FORMULATION AND EVALUATION OF CHITOSAN/ PLURONIC F-127 HYDROGEL CONTAINING PHYCOCYANIN IN TREATMENT OF GINGIVITIS

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MASTER OF ENGINEERING

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31 October 2022

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Chawan Manaspon for his kindness guidance counselor. Reading and proofing the thesis, and guiding me how to be a good researcher to finish this research. Moreover, I would like appreciative thanks for Dr. Panwong Kuntanawat for his extract samples and information. I would like to acknowledge Prof Dr. Thanaphum Osathanon, DDS, for providing the human gingival fibroblasts cells.

Furthermore, I would like to express my sincere gratitude to Dr. Pathinan Paengnakorn for her suggestions and help. I would like to special acknowledge Mr. Patipat Kamdenlek, a doctor's degree student in Institute of Biomedical Engineering, Chiang Mai university for his great help on data acquisition, statistical analysis, and manuscript drafting.

Finally, I am grateful to my parents, they always give me a support about my studying with love and take care me all the time. I hope my parents would be pound in me when I become successful.

Mingxing Li

หัวข้อวิทยานิพนธ์	การเตรียมไฮโดรเจลจากไคโตซานและพลูโรนิก เอฟ-127 ที่บรรจุสารสกัดไฟโคไซยานินในการรักษาโรคเหงือกอักเสบ
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บทคัดย่อ

อาการเหงือกอักเสบในมนุษย์เป็นโรคทางช่องปากชนิดหนึ่งที่ก่อให้เกิดปัญหาสุขภาพในช่องปาก การรับการรักษาไม่ทันท่วงทีจะส่งผลให้เกิดการอักเสบรุนแรง แพ้ บวม แดง ถึงขั้นที่จะสูญเสียฟันได้ ไฟโคไซยานินจัดเป็นรงควัตถุชนิดหนึ่งที่ได้จากการสกัดจากสิ่งมีชีวิตจำพวกไซยาโนแบคทีเรีย มีฤทธิ์ในการต้านการอักเสบได้ งานวิจัยนี้จึงทำการพัฒนาไฮโดรเจลที่ทำการบรรจุสารสกัดไฟโคไซยานินที่สกัดได้จากไซยาโนแบคทีเรีย *Arthrospira platensis* (สายพันธุ์ C005H และ C005L) และ *Chlorella sp* เพื่อใช้เป็นระบบนำส่งเพื่อการรักษาโรคเหงือกอักเสบ คุณสมบัติทั่วไปของสารสกัดไฟโคไซยานินจากไซยาโนแบคทีเรียทั้ง 3 สายพันธุ์ มีค่าปริมาณฟีนอลทั้งหมดอยู่ในช่วง 2 ถึง 7 μg GAE/100g ค่าความบริสุทธิ์ของไฟโคไซยานินอยู่ในช่วง 2 ถึง 5 เปอร์เซ็นต์ เมื่อทำการทดสอบฤทธิ์ในการต้านการอักเสบด้วยวิธีการยับยั้งการเสียสภาพของโปรตีนและการยับยั้งการทำงานของเอ็มไซม์ลิโพออกซิจิเนส พบว่า สารสกัดไฟโคไซยานินมีคุณสมบัติในการยับยั้งการเสียสภาพของโปรตีนได้มากกว่า 85 เปอร์เซ็นต์ และยับยั้งการทำงานของเอ็มไซม์ลิโพออกซิจิเนสได้สูงถึง 70 เปอร์เซ็นต์ ในส่วนระบบนำส่งแบบไฮโดรเจลได้ถูกสร้างขึ้นจากไคโตซานและพลูโรนิก เอฟ-127 อาศัยความแตกต่างของประจุในการขึ้นรูปเจลที่สามารถบรรจุสารสกัดไฟโคไซยานินได้ปริมาณที่สูง รวมถึงมีความเสถียรของเจลถึง 2 เดือน เมื่อทำการเก็บรักษาไว้ที่อุณหภูมิห้อง โดยไฮโดรเจลที่ทำการบรรจุสารสกัดไฟโคไซยานินทั้งสามชนิดมีค่าการปลดปล่อยในสารละลายน้ำลายเทียมที่ 6 ชั่วโมง เมื่อเทียบกับปริมาณที่บรรจุเริ่มต้นถึง 70 เปอร์เซ็นต์ โดยไฟโคไซยานินที่ปลดปล่อยออกมาจากระบบไฮโดรเจลมีฤทธิ์ในการยับยั้งการทำงานของเอ็มไซม์ลิโพออกซิจิเนสได้ในช่วง 15 ถึง 47 เปอร์เซ็นต์ ทั้งนี้ระบบไฮโดรเจลนี้ไม่เป็นพิษต่อเซลล์สายพันธุ์ชนิด L929 และเซลล์เหงือกปฐมภูมิจากมนุษย์ โดยรวมระบบนำส่งสารสกัดไฟโคไซยานินชนิดไฮโดรเจลที่ทำจากไคโตซานและพลูโรนิก เอฟ-127 ไม่มีความเป็นพิษและยังคงมีฤทธิ์ในการยับยั้งการอักเสบได้ จัดได้ว่าเป็นอีกทางเลือกหนึ่งสำหรับการพัฒนาไฮโดรเจลเพื่อใช้ในการรักษาโรคเหงือกอักเสบต่อไป

Thesis Title	Formulation and Evaluation of Chitosan/Pluronic F-127 Hydrogel Containing Phycocyanin in Treatment of Gingivitis
Author	Mr. Mingxing Li
Degree	Master of Engineering (Biomedical Engineering)
Advisor	Dr. Chawan Manaspon

