PRODUCTION AND BIOLOGICAL ACTIVITIES OF OLIGO PECTIN FROM EARLY IMMATURE FRUIT OF *Durio zibethinus* L.

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A Thesis Submitted in Partial Fulfillment of the Requirements
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การผลิตและฤทธิ์ทางชีวภาพของออลิโกเพกตินจากผลอ่อนระยะต้นของทุเรียน *Durio zibethinus* L.

น.ส.อัญญ์ชิยา พิทักษา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	PRODUCTION AND BIOLOGICAL ACTIVITIES OF OLIGO PECTIN FROM EARLY IMMATURE FRUIT OF *Durio zibethinus* L.
By	Miss Aunchiya Pitaksa
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Associate Professor SUPAART SIRIKANTARAMAS, Ph.D.

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อัญญ์ชิยา พิทักษา : การผลิตและฤทธิ์ทางชีวภาพของออลิโกเพกตินจากผลอ่อนระยะต้นของทุเรียน *Durio zibethinus* L.. (PRODUCTION AND BIOLOGICAL ACTIVITIES OF OLIGO PECTIN FROM EARLY IMMATURE FRUIT OF *Durio zibethinus* L.) อ.ที่ปรึกษาหลัก : รศ. ดร.ศุภอรรจ ศิริกันทรมาศ

ทุเรียนระยะตัดแต่งผล หรือ EID มีขนาดความยาว 6-12 ซม. และเกิดขึ้นหลังการผสมเกสร 30-45 วัน ถือเป็นวัสดุเหลือใช้ทางการเกษตรจากการเพาะปลูก เป็นที่ทราบกันดีว่าเปลือกทุเรียนของผลสุกมีสารเพกทินเป็นองค์ประกอบ แต่ยังไม่มีรายงานเกี่ยวกับปริมาณเพคตินของผลทุเรียนระยะตัดแต่งผลดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อยืนยันการมีอยู่เพคตินในทุเรียนระยะนี้ การสกัดเพคตินจาก EID โดยใช้น้ำที่อุณหภูมิต่างกันที่ (25°C, 50 °C และ 75 °C) เป็นเวลา 1 ชั่วโมงเพื่อยืนยันการมีอยู่ของเพกทินโดยวิเคราะห์ด้วยเครื่องมือ X-ray diffraction (XRD) และ Fourier Transform Infrared Spectrometer (FT-IR) นอกจากนี้ยังมีการใช้เอนไซม์ทางการค้า Pectinex Ultra SP-L® exo-Polygalacturonase, and Viscozyme® เพื่อไฮโดรไลซิสเพกทินให้เป็นเพกทินออลิโกกาแซ็คคาไรด์ที่มีฤทธิ์ทางชีวภาพที่ดีขึ้น โดยผลิตภัณฑ์ที่ได้หลังจากการย่อยถูกนำไปตรวจสอบด้วยวิธีทางโครมาโตกราฟีแบบชั้นบาง (TLC) และ โครมาโทกราฟีของเหลวสมรรถนะสูง (HPLC) ฤทธิ์ต้านอนุมูลอิสระของเพกทินและเพกทินออลิโกกาแซ็คคาไรด์ได้รับการประเมินโดยการวัดความสามารถในการกำจัดอนุมูลอิสระโดยใช้การทดสอบ 1,1-diphenyl-2-picrylhydrazyl (DPPH) และ azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) เป็นการทดสอบฤทธิ์ต้านอนุมูลอิสระของเฟอริก (FRAP) นอกจากนี้ทั้งเพกทินและเพกทินออลิโกกาแซ็คคาไรด์มีความสามารถยับยั้งกระบวนการเกิดไกลเคชั่นที่เหมือนกัน แต่ความสามารถในการต้านอนุมูลอิสระและความสามารถยับยั้งกระบวนการเกิดไกลเคชั่นของเพกทินออลิโกกาแซ็คคาไรด์นั้นสูงกว่าเพกทินที่ความเข้มข้นเดียวกัน มากไปกว่านั้นมีการทดสอบฤทธิ์ทางชีวภาพในเซลล์เพาะเลี้ยงพบว่าเพกทินออลิโกกาแซ็คคาไรด์มีการส่งเสริมการเพิ่มจำนวนของเซลล์รากผมและให้ผลการป้องกันความเสียหายจากความเครียดจากปฏิกิริยาออกซิเดชันที่เกิดจาก H_2O_2 ดังนั้นเราจึงประสบความสำเร็จในการพัฒนาวิธีการสกัดเพกทินจากทุเรียนระยะตัดแต่งผลและกระบวนการไฮโดรไลซิสเพื่อเพิ่มฤทธิ์ทางชีวภาพของสารสกัดทำให้เหมาะสำหรับการนำไปใช้ประโยชน์ในผลิตภัณฑ์เครื่องสำอาง

สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อนิสิต .....
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KEYWORD: Early immature durian (EID) fruits Pectin Enzyme Pectin oligosaccharide Biological activities

Aunchiya Pitaksa : PRODUCTION AND BIOLOGICAL ACTIVITIES OF OLIGO PECTIN FROM EARLY IMMATURE FRUIT OF *Durio zibethinus* L.. Advisor: Assoc. Prof. SUPAART SIRIKANTARAMAS, Ph.D.

Early immature durian (EID) fruits, 30-45 days after anthesis with 6-12 cm in length, are considered agricultural waste from cultivation. It is known that durian rinds of mature fruits contain pectin, but there have been no reports on the pectin content of early immature durian (EID) fruits. Therefore, the objective of this study was to determine the pectin content in EID fruits. We extracted pectin from EID fruits using distilled water at different temperatures (25℃, 50℃, and 75℃) for 1 h to investigate pectin content. Instrumental analyses, including X-ray diffraction (XRD) and Fourier Transform Infrared Spectrometer (FT-IR), confirmed the presence of pectin in the EID extract. To enhance the biological activity, we employed Pectinex Ultra SP-L® exo-Polygalacturonase, and Viscozyme® L, a mixture of carbohydrases, to degrade pectin into pectin-oligosaccharides (POSs). Moreover, Thin-layer chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) were used for the qualification analysis of POSs. The antioxidant activity of the extract was evaluated by measuring its free radical scavenging capacity using DPPH and ABTS assays, as well as the ferric-reducing antioxidant power (FRAP) assay. Additionally, both POSs and pectin demonstrated antiglycation activity. The antioxidant and antiglycation activities of POSs were found to be stronger than those of pectin. Moreover, POSs exhibited hair proliferation promotion and provided protective effects against H_2O_2-induced oxidative stress damage. Consequently, we have successfully developed an extraction method and hydrolysis process to enhance the biological activity of the extract, making it suitable for potential utilization in cosmetic products.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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Aunchiya Pitaksa

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CHAPTER I

INTRODUCTION

In Thailand, durian (*Durio zibethinus*. L), known as the king of fruits, is widely consumed. Many of the early immature durian fruits (EID) (30-45 days after anthesis with a length of 6-12 cm) are thinned to retain the fruit's qualities, including size and flavor. Thus, farmers prefer to cut the fruit at this stage. Therefore, it is important to convert this agricultural waste into a value-added product.

According to a previous study by (Pewlong et al., 2021), it was found that the ethanolic extract of EID exhibited antioxidant activities and can be used as a cosmetic ingredient. However, water extract of EID has not been investigated. It was interesting to find that the water extract formed a gel-like structure, and we hypothesized in this study that the water extract of early immature durian might contain pectin which requires further confirmation. It has been known that discarded fruit-processing industry waste, such as citrus peel, mango peel, apple pomace and banana peel are a major source of pectin (Panchami & Gunasekaran, 2017; L. Tian et al., 2020). Pectin refers to a collection of plant cell wall polysaccharides that are widely distributed and consist of linked galacturonic acids. The purity of pectin is estimated by galacturonic acid (GalA), and a minimum purity of 65% GalA is required for commercial applications (Nadar et al., 2022). However, there has not been reported whether the EID contains pectin.

In recent years, pectin oligosaccharide (POSs) has been applied in various fields, notably because of their specific biological activities. POSs can be obtained through both chemical and enzymatic processes (Dranca & Oroian, 2018). The increasing use of POSs in food, medicine, pharmacy, and cosmetics industry is because POSs has better biological activities than pectin (Yeung et al., 2021). The biological activity of POSs derived from durian pectin has not been previously studied. Therefore, the aim of this study is to investigate the extraction of pectin from EID using water, referred to as durian pectin, and enzymatically hydrolyze it into POSs to enhance biological activity. This process provides an environmentally

friendly condition and is suitable for use in the cosmetic industry. Additionally, this chapter reviews further study and information related to these topics.

. 1.1 Early immature durian (EID)

Durian (*Durio zibethinus*) is one of the most popular and economically important fruit crops in South-East Asia. There are several durian cultivars that exhibit significant variations in taste, aroma, texture, nutritional composition, flesh color, as well as variations in the size and shape of the fruit. The fruit is exported in the form of whole fruits, either fresh or frozen, and the ripening stage of the fruit is a major criterion that greatly influences their quality. Various indicators are utilized to assess the ripeness of durian, including methods tapping the durian husk to determine the sound, analysis of specific volatile compounds, observation of the color and elasticity, counting the number of days after anthesis. To preserve the quality of durian, thin the branches by cutting early immature durian fruits to maintain the quality of durian for export (over 200-300 fruits per tree) that are 45 days old after pollination, and these unutilized fruits are classified as agricultural waste.

The early immature durian (EID) fruits, as shown in Figure 1, are typically 6-12 cm in length. They are smaller in size and have a different texture and flavor compared to fully ripe durian, especially without the strong and unique sulfur-containing odor. The immature fruit has been used to create many values in previous studies, such as the extraction of pectin from immature melons (Yuliarti et al., 2015) and pectin extract from immature Vietnamese mango peel(Nguyen et al., 2019). Moreover, pectin is extracted from immature persimmon waste, there have significant quantities of pectin with potential bioactive properties such as very high total phenolic content and antioxidant activity (Méndez et al., 2022). There is also extraction of pectin from ripe durian peel (Wai et al., 2009). Therefore, in this study, pectin was extracted from EID fruits, and evaluated biological activity. Which serves as an interesting aspect of this study.

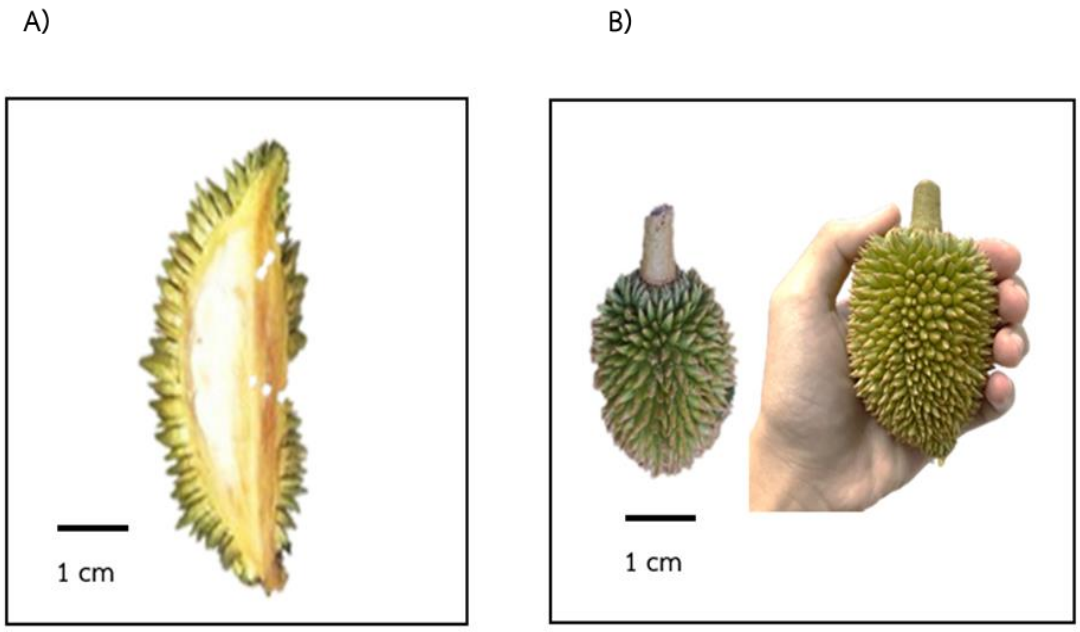

Figure 1. The early immature durian fruit used in this study.

A) The cross-section shows the interior of EID fruit, B) which is roughly the size of a handful. The early immature durian fruit was used in this study.

1.2 Pectin and pectin oligosaccharide (POSs)

1.2.1 Pectin

Plant cell walls consist of carbohydrates, protein, and aromatic compounds, and are essential to the proper growth and development of plants. The carbohydrate components, such as pectin, cellulose, and hemicellulose, are crucial constituents that provide dietary fibers, vitamins, minerals, carotenoids, and polyphenols (Cui et al., 2019; Saini et al., 2015; Santhakumar et al., 2018). A family of complex polysaccharides rich in galacturonic acid (GalA) has generally been referred to as pectin (Buggenhout et al., 2015). Pectin is a complex polysaccharide that is naturally present in the cell walls of fruits, particularly in the peels and pulps. Sources of pectin include citrus fruits, apples, sugar beet and peaches, etc.

The structure of pectin which is most abundant in the plant primary cell walls and the middle lamellae, is a family of heterogeneous polysaccharides as explained in Figure 2 from(Liu et al., 2020). The most abundant pectin polysaccharide is homogalacturonan (HG), a linear homopolymer of α-1,4-linked galacturonic acid that comprises ∼65% of pectin. HG is partially methylesterified at the C-6 carboxyl, may be O-acetylated at O-2 or O-3, and may contain other potentially crosslinking esters of uncertain structure. HG is characterized by their high degree of methylation, meaning that many of the galacturonic acid units are esterified with methyl groups (Pelloux et al., 2007). Rhamnogalacturonan I (RG-I) represents 20–35% of pectin. RGI differs from the structures discussed above because its backbone comprises many repeating [(1⟶2)-α-l-rhamnose-(1⟶4)-α-d-galacturonate] $_n$ disaccharide units, where n can be larger than 100 (Willats et al., 2001).

