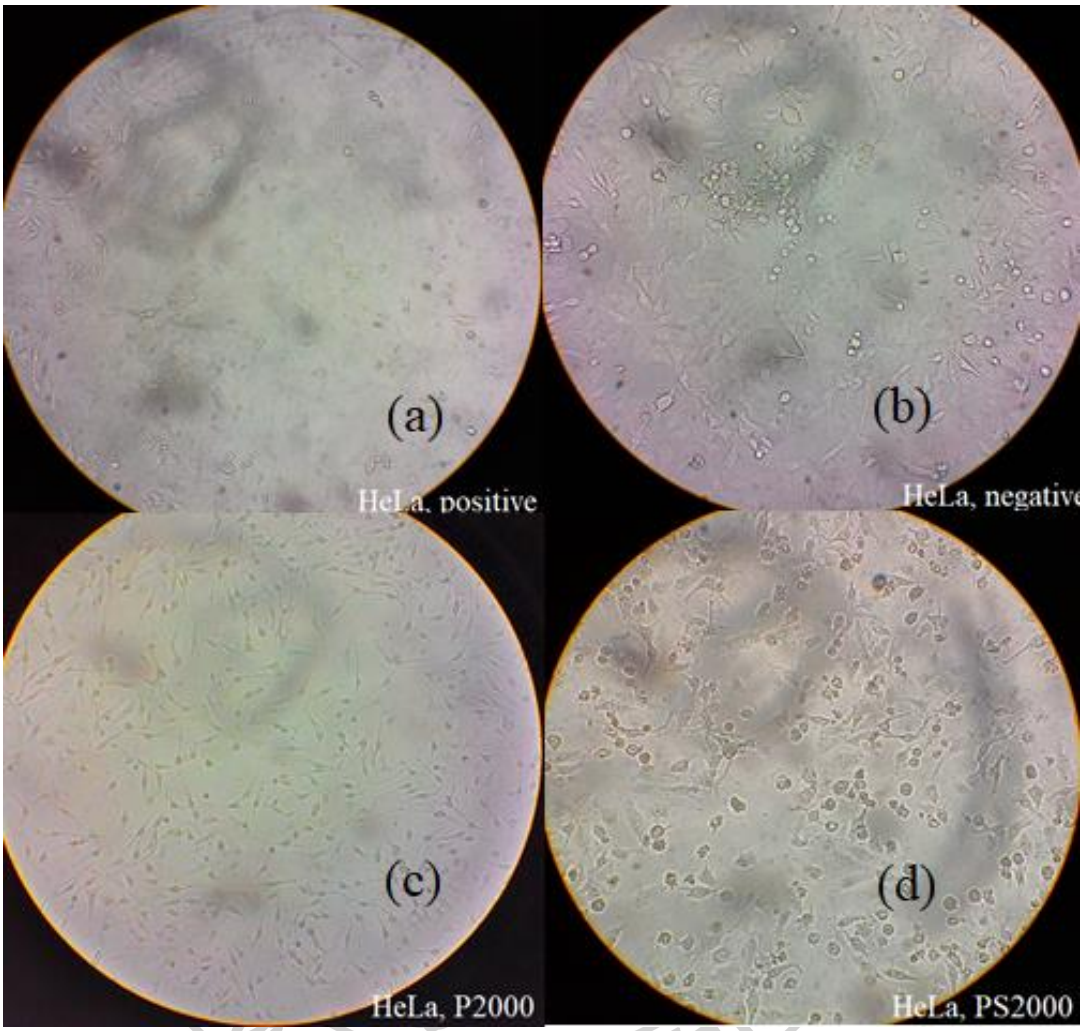

Figure 29. Effect of Pec and PecS at concentration 2000 µg/mL on HeLa cell line after cultivation for 48 h. (a) Positive control, (b) Negative control, (c) Pec at conc. 2000 µg/mL at 48 h, and (d) PecS at conc. 2000 µg/mL at 48 h.