ABSTRACT

Gingivitis is one of the gum inflammatory diseases affecting the health of the human oral cavity. Delay treatment initiation of gingivitis increases progression (irritation, redness, and swelling) and development of periodontitis which may lead to cause tooth loss or drop. Phycocyanin (PC) in the cyanobacterial crude extracts is a major pigment constituent with significant anti-inflammatory property to treat gingivitis. This thesis aimed to develop and evaluate biocompatible hydrogels encapsulating crude extracts from *Arthrospira platensis* (C005H and C005L) and *Chlorella sp* as a drug delivery system for the treatment of gingivitis. The results showed the total phenolic content (TPC) value of the crude extracts was in the range of 2 to 7 μg GAE/100g, the purity was in the range of 2~5%, and it exhibited more than 85% inhibition in the protein denaturation inhibition assay, and over 70 % inhibition tested using lipoxygenase (LOX) assay. Hydrogels containing PC crude extracts were perfectly prepared by the electrostatic interaction between chitosan and pluronic F-127. The stability of the chitosan/pluronic F-127 hydrogels were confirmed upto 2 months at room temperature. PC-loaded hydrogels showed an over 70% cumulative release within 6 hours under artificial saliva conditions. Moreover, the release from PC-loaded hydrogel still presented anti-inflammatory

potential. The anti-inflammatory activity of the supernatant of the released solution from the hydrogels after 6 hours was approximately 15-47 % by LOX inhibition assay. The hydrogel carrier and loaded hydrogel did not exhibit any cytotoxic on mouse fibroblast cell lines (L929) and human gingival fibroblast cells. This study showed that crude extract PC-loaded chitosan/pluronic F-127 hydrogels were biocompatible with those cells and indicated stability at room temperature for up to 2 months. Overall results suggested that the crude extract PC-loaded chitosan/pluronic F-127 hydrogels were biocompatible, inexpensive, effective, and could be used as an alternative application for treating gingivitis.

CONTENT

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

CH	*Chlorella sp*
C005H	*Arthrospira platensis*, wild type spirulina, helical trichomes
C005L	*Arthrospira platensis*, straight trichomes or developed strain
DMSO	Dimethyl sulfoxide
GAE	Gallic acid equivalents
GF	Human gingival fibroblasts
LOX	Lipoxygenase
L929	L929 cell line
MTT	3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide
PBS	Phosphate buffer saline
PC	Phycocyanin
PEG	Polyethylene glycol
TPC	Total phenolic compounds
UV	Ultraviolet
UV/VIS	Ultraviolet–visible

CHAPTER 1

INTRODUCTION

1.1 Gingivitis

Gingivitis or gingival inflammation is a kind of non-destructive disease that causes inflammation of the gums. The progress of this symptom is caused by the endogenous gram-negative periodontal bacteria which is attached to the surface of the tooth. This kind of bacteria will make the gum shrink along with the teeth upwards, a pocket is formed between the teeth and gums [1]. The bacteria can hide in these pockets, making the bones around the teeth fragile and even causing the teeth dropping (periodontitis).

One of the causes of gingivitis is induced by dental plaque (bacterial plaque), which can trigger a host reaction in the body. This infection may lead to the destruction of gum tissue, which in turn may lead to the destruction of periodontal attachment. The structure of periodontal teeth has been shown in Figure 1.1. It is a small pocket between teeth, where the dental plaque accumulates inside and finally resulting in situations including large, overhanging repair edges, hooks for removable partial dentures, and calculus (tartar) formed on the teeth [2]. These changes are actually due to plaque bacteria producing chemical substances such as degrading enzymes and toxins that can cause inflammation, the inflammation leads gums to get periodontal diseases.

Figure 1.1 Structure of periodontal teeth [3].

1.2 Treatment of the gingivitis

The treatment of gingivitis entails the alleviation of the acute symptoms and elimination of all other periodontal diseases, both chronic and acute, throughout the oral cavity [4]. The treatment is not complete if the periodontal pathologic changes or factors capable of causing them are still present. Eliminating inflammation is a very important part of the treatment process.

Gingival inflammatory means inflammation of the gums or gingiva. The most common cause of gingivitis is the accumulation of bacterial plaque between and around the teeth. The plaque triggers an immune response, which, in turn, can eventually lead to the destruction of gingival, or gum, tissue [5]. It may also, eventually, lead to further complications, including the loss of teeth. Whereas, dental plaque is a biofilm that accumulates naturally on the teeth. It is usually formed by colonizing bacteria that are trying to stick to the smooth surface of a tooth. It can cause tooth decay and periodontal problems such as gingivitis and chronic periodontitis.

Thus, anti-inflammatory drugs can effectively treat gingival inflammatory diseases. There are some existing products including Kanolone®, Peridex™, Arestin®, that can use topically or orally to eliminate bacterial infections. However, they more or less have side effects such as nausea, vomiting, and rash. Kanolone® is widely used for the treating of gingival inflammation in Thailand. It is a local drug delivery system contains triamcinolone acetonide which is a synthetic corticosteroid drug. Triamcinolone acetonide suppresses the inflammation by inhibiting leukocyte and monocyte migration and decreases the expression of cyclooxygenase and lipoxgenase. [6, 7]. It should be noted that patientswith allergic to adrenal corticosteroids are not suitable for this drug [8].

1.3 Subgingival drug delivery system

Traditional drug delivery methods such as an oral administration do not give gingival effective treatment due to the duration of the drug in the oral cavity and the first-pass effect that has been found in the livery. Therefore, the local drug delivery system which is used to add more time for an interaction between drug and target is introduced as an alternative approach [9]. Using delivery systems, drugs or compounds are protected by the carrier from environments such as saliva washout, heat, or solution. Another benefit of the delivery system is maintaining the concentration of drugs within the therapeutic index.

An alternative approach, nowadays, is the direct delivery of agents to the tissues by controlled release or infusion. Polymeric delivery systems can be applied at specific sites, localizing therapy to the oral region. Polymers that for controlled release drugs and be degraded in biological environments are required for this system.