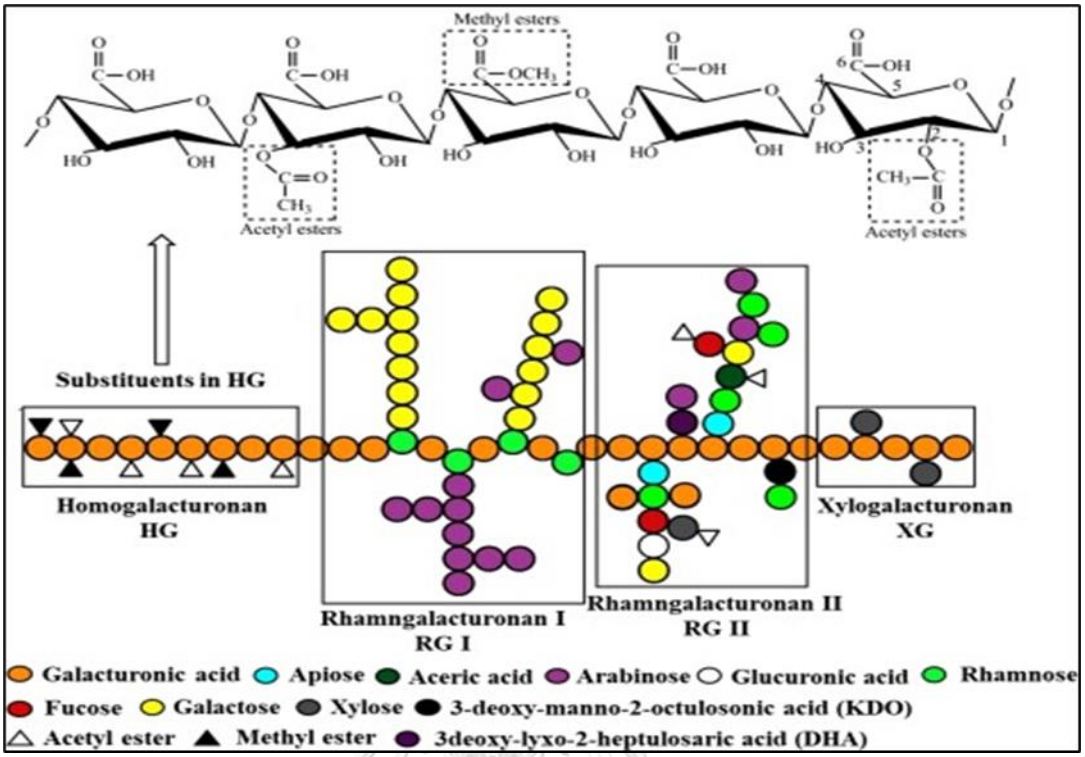

Figure 2. Schematic representation of pectin structure.

Galacturonic acid comprises approximately 70% of pectin, and all the pectic polysaccharides contain galacturonic acid linked at the O-1 and the O-4 position (Mohnen, 2008). Homogalacturonan (HG) is the main component of pectin and is composed of a linear chain of galacturonic acid residues. Rhamnogalacturonan I (RG-I) is a linear polysaccharide composed of side chains containing various sugars such as rhamnose, galactose, and arabinose. RG-II, on the other hand, is a highly branched and complex polysaccharide with a unique structure. Xylogalacturonan (XG) consists of a backbone of galacturonic acid residues, similar to pectin, which are interspersed with xylose residues.

Pectin characterization involves the analysis and description of the properties, structure, and functionality of pectin. It encompasses a range of techniques and methods aimed at understanding the physical, chemical, and biological characteristics of pectin molecules. The widely used methods for pectin characterization include analyzing the chemical composition, determining the molecular weight, assessing the degree of esterification (DE), conducting structural analysis, and evaluating the functional properties. Techniques such as X-ray diffraction (XRD), Fourier transform-infrared (FT-IR) spectroscopy, scanning electron microscopy (SEM), and High-performance liquid chromatography (HPLC) are commonly employed for analyzing the chemical composition or monosaccharide composition (Lin et al., 2022). XRD spectra are particularly useful for determining the atomic structure of pectin or crystalline pectin. FT-IR can provide information about the degree of methylation of pectin by identifying characteristic absorption bands of functional groups, such as methoxyl (CH_3O) groups (Rahmani et al., 2020). Functional attributes of pectin, including its gelling, thickening, stabilizing, and emulsifying capabilities, have been evaluated in previous studies (Huang et al., 2020).

Pectin is mostly used as a gelling agent in jams and jellies, and it is also known for its effective ability to stabilize fruit juices and acidified milk drinks. (Srivastava et al., 2011). Other relevant uses are in cosmetics, personal care (paints, toothpaste, and shampoos) and pharmaceutical (gel caps) (Ciriminna et al., 2016). In cosmetic and in personal products, besides their use as natural texturizer for ointments, oils, and creams, and as an effective thickener and stabilizer for shampoos, lotions, and hair tonics, Moreover, pectin is now recognized for its effectiveness as a skin anti-aging agent (Decoster et al., 2011). Recently, innovative enzymatic techniques have been used to hydrolyze pectin into pectin oligosaccharides (POSs) to enhance their biological activity.

1.2.2 Pectin oligosaccharide (POSs)

POSs are derived from pectin through the process of either acid or enzymatic hydrolysis as shown in Figure 3. POSs can be obtained through enzymatic hydrolysis of pectin. Enzymes such as pectinases or specific pectinolytic enzymes are used to break down the pectin molecules into smaller oligosaccharides. These oligosaccharides retain some of the structural characteristics and functional properties of pectin but have a shorter chain length.

Various enzymes have been widely used to produce POSs because of their specificity and selectivity. In addition, the use of enzymes over other pre-treatment methods is regarded as safe due to minimum adverse chemical modifications of products (Kim & Rajapakse, 2005). Exo-polygalacturonase (Exo-PG) is an enzyme that catalyzes the hydrolysis of pectin at glycosidic bonds between galacturonic acid residues (Gal A) residues from HG chains (Kester et al., 1999). The present study investigates the individual efficiency of commercial pectinases such as Viscozyme® L and Pectinex Ultra SP-L® to produce pectin oligosaccharides. These processes are suitable for producing low molecular weight POSs and achieving their potential prebiotic properties (Prandi et al., 2018). Moreover, the production of pectin oligosaccharides (POSs) using pectinase and their detection by thin layer chromatography (TLC) is a new approach for producing highly active compounds from fruit wastes. This method has been found that POSs has antioxidant efficiency better than pectin (Hosseini Abari et al., 2021). Various methods can be used for the characterization of pectin oligosaccharides (POSs). Commonly employed techniques include high-performance liquid chromatography (HPLC). These methods provide valuable information about the structural composition, molecular weight distribution, monosaccharide composition, and other physicochemical properties of POSs. The analysis of monosaccharide composition was analyzed using HPLC and UV detection was 250 nm previously reported by (Lv et al., 2009). While chemical hydrolysis has not been studied extensively. They are generally dangerous for the environment and there is also a limitation to achieve the desired degree of polymerization (Kim & Rajapakse,2005).

POSs have various applications as they serve as important signal molecules in plant defenses and play roles in plant growth and development processes (Baldan et al., 2003; Ridley et al., 2001) and in food industry as potential ingredients (Willats et al., 2006). Accordingly, Enzymatic methods are considered safe and mild means over acidic and hydrothermal treatments for POSs production (Babbar, Dejonghe, Gatti, Sforza, & Elst, 2016) (Babbar et al., 2016). Therefore, the production of POSs from fruits in the early immature stage is a novel approach.

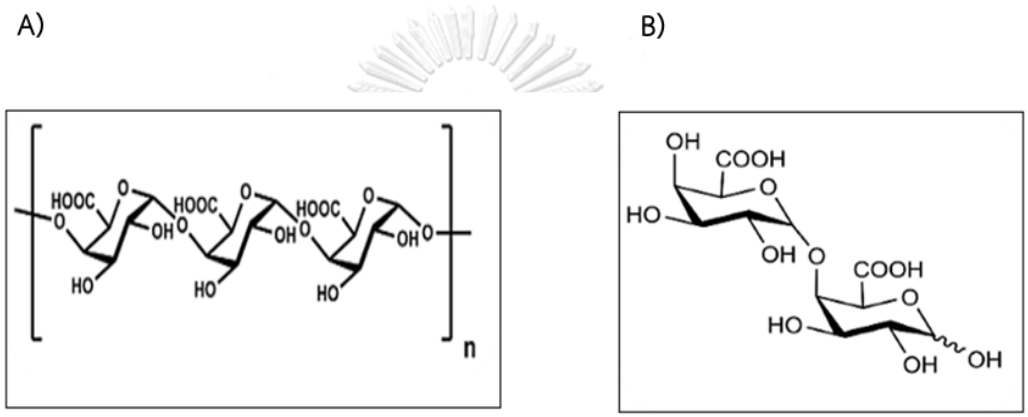

Figure 3. Pectin digestion process into POSs.

A) pectin structure, B) The structure of digalacturonic acid is obtained from the hydrolysis of pectin, which is an oligomer of pectin.

1.2.3 Drying method

There are several methods commonly used for drying plant raw materials, including freeze drying, oven drying, sun drying, and air drying. These methods can affect the bioactive compounds in plant materials. Plant-derived bioactive compounds are extensively studied as functional and nutraceutical ingredients (Yeung et al., 2018). Drying plays a crucial role in reducing moisture content and can also be used to concentrate plant extracts, impacting various aspects of the final dried products, such as the content of bioactive compounds (Belwal et al., 2022). Bioactive compounds derived from plant materials possess a wide range of properties including antioxidants, antimutagenic, anticancer, antiemetic, antifungal, antibacterial, etc. These substances are utilized for the treatment and alleviation of various diseases and find applications in diverse sectors such as food, cosmetics, pharmaceuticals, agriculture, and other industries across the economy (Romanik et al., 2007; Zygmunt & Namieś**ś**nik, 2003). Therefore, the method of drying the plant material widely depends on the active ingredients that contains.

Drying plays an important role in the production of pectin, which can affect the quality of pectin because pectin are susceptible to heat degradation during processing (Thakur et al., 1997). Many previous studies have been reported the drying method that effect on structural and functional properties of pectin (Huang et al., 2017). Freeze-Drying (Lyophilization) removes water from a material using sublimation, i.e., a solid-to-gas transition by passing the liquid phase (Krakowska-Sieprawska et al., 2022). The results of antioxidant capacity show that freeze-dried raw material has a better antioxidant capacity compared to oven-dried material. (Mendeley et al., 2019)(Qin et al., 2019). Recently, the effect of drying on the raw material of EID fruits has not been studied. Therefore, in this study, the oven-drying and freeze-drying methods were compared to determine the highest percentage yield in pectin extraction. Additionally, their biological activities, such as antioxidant activity measured by DPPH and ABTS assays, were evaluated.

1.3 Biological activity of POSs and pectin

The biological activity of POSs includes a range of potential health benefits such as antioxidants, antiglycation and anti-oxidative in skin cells that are described in this part.

1.3.1 Antioxidant activity

Antioxidant activity refers to the ability of a substance to inhibit or neutralize the harmful effects of free radicals. Free radicals are highly reactive molecules that can be generated through normal metabolic processes or can be introduced from external sources such as pollution, smoking, or certain medications. When an excess of free radicals cannot gradually be destroyed, their accumulation in the body generates a phenomenon called oxidative stress (Pham-Huy et al., 2008). During cellular energy production, specifically through the generation of ATP (adenosine triphosphate) by mitochondria, the essential element oxygen is utilized, resulting in the formation of free radicals. The cellular redox process gives rise to reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are typically the by-products with high reactivity (Halliwell et al., 2015). Cells can produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) through two distinct mechanisms: enzymatic reactions and non-enzymatic reactions. Enzymatic reactions that produce free radicals encompass processes such as the respiratory chain, phagocytosis, and prostaglandin synthesis. Several cellular oxidase systems, including NADPH oxidase, xanthine oxidase, and peroxidases, are responsible for the production of the superoxide anion radical ($O2^{\bullet-}$). This radical, once generated, engages in multiple reactions leading to the formation of various reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as hydrogen peroxide and the hydroxyl radical ($OH^{\bullet}$) (Valko et al., 2004). The non-radical hydrogen peroxide (H_2O_2) is generated through the activity of various oxidase enzymes, such as amino acid oxidase and xanthine oxidase. Non-enzymatic reactions involving oxygen and organic compounds, as well as reactions induced by ionizing radiation, can lead to the formation of free radicals. Additionally, this nonenzymatic process can take place within the

mitochondria during oxidative phosphorylation, the process of aerobic respiration (Droge, 2002)

Following, the hydroxyl radical has the ability to directly interact with DNA, resulting in oxidative damage that can be permanent. This damage has the potential to induce mutations in the DNA sequence (Imlay, 2013). Protein carbonyls are formed through the reaction of the hydroxyl radical ($OH^{\bullet}$) with specific amino acids, including lysine, arginine, proline, and histidine, at the carboxylic acid functional group. This reaction leads to the incorporation of carbonyl groups into the amino acid residues of proteins. Furthermore, the oxidation of histidine residues within proteins can result in the formation of 2-oxo-histidines (Dickinson & Chang, 2011).

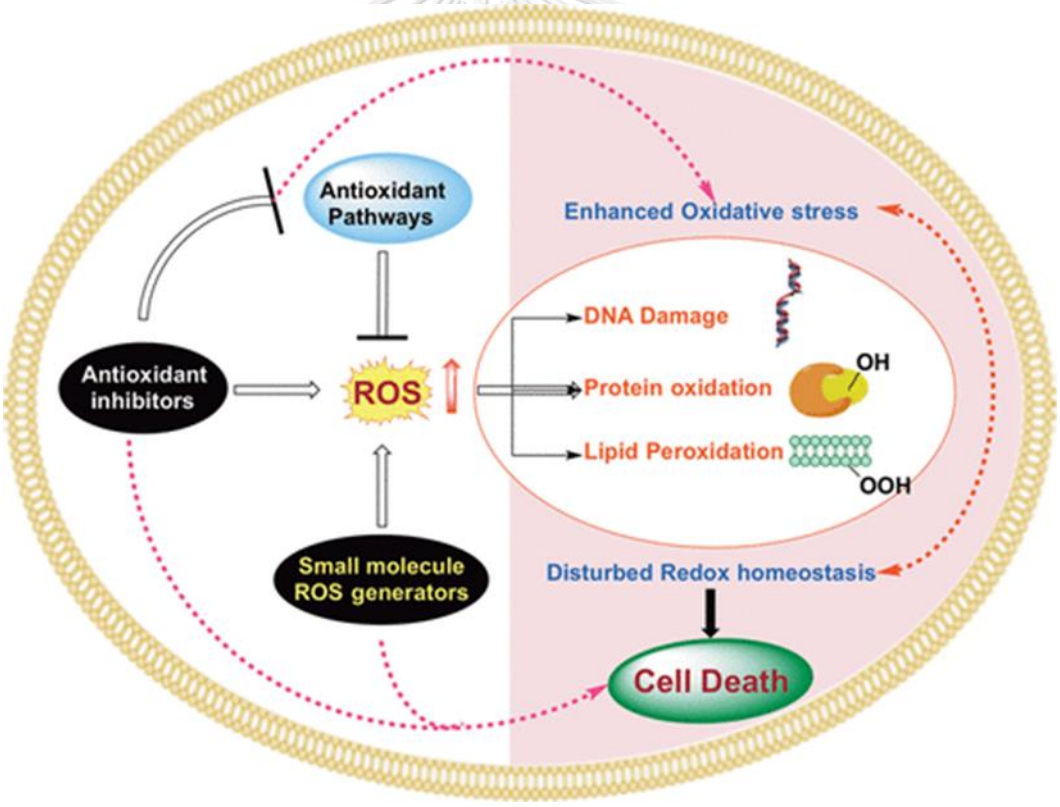

Figure 4. Role of reactive oxygen species (ROS).