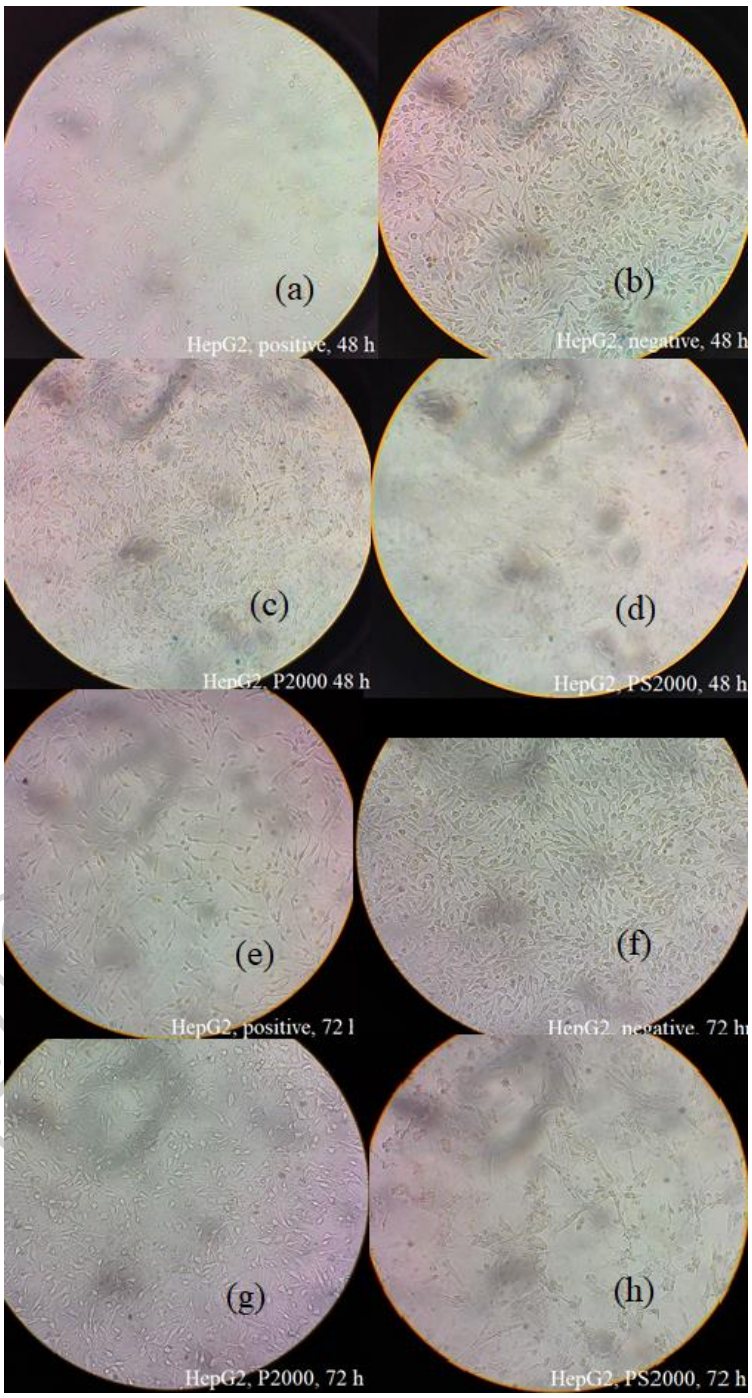

Figure 30. Effect of Pec and PecS at concentration 2000 µg/mL on HepG2 after cultivation for 48-72 h. (a) Positive control, 48 h (b) Negative control, 48 h, (c) Pec at conc. 2000 µg/mL at 48 h, (d) PecS at conc. 2000 µg/mL at 48 h, (e) Positive control, 72 h (f) Negative control, 72 h, (g) Pec at conc. 2000 µg/mL at 72 h., and (h) PecS at conc. 2000 µg/mL at 72 .

4.3.4 Biochemical blood analysis and Serology test

Table 7 and Figure 29 were displayed the ability of PecS to be anticoagulant activity compare to commercially anticoagulant including lithium heparin, clot activator (silica dioxide) and sodium fluoride. PecS in the range of concentration between 10-200 µg/mL no significant difference in result of biochemical blood analysis. Furthermore, the result of serology test showed that PecS was not interrupted the measurement of blood specimens, demonstrated that PecS can be used as anticoagulant without inhibiting of the action of the test substance.