In this work, we reported the preparation of hydrogel containing phycocyanin for anti-inflammation as a local delivery system. The phycocyanin-loaded hydrogel could be applied on gum tissue within 6 hours to treat gingivitis. Encapsulation and *in vitro* characterization studies were performed in phosphate buffer saline (PBS) and artificial saliva. *In vitro* cytotoxicity was examined using fibroblast cell line (L929) and gingival fibroblast from the human (primary cell). Here, we believed that the developed drug delivery system can provide patients with more comfortable and effective treatment.

1.4 Objective and outcomes

Objective

- To investigate the anti-inflammatory efficacy of phycocyanin from cyanobacteria (*Arthrospira platensis* (Spirulina), developed *Arthrospira platensis* strain, and *Chlorella sp*)
- To fabricate and characterize the hydrogel made from chitosan and pluronic F-127
- To evaluate the properties of phycocyanin-encapsulated hydrogels and *in vitro* cytotoxicity of the hydrogel using L929 cell line and human gingival fibroblast cells.

Outcome

- Hydrogels made from chitosan and Pluronic F-127 were prepared and incorporated with the phycocyanin crude extract. The phycocyanin-loaded hydrogel could be used to treat gingivitis

CHAPTER 2

LITERATURE REVIEWS

2.1 Cyanobacteria & Phycocyanin

2.1.1 Cyanobacteria

Cyanobacteria are the earliest photosynthetic oxygen-evolving organisms and have played a huge role in changing the surface of the earth from an oxygen-free atmosphere to an aerobic environment. Since thousands of years ago, Southeast Asia people have had a habit and history of eating cyanobacteria, which are used to treat and prevent diseases. In recent years, researchers have paid attention to cyanobacteria due to its potential and less side effects to human. Cyanobacteria have a high value in biotechnology as human and animal food, food coloring, and dietary supplements [10]. Recent research results show that cyanobacteria drugs have shown great potential as anticancer agents, antibiotics, anti-inflammatory agents, and anti-aging agents. It is a natural resource that can be used.

In Table 2.1, the general information and characteristics of the following cyanobacteria will be investigated for this study. *Arthrospira platensis* (C005H, wild type spirulina, helical trichomes) and *Chlorella sp* are usually found in a local environment in Thailand. Thai people also use them for additional food substances. However, Asst Prof Panwong Kuntanawat, Ph.D. and team from Suranaree University of Technology have developed novel strain of *Arthrospira platensis* (C005L, straight trichomes or developed strain) using his biological approach [11], straight trichomes were found immerged from the C005H culture, a single trichrome was reisolated and renamed as C005L. They found the pigments which are the main components in cyanobacteria of the new strain might be used in the medicine field. The

pigment they reported is phycocyanin or PC. The images of *Arthrospira platensis* strain C005H and C005L are shown in Figure 2.1.

Table 2.1 Appearance characteristics of four species of cyanobacteria [11-15].

Species	**C005H**	**C005L**	***Chlorella sp***
Length (μm) (Median ± SD)	$37 \pm 58 \times 10^3$	$2 \pm 3 \times 10^3$	2 - 10
Shape	Helical trichomes	Straight trichomes	Round
Color	Blue-green	Blue-green	Green

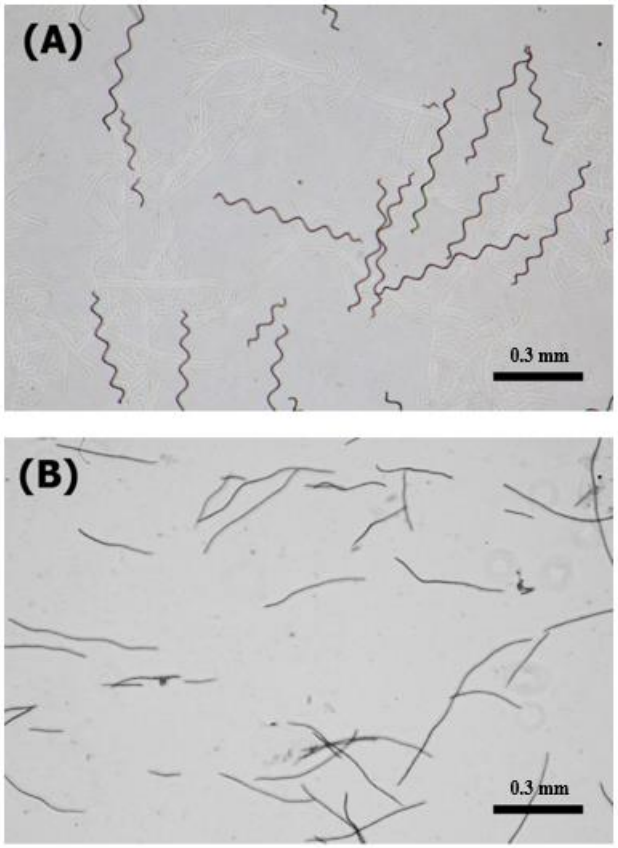


Figure 2.1 Morphology structure of *Arthrospira platensis* (A) C005H strain and (B) C005L strain [11].

2.1.2 Phycocyanin

Phycocyanin (PC) is one of the major pigment constituents of *Spirulina* sp, cyanobacteria used in many countries as a dietary supplement, its nutritional and therapeutic values have been very well documented around the world. PC can be directly extracted from the cyanobacteria using different techniques such as the acid extraction method and ultrasonic-associated extraction method [16]. The chemical structure of phycocyanin is shown in Figure 2.2.

Phycocyanin powder is stable at ambient temperature (25 $\pm$ 2°C). The effect of temperature on the stability of phycocyanin shows that it is very unstable at 45°C and higher. It is stable for a long time at 10°C and 4°C. Below and above the pH in the range of 5-7.5, the phycocyanin gradually loses its color. This information indicates that fresh biomass is most suitable for the extraction of phycocyanin. Acid extraction can be used to directly extract the PC from cyanobacteria [17, 18].