ROS, which are generated as natural byproducts during aerobic respiration, play an important role in maintaining the balance of oxidation and reduction processes within cells. Excessive levels of free radicals can damage cell membranes, proteins,

and DNA, potentially leading to various diseases and accelerating the aging process. (Taken from (Dharmaraja, 2017)

Antioxidant activity evaluated from plant materials are widely studied (Mensor et al., 2001; Nantz et al., 2006). In addition, the several assays commonly used to measure antioxidant activity are 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) ABTS assays. Firstly, The FRAP assay is a widely used method to measure the antioxidant capacity of a sample based on its ability to reduce ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). The reduction of Fe^{3+} to Fe^{2+} is accompanied by a change in color, from blue to colorless, which can be quantified spectrophotometrically. The decrease in absorbance at a specific wavelength (usually around 593 nm) is directly proportional to the reducing power of the antioxidants in the sample (Munteanu & Apetrei, 2021). Next, DPPH assay is the simple and extensively used to measure antioxidant activity. In this assay, the purple chromogen radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) is reduced by antioxidant/reducing compounds to the corresponding changes from purple to yellow hydrazine. This color change can be measured spectrophotometrically at a specific wavelength at 517 nm (Boligon et al., 2014).

The ABTS or trolox equivalent antioxidant capacity (TEAC) assay based on ABTS is oxidized by a chemical oxidizing agent to generate the ABTS radical cation ($ABTS^{\bullet+}$). This radical cation is blue-green in color. The ABTS assay quantifies the capacity of an antioxidant to scavenge the $ABTS^{\bullet+}$ radical produced in an aqueous solution, relative to a standard antioxidant called Trolox, which is a water-soluble analogue of vitamin E. (Boligon et al., 2014). The chemical reaction for measuring the antioxidant capacity in the assay is explained in Figure 5.

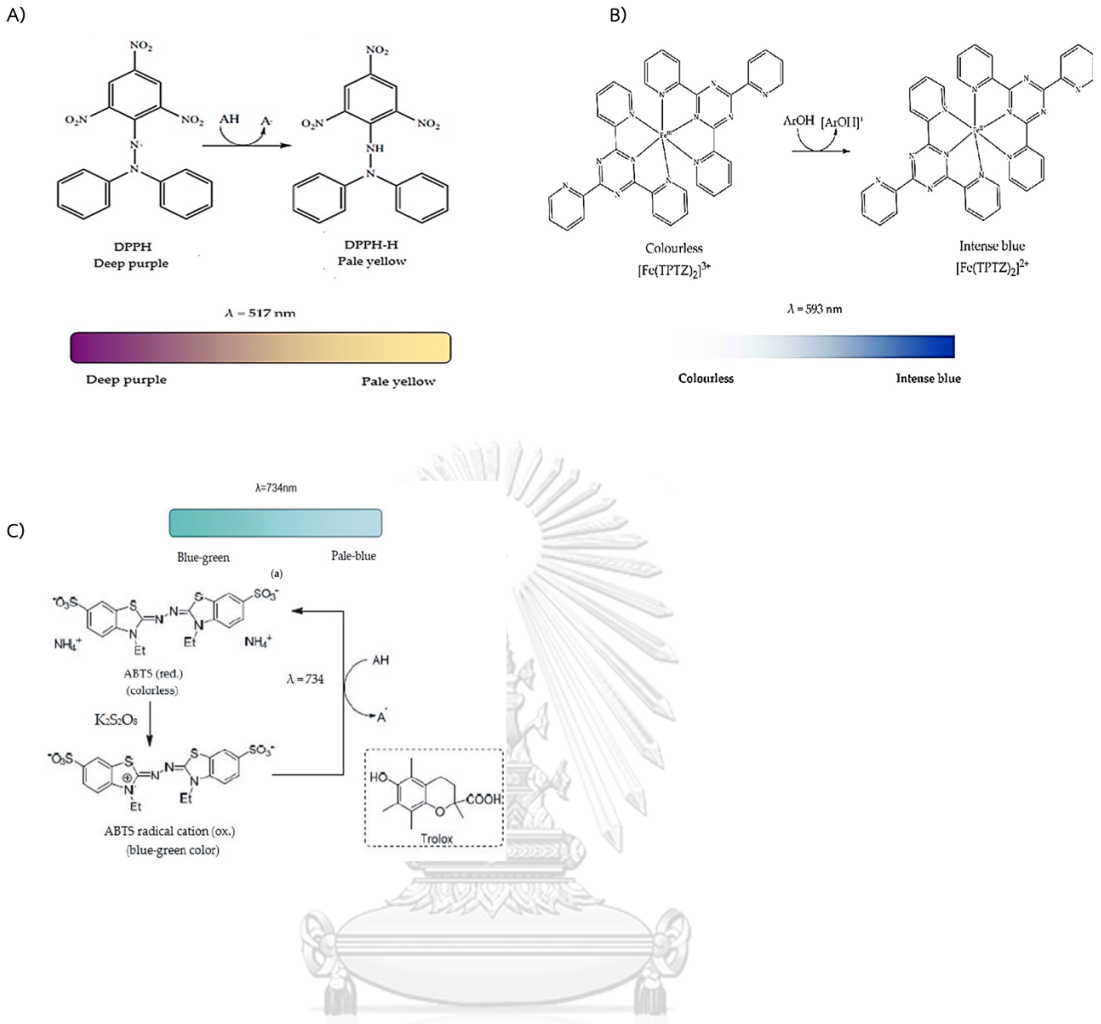

Figure 5. The chemical mechanisms of the DPPH, FRAP, and ABTS assays.

A) The DPPH assay, as described by (Munteanu & Apetrei, 2021), involves a change in the color of the DPPH radical during the reaction, which can be measured at 517 nm. B) The FRAP assay, as described by (Bibi Sadeer et al., 2020), involves the reduction of blue Fe^{2+} ions to colorless Fe^{3+} ions, which can be measured at 593 nm. C) The ABTS assay, as described by (Munteanu & Apetrei, 2021), evaluates the ability of antioxidants to counteract the effects of the stable radical cation ABTS•+, which has a blue-green color and absorbs light at 734 nm.

In addition, in this study, to evaluate the antioxidant activity of antioxidants in EID fruits the DPPH, ABTS, and FRAP assays were performed.

1.3.2 Antiglycation

Glycation is a non-enzymatic chemical reaction that occurs when sugars, such as glucose or fructose, react with proteins, lipids, or nucleic acids. During the glycation process, fructosamines and advanced glycation end products (AGEs) are produced. The term glycoxidation is used to describe the glycation process that involves oxidative reactions leading to the formation of advanced glycation adducts (Thornalley, 2002). Glycation plays a role in both diabetes and aging, as the buildup of glycation products can result in adverse effects. In the initial stage of a glycation reaction, an unstable Schiff base is formed, which subsequently undergoes rearrangement to yield a more stable Amadori product. The Amadori product can further participate in reactions to generate irreversible cross-linked products known as advanced glycation end products (AGEs). Apart from lysine residues, AGEs have also been identified to occur on the side chains of arginines and histidines (Bidasee et al., 2004). AGEs have been found in various cellular components, including the nuclei, nuclear envelope, mitochondria, endoplasmic reticulum, Golgi complexes, endocytic vesicles, lysosomal vacuoles or granules, secretory granules, cytosol, and cell membranes. Additionally, they have also been detected in the extracellular matrix of human cells (Ling et al., 1998). The intracellular pathway is intricate as glucose metabolites participate in the formation of AGEs. Glyoxal, methylglyoxal, and 3-deoxyglucosone are significant glucose metabolites that play a role in the generation of AGEs (Verbeke et al., 2000). The implications of glycation in the aforementioned diseases have generated considerable medical interest in the prevention of protein glycation in humans. That important to know about the mechanism of the glycation reaction. The mechanism of AGES product formation is shown in Figure 6.

Recently, a previous study demonstrated the presence of pectic oligosaccharides (POSs) derived from Actinidia arguta. The results indicated that POSs exhibits prebiotic and antiglycation activities, with the effectiveness of POSs being correlated to the content of galacturonic acid in the low molecular weight range. Therefore, the aim of this study is to confirm whether POSs derived from EID fruits can also inhibit the formation of advanced glycation end products (AGEs).

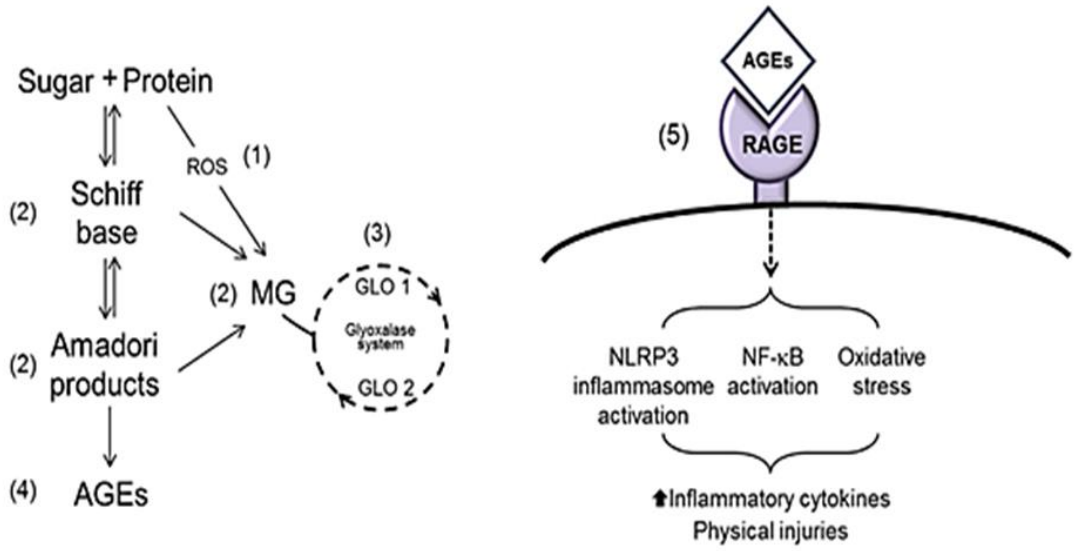

Figure 6. Antiglycation mechanisms.

1) Preventing the formation of reactive oxygen species (ROS) during glycation. 2) Hindering the formation of Schiff base, Amadori products, and subsequent dicarbonyl groups. 3) Detoxifying the precursor of advanced glycation end products (AGEs), methylglyoxal (MG), through the glyoxalase system. 4) Inhibiting the formation of AGEs. 5) Blocking the interaction between AGEs and their receptor (RAGE) to inhibit oxidative stress, generation of ROS, inflammatory responses, and physical damage via various pathways (Taken from (Yeh et al., 2017).

1.3.3 Cytotoxicity

Before commencing the cell culture experiment, it is necessary to assess the toxic effects of the extract or agents on cells. The cells are monitored over a specific period, and their viability, proliferation, or other relevant parameters are measured. The MTT assay, shown in Figure 7, is a commonly used assay in cytotoxicity testing. These methods measure viability, cell membrane integrity, cell proliferation, and metabolic activity (Tolosa, Donato et al., 2015). Cell viability can be assessed through observations of morphological alterations, changes in membrane permeability, and the physiological state inferred from the exclusion or uptake and retention of specific dyes (Stoddart, 2011). One of the most widely recognized metabolic dyes is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and the introduction of a 96-well assay format significantly enhanced its capacity (Mosmann et al., 1983). This particular assay relies on the reduction of soluble tetrazolium into insoluble blue formazan crystals. Traditionally, it has been believed that this conversion takes place within the mitochondria. The principle of this assay involves the reduction of soluble tetrazolium to form insoluble blue formazan crystals. It has been conventionally accepted that this conversion primarily occurs within the mitochondria. However, there is a growing body of evidence suggesting that this process may not be exclusively limited to mitochondria. The resulting solution is then subjected to colorimetric assays, where a biochemical marker is detected and measured to serve as an indicator of cellular metabolic activity.

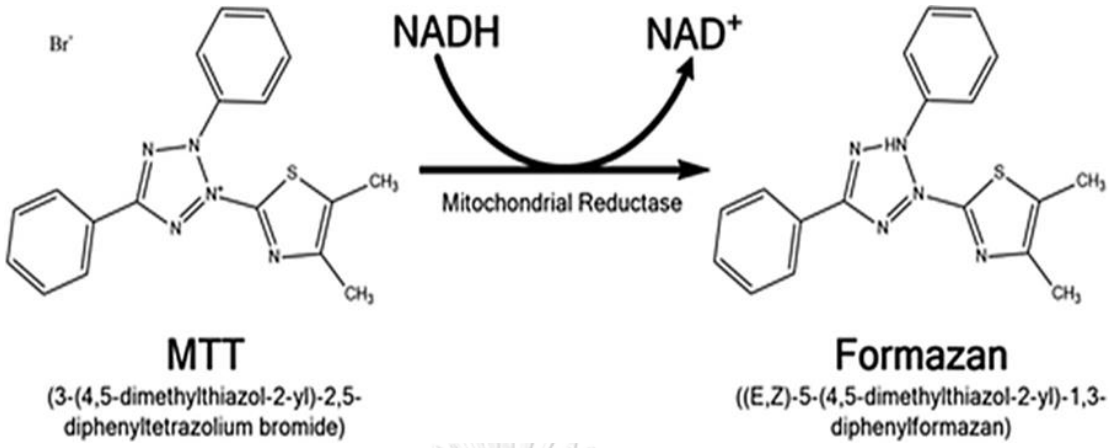

Figure 7. Reduction of MTT to formazan crystals.

In MTT assay, the tetrazolium salt is reduced to insoluble formazan dye by dehydrogenase enzyme present in the viable cells at 37° C (Taken from (Kamiloglu et al., 2020)

1.3.4 Oxidative stress in skin cell

Oxidative stress in cells occurs due to an imbalance between the production of reactive oxygen species (ROS). Reactive oxygen species, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are natural byproducts of cellular metabolism.