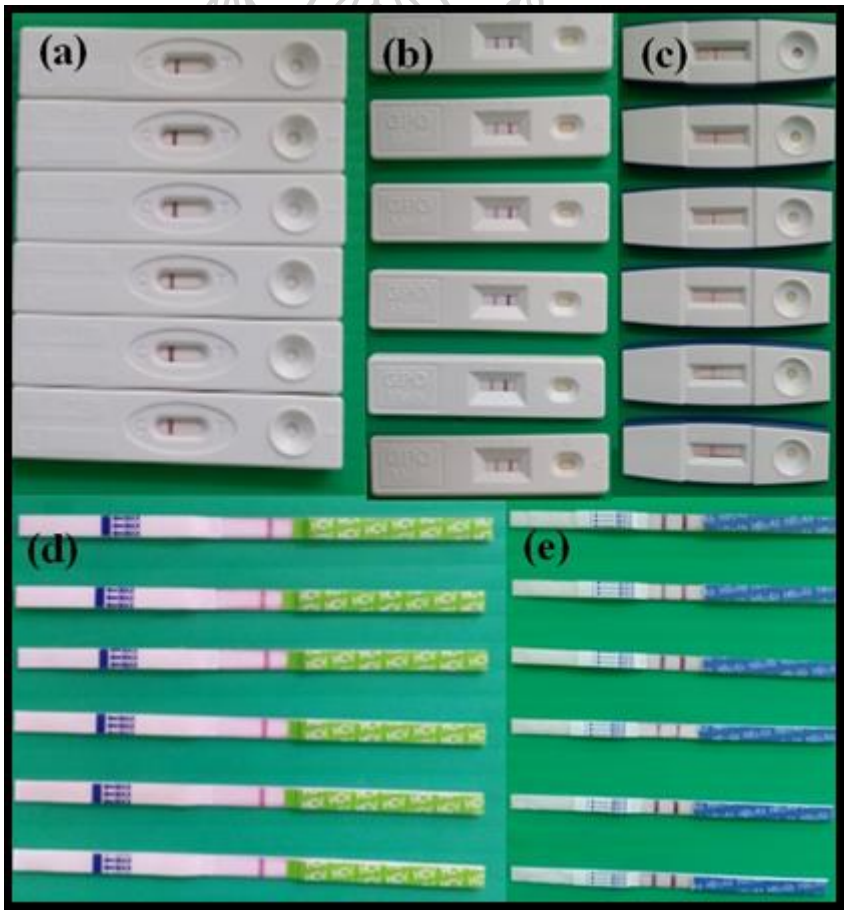

Figure 31. Serology test of PecS at the difference concentrations compere to clot activator. (From the top to the below were control, 10, 50, 100, 200 and 500 µg/mL) (a) HBsAg, (b) methamphetamine, (c) HIV, (d) HCV and (e) HBcAb

Table 7. Anticoagulant activity of PecS in the difference concentrations compare to commercially anticoagulant on biochemical blood analysis.

Test item	units	control	10 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	Reference range
Blood glucose**	mg/dL	92	86	89	90	89	70-110
Blood urea nitrogen*	mg/dL	8.8	8.9	8.9	9	8.8	5-25
Creatinine*	mg/dL	0.6	0.6	0.6	0.6	0.6	0.5-1.5
Uric acid*	mg/dL	4	3.9	3.9	4	3.9	2.3-8.2
Cholesterol*	mg/dL	210	200	200	208	207	0-200
Triglyceride*	mg/dL	51	46	49	49	52	0-200
High-density lipopolysaccharide*	mg/dL	74	72	71	72	69	35-75
Low-density lipopolysaccharide*	mg/dL	118	120	118	120	120	0-130
Albumin*	g/mL	4.3	4.2	4.1	4.3	4.2	3.5-5.2
Globulin*	g/mL	2.5	2.0	1.1	2.3	2.3	1-5
Total bilirubin*	mg/dL	0.4	0.36	0.34	0.34	0.37	0.5-1.5
Direct bilirubin*	mg/dL	0.2	0.18	0.05	0.19	0.21	0-0.5
Glutamic oxaloacetic transaminase*	U/L	15	16	2	15	14	0-37
Glutamic pyruvic transaminase*	U/L	7	5	4	6	6	0-41
Alkaline phosphatase*	U/L	56	56	53	56	54	53-128

Note: ** was referred to use sodium fluoride as anticoagulant.

* was referred to use lithium heparin as anticoagulant.

CONCLUSION

The pectin was submitted to chemical modification specifically sulfation and bioactivities of the sulfated pectin was investigated. Successful replacement of hydroxyl groups of pectin with sulfate groups was confirmed by degree of sulfation (DS) and Fourier fransform infrared spectroscopy (FTIR) spectra of absorption bands at 830 (S=O) and 1242 (C-O-S) cm^{-1} form Pec and peaks at 826 and 1251 cm^{-1} from PecHS.

Sulfated pectin (PecS) with MW less than 90 KDa and Ds of 1.45 and hydrolyzed sulfated pectin (PecHS) with MW less than 50 KDa and DS of 1.27 demonstrated significantly prolonging activated partial thrombosis time (APTT) of 179 and 63.5 seconds, respectively, at a concentration of 50 µg/mL. The PecS elevated prothrombin time (PT) to 54 seconds at a concentration of 250 µg/mL compared to native pectin that had no significant anticoagulant effect. The result indicated the inhibition of intrinsic pathway according to the prolonged APTT. The extrinsic coagulation pathways could be inhibited with PecS at high concentrations according to an increase of prolonged PT. Pectin and its derivative were not inhibited all of bacteria in this study.