PC presents obvious cell and tissue protection potential, especially against oxidative stress, which is particularly associated with inflammation, neurodegenerative diseases, cancer, and reperfusion injury diseases. It shows an excellent anti-inflammatory potential, which is a series of multi-site effects such as removing various reactive oxygen species (ROS) and inhibiting the protein denaturation or enzyme [19]. PC also shows low cytotoxicity in rats and mice, the lethal dose (LD) 50 values was estimated to be greater than 3 g/kg for rats [20].

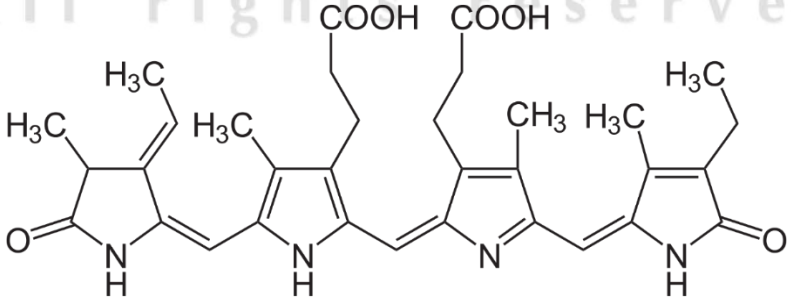


Figure 2.2 Chemical structure of phycocyanin [18].

2.2 Total phenolic compound (TPC)

Phenolic compounds are secondary metabolites naturally produced during soaking and germination. Phenolic compounds have antioxidant properties such as reactive oxygen species scavenging and inhibition, electrophilic scavenging, and metal chelation. Phenolic hydroxyl groups are good hydrogen donors and can react with organic radicals of oxygen and nitrogen. This reaction forms stable free radicals, delaying or destroying the oxidation of organic free radicals. Due to their antioxidant properties, phenolic compounds are beneficial in diabetes, cardiovascular, and neurodegenerative diseases, mutagenesis, and carcinogenesis [21]. Antioxidant properties are also closely related to anti-inflammatory capabilities. Oxidative stress is seen as an imbalance between the production of reactive oxygen species (ROS) and their elimination through protective mechanisms, which can lead to chronic inflammation. Therefore, the stronger the antioxidant capacity, the stronger the anti-inflammatory capacity. Measuring total phenolic compounds can help study anti-inflammatory capacity. For example, Hidayati JR et al. (2019) reported the total phenolic compound of *Tropical Sargassum sp.* extract and measured this value using gallic acid reagents as a standard compound. They used an extract solution in ethanol following with the addition of Folin Ciocalteu reagents. The solution was left for 5 minutes then added 7.5% Na_2CO_3 solution and incubated at room temperature for 2 hours in dark conditions, finally measured absorbance at 720 nm using a spectrophotometer. Results showed the highest content was found in ethyl acetate extract with a value of 120.29 $\pm$ 0.404 mg GAE/g sample [22].

Johari MA and Khong HY (2019) measured the TPC of *Pereskia bleo* extract which was performed by combining their extract solution (1 mg/ml) with Folin–Ciocalteu reagent. The mixture was then added Na_2CO_3 solution. After 90 minutes, the absorbance of the mixture was measured using the UV-Vis spectrophotometer at 725 nm. The results showed that the methanolic extract exhibited higher TPC as compared to the chloroform and hexane extracts which are approximately about 40.82 mg GAE/g for methanolic extract, 31.91 mg GAE/g for chloroform extract, and 25.2 mg GAE/g for hexane extract [23].

2.3 Anti-inflammation

2.3.1 Inflammation

Inflammation is a common pathology that can occur in various organs of the body. This is a protective strategy, which in the response to the invasion of a harmful substance, and is shown as redness, swelling, heat, and pain. Inflammation can be classified as an infectious inflammation that is caused by infection or non-infectious inflammation caused by other responses. Inflammation can restore cellular homeostasis when the body faces any destructive situation. Inflammation is beneficial in most cases, but sometimes inflammation can also have harmful effects, there will be abnormal immune function, taking the body tissues as foreign, and producing antibodies or immune cells attacking host cells or tissues. This response is called an autoimmune response. Inflammation occurs very quickly by any stimulants (microbial infection, foreign invaders, or any irritant) and leads to inflammation in minutes [24].

2.3.2 Mechanism of inflammation

The metabolism of arachidonic acid plays an important role in the mechanisms of inflammation. It is metabolized in two different pathways: one is the Cyclooxygenase (COX) pathway to prostaglandins and thromboxane A2; the other is the 5-Lipoxygenase (5-LOX) pathway to eicosanoids and leukotrienes (LTs), which acts as chemical mediators in a series of inflammatory events [24, 25]. Figure 2.3 shows the pathway of inflammation.

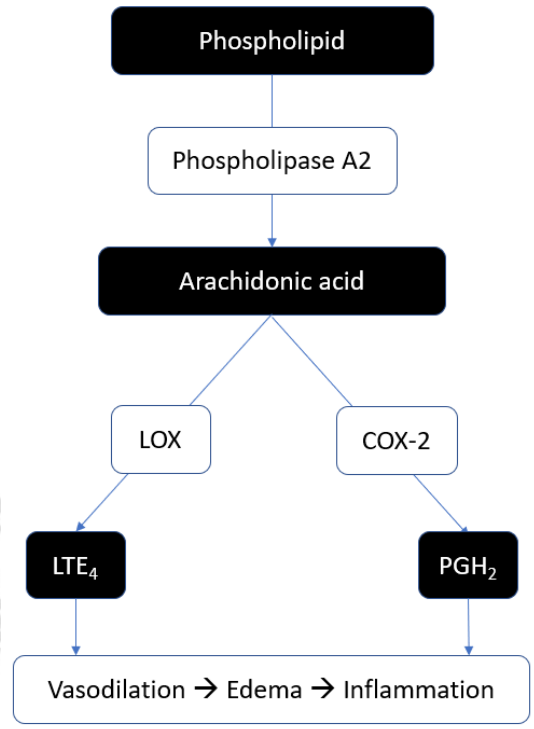


Figure 2.3 Pathway of inflammation [24].