HaCaT human keratinocytes are a well-established and widely used immortalized cell line derived from human skin. Keratinocytes, which make up the main cellular composition of the epidermis, are highly prone to oxidative damage, leading to the development of notable skin-related disorders and plays an important role in defense against invading pathogens (Awad et al., 2018). Similar to other organs, the human skin experiences inherent aging (chronological aging) and external aging (photoaging), primarily caused by prolonged exposure to ultraviolet (UV) radiation from the sun (Fisher et al., 2002). Exposure to UV radiation, encompassing UVA and UVB wavelengths, results in DNA damage and the generation of reactive oxygen species (ROS), both of which contribute to inflammation and the development of tumors. (Calleja-Agius et al., 2013; Pillai et al., 2005; Yaar and Gilchrest, 2007; Bachelor and Bowden, 2004; Hussain et al., 2003). There mentioning that reactive oxygen species (ROS), DNA damage, and disturbances in cellular equilibrium can serve as stimuli for the activation of intracellular protein complexes called inflammasomes (Rho et al., 2005). There mentioned that reactive oxygen species (ROS) as shown in Figure 4, DNA damage, and disturbances in cellular equilibrium can serve as stimuli for the activation of intracellular protein complexes called inflammasomes. Hydrogen peroxide (H_2O_2)-induced cytotoxicity refers to the harmful effects caused by the exposure of cells to excessive levels of hydrogen peroxide and DNA damage by blocking ROS accumulation (Park et al., 2020). Reactive oxygen species (ROS) can induce cell apoptosis, which is a programmed cell death process. Apoptosis is a tightly regulated physiological process that plays crucial roles in normal development, tissue homeostasis, and the elimination of damaged (Park et al., 2020). The mechanism of oxidative stress in HaCaT human keratinocytes as shown in Figure 8.

In this study, we aimed to investigate the protective effects of POSs and pectin from EID fruits against oxidative stress in HaCaT human keratinocytes.

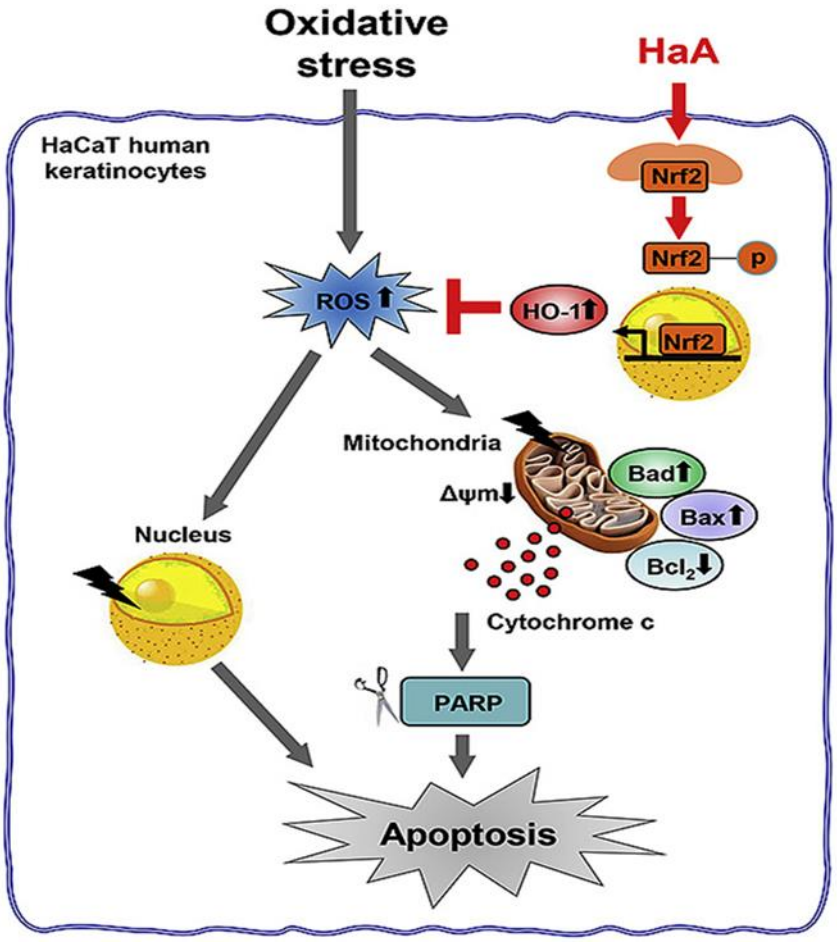

Figure 8. Mechanism of oxidative stress in HaCaT human keratinocytes.

The interaction between reactive oxygen species (ROS) and oxidative stress is closely linked. ROS are highly reactive molecules produced naturally during cellular metabolism. ROS can cause damage to cellular components, including lipids, proteins, and DNA, through oxidative reactions (Taken from (Park et al., 2020).

1.3.5 Hair proliferation

The hair growth cycle consists of three main phases: anagen (growth phase), catagen (transition phase), and telogen (resting phase). During the anagen phase, the hair follicles are actively producing new cells and hair fibers, resulting in visible hair growth. The duration of the anagen phase determines the maximum potential length of the hair (Rho et al., 2005). These repetitive variations involve rapid reconfiguration of both the cellular and structural elements within the hair follicles, including the epithelial and dermal components (Stenn & Paus, 2001). The dermal papilla (DP), positioned at the bottommost part of the hair follicle, serves as the primary mesenchymal element. There is believed to have crucial functions in stimulating the formation of new hair follicles and sustaining hair growth (Oliver, 1967). In addition to growth factors, androgens are recognized as the primary and well-established contributors to hair loss. Androgens have been found to induce hair regression and contribute to balding in individuals with a genetic predisposition. The hair growth cycle is shown in Figure 9.

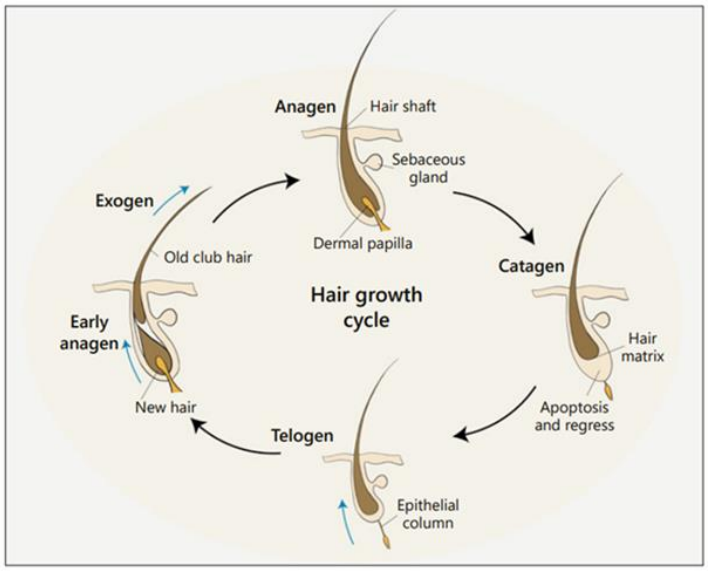

Figure 9. The hair growth cycle.

Three phases of hair growth cycle include anagen, catagen, and telogen (Taken from Houschyar et al., (2020).

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

Citric acid (Q RëC™, Auckland, New zealand)

Sodium hydroxide (Q RëC™, Auckland, New zealand)

Absolute ethanol (Q RëC™, Auckland, New zealand)

Pectinex Ultra SP-L® (Novozymes, Bagsværd, Denmark)

exo-Polygalacturonase (Megazyme, Ireland)

Viscozyme® L (Merck KGaA, Darmstadt, Germany)

Ethyl acetate (RCI Labscan Limited, Thailand)

Butan-1-nol (n-butanol) (Thermo ScientificTM, USA)

Acetonitrile (HPLC grade) (RCI Labscan, Thailand)

Orcinol (Merck, Germany)

Sulphuric acid (Q RëC™, Auckland, New zealand)

Silica gel 60 F254 (Merck, Germany)

Standard Galacturonic acid (TCI®, Japan)

Standard Digalacturonic acid (Megazyme, Bray, Ireland)

Standard Trigalactyronic acid (Megazyme, Bray, Ireland)

Standard Tetragalacturonic acid (Megazyme, Bray, Ireland)

Sodium phosphate anhydrous (Q RëC™, Auckland, New zealand)

2, 2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St, Louis, USA)

Absolute methanol (Grade AR, Qrec, New Zealand)

TPTZ (2, 4, 6- tripyridyl-s-triazine)

Acetic acid (Sigma-Aldrich, St, Louis, USA)

Hydrochloric acid (Sigma-Aldrich, St, Louis, USA)

$FeCl_3 \cdot 6H_2O$ (Sigma-Aldrich, St, Louis, USA)

2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich, USA)

Glucose (Q RëC™, Auckland, New zealand)

Sodium azide (Sigma-Aldrich, St, Louis, USA)

AG (guanidine) (Sigma-Aldrich, St, Louis, USA)

Dimethyl sulfoxide (RCI Labscan, Thailand)

Phosphate-buffered saline (PBS) (Gibco, New York USA)

2.1.2 Cell culture

Human hair dermal papilla cells (HHDPC) (Sciencell Research Laboratories, USA)

Human keratinocyte (HaCaT) (ATCC, USA)

Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York USA)

Fetal bovine serum (FBS) (Gibco, New York USA)

Trypsin-EDTA (0.25%), phenol red (Gibco, New York USA)

GlutaMAX Supplement (Gibco, New York USA)

Penicillin-streptomycin (Gibco, New York USA)

MTT (Sigma-Aldrich, St, Louis, USA)

Hydrogen peroxide (Merck, Germany)

Phosphate buffered saline solution (PBS) (Gibco, New York USA)

2.1.3 Glasswares and plasticwares

1.5-ml microcentrifuge tube (Axygen Hayward, USA)

10-, 100-, 1000-µl pipette tips (Axygen Hayward, USA)

0.45-micron nylon membrane filter (Whatman, Germany)

Glass bottles (Duran®, USA)

12x32mm clear glass screw thread standard mouth vials (Vertical chromatography, Thailand)

Black open-top cap with PTFE/white silicone septa (Vertical chromatography, Thailand)

NIPRO disposable, syringe (Nissho, Japan)

Nylon syringe filters (CNW Technologies, China)

Cell culture dishes (Thermo ScientificTM, USA)

6-,96-well plate (Corning®, USA)

96 well black polystyrene microplate (Corning®, USA)

Cryovial tube (SPL Life Sciences Co., Ltd, Korea)

2.1.4 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Elcectric Co., Ltd., Japan)

Balance: PB303-L (Mettler Toledo, USA)

Drying oven: Heraeus UT 5402 (Heraeus, USA)

Forma Series II 3140 (Thermo ScientificTM, USA)

Lyophilizer: Freezone 2.5 (Labconco, USA)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

Microwave oven: R-362 (SHARP, Thailand)

Mixer mill MM400 (Retsch®, Germany)

pH meter: S220 Seven compactTM pH/ion (Mettler Toledo, USA)

Refrigerator: Ultra-low temperature freezer (New Brunswick Scientific, UK)

Refrigerated centrifuge: Legend XTR (Thermo Scientific, USA)

ThermoMixer® C (Eppendorf, Germany)

Fourier-transform infrared (FT-IR) (Thermo scientific, USA)

HPLC Shimadzu: LC-20A (Shimadzu, Japan)

Shodex Asahipak NH2P-50 4E (Showa Denko, Japan)

X-ray diffractometer (Bruker™, USA)

Laminar flow: Bio Clean Bench (SANYO, Japan)

Spectrophotometer: BioSpectrometer® basic (Eppendorf, Germany)

Synergy H1 Multi-Mode Reader (Biotek, USA)

2.1.5 Software

GraphPad Prism 8 software (San Diego, CA)

LabSolutions™ lcms software (Shimadzu, Japan)

OMNIC spectra (Thermo fisher scientific, USA)

DIFFRAC.SUITE Software (Bruker, USA)

2.2 Methods

2.2.1 Raw material preparation

The pruned-early immature durian fruits (~20 g of each, with the length of fruit 6-12 cm after anthesis 30-45 days, Mon Thong cultivar) from Sangpong durian orchard in Chanthaburi province, eastern Thailand. The fruits were selected according to the regularity of size, free from defects. To remove contamination and dust, the early immature durian fruits were washed with water. Next, the fruits were cleaned, the thorny rind was removed, and the stems were discarded. The white pulp was then sliced into small pieces using a kitchen knife.

The samples to be used in this study were dried for further analysis. Use freeze-drying (-48°C, 0.390 hPa using Freezone 2.5 lyophilizer (Labconco, USA)) and oven drying method (60°C in Heraeus UT 5402 (Heraeus, USA)) for long storage of samples. The two drying methods were compared in the water extraction procedure of early immature durian. In addition, dried samples were ground to make a fine powder using a Mixer Mill MM400 (Retsch®, Germany) at 30 Hz for 30 seconds. All samples were stored in sealable bags and maintained in a dry environment prior to the experiments.

2.2.2 Pectin extraction procedure

The early immature durian powder was dried and then extracted with distilled water. The extraction was conducted under different conditions, including varying temperatures (25, 50, and 75°C), solid-liquid ratios (20, 30, and 50 mg/ml) and pH value (3, 4, and 5). The resulting extract was shaken in a ThermoMixer® C (Eppendorf, Germany) at 1400 RPM and 25°C for 1 hour. After extraction, the supernatants were collected using a Legend XTR (Thermo Scientific, USA) for 10 minutes at 10,000 g. The supernatants were then precipitated with a 1:2 ratio of 70% ethanol and stirred gently with a magnet. The sediment was subsequently evaporated in a fume hood until the solvent was completely removed, and the sample was dried before further analysis.

2.2.3 Pectin confirmation

To investigate the presence of pectin in early immature durian (EID) extract, instrumental analyses including X-ray diffraction (XRD) and Fourier-transform infrared spectroscopy (FT-IR) were conducted. These analyses suggested the possible existence of pectin in the extract. Additionally, HPLC analysis of hydrolyzed EID extract identified oligomers of the pectin polymer, specifically oligogalacturonic acid.

2.2.3.1 XRD investigation

X-ray diffraction analysis (XRD) is a technique used to study the crystalline structure of a substance. It involves directing a beam of X-rays onto a sample, which diffracts the X-rays in a characteristic pattern that provides information about the arrangement of atoms or molecules in the sample. In this study, 1 g of EID powder was irradiated with filtered Cu-K(α) X-rays at a voltage of 40.0 kV and a current of 40.0 mA. The diffraction angle 2θ was scanned from 5° to 60° at a scanning rate of 2° per minute. (Wathoni et al., 2019).