Both Pec and PecS were not cause of cell death in all of cells in this study. the half maximal inhibitory concentration (IC_{50}) of Pec for L929, PBMC, MK, HeLa and HepG2 cells were 324,927 µg/mL, 232,925 µg/mL, 16,427 µg/mL, 13,884 µg/mL and 8,389 µg/mL, respectively and IC_{50} of PecS were 925,770 µg/mL, 29,062 µg/mL, 26,746 µg/mL, 10,223 µg/mL and 8,245 µg/mL, respectively. The high of IC_{50} clearly demonstrated that Pec and PecS were not toxic to both of normal cells and cancer cells, but effected to cell extension and the changing the morphology of cells for HeLa and HepG2 cells.

PecS to be anticoagulant activity compare to commercially anticoagulant in the range of concentration between 10-200 µg/mL no significant difference in result of biochemical blood analysis. Furthermore, the result of serology test showed that PecS was not interrupted the

measurement of blood specimens, demonstrated that PecS can be used as anticoagulant without inhibiting of the action of the test substance.

This research suggestes potential anticancer application and in vitro use for substitution of traditional anticoagulant using for biochemical analysis in blood. In addition, at high concentration, PecS prone to promote cell proliferation. Nevertheless, this requires to determine mechanisms occurring in mentioned cell processes.

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APPENDIX A

Cell culture media

1. **Media for cell line culture**

D-MEM (Gibco)	10.0	g
10% Fetal bovine serum	100.0	mL
Pennicillin	100.0	unit/mL
Streptomycin	100.0	µg/mL
10% $NaHCO_3$	1.5	mL

Add D-MEM power into water to make 90% (w/v), mix throroughly and then adjust pH using 1M HCl and 1M NaOH to contribute of pH 7.2 ± 0.02. Then add all other chemicals into the solution. Adjust the volume to be 1000 mL. After than the solution was filtered through membrane unit filter with pore size 0.22 µ. Separate solution in the containers and store at 4°C. until use.

APPENDIX B

Chemical preparation

1. 3 M hydrochloric acid (HCl) solution

Measure 26.29 mL of HCl 35% w/w and slowly and gradually add into 73.70 mL of water. Mix until homogenous solution was obtained.

2. 8% (w/v) sodium hydrogen carbonates ($NaHCO_3$) solution

Preparation was carried out by weight 8 g of $NaHCO_3$ and dissolved in 100 mL of distilled water.

3. 1M Sodium hydroxide solution

Weight 4 g of NaOH 4 and then dissolved in 100 mL of distilled water.

4. Preparation of chemical using in sulfate analysis

4.1 gelatin-barium chloride solution

Weight 0.5 g of barium chloride and 0.5 g of gelatin 0.5, then dissolve them both in 100 mL of distilled water.

4.2. 8% (w/v) trichloroacetic acid solution

Weight 8 g of trichloroacetic acid and then dissolve it in distilled water and finally adjust the volume to be 100 mL.

5. Preparation of chemicals in molecular weight analysis

5.1 1.5 M Tris - hydrochloric acid solution with pH to 8.8

Weight 18.17 g of Tris base and then dissolve in 80 mL of distilled water. Then add concentrated hydrochloric acid to adjust pH at 8.8 and finally adjust volume to be 100 mL.

5.2 0.58 M Tris - hydrochloric acid pH 6.8

Weight 7.02 g of Tris base and then dissolve in 80 mL of distilled water. Then add concentrated hydrochloric acid to adjust pH at 6.8 and finally adjust volume to be 100 mL.

5.3 10% (w/v) ammonium persulfate

Weight 10 g of ammonium persulfate and then dissolve in 100 mL of distilled water.

5.4 loading dry

Dissolve sucrose in 1X TBE buffer to make 2 M concentration and then dissolve bromphenol blue into the solution to acieve the concentation of 0.2% (w/v).

5.5 Staining (Alcain blue)

Weight 0.5 g of Alcine blue and then dissolve in 100 mL of 2% (v/v) acetic acid.