Reactive oxygen species (ROS) are involved in a diversity of important pathological processes in medicine including among others: inflammatory and neurodegenerative diseases, atherosclerosis, cancer, and reperfusion injury. Some studies have shown through different experimental methods that PC is an effective oxygen radical scavenger and can also react with other pathological oxidants such as HOCl and $ONOO^-$ [26]. Many diseases are accompanied or even caused by oxidative stress, which is characterized by excessively formed ROS, and these oxidations cannot be offset by the organism's antioxidant defense system. Therefore, the therapeutic use of natural or synthetic antioxidants seems promising.

2.3.3 The mechanism of phycocyanin in the anti-inflammatory process

Phycocyanin is a phycobiliprotein; antioxidant, anti-inflammatory, neuroprotective, and liver-protective effects have been experimentally attributed. It is evaluated as an antioxidant *in vitro*, it can scavenge alkoxy, hydroxyl, and peroxy radicals, which can react with peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl). Phycocyanin also inhibits microsomal lipid

peroxidation induced by Fe^{2+}-ascorbic acid or the free radical initiator 2,2 azobis(2-amiylpropane) hydrochloride (AAPH). PC reduces edema, histamine (Hi) release, myeloperoxidase (MPO) activity, and prostaglandin (PGE2) and leukotriene (LTB4) levels in inflamed tissues. These anti-inflammatory effects of PC may be due to its scavenging properties of oxygen-responsive species (ROS) and its inhibitory effect on COX-2 and LOX activity and the release of Hi from mast cells [17, 18].

2.3.4 Subgingival disease or gingivitis

Subgingival disease is caused by inflammatory responses. It is a disease that troubles a large number of people. According to the statistical data, around 8.52% of adults aged 20 to 64 face gingivitis [27]. Gingivitis is one of the most common diseases in the oral cavity, which is an infection and inflammation of the gingiva. If not detected and treated in time, gingivitis may get worsen and become periodontitis, and the inflammation may also spread to the periodontal ligament and alveolar bone. An alteration in the composition of subgingival microbiota is one of the most common causes of gingivitis and smoking increases the risk of gingivitis. Gingivitis should be treated with caution. If it is not treated in time, it will become more serious. The gums will be pulled away from the teeth, forming periodontal pockets, teeth will loosen and fall off, and alveolar bone loss will occur.

The traditional treatment for gingivitis is to use scaling to remove tartar and bacteria from the tooth surfaces and gums. Antibiotics, including erythromycin, spiramycin, and azithromycin, can be used topically or orally to eliminate bacterial infections. Figure 2.4 shows the introduction of scaling and root planning. However, the inflammation at the gingiva could be treated either due to the progress of inflammation at the gum will lead to periodontal disease in patients.

Figure 2.4 Scaling and root planing [28].

2.4 Local delivery system and its properties

2.4.1 Local drug delivery system

A local drug delivery system (LDDS) is defined as a preparation or device which makes the substance reach its site of action and does not reach the non-target cells [29]. It is an emerging research field with minimal to no side-effects of vehicles for drugs or compounds used in humans [30]. The LDDS can provide a 100-fold higher concentration of drug at the target area compared to oral dosage. Thus, LLDS can reduce the total dose of patients by more than 100 times, thereby reducing the potential problems of using systemic antibiotic drug treatment programs and producing resistant bacterial populations. Compared with oral administration, local drug delivery in the oral cavity might skip the elimination or first-pass effect of the gastrointestinal tract, mainly the liver, which can maintain the efficacy for a long time and reduce side-effects for some cases.

A hydrogel is planned to use as the carrier in this study. It is a kind of polymer with a three-dimensional network structure that has hydrophilic groups and can be swelled by water but is insoluble in water. It can absorb a large amount

of water in water and swell significantly, and can continue to maintain its original structure without being dissolved after swelling; good biocompatibility, excellent physical and mechanical properties, and long-term implant stability. It generally uses to sustain or provide slowly release of entrapped drugs. Herein, there are two polymers used in this work, pluroinic F-127 and chitosan, which are mentioned below.

Pluronic F-127**,** also called as Poloxamer 407, is a hydrophilic non-ionic surfactant, which is a relatively common copolymer. It is composed of polyoxyethylene (A) and polyoxypropylene (B) to form an ABA triblock copolymer. The approximate length of the two polyethylene glycol (PEG) blocks is 101 repeating units, while the approximate length of the propylene glycol block is 56 repeating units. Pluronic has been approved by the US Food and Drug Administration (FDA) for use in humans and has been widely used as a drug carrier and tissue engineering [31]. It is mainly used for preparing hydrogels. The gelation mechanism of pluronic F-127 is believed to occur through the formation of micelles at a critical temperature, leading to dehydration. The photo and chemical structure of pluronic F-127 are shown in Figure 2.5. Turabee MH et al. (2019) fabricated docetaxel-loaded hydrogels from pluronic F127 and N,N,N-trimethyl chitosan to treat brain tumors. They investigated the hydrogels under different pH to sustain the release of docetaxel. The released docetaxel significantly inhibited the glioblastoma (U87MG) xenograft tumor growth in mice [32]. Deliormanlı AM and Türk M (2020) reported thermo-responsive injectable hydrogels prepared using pluronic F127 block-copolymer and 13-93B3 bioactive glass particles for bone tissue engineering applications. The hydrogels presented the sol-gel transition at around 25 °C. Drug release studies showed that the addition of bioactive glass and carbon-based nanoparticles improved the drug release behavior of the prepared hydrogels [33]. Youn J et al. (2021) developed a pluronic F-127/silk fibroin hydrogel which enhanced the intermolecular interaction by physical crosslinking. The pluronic F-127/silk fibroin hydrogel showed sol-gel transition at 4 and 37 °C, and the cumulative release of pure pluronic F-127 was improved by the intermolecular interaction

of silk fibroin [34]. Liu G et al. (2020) fabricated a baicalin/pluronic F-127 hydrogel for skin wound treatment. The hydrogel showed excellent cytocompatibility and *in vivo* experiments exhibited the ability of baicalin/pluronic F-127 hydrogel to accelerate wound healing [35].