2.2.3.2 FT-IR analysis

Information regarding the chemical structure of the extracted pectin, including its functional groups, was obtained using FTIR analysis. The sample can absorb or transmit specific wavelengths of the IR radiation based on its molecular composition and the vibrational energies of its chemical bonds. FTIR measures the molecular composition of a sample by analyzing its interaction with infrared light, causing the molecules to vibrate in characteristic ways that reflect their molecular structure. In this study, a powder sample weighing 1 g was scanned in the range of 400–4000 cm^{-1}, following modifications made by (Abang Zaidel et al., 2017).

2.2.4 POSs production

Pectin (50:1 v/w) obtained from early immature durian fruit was hydrolyzed into pectin-oligosaccharides (POSs) using Pectinex Ultra SP-L®, exo-Polygalacturonase (exo-PG), and Viscozyme® L, which is a mixture of carbohydrates. The hydrolysis was conducted using 1% (v/v) of the diluted enzyme/pectin solution. The hydrolysis process took 10 min at 55°C, and the enzymes were inactivated by thermal treatment at 95°C for 5 minutes (Babbar et al., 2016). The resulting POSs were further analyzed using TLC and compared with standard oligo galacturonic acid. The POSs were dried before investigating their biological activities.

2.2.5 POSs characterization

2.2.5.1 TLC analysis

Thin-layer chromatography (TLC) is a commonly used analytical technique for the separation and identification of chemical compounds. POSs production after hydrolysis was analyzed using silica gel 60 F254 from Merck (Darmstadt, Germany). Chromatography was performed twice using ethyl acetate: n-butanol: acetonitrile: water (1:1:2:1 by volume) as a mobile phase. For visualization, the dried spots on the plates were sprayed with orcinol/sulfuric acid reagent (8 mg orcinol in 10 ml of 70% sulfuric acid) and then heated at 100°C for 5 minutes. (Modified from Abari et al., 2018)

2.2.5.2 Determination of POSs using HPLC analysis.

The identity and purity of oligo galacturonic acid were verified using HPLC (Shimadzu, Japan) with an Asahipak NH2P-50 4E column (Shodex Asahipak NH2P-50 4E, 250 × 4.6 mm, Japan). The analysis was carried out at a flow rate of 1.2 ml/min in 0.3 M phosphate buffer with a pH of 4.4, and the measurement was taken at 210 nm and 40°C (modified from Flodrová et al., 2009).

2.2.6 Evaluation antioxidant activity

2.2.6.1 DPPH assay

The antioxidative effects of obtained pectin oligosaccharides were assessed at various concentrations ranging from 1.25 to 80 mg/ml A 0.5 ml of POSs and pectin was added to 2 ml of 0.2 mM methanolic solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). A stock solution of DPPH was performed by dissolving 24 mg of DPPH (Sigma Aldrich, Singapore) with 100 ml methanol and the working solution was prepared by mixing 10 ml of the stock solution with 45 ml methanol. The reaction tubes were placed for 30 min at 25 °C in darkness. After that, the absorbance of the samples was examined at 517 nm. All samples were analyzed in triplicate and inhibitory activity (%) was calculated using the following equation modified from (Abari et al., 2021). Inhibition ratio (%) = $(A_1 - A_2) \times 100/A_1$, where A_1 is the absorbance of the addition of ethanol instead of testing sample and A_2 is the absorbance of testing sample solution.

2.2.6.2 ABTS assay

To evaluate 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging activities of OP and POSs, the method of (Xu, Shen, et al. (2018) was used with some modifications. Briefly, 7 mM of ABTS solution was allowed to react with 2.45 mM of potassium sulfate (1:1, v/v) in darkness for 12–16 h to generate ABTS radical cation. The solution was diluted with deionized water to obtain an absorbance of 0.700 ± 0.02 at 734 nm. After adding 0.8 ml ABTS solution to 0.2 ml sample (1–5 mg/ml), the mixture was incubated in darkness for 6 min. Absorbance of each sample was then read at 734 nm modified from (Jayesree et al., 2021). All samples were analyzed in triplicate and inhibitory activity (%) was calculated using the following equation (Miller & Rice-Evans, 1997).

ABTS radical scavenging activity (%) = $(A_{sample} - A_{blank})$ x100

2.2.6.3 FRAP assay

The FRAP assay is a method used to measure the antioxidant activity in a sample. FRAP stands for ferric reducing antioxidant assay, and it involves the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) by an antioxidant in the sample. The assay is based on the antioxidant's ability to donate electrons and reduce the yellow ferric tripyridyl-triazine complex (Fe (III)-TPTZ) to a blue ferrous complex (Fe (II)-TPTZ). The FRAP reagent was prepared by mixing 2.5 ml of a 10 mmol/L TPTZ solution in 40 mmol/L HCl with 5 ml of a 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution and 25 ml of a 0.3 mol/L acetate buffer (pH 3.6). To determine the antioxidant capacity, 20 µL of the sample solution and 280 µL of the FRAP reagent were mixed and incubated in the dark at room temperature for 10 minutes before measuring the absorbance at 593 nm. A calibration curve with six trolox standards was measured in the range of 0-1000 mM modified from (Benzie & Strain, 1996) (Picot-Allian et al., 2022)

2.2.7 Evaluation antiglycation activity

Antiglycation is a term used to describe the process of inhibiting or reducing the formation of advanced glycation end products (AGEs) within the body. To determine the ability of the samples to inhibit glycation, the BSA/glucose model was used. The mixture included BSA (20 mg/ml), glucose (500 mmol/L), sodium azide (1%), phosphate buffer (200 mmol/L, pH 7.4), and oligosaccharide samples with varying concentrations (0.03-1 mg/ml). As a negative control, no AG was used, and aminoguanidine (AG) (10mg/ml dissolved in 50% DMSO) was used as a positive control. The mixtures were incubated at 37°C for four days in Heraeus UT 5402 (Heraeus, USA), and the fluorescence of the solution was measured using a spectrofluorometer (BioTek, USA) at an excitation/emission wavelength at 335/385 nm, which is characteristic of the formation of AGEs modified from (Zhu, Wang, et al., 2019). The equation below was utilized to compute the relative fluorescent unit (RFU)

Relative fluorescent unit (RFU) = $(F_{sample} - F_{blank})$

Where F_{blank} is the fluorescent intensity of the BSA and F_{sample} is the fluorescent intensity of the test sample

2.2.8 Cell culture

2.2.8.1 Cytotoxicity assay of POSs and pectin on HHDPC and HaCaT cells

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was utilized to assess cell viability. The cells were seeded in 96-well culture plates and incubated in a CO_2 incubator at 37°C with 5% CO_2. HHDPC and HaCaT cells were plated overnight at a density of $8x10^3$ cells/well. The cells were then treated with various concentrations of extract (100, 200, 300, 500, and 1000 µg/ml) and H_2O_2 (50, 100, 200, 300, 400, and 500 µM) for 24 hours. Subsequently, 80 µL of MTT solution (5 mg/ml) was added to each well, and the cells were incubated in the dark at 37°C for 2 hours. After removing the medium, the formazan crystals produced by the cells were dissolved with 100 µL of dimethyl sulfoxide (DMSO).

The percentage of cell viability was calculated by comparing the treated cells to the non-treated group, and the cell viability was measured at 570 nm using a multi-mode reader. The calculation was carried out using the following equation. This method was modified from (Ashrafian & Hosseini-Abari, 2022)

$$\% \text{ cell viability} = [(A_{sample})/(A_{control})] *100$$

Where $A_{control}$ is the cell viability of the non-treated sample

2.2.8.2 Examined POSs and pectin extract increased proliferation of human hair dermal papilla cells (HHDPCs)

The effect of POSs and pectin on cell proliferation was tested using MTT assay. Generally, methods used to determine viability are also common for the detection of cell proliferation. Cell cytotoxicity and proliferation assays are generally used for drug screening to detect whether the test molecules have effects on cell proliferation or display direct cytotoxic effects. Regardless of the type of cell-based assay being used, it is important to know how many viable cells are remaining at the end of the experiment (Adan et al., 2016). Briefly, HHDPC cells were seeded onto 96-well plates in a density of $4x10^3$ cell/well for overnight. Then various concentrations of POSs and pectin extract (100, 200, 300 and 500 µg/ml) were incubated with the cells for 48 h. The cell viability was determined using an MTT assay as previously described.

2.2.8.3 Evaluate effect of different concentrations of H_2O_2 on HaCaT cell viability.

HaCaT cells were pretreated with 25, 50, 100, and 150 µg/ml of POSs and pectin extract on 96-well plate 24 hours prior to oxidative damage. Subsequently, the cells were exposed to H_2O_2 at an optimal injury concentration of 100 µM for 24 hours. A significant reduction in cell viability, compared to the control group, was defined as a 50% decrease, and the viability of HaCaT cells was determined using the MTT method. The results were expressed as a fluorescence percentage in control cells.

2.2.8.4 Measurement of Intracellular ROS Generation

ROS generation refers to the process of producing reactive oxygen species (ROS) in living organisms. However, excessive ROS production can lead to oxidative stress, which can damage cellular components such as DNA, lipids, and proteins. This damage has been linked to aging. The presence of ROS was evaluated by the compound 2', 7'-dichlorofluorescein diacetate (DCFH-DA). HaCaT were seeded in a 96 well black polystyrene microplate with the non-cytotoxic concentrations of crude extract for 24 h. After 24 h, the medium was removed, and cells were washed with PBS. Then the cells were incubated with H_2O_2 (100 µM) for 6 h to induce ROS regeneration. Afterward the cell was replaced with 100 µl of medium containing 50 µM of DCFH2-DA for 30 min. Subsequently, the medium was replaced with 100 µl PBS to measure fluorescent intensity using the BTH1M spectrophotometer (BioTek, USA) at the excitation/emission wavelengths at 485/535 nm. The ROS level (%) production was calculated using the following equation modified from (Ramanauskien et al., 2015)

$$\% \text{ ROS production} = (F_{sample} / F_{blank}) * 100$$

Where F_{blank} is the fluorescent intensity of the non-treated sample and F_{sample} is the fluorescent intensity of the test sample

CHAPTER III

RESULTS

3.1 Structural properties of durian pectin

The pectin extracted from early immature durian fruit powder was analyzed using X-ray diffraction (XRD) spectroscopy to obtain further information about the pectin structure. Fourier transform-infrared (FT-IR) spectroscopy was also commissioned to confirm and investigate the pectin structure.

3.1.1 X-ray diffraction (XRD) spectroscopy of durian pectin

The presence of sharp signals at 14.27, 20.47, 32.15, and 40.21 (2θ) indicates the crystal structure and molecular arrangement of pectin. There is widely accepted that pectin is always present in an amorphous state or less crystalline, as shown in figure 10. Similar results have been previously reported for pectin from different sources. There is a possibility that the extract contains pectin as a constituent.

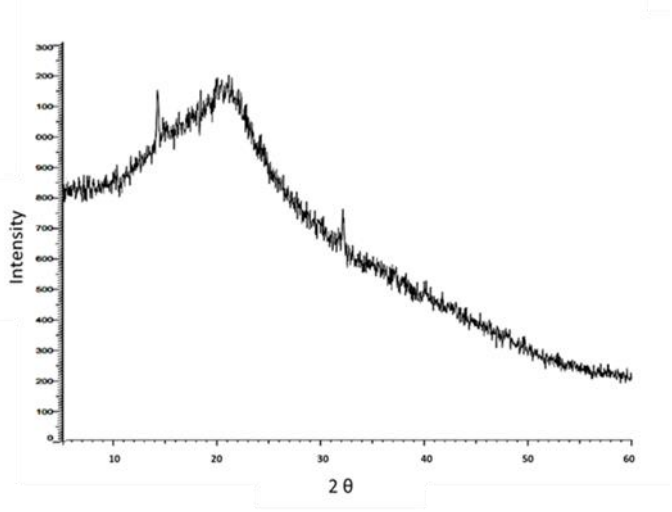

Figure 10. XRD spectra of durian pectin.

The peak at 2θ = 20° shows that the membrane was amorphous. XRD spectra were obtained from 1 g of pectin extracts derived from early immature durian using the oven dry method.

3.1.2 Fourier transform-infrared (FT-IR) analysis of durian pectin.

Based on the preliminary XRD analysis, further confirmation of pectin as a constituent was required through FT-IR analysis. Pectin is composed of various functional groups, such as hydroxyl (-OH), ester (-COO-), and carboxyl (-COOH) groups. The FTIR spectra of durian pectin samples exhibit characteristic peaks at 3276.01, 2931.44, 1700, and 1031.76 cm^{-1}, corresponding respectively to –OH, –CH, C=O of ester, and C–O of galacturonic acid, as shown in Figure 11. The low intensity bands at 1239 proved that the pectin samples were acetylated. The region of 1200–1000 cm^{-1} contains skeletal C–O and C–C vibration bands of glycosidic bonds. Therefore, we can confirm that the FT-IR spectra of the extract contains pectin.

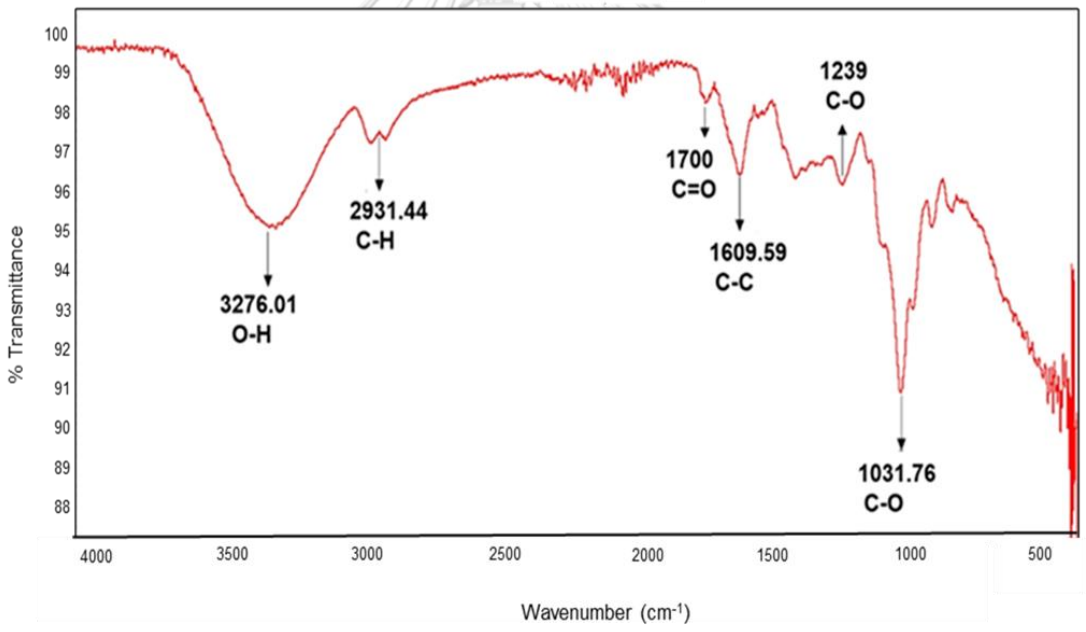

Figure 11. FT-IR spectra of durian pectin.