6. Griess's reagent

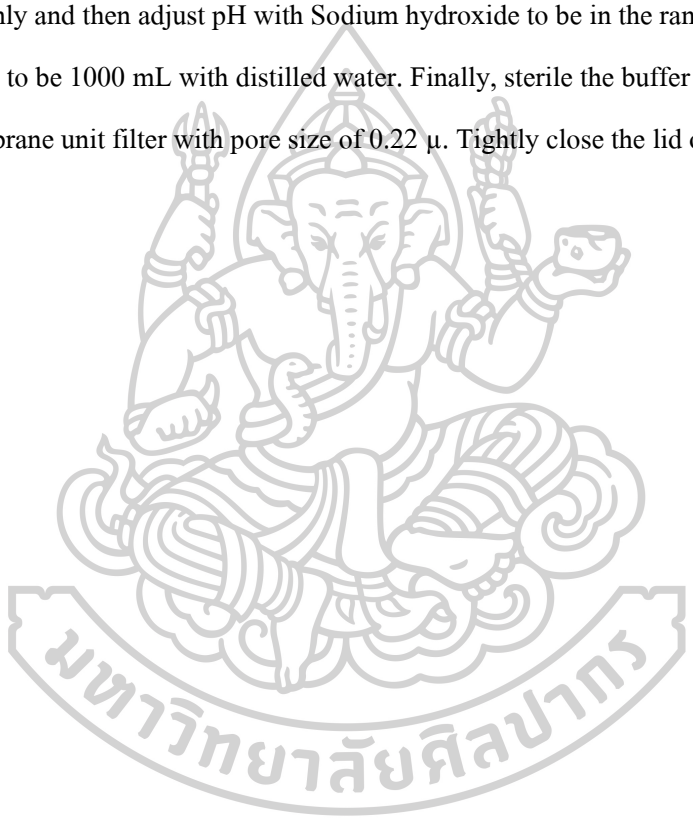
NED	0.01	g
Sulfanilamide	0.1	g
O-Phosphoric acid	0.5	mL
H_2O	9.5	mL

7. Hank' s Balanced Salt Solution (HBSS)

Sodium chloride	8.0	g
Potassium chloride	0.4	g
Potassium dihydrogen phosphate	0.06	g

Disodium hydrogen phosphate	1.44	g
D-Glucose	1.0	g
Sodium bicarbonate	0.35	g
H_2O	900	mL

Weight all composition of solution and then add 900 mL of distilled water into them. Mix throroughly and then adjust pH with Sodium hydroxide to be in the range of 7.12-7.2. Next, adjust volume to be 1000 mL with distilled water. Finally, sterile the buffer solution by filtering through membrane unit filter with pore size of 0.22 μ. Tightly close the lid of the container.



APPENDIX C

Standard curve and analysis methods

1. Sulfate content analysis with barium sulfate nephelometry method (Fan et al., 2011: 1797-1803)

Standard solution preparation

1.1 Preparation of standard solution at ranged concentration of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/L at volume of 250 mL in test tube.

1.2 Add 1 mL of distilled water into the test tube.

1.3 Add 625 µl of gelatin-barium chloride solution into the test tube.

1.4 Add 350 µl of 3 % (v/v) trichloroacetic acid into the test tube.

1.5 Carefully shake the test tube for 1 min and leave for the reaction to completely occure for 30 mins.

1.6 Measure the absorbance at wavelength of 360 nm.

Table 8. Relationship between sulfate content and absorbance at wavelength 360 nm

Concentration of sulfate group (µg/mL)	**OD 360 nm**
0.0	0.000
0.1	0.227
0.2	0.434
0.3	0.598
0.4	0.724
0.5	0.876

2. Standard graph of sulfate analysis

Standard graph obtained is linear equation where y = 0.5381x is concentration of sulfate (mg/mL) and x is absorbance at 360 nm (OD_{360}).