Chitosan is a cationic polysaccharide, which is derived through the deacetylation of chitin. It is a low-toxic, inexpensive, hydrophilic, biodegradable, & biocompatible biopolymer. Thus, chitosan polymer is widely used in tissue engineering and drug delivery application research [36]. It is also widely used in tissue engineering and drug delivery. Chitosan is obtained from chitin and can be extracted from crabs, shrimps, and mushrooms, so its use in technical products is of ecological and sustainable importance. In addition, Chitosan also has important functions such as antibacterial, anti-cholesterol, antitumor, hemostatic, and antioxidant activities. Since it is extracted from the shells of marine arthropods such as shrimps and crabs, it cannot be used by people allergic to seafood [37]. The photo and chemical structure of chitosan are shown in Figure 2.6. Liu Z et al. (2022) reported the various preparation of chitosan-based hydrogels such as crosslinked networks ionic complexes or self-assembling vehicles for drug delivery of a therapeutic payload. Here, the developed chitosan hydrogels could control the release to high bioavailability at the site of action [38]. Wei Q et al. (2022) developed a crosslinking-based hydrogel from carboxymethyl chitosan (CMCS) and polyethylene glycol (PEG) as a wound dressing. Based on CMCS backbone of the hydrogel, the grafting of antioxidant and antibacterial agents were also confirmed using Fourier Transform Infrared Spectroscopy (FTIR) and ^{1}H NMR. Results showed that the hydrogel exhibited good bovine serum albumin (BSA) adsorption capacity, cytocompatibility, and hemostatic properties. Suggested that the CMCS/PEG hydrogel can be used as a promising wound dressing for other complex wounds such as chronic diabetic wounds [39]. Bagher Z et al. (2020) evaluated the skin wound healing potential of alginate/chitosan hydrogel. *In vitro* cell growth study confirmed that the cells of the test group had high cell proliferation than the control group. The test group exhibits almost 95% of

the wound was healed after 14 days for the *in vitro* results, suggesting that the prepared alginate/chitosan hydrogel was promising for successful wound treatment [40]. Xu Q et al. (2019) reported a cellulose nanocrystal/chitosan hydrogel used as a carrier for the controlled delivery of theophylline. The cellulose nanocrystal (CNC)/chitosan hydrogel presented excellent drug-controlled release behaviors which released approximately 85% at pH 1.5. Therefore, CNC/chitosan hydrogel has application potential as a theophylline carrier for gastric-specific drug delivery [41].

The interaction between chitosan and pluronic F-127 is electrostatic interaction due to the different polar charges of those polymers. Chitosan shows a positive charge, whereas pluronic F-127 has a negative surface charge [42]. The opposite charges lead to attracting force and pulling them towards each other so that the electrostatic interaction leads to the hydrogel formulation. A literature review table of previous works with hydrogel prepared by chitosan and pluronic F-127 is described in the Table 2.2.

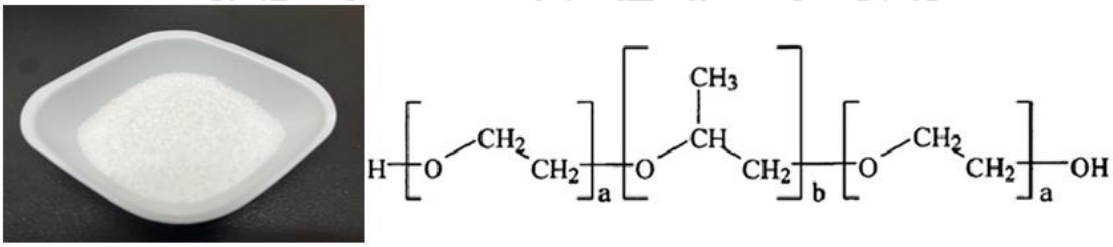


Figure 2.5 Photo and chemical structure of pluronic F-127 [43].

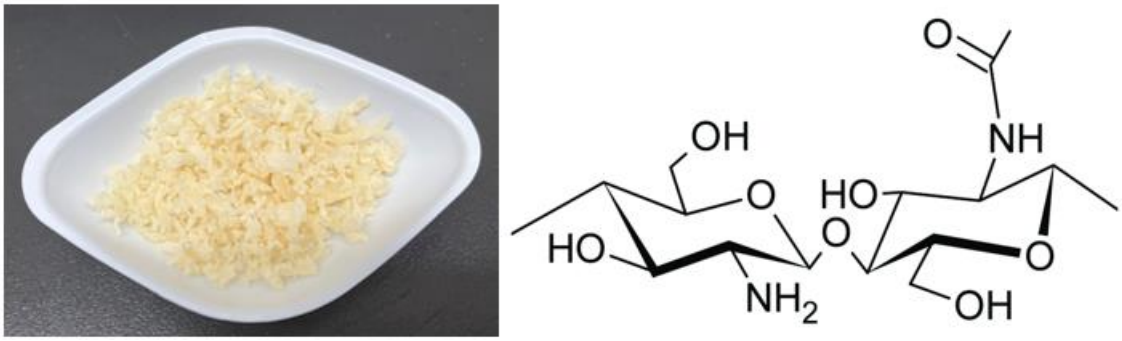


Figure 2.6 Photo and chemical structure of chitosan [44].

Table 2.2 Summary of chitosan/pluronic F-127 hydrogels.