Obtained from 2 g of pectin extracts derived from early immature durian using the freeze dry method.

3.2 Effect of extraction on pectin yield

To evaluate drying method, freeze-drying, and oven-drying were performed. Figure 3 A) showed that the drying method of the raw material yielded significantly different percentages, namely 19.87% and 10.69% for freeze-dried and oven-dried process, respectively. After that, the amount of raw material varies, as shown in Figure 12 C). As a result, the % yield increased with increasing powderize durian amount, with 20 and 30 mg/ml not significantly different, but significantly different from 50 mg/ml. Moreover, at 50 mg/ml, the highest % yield was obtained. Afterward, the effect of different temperatures (25, 50, and 75 °C) on pectin extraction was analyzed, as shown in Figure 12 B). As the extraction temperature increases, the % yield also increases, but the difference is not statistically significant. Lastly, by varying the pH of the extraction solvent (pH 3, 4, and 5) shown in Figure 12 D), there was found that pH 3 gave the lowest % yield of 9.16. At pH 4 and 5, the % yields were 19.91 and 17.59, respectively. Therefore, based on the observed effects on pectin extraction, we selected to utilize freeze-drying at a concentration of 50mg/ml, pH 5, and 25℃ for the entire experiment.

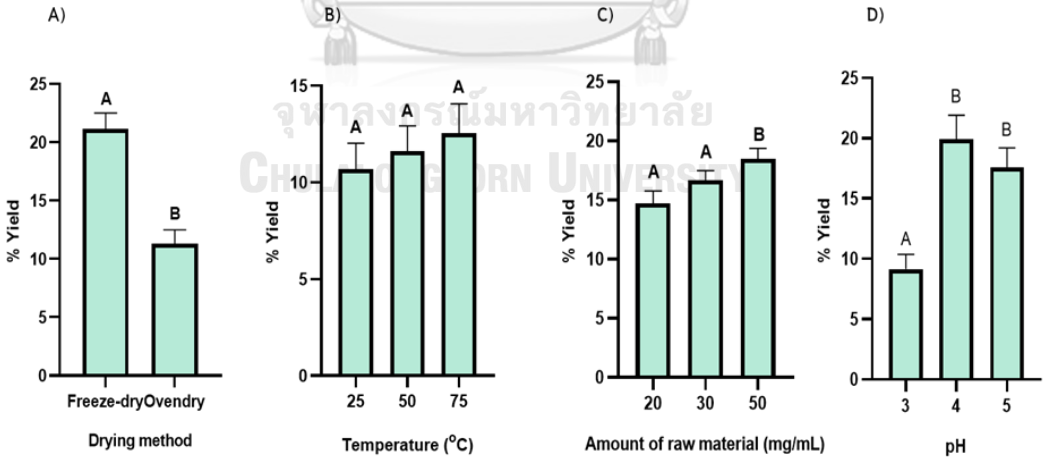

Figure 12. The effects of variations on pectin extraction.

A) The drying method for the raw material, B) Temperature, C) Amount of raw material, and D) pH can affect pectin yield. Student's t-test, n=3 (technical replicates), mean ± SD

3.3 Characterization of POSs

After pectin extraction, enzymes Pectinex Ultra SP-L®, Viscozyme® L and exo-PG were treated at a concentration of 1% (v/v) and incubated for 10 minutes. These enzymes have been used for POSs production (Combo et al., 2012a, 2012b).

3.3.1 TLC analysis after pectin hydrolysis

Treated durian pectin was performed using a solvent mixture of n-butanol, acetic acid, and H_2O (4/6/3, v/v/v) and spotted with 50% sulfuric acid.

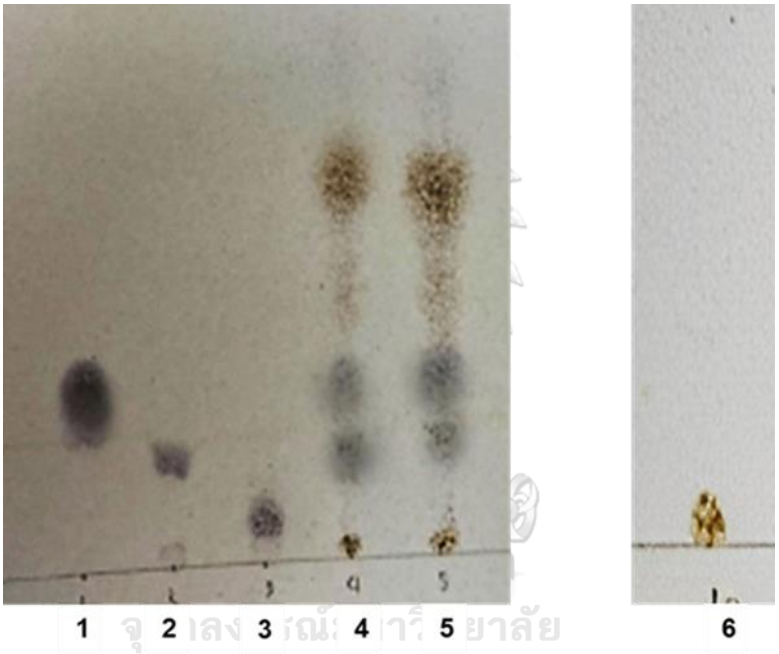

Figure 13. Revealed that digalacturonic acid and trigalacturonic acid were the end products of pectin oligosaccharides extracted from durian pectin.

The results showed different spots representing the various components present in the hydrolyzed pectin product, which were compared with a standard before further analysis by HPLC. TLC analysis of pectin oligosaccharide produced by enzymatic hydrolysis. TLC pattern of the obtained products, 1: standard solution of monogalacturonic acid, 2: digalacturonic acid, 3: trigalacturonic acid, 4: durian pectin after enzyme treatment for 5 min, 5: durian pectin after enzyme treatment for 10 min and 6: durian pectin without enzyme treatment.

3.3.2 HPLC chromatograms after pectin hydrolysis

POSs analysis with HPLC confirms the product obtained after pectin hydrolysis. The sample was analyzed using commercial standards through peak area and retention time. The resulting chromatograms are displayed in Figure 14. The retention time of digalacturonic acid is shown at 5.5 minutes, and the retention time of trigalacturonic acid is shown at 7.2 minutes.

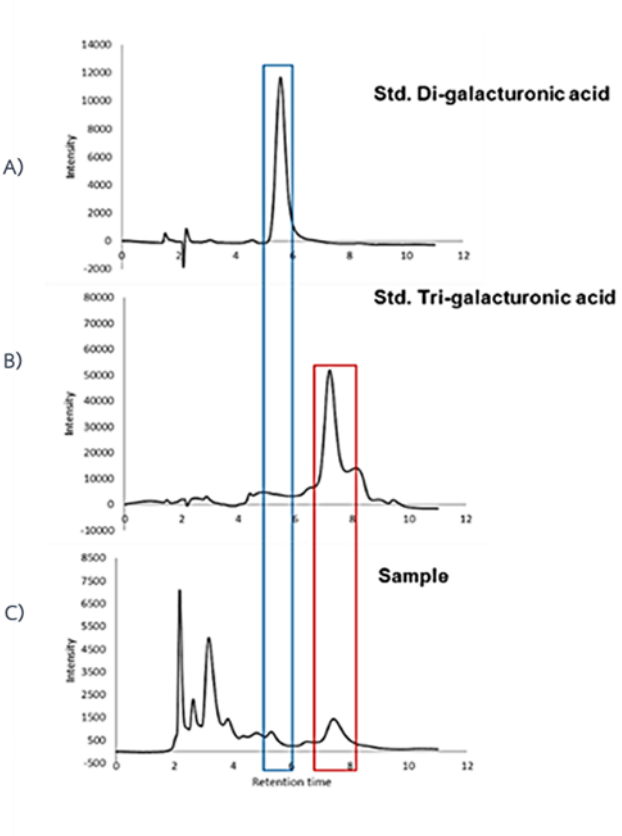

Figure 14. HPLC chromatograms of standard oligogalacturonic acid and durian pectin treated with Pectinex Ultra SP-L® for 10 min.

HPLC chromatograms of 0.05 mg/ml (w/v) pectin extracts from freeze dry methods and detected at 255 nm.

3.4 Effect of enzyme selection on antioxidant activity

To select the most effective enzymes. We selected from antioxidant efficacy studies to be used in further experiments.

The results showed that exo-PG had the highest inhibition percentage, followed by Pectinex Ultra SP-L® and Viscozyme® L, respectively. These enzymes (Pectinex Ultra SP-L®, exo-PG, and Viscozyme® L) were analyzed using the DPPH method when used to digest pectin into POSs, and had the inhibition percentage equal to 59.26, 62.1, and 46.12 respectively. Viscozyme® L was significantly less, different than Pectinex Ultra SP-L® and exo-PG as shown in figure 15. Therefore, we selected to use Pectinex Ultra SP-L® to hydrolyze pectin into POSs for further experiments.

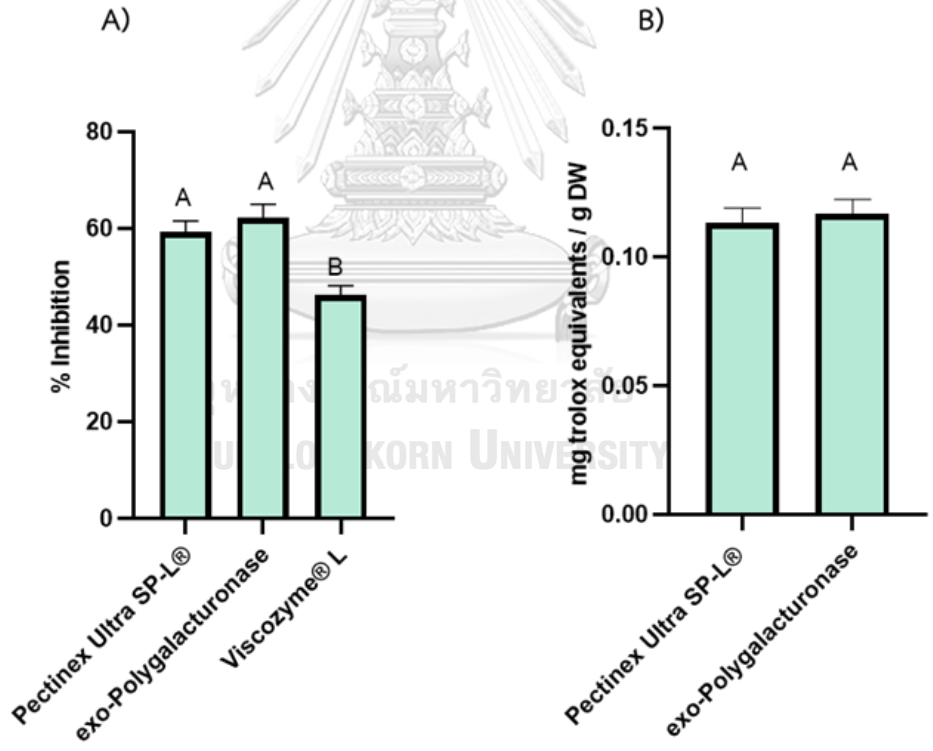

Figure 15. The antioxidant capacity of POSs.

Durian pectin after enzyme treatment was evaluated using three assays: DPPH A) and FRAP B). Student's t-test, n=3 (technical replicates), mean ± SD

3.4.1 Antioxidant inhibition effect between POSs and durian pectin

After completing POSs production with Pectinex Ultra SP-L®. Many previous studies have demonstrated that POSs is more effective as an antioxidant than pectin (Yeung et al., 2021) (Zheng et al., 2022). Accordingly, we should confirm that POSs has a higher antioxidant capacity than pectin.

ABTS and DPPH assays were performed to determine antioxidant capacity of POSs and durian pectin. Comparison of the antioxidants from each enzyme used to hydrolyze durian pectin revealed that, at the same concentration of 0.1 mg/ml. The results of the ABTS assay shown in Figure 16 A) show a mean % inhibition of 61 for POSs and 34 for pectin. Furthermore, in the DPPH assay shown in Figure 16 B), POSs exhibited a significantly higher mean % inhibition and stronger antioxidant activity, including scavenging, compared to pectin. The values for POSs and pectin were 46 and 30, respectively.

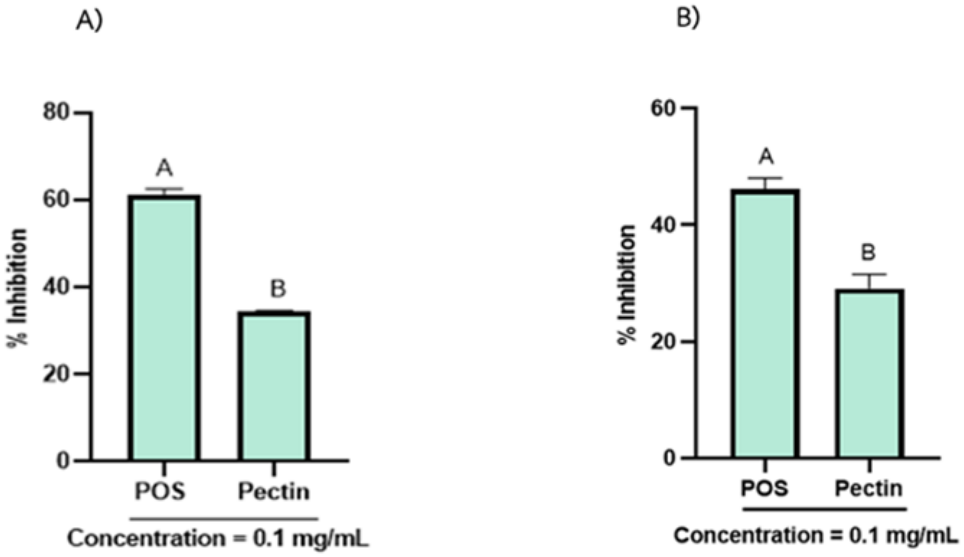

Figure 16. POSs and pectin showed antioxidant activity.