Sulfate content (mg/mL) = OD_{360} 0.5381x

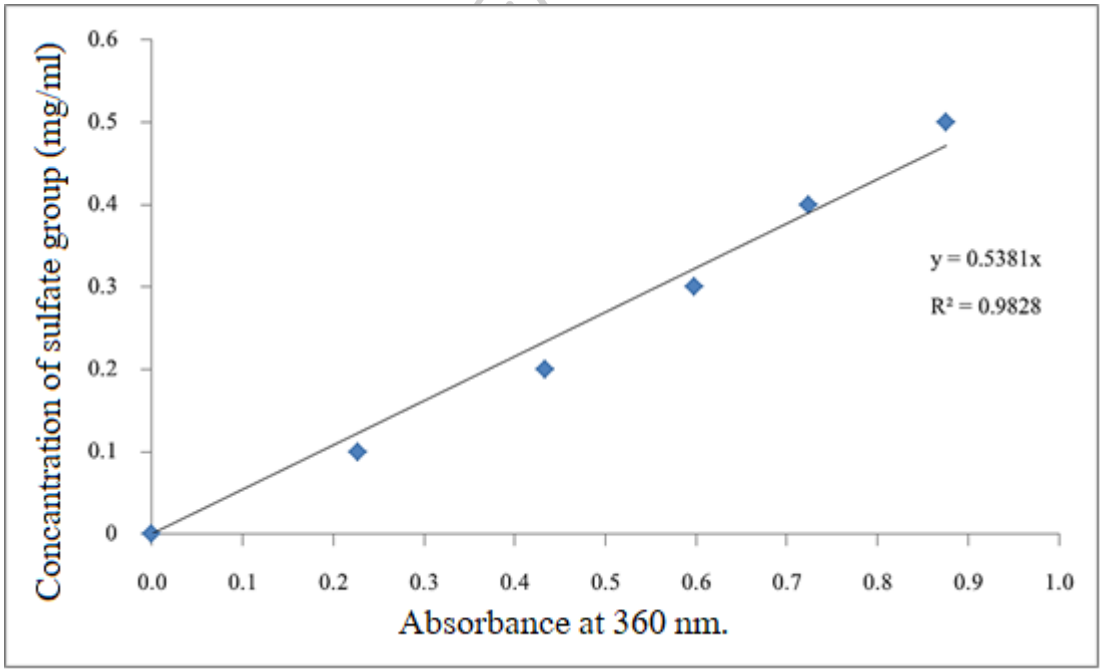


Figure 32. Standard graph for sulfate analysis by measuring optical density at wavelength 360 nm

APPENDIX D

Table 9. Minimal inhibitory concentration: MIC of Ampicillin and Chloramphenical.

Atibiotics	**Bacterias**	**Concentration (µg/mL)**										
		512	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**
Ampicillin	*E.coli*	+	+	+	+	+	+	+	+	+	+	+
	S.marcescens	-	-	-	-	-	-	-	-	-	-	-
	P.aeruginosa	-	-	-	-	-	-	-	-	-	-	-
	Salmonella sp.	-	-	-	-	-	-	-	-	-	-	+
	B.cereus	-	-	-	-	-	-	-	-	-	-	-
	S.aurese	-	-	-	-	-	-	-	-	-	-	-
Chloramphenical	*E.coli*	+	+	+	+	+	+	+	+	+	+	+
	S.marcescens	+	+	+	+	+	+	+	+	+	+	+
	P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+
	Salmonella sp.	+	+	+	+	+	+	+	+	+	+	+
	B.cereus	-	-	-	-	-	-	-	-	-	-	+
	S.aurese	-	-	-	-	-	-	-	-	+	+	+

Table 10. Minimal inhibitory concentration: MIC of Native pectin and Pectin sulfate.

Atibiotics	**Bacterias**	**Concentration (µg/mL)**										
		512	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**
Native pectin	*E.coli*	+	+	+	+	+	+	+	+	+	+	+
	S.marcescens	+	+	+	+	+	+	+	+	+	+	+
	P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+
	Salmonella sp.	+	+	+	+	+	+	+	+	+	+	+
	B.cereus	+	+	+	+	+	+	+	+	+	+	+
	S.aurese	+	+	+	+	+	+	+	+	+	+	+
Pectin sulfate	*E.coli*	+	+	+	+	+	+	+	+	+	+	+
	S.marcescens	+	+	+	+	+	+	+	+	+	+	+
	P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+
	Salmonella sp.	+	+	+	+	+	+	+	+	+	+	+
	B.cereus	+	+	+	+	+	+	+	+	+	+	+
	S.aurese	+	+	+	+	+	+	+	+	+	+	+

Table 11. Minimal inhibitory concentration: MIC of Hydrolyzed pectin and Sulfated hydrolyzed pectin.

Atibiotics	**Bacterias**	**Concentration (µg/mL)**										
		512	**256**	**128**	**64**	**32**	**16**	**8**	**4**	**2**	**1**	**0.5**
Hydrolyzed pectin	*E.coli*	+	+	+	+	+	+	+	+	+	+	+
	S.marcescens	+	+	+	+	+	+	+	+	+	+	+
	P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+
	Salmonella sp.	+	+	+	+	+	+	+	+	+	+	+
	B.cereus	+	+	+	+	+	+	+	+	+	+	+
	S.aurese	+	+	+	+	+	+	+	+	+	+	+
Sulfated hydrolyzed pectin	*E.coli*	+	+	+	+	+	+	+	+	+	+	+
	S.marcescens	+	+	+	+	+	+	+	+	+	+	+
	P.aeruginosa	+	+	+	+	+	+	+	+	+	+	+
	Salmonella sp.	+	+	+	+	+	+	+	+	+	+	+
	B.cereus	+	+	+	+	+	+	+	+	+	+	+
	S.aurese	+	+	+	+	+	+	+	+	+	+	+

APPENDIX E

Table 12. The absorbance at wavelength 540 nm of MTT assay (L929 cells).