Applications	Model Drug	Preparation method	References
Periodontitis	Prilocaine hydrochloride	Chitosan solution (PBS, pH = 5.5-6) was mixed with pluronic F-127 (0.9% NaCl, pH = 7.4) and prilocaine hydrochloride at 37°C	[29]
Topical Nitric oxide delivery	S-nitrosoglutathione	Pluronic F-127 powder and S-nitrosoglutathione were added to chitosan solution (2% lactic acid) under gentle magnetic stirring at 4 °C until complete dissolution. The mixture was kept at room temperature for hydrogel formulation.	[45]
Trichomonas vaginalis infections	metronidazole	Pluronic F-127 powder was gradually dissolved in the chitosan solution (lactate buffer) and stirred at 4 °C until complete dissolution of pluronic F-127 powder.	[46]
Colon Cancer treatment	5-Fluorouracil	Pluronic F-127 (powder form) was gradually added to chitosan solution (2% acetic acid) under constant stirring overnight.5-fluorouracil and glutaraldehyde were added and stirred for 2 hours. The gels were separated by filtration and washed with n-hexane.	[47]
Ocular diseases like glaucoma, dry eye syndrome	Timolol maleate	Chitosan solution (0.9% NaCl, pH = 5.5-6) was mixed with pluronic F-127 (0.9% NaCl, pH = 7.4).Timolol maleate was then added and mixed at 37°C	[48]

2.4.2 *In vitro* properties of the local drug delivery

1) Anti-inflammatory assay

In vitro studies help to study cellular responses in closed systems that maintain experimental conditions. These *in vitro* studies help to improve the understanding of the mechanism of the anti-inflammatory activity of herbal ingredients.

1.1) Protein denaturation assay

Protein denaturation assay is one of the most commonly used method for measuring anti-inflammatory activity. The denatured protein has the same primary structure as the original protein. The weak forces between charged groups and the weaker forces of the mutual attraction of nonpolar groups are disrupted at elevated temperatures, as a result, the tertiary structure of the protein is lost [49]. By measuring the absorbance using a spectrophotometer, the anti-inflammatory ability of the substance can be shown. Protein denaturation is associated with the formation of inflammatory diseases such as rheumatoid arthritis, diabetes, and cancer. Therefore, the ability to prevent protein denaturation may also help to prevent inflammatory disorders [50].

Generally, natural protein is affected by physical or chemical factors, and the original specific conformation of the molecule changes, resulting in partial or total loss of its properties and functions. This effect is called protein denaturation. Chemical methods that can denature proteins include strong acids, strong bases, heavy metal salts, urea, acetone, etc.; physical methods that can denature proteins include heating (high temperature), ultraviolet, and X-ray irradiation, ultrasound, vigorous shaking or stirring, etc. Literature reviews show that protein denaturation is closely related to the occurrence of inflammatory activity and

leads to various inflammatory diseases including arthritis [51]. Therefore, the ability of a substance to prevent protein denaturation may also help to prevent the inflammatory disorder. The absorbance of the protein denaturation inhibition assay will be measured at 660 nm because it is used to measure the turbidity of protein. Turbidity is a measure of the degree to which the water loses its transparency due to the presence of suspended particulates, and it is usually measured at 660 nm [52]. The lower the turbidity, the lower the absorbance, so the better the anti-inflammatory activity.

Shortly, the protein denaturation assay uses bovine serum albumin (BSA) or egg albumin as a mock protein, the denaturation reaction is induced by heating. Both heating at 54℃ for 5 minutes or 37℃ for 15 minutes can cause protein denaturation. Leelaprakash G and Dass SM (2011) used the protein denaturation assay to measure the anti-inflammatory performance of *Enicostemma axillare* extract. They used aspirin as a control. The albumin was used as a model protein, the extracts were incubated at 37 ºC for 20 min and then heated to 51℃ for 20 min [53]. They measured the solution by a spectrophotometer at 600 nm. The results showed that *Enicostemma axillare* extract presented maximum inhibition at 500 µg/ml, inhibiting rate was 71%, while the standard anti-inflammatory drug aspirin showed maximum inhibition of 68% at 100 µg/ml, respectively. The extract from *E. axillare* was considered as an effective compound in inhibiting heat-induced albumin degeneration. Chopade AR et al. (2012) measured the anti-inflammatory of *Phyllanthus amarus* Linn, a perennial annual herb by protein denaturation inhibition assay. The group with no drug was used as a control group. Results showed that *Phyllanthus amarus* Linn extract (50-200 µg/ml) significantly inhibits the degeneration of egg whites in a concentration-

dependent manner [54]. Compared with the control group, the *Phyllanthus amarus* Linn extract at a concentration of 200 µg/ml and the indomethacin at a concentration of 100 µg/ml showed significant inhibitory effects on protein denaturation, inhibiting 54.16% and 82.83%, respectively.

1.2) LOX inhibition assay

LOX inhibition assay is another method for measuring anti-inflammatory performance, and it is also widely used by researchers. As the inflammation reaction involves 5-Lipoxygenase, the mechanism of metabolism into pro-inflammatory substances (leukotriene, Leukotriene A4, LTA 4), which can be used to screen the anti-inflammatory activity of comparative drugs by inhibiting 5-LOX in the reaction, can be used to compare the anti-inflammatory activity of drugs. Lipoxygenase is an oxidoreductase containing non-heme iron that specifically catalyzes the double addition of polyunsaturated fatty acids with cis and cis-1,4-pentadiene structures such as arachidonic acid and linoleic acid (LA) Oxygen reacts to produce leukotrienes, hydroxytetracotetraenoic acid, and other conjugated unsaturated fatty acid hydroperoxides.