The antioxidant capacity was assessed using the DPPH assay A) and ABTS assay B). Student's t-test, n=3 (technical replicates), mean ± SD

3.5 Antiglycation activities

Our hypothesis is based on previous studies that demonstrated the effectiveness of pectin and POSs in reducing the formation of advanced glycation end products (AGEs) (Zhu et al., 2022). Therefore, we are interested in confirming the efficacy of POSs and pectin in inhibiting AGEs product in this experiment.

In this study, we utilized an antiglycation assay to investigate the formation of advanced glycation end products (AGEs) resulting from the Maillard reaction between glucose and BSA. The results represent the description of the relative fluorescent levels of advanced glycation end products (AGEs) obtained from the POSs as shown in Figure 17, with aminoguanidine (AG) as the reference standard. IC_{50} value of crude POSs and pectin were 41 and 53, respectively. The results indicate that POSs and pectin can reduce the level of AGEs fluorescent product in dose-dependent manner. POSs and pectin at 1000 μg/ml showed the most potent effect.

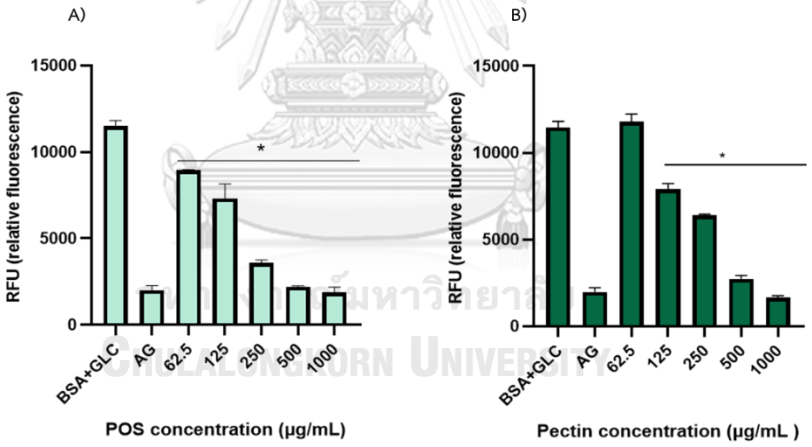

Figure 17. Inhibitory effect of POSs and durian pectin

A) durian pectin B) POSs inhibits AGE formation. AGEs were detected using fluorescent intensity at an excitation wavelength at 335 nm and emission wavelength at 385 nm. Dunnett's multiple comparisons test, n=3 (biological replicates), mean ± SD

3.6 Cytotoxicity of POSs and pectin on HaCaT and HHDPCs cells

HaCaT and HHDPC cells were incubated with POSs and pectin at concentration (100, 200, 500 and 1,000 μg/ml) for 24 h, and cytotoxicity was analyzed, shown in Figure 18. The results indicate that POSs and pectin, when tested at concentrations of 200-1000 μg/ml on HaCaT cells, led to a slight decrease in cell viability or exhibited cytotoxic effects. Conversely, concentrations of 100-200 μg/ml were found to be non-cytotoxic, and the cell viability slightly increased in HHDPC cells when treated with the extracts. Therefore, concentrations below 200 μg/ml were chosen for further analysis. In Figure 18 A) demonstrates that pectin at concentrations below 200 μg/ml exhibited toxicity to HaCaT cells and decreased in a dose-dependent manner. However, no toxicity was observed on HHDPCs at concentrations of 100-200 μg/ml. Furthermore, the cell viability increased, indicating that this concentration range was selected for further cell proliferation testing.

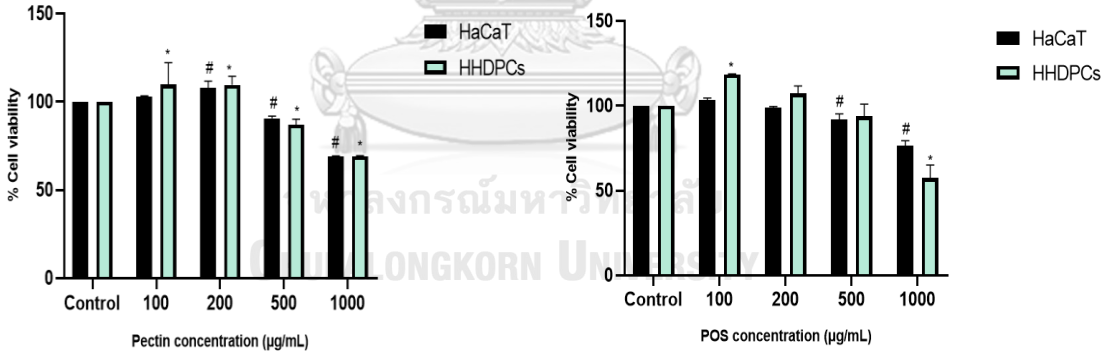

Figure 18. Cytotoxicity of POSs and pectin on HHDPCs and HaCaT cell

The cytotoxic effects of A) pectin and B) POSs on HaCaT and HHDPC cells. Cell viability was examined by MTT assay. *P < 0.05 versus control (non-treat group) for HHDPCs and #P < 0.05 versus control (non-treat group) for HaCaT. Dunnett's multiple comparisons test, n=3 (biological replicates), mean ± SD

3.6.1 POSs and pectin promote cell proliferation of human hair dermal papilla (HHDPCs)

After evaluating the cytotoxicity of POSs and pectin on HHDPCs cells as shown in Figure 18. There was observed that concentrations ranging from 25 to 200 µg/ml resulted in cell proliferation. Consequently, these concentrations were further evaluated for their effects on cell proliferation in HHDPC cells.

HHDPC cells were exposed to the POSs and durian pectin at concentrations of 25, 50, and 100 µg/ml for 24 hours. POSs and pectin significantly promoted the proliferation of HHDPC cells compared to the control (non-treated) shown in Figure 19. Furthermore, POSs exhibits a significantly higher proliferative effect than pectin.

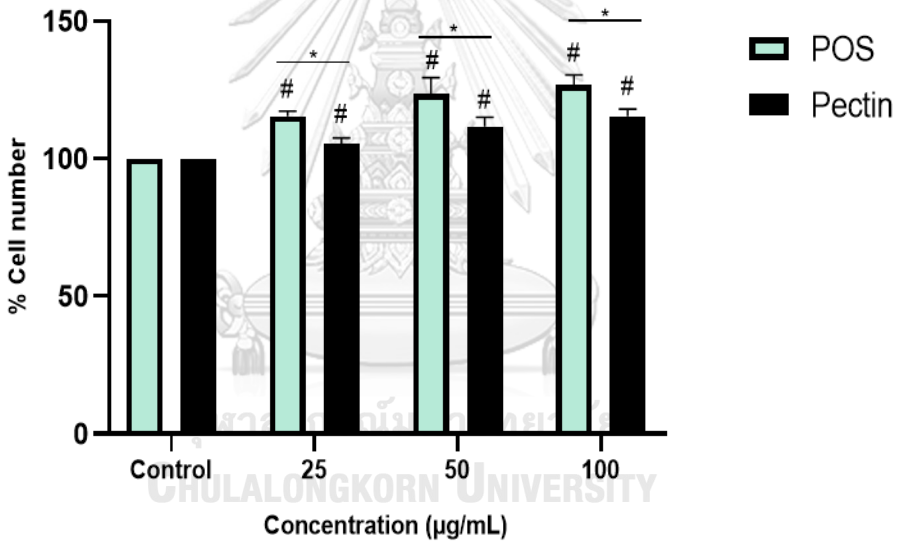

Figure 19. POSs and pectin promote cell proliferation on human hair dermal papilla (HHDPCs).

*P < 0.05, versus pectin-treated group, #P < 0.05, versus control (non-treated). Student's t-test, and two-way ANOVA (biological replicates), mean ± SD.

3.7 Effect of different concentrations of H_2O_2-induced cytotoxicity on HaCaT cell

H_2O_2 was utilized to test the treatment of injured HaCaT cells. Various concentrations of H_2O_2 (ranging from 50 to 500 μM) were applied for 24 hours. Cell viability was assessed using the MTT method. Figure 20 shows a decrease in HaCaT cell viability in response to H_2O_2 in a dose-dependent manner. Cell viability decreased gradually with increasing H_2O_2 concentration. Additionally, at 100 μM, cell viability was significantly reduced by 50%, hence this H_2O_2 concentration was selected for further analysis.

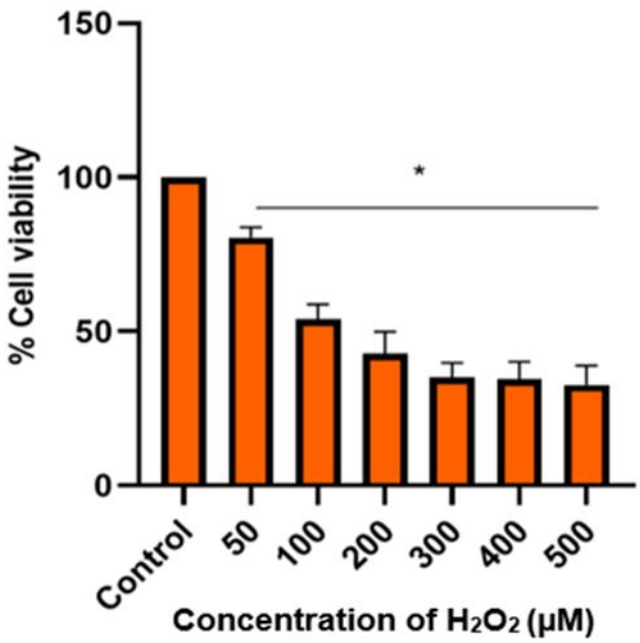

Figure 20. Effect of different concentrations of H_2O_2-induced cytotoxicity on HaCaT cells.

*P < 0.05, versus control (non-treated), Student's t-test, n=3 (biological replicates), mean ± SD

3.7.1 Protective effects of POSs and pectin against H_2O_2-induced oxidative stress damage on HaCaT cells.

To elucidate the protective effect of POSs and pectin in protecting HaCaT cells against injury from H_2O_2 treatment. Figure 21 A) shows pretreatment with 25, 50, 100 and 150 µg/ml of POSs for 24 h slightly increased, Average of %inhibition of POSs and pectin were 40.76 and 33.82 respectively. And their restores cell viability in a dose-dependent manner compared to H_2O_2-treated alone group. Therefore, POSs demonstrated a better protective effect against H_2O_2-induced skin damage compared to pectin. Figure 21 B) correspondingly shows that pectin exhibited the highest restored cell viability after a 24-hour pretreatment, particularly at a concentration of 100 µg/ml.

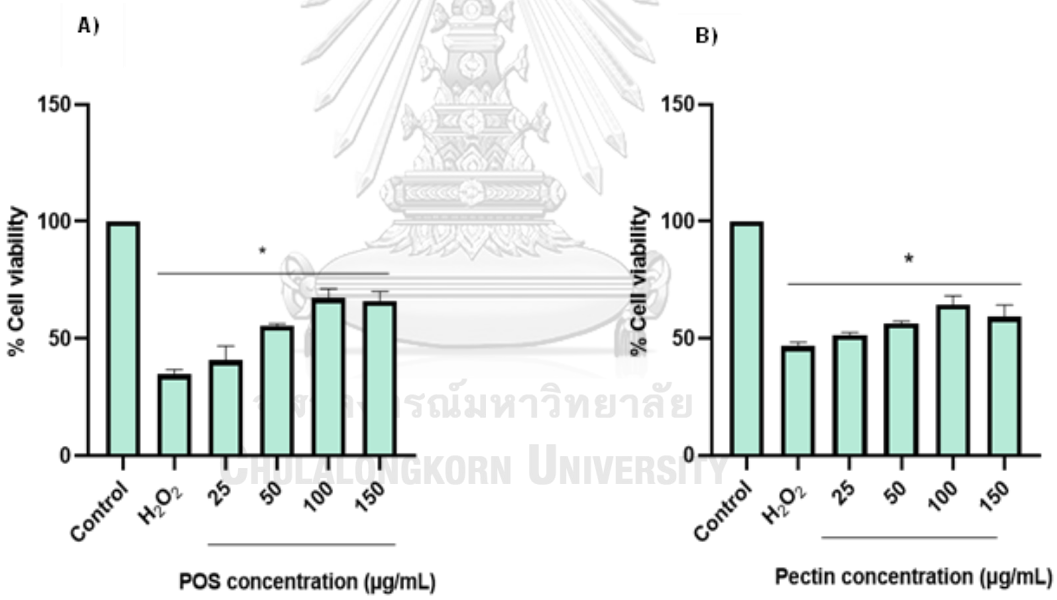

Figure 21. Protective effects of POSs and pectin against H_2O_2-induced oxidative stress damage on HaCaT cells.

*P < 0.05, Dunnett's multiple comparisons test, n=3 (biological replicates), mean ± SD

CHAPTER IV
DISCUSSION

4.1 Confirmation of pectin extract from EID

There are several methods available for confirming pectin through analysis, including X-ray diffraction (XRD) analysis, as shown in Figure 10, and Fourier transform-infrared (FT-IR) spectroscopy, demonstrated in Figure 11. The use of these methods, referring to (FT-IR and XRD), has become widespread in recent published literature. There is literature related to the extraction of pectin from different parts such as sweet lemon peel. The physicochemical and structural of pectin from sweet lemon peel was rich in galacturonic acid, high in molecular weight and low in degree of esterification. Also, the FT-IR results confirm the major presence of low methylated galacturonic acid rich structure in the isolated samples (Rahmani et al., 2020). The FT-IR spectrum in our study is very similar to previous studies, showing that the broad and extensive signals ranged from 3000 to 3500 cm^{-1} are attributed to intramolecular hydroxyl groups. The characteristic signals at 1700 and 1609 cm^{-1} were due to the esterified and free carboxyl groups of GalA units. These two peaks represent the DE value of durian pectin. Assuming DE measurement results and indicating that durian pectin similar to sweet lemon peel can be considered as low methoxyl (LM; DE < 50%) pectin. Based on previous studies (Rahmani et al., 2020), there was confirmed that the extracted samples exhibit a dominant presence of a low methylated galacturonic acid-rich structure. Moreover, the FT-IR spectrum of durian pectin corresponds to the spectrum of standard pectin that was purchased from Yarrow Chem (Mumbai, India). It showed characteristic absorption peaks at 1745 cm^{-1} and 1641 cm^{-1}, corresponding to the carboxyl ester and carboxylate groups, respectively (Lijun Tian et al., 2020), which are close to our study. FT-IR spectra provide useful information about chemical changes in cell wall components during the plant development stage (Manrique & Lajolo, 2002).

The results showed that pectin methyl esterase activity increases during ripening. In addition, Probably, pectin are the cell wall components that change most during fruit softening, but their role in fruit firmness and softening is considered controversial (Paniagua et al., 2014). It is also possible that the FT-IR spectrum of durian pectin in this study was affected by the methyl group and ripening stage, resulting in differences compared to other fruits.