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	0.332	0.332	0.331	0.329	0.326	0.324	0.325	0.327	0.335	0.338
	2	0.31	0.32	0.29	0.324	0.341	0.333	0.299	0.321	0.321	0.324
	3	0.343	0.351	0.347	0.342	0.341	0.340	0.340	0.344	0.345	0.346
Pectin sulfate	**1**	0.306	0.308	0.308	0.308	0.308	0.306	0.309	0.308	0.311	0.308
	2	0.326	0.325	0.319	0.321	0.322	0.322	0.321	0.322	0.326	0.323
	3	0.315	0.316	0.311	0.314	0.310	0.314	0.314	0.312	0.313	0.318

Table 13. % survival of L929 cells.

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	98.22	98.22	97.93	97.34	96.45	95.86	96.15	96.75	99.11	100.00
	2	91.72	94.67	85.80	95.86	100.89	98.52	88.46	94.97	94.97	95.86
	3	101.48	103.85	102.66	101.18	100.89	100.59	100.59	101.78	102.07	102.37
	Aver.	97.14	98.92	95.46	98.13	99.41	98.32	95.07	97.83	98.72	99.41
	SD	4.97	4.62	8.70	2.75	2.56	2.37	6.14	3.53	3.57	3.29
Pectin sulfate	**1**	90.53	91.12	91.12	91.12	91.12	90.53	91.42	91.12	92.01	91.12
	2	96.45	96.15	94.38	94.97	95.27	95.27	94.97	95.27	96.45	95.56
	3	93.20	93.49	92.01	92.90	91.72	92.90	92.90	92.31	92.60	94.08
	Aver.	93.39	93.59	92.50	93.00	92.70	92.90	93.10	92.90	93.69	93.59
	SD	2.96	2.52	1.68	1.92	2.24	2.37	1.78	2.13	2.41	2.26

Table 14. The absorbance at wavelength 540 nm of MTT assay (MK cells).

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	0.258	0.229	0.227	0.233	0.205	0.221	0.215	0.223	0.201	0.191
	2	0.251	0.249	0.245	0.263	0.256	0.229	0.242	0.240	0.223	0.241
	3	0.252	0.248	0.257	0.261	0.230	0.231	0.230	0.235	0.237	0.223
Pectin sulfate	**1**	0.234	0.214	0.226	0.226	0.227	0.227	0.217	0.215	0.217	0.212
	2	0.233	0.224	0.221	0.220	0.208	0.211	0.222	0.205	0.203	0.190
	3	0.237	0.230	0.230	0.239	0.232	0.233	0.241	0.233	0.225	0.204

Table 15. % survival of MK cells.

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	76.33	67.75	67.16	68.93	60.65	65.38	63.61	65.98	59.47	56.51
	2	74.26	73.67	72.49	77.81	75.74	67.75	71.60	71.01	65.98	71.30
	3	74.56	73.37	76.04	77.22	68.05	68.34	68.05	69.53	70.12	65.98
	Aver.	75.05	71.60	71.89	74.65	68.15	67.16	67.75	68.84	65.19	64.60
	SD	1.12	3.33	4.47	4.96	7.54	1.57	4.00	2.58	5.37	7.49
Pectin sulfate	**1**	69.23	63.31	66.86	66.86	67.16	67.16	64.20	63.61	64.20	62.72
	2	68.93	66.27	65.38	65.09	61.54	62.43	65.68	60.65	60.06	56.21
	3	70.12	68.05	68.05	70.71	68.64	68.93	71.30	68.93	66.57	60.36
	Aver.	69.43	65.88	66.77	67.55	65.78	66.17	67.06	64.40	63.61	59.76
	SD	0.62	2.39	1.33	2.87	3.75	3.36	3.75	4.20	3.29	3.29

Table 16. The absorbance at wavelength 540 nm of MTT assay (PBMC cells).

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	0.114	0.126	0.116	0.121	0.124	0.122	0.115	0.130	0.115	0.138
	2	0.115	0.111	0.108	0.099	0.112	0.100	0.113	0.114	0.117	0.112
	3	0.110	0.114	0.113	0.106	0.098	0.109	0.102	0.107	0.115	0.126
Pectin sulfate	**1**	0.105	0.103	0.095	0.097	0.100	0.105	0.101	0.113	0.112	0.114
	2	0.108	0.121	0.111	0.120	0.117	0.108	0.115	0.125	0.126	0.128
	3	0.107	0.090	0.093	0.107	0.115	0.105	0.101	0.115	0.105	0.109

Table 17. % survival of PBMC.

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	93.44	103.28	95.08	99.18	101.64	100.00	94.26	106.56	94.26	113.11
	2	94.26	90.98	88.52	81.15	91.80	81.97	92.62	93.44	95.90	91.80
	3	90.16	93.44	92.62	86.89	80.33	89.34	83.61	87.70	94.26	103.28
	Aver.	92.62	95.90	92.08	89.07	91.26	90.44	90.16	95.90	94.81	102.73
	SD	2.17	6.51	3.31	9.21	10.67	9.07	5.74	9.66	0.95	10.67
Pectin sulfate	**1**	86.07	84.43	77.87	79.51	81.97	86.07	82.79	92.62	91.80	93.44
	2	88.52	99.18	90.98	98.36	95.90	88.52	94.26	102.46	103.28	104.92
	3	87.70	73.77	76.23	87.70	94.26	86.07	82.79	94.26	86.07	89.34
	Aver.	87.43	85.79	81.69	88.52	90.71	86.89	86.61	96.45	93.72	95.90
	SD	1.25	12.76	8.09	9.45	7.62	1.42	6.63	5.27	8.76	8.07

Table 18. The absorbance at wavelength 540 nm of MTT assay (HeLa cells).

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	0.232	0.281	0.286	0.297	0.303	0.296	0.300	0.297	0.310	0.317
	2	0.218	0.216	0.222	0.236	0.229	0.228	0.235	0.242	0.243	0.251
	3	0.280	0.263	0.283	0.288	0.272	0.284	0.305	0.299	0.304	0.306
Pectin sulfate	**1**	0.224	0.245	0.263	0.261	0.248	0.253	0.270	0.278	0.257	0.274
	2	0.229	0.260	0.288	0.290	0.253	0.278	0.286	0.297	0.298	0.313
	3	0.232	0.255	0.249	0.264	0.286	0.285	0.287	0.289	0.296	0.304

Table 19. % survival of HeLa cells.

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	72.05	87.27	88.82	92.24	94.10	91.93	93.17	92.24	96.27	98.45
	2	67.70	67.08	68.94	73.29	71.12	70.81	72.98	75.16	75.47	77.95
	3	86.96	81.68	87.89	89.44	84.47	88.20	94.72	92.86	94.41	95.03
	Aver.	75.57	78.67	81.88	84.99	83.23	83.64	86.96	86.75	88.72	90.48
	SD	10.10	10.42	11.22	10.23	11.54	11.27	12.13	10.05	11.51	10.98
Pectin sulfate	**1**	69.57	76.09	81.68	81.06	77.02	78.57	83.85	86.34	79.81	85.09
	2	71.12	80.75	89.44	90.06	78.57	86.34	88.82	92.24	92.55	97.20
	3	72.05	79.19	77.33	81.99	88.82	88.51	89.13	89.75	91.93	94.41
	Aver.	70.91	78.67	82.82	84.37	81.47	84.47	87.27	89.44	88.10	92.24
	SD	1.26	2.37	6.14	4.95	6.41	5.22	2.96	2.96	7.18	6.34

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Table 20. The absorbance at wavelength 540 nm of MTT assay (HepG2 cells).

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	0.161	0.197	0.220	0.222	0.222	0.229	0.238	0.229	0.240	0.240
	2	0.169	0.213	0.220	0.239	0.221	0.226	0.236	0.239	0.241	0.244
	3	0.195	0.198	0.198	0.205	0.207	0.209	0.208	0.216	0.217	0.230
Pectin sulfate	**1**	0.146	0.163	0.174	0.169	0.169	0.173	0.179	0.186	0.209	0.220
	2	0.118	0.120	0.114	0.157	0.127	0.136	0.133	0.140	0.154	0.178
	3	0.142	0.173	0.186	0.167	0.161	0.181	0.187	0.184	0.194	0.219

Table 21. % survival of HepG2 cells.

Sample	Time	Concentration (µg/mL)									
		2000	**1000**	**500**	**250**	**125**	**62.5**	**31.3**	**15.6**	**7.8**	**3.9**
Native pectin	**1**	60.07	73.51	82.09	82.84	82.84	85.45	88.81	85.45	89.55	89.55
	2	63.06	79.48	82.09	89.18	82.46	84.33	88.06	89.18	89.93	91.04
	3	72.76	73.88	73.88	76.49	77.24	77.99	77.61	80.60	80.97	85.82
	Aver.	65.30	75.62	79.35	82.84	80.85	82.59	84.83	85.07	86.82	88.81
	SD	6.63	3.34	4.74	6.34	3.13	4.02	6.26	4.30	5.07	2.69
Pectin sulfate	**1**	54.48	60.82	64.93	63.06	63.06	64.55	66.79	69.40	77.99	82.09
	2	44.03	44.78	42.54	58.58	47.39	50.75	49.63	52.24	57.46	66.42
	3	52.99	64.55	69.40	62.31	60.07	67.54	69.78	68.66	72.39	81.72
	Aver.	50.50	56.72	58.96	61.32	56.84	60.95	62.06	63.43	69.28	76.74
	SD	5.65	10.51	14.39	2.40	8.32	8.96	10.87	9.70	10.61	8.94

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