According to the X-ray diffraction (XRD) spectroscopy was applied to obtain further information about the structure of pectin. The XRD pattern of pectin from sweet lemon peel was showed structural analysis (crystalline or amorphous). The sharp signals observed at 14.91, 19.61, 21.36, and 32.46 (2θ) are attributed to the crystallinity of certain portions in the pectin structure (Rahmani et al., 2020). Previous research based on the XRD effect has found that durian pectin in this study exhibits very similar sharp signals at 14.27, 20.47, 32.15, and 40.21 (2θ). Previously, the crystallinity of apple pomace pectin (Kumar & Chauhan, 2010), *Musa sapientum* L. pectin (Suvakanta, Narsimha, Pulak, Joshabir, & Biswajit, 2014), *Tamarindus indica* L. pulp pectin (Sharma et al., 2015), citrus peel pectin (Jiang et al., 2012) and grapefruit peel pectin (Wang et al., 2016) were also reported. We suggest that the decrease in pectin molecular weight primarily influences the XRD patterns. This is because the arrangement of atoms or molecules in the crystal lattice affects the XRD pattern, and different crystal structures generate distinct diffraction patterns.

4.2 Optimal condition for pectin extracted from EID

The yield of pectin is significantly influenced by various extraction parameters, including pH, temperature, extraction time, amount of raw material, and the drying of raw materials, as shown in Figure 12. The extraction of pectin from natural sources can be a laborious and time-consuming procedure due to the demanding nature of the raw materials involved in the extraction process. Firstly, the effect of drying method on pectin extraction from grape pomace was studied to investigate the impact of different drying methods. This study was conducted considering that grapes are a rich source of phenolic acids and pectin content. The results revealed that freeze-dried red grape pomace peels exhibited a higher yield compared to oven-dried samples (Tseng & Zhao, 2012). Several previous studies have demonstrated that the freeze-drying method produces a higher yield percentage of extracted pectin from raw materials compared to the oven-drying method such as (Boateng & Yang, 2021), (Qin et al., 2019) and (Monsoor, 2005). Meanwhile, previous studies have defined pectin extraction with acids such as nitric, hydrochloric, sulfuric, phosphoric, and citric acid. Pectin was extracted under various conditions (pH 7.0, 4.0, 2.5, 1.5, and 1.0; extraction periods 1–3 h) the highest yield is obtained after 1 h of extraction at pH 2.5 (Mollea et al., 2008). In addition, subsequent water extraction of pectin was used as a tool to release and gently dissolve pectin and hemicellulose from the insoluble cell wall matrix. The investigation into the solubilization of cell wall polysaccharides revealed novel characteristics of the primary plant cell wall. Pectin from carrot, tomato, and strawberry, the quantity of water-soluble material increased as the extraction time was increased (Broxterman & Schols, 2018). Although the acid pectin extraction yields large amounts of pectin, it may be an inappropriate and dangerous condition for further cell culture assays and pH of the extract is 5, which is the active enzyme range. So, it's easy to try the next experiment. Most pectin is typically extracted at high temperatures, but in our study, pectin extraction was conducted at room

temperature. This approach was chosen as it is considered a green method similar to the method of (Roy et al., 2023), (Du et al., 2023). Additionally, pectin extraction yield increases following an increase in the solid to liquid ratio through an increase in the dissolution capacity (Belkheiri et al., 2021). Similarly to our study, the results showed that increasing the amount ratio of raw material, as described in Figure 12 C), also resulted in an increased percentage yield. Currently, the use of fruit and vegetable waste is studied to obtain bioactive compounds, through non-conventional techniques, also known as green extraction techniques. These extraction techniques yield higher quantities, require less extraction time, and enhance the overall efficiency of the process for obtaining bioactive compounds. These compounds can be utilized in the cosmetic, pharmaceutical, or food industry. Furthermore, pectin extraction from early immature durian fruit using water has not been studied yet. Our study is interesting because it can obtain pectin content that is comparable to that obtained from alternative sources.

4.3 POSs production using enzymatic

There have been many previous studies using enzymatic hydrolysis of pectin. Much of the study has been done on pectin from the hearts of sunflower, apple, orange and hawthorn and okra. Currently, pectin oligomers can be acquired through various approaches such as physical methods (ultrasonic or high-pressure treatment), chemical methods (acidic hydrolysis), and enzymatic methods (using pectinase). Among these techniques, pectinase treatment is favored for its gentle depolymerization process and the generation of consistent oligomers. (Guo et al., 2023). Characterization of the pectin oligosaccharide (POSs) structures, including molecular weight distribution (kDa), uronic acid content (wt%), degree of esterification (DE %), and neutral sugar composition (wt%), has been shown in previous studies. However, in our study, we have confirmed the presence of pectin and the amount of POSs through enzymatic digestion. In addition, the incubation time of 0.3-3 hours was studied and, found that GalA was also administered at 0.3 hours (Babbar et al., 2017). Therefore, in our study, a pectin digestion time of less than 0.3 h was found to allow the product to be POSs as well.

Pectin oligosaccharides (POSs) were obtained through enzymatic hydrolysis using Pectinex Ultra SP-L® (Novozymes, Bagsværd, Denmark), exo-Polygalacturonase (Megazyme, Ireland), and Viscozyme® L (Merck KGaA, Darmstadt, Germany) at a concentration of 1% (v/v) for 10 minutes. The TLC results from Figure 4 confirm that under these conditions, pectin can be completely digested into POSs. When comparing the characteristics of the pectin extract digested for different durations of 5, 10, 20, and 30 minutes, it was observed that the resulting pectin oligosaccharides (POS) were the same. Furthermore, after a 24-hour digestion time, the pectin extract was successfully converted to monomers. However, the extract digested for 5 minutes exhibited higher viscosity compared to the extract digested for 10 minutes using Pectinex Ultra SP-L®. This condition takes less time and gives both digalacturonic acid and trigalacturonic acid. Therefore, a digestion time of 10 minutes

was selected for the entire experiment. In previous study, have used mobile phase with containing 2:1:1 ratio of ethyl acetate/acetic acid/water in the TLC analysis and then the hydrolysis reaction products and standard oligo-galacturonate of different pectic substrates were analysed by HPLC using Zorbax-SAX column (200 ×10.0 mm, Agilent) in 0.3M sodium acetate (pH 5.0) at a flow rate of 0.9 ml/min at 40°C (Jovanović et al., 2020). Figure 14 shown the chromatographic analysis of enzymatic hydrolysates of polygalacturonic acid (PGA) and pectin substrates using HPLC and TLC revealed tri and tetra-galacturonates as the end products (Rafique et al., 2021). The use of enzymes to digest pectin extracts from various fruits into pectin oligosaccharides (POSs) has been confirmed in previous studies using TLC and HPLC analysis. This study aims to investigate the microbial degradation of pectin in apple waste, as well as the production of bioactive compounds. Furthermore, the study investigates the utilization of pectin-digesting enzymes as POSs to enhance biological activities. The generation of oligosaccharides through pectinase activity was generally identified through thin-layer chromatography (TLC) (Hosseini Abari et al., 2021). The enzymatic hydrolysis of pectin into pectin oligosaccharides (POSs) is carried out to enhance biological activities. This can be explained by the fact that Pectinex Ultra SP-L® degrades the glycosyl bonds of pectin and removes certain carbohydrates from fruit tissue.

4.4 POSs exhibits higher antioxidant than pectin

Pectin is hydrolyzed into pectin oligosaccharides (POSs) to enhance biological efficacy. Previous studies have focused on the generation of oligosaccharides from various pectin-rich feedstocks, such as citrus peel, sugar beet pulp, and potato pulp (Rabetafika et al., 2014) (Gómez et al., 2016) (Khodaei et al., 2018) (Mandalari et al., 2007). In general, pectin oligosaccharides have been investigated for their potential immunomodulatory, antioxidant, cancer cell proliferation inhibition effect of citrus pectin-oligosaccharide, and prebiotic effects (Kang et al., 2006). As we mentioned before, the biological activities of pectin oligosaccharides (POSs) and pectin derived from different sources have been explored. However, the biological activities of POSs and pectin derived from early immature durian fruit have not been studied, which is interesting for further study.

4.4.1 Antioxidant activity of POSs and pectin

To describe the optimal conditions to produce pectin oligosaccharides (POSs) with excellent antioxidant activity displayed in Figure 15. After pectin is hydrolyzed to POSs using enzyme. POSs had higher antioxidant activities (DPPH and ABTS radical scavenging activities) than pectin shown in Figure 16. We suggest that the enzyme breaks glycosidic bonds, resulting in lower molecular weights. Furthermore, studies have indicated that the structural properties of polysaccharides including glycosidic bonds and the types of sugars play an important role in antioxidant activity. Pectin is a high molecular weight polysaccharide including linear homogalacturonan (HG), highly branched rhamnogalacturonan-I (RG-I), and rhamnogalacturonan-II. Although, the high molecular weight of polysaccharide exhibits a complex structure and poor water solubility, thus limiting its biological activities (Zhu et al., 2016). Additionally, Xu et al. (2019)(Xu et al., 2019) have reported that structural characteristics such as glycosidic bonds and sugar types of polysaccharides also influence their biological activities such as prebiotic.

Another factor that we believe affects antioxidants is the presence of electron-donating groups in the structures of pectin and POSs, such as carboxyl and hydroxyl groups. These groups can enhance the antioxidant activity of phenolic compounds.

4.4.2 POSs and pectin inhibition of AGEs formation.

Pectin oligosaccharide (POSs) derived from hawthorn (*Crataegus pinnatifida* Bunge. Var.major) pectin exhibits the highest antiglycation activity, as reported by (Zhu, Wang, et al., 2019; Zhu, Zhang, et al., 2019). Previous studies have shown that free radical depolymerized polysaccharides demonstrate even stronger antioxidant and antiglycation activities compared to pectin. Furthermore, studies shown by (Ogutu & Mu, 2017) and (Hafsa et al., 2017) also support these findings. Based on the previous study, it was shown that pectin oligosaccharides (POSs) exhibit better antiglycation properties than pectin. Therefore, in our study, we observed that POS inhibits the formation of advanced glycation end products (AGEs) more effectively than pectin explained in Figure 17. This difference could be attributed to the presence of functional groups, such as carboxyl and methyl groups, as well as the molecular weight of POSs. Moreover, the bioactive compound content in POSs and pectin was not equivalent to that of the positive control, AG (aminoguanidine) was known inhibitor of AGEs. However, the exact degree of differential antiglycation activity may vary depending on the specific properties and constituents of POSs and pectin to be studied further.

4.4.3 POSs and pectin promote cell proliferation on HHDPC cells.

Dermal papilla cells are an important component of the hair follicle structure and play a crucial role in regulating hair growth and hair follicle development. In our study, as shown in Figure 19, both POS and pectin extracted from early immature durian were tested, and there were found to potentially contain polyphenols as constituents should be further analyzed. While pectin is not classified as a polyphenol, certain fruits and vegetables that contain pectin also contain polyphenols such as apple pectin and a polyphenol-rich apple (Aprikian, 2003). Moreover, polyphenol stimulates hair follicle proliferation (Kubo et al., 2020). Therefore, we suggest that these polyphenols in pectin extract from early immature durian fruit activate the transition from telogen to anagen phase. Preferably, in a study by (Huang et al., 2022), there was found that oligosaccharides with a lower molecular weight were more effective in increasing epidermal papilla cell proliferation and the expression of growth factor-related genes, specifically Insulin-like growth factor (IGF)-1, which plays an important role in regulating cellular proliferation and modulating hair growth. These effects on hair follicle cells suggest that oligosaccharides have the potential to support hair growth more effectively than pectin, possibly due to their molecular weight or their ability to promote the anagen growth phase of the hair follicle cell cycle. Additionally, our study's purpose to confirm the expression of genes involved in promoting cell proliferation.

4.4.4 Protective effects of POSs and pectin against H_2O_2-induced oxidative stress damage on HaCaT cells.

There are several ways to effectively decrease ROS (reactive oxygen species) levels in an H_2O_2-induced oxidative stress model using HaCaT cells. There are commonly used as a study model for investigating the effects of various compounds or treatments on oxidative stress and cellular responses (Cheng et al., 2021). Previous studies have investigated chito oligosaccharides with antioxidant properties against H_2O_2-induced cell injury in HaCaT cells (Zhang et al., 2020). However, POS and pectin from early immature durian fruit have not been reported the effects of pectin oligosaccharides that protect the skin from damage caused by H_2O_2 on HaCaT cell, as described in Figure 21. The antioxidant capacity of pectin can play a role in protecting against H_2O_2-induced skin damage. H_2O_2 is a type of reactive oxygen species (ROS) that can cause oxidative stress and damage to skin cells. Pectin from citrus has been shown to possess antioxidant activity and can scavenge ROS, including H_2O_2 (Wang et al., 2022). Therefore, the antioxidant capacity of pectin can contribute to the prevention of H_2O_2-induced skin damage. Consequently, the present study has demonstrated that oligosaccharides possess a significant protective effect against H_2O_2-induced oxidative damage in HaCaT cells. Furthermore, the inhibitory actions of oligosaccharides may be attributed to their modulation of the MAPKs and Nrf2/ARE signaling pathways, as well as the mitochondrial apoptotic pathway, which are associated with oxidative stress. We suggest that POSs provide better protection against H_2O_2-induced skin damage compared to pectin. This may be due to their low molecular weight, which is associated with decreased cell apoptosis. Therefore, further exploration is needed to understand the regulation of genes and their antioxidant role.

CHAPTER V

CONCLUSION

In this study, we conclude that the early immature durian fruit (EID) contains pectin based on instrumental analysis using Fourier-transform infrared spectroscopy (FT-IR) and X-ray diffraction. Additionally, we successfully extracted pectin using freeze-drying at a concentration of 50mg/ml, pH 5, and 25℃ throughout the entire experiment. We successfully hydrolyzed the pectin into POSs using commercial enzymes. The resulting POSs were further analyzed using TLC and HPLC. We performed experiments using POSs and pectin enriched with bioactive compounds, and assessed their biological activities, including antioxidant, antiglycation, protective effects against H_2O_2-induced oxidative stress on HaCaT cells, and promotion of proliferation in HHDPC cells. The results indicate that the POSs exhibited better efficacy in terms of biological activity compared to pectin. We suggest that the observed effect, which can be attributed to the smaller molecular size of the POSs, is responsible for the superior biological activity. In conclusion, pectin extracted from EID fruits using the green method and hydrolyzed POSs demonstrate effectiveness as ingredients in cosmetic products.

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