5-LOX catalyzes the metabolism of Arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and Leukotriene A4 (LTA_4) 12, PhospholipaseA2 (phospholipase) in mammals A2) After hydrolysis, LTB4 is produced, which promotes the adhesion of neutrophils to the walls of veins and capillaries, increasing the degree of inflammation [55]. Therefore, inhibition of 5-LOX activity can inhibit the metabolism of arachidonic acid, treat inflammation, and allergies related diseases. LOX inhibition assay uses linoleic acid to react with 5-LOX [56]. 5-LOX catalyzes the oxidation of Linoleic acid to convert 1,4-diene structure to 1,3-diene conjugated peroxide 1, 3-

hydroperoxylinoleic acid has a characteristic absorption at 234 nm. The degree of oxidation of linoleic acid can be determined by measuring the absorbance value. When the inhibitor is present, it will reduce the activity of 5-LOX, so that the absorbance value of the detected product at 234 nm decreases, reflecting the inhibition ability and also representing the anti-inflammatory activity. Figure 2.7 shows the pathway of 5-Lipoxygenase.

Leelaprakash G and Dass SM (2011) used linoleic acid as substrate, lipoxidase as enzyme, and indomethacin as control. They mixed the sodium borate buffer and lipoxidase enzyme solution, and incubated with extract in a 1 ml cuvette at room temperature (30 ± 2 ^{o}C) for 5 min [53]. The reaction was initiated by the addition of linoleic acid substrate, the absorbance of the reaction solution was measured at 234 nm using a UV spectrometer. The results showed the strongest inhibitory effect was obtained at a concentration of 500 µg/ml. Standard indomethacin showed 86% inhibition at a concentration of 100 µg/ml. Compared with the control, EAME did not show a significant difference (p>0.05) at the concentrations of 100 and 200 µg/ml. These showed the extracts inhibited the lipoxygenase enzyme activity.

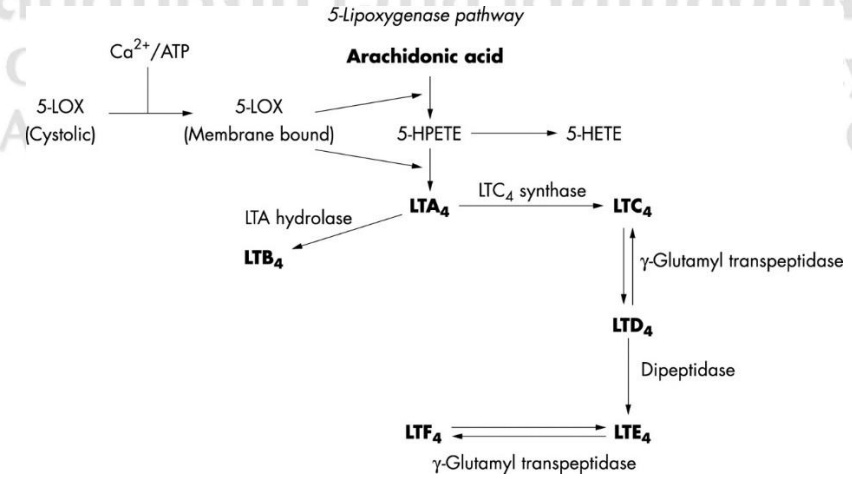


Figure 2.7 Pathway of 5-lipoxygenase [57].

2) *In vitro* cytotoxicity assay

Cytotoxicity is the quality of being toxic to cells. Before preparing a drug that can be used in the human body, its cytotoxicity must be measured to ensure that it does not cause harm to cells. Treating cells with a cytotoxic compound can result in a variety of cell fates. The cells may undergo necrosis, lose membrane integrity, and die rapidly as a result of cell lysis [58]. The cells may become apoptosis, an activated genetic program of controlled cell death. Cytotoxicity assay is a test for analyzing the cytotoxic effects of a material or medical device on a living organism. It is the earliest and simplest *in vitro* technique designed for biocompatibility evaluation.

The MTT assay is a sensitive and reliable indicator of cell metabolic activity. The MTT assay is a colorimetric assay and can be used to measure the cytotoxicity or cytostatic activity of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light [59]. It is accurate and simple enough to be the most commonly used method. This assay relies on the reduction of the yellow water-soluble tetrazole dye MTT, to purple formazan crystals mainly by mitochondrial dehydrogenase. After being dissolved in DMSO, the formazan product is analyzed by spectrophotometry at a certain wavelength (usually570 nm). The difference in absorbance of treated cells and untreated cells give an estimate of the degree of cytotoxicity. Greater the formazan concentration the deeper the purple color and thus the higher the absorbance [60]. For example, Oshiro A et al (2014) used MTT assay to measure the cytotoxicity of pluronic F-127/L-81 binary hydrogels in their project. The result showed that the hydrogel formulation only contained pluronic F-127 did not exhibit cytotoxicity. But when increasing the ratio of pluronic L-81 to 0.6%, it showed a slight effect on the cell viability (~20%) [61].

3) Drug release from the system

The drug release profile is a key factor in the performance of a drug delivery system. Drug release is a phenomenon controlled by diffusion or dissolution or both. The function of drug controlled release can be achieved through membrane permeation control system and body diffusion system. The targeted release function of drugs can be implemented by means of biological recognition mechanism, permeation mechanism, and in vitro control [9].For drug release profile of hydrogel, it can be measured by suspending the hydrogel in a glass container containing buffer and incubating on a 37°C shaker, shaking at 80 rpm. The aliquot of the solution was taken at regular time intervals, and the amount of drug released by the drug-loaded hydrogel will be evaluated [45]. Another method is to use the dialysis technique by placing the hydrogel in a dialysis tube, which suspends in a beaker containing 100 ml simulated fluid pH 7.4 and 37°C under continuous stirring. At different time intervals, aliquots of the medium were removed, and an equal volume of fresh medium was added to replace the samples taken. Dilute the taken sample appropriately, and measure the absorbance by UV. The amount of drug released by the drug-loaded hydrogel will be evaluated [29].

The formula for determination of the percentage of released drug from in vitro dissolution testing is listed below:

$$\%\ \text{Drug release} = \frac{\text{Amount of drug release (mg)}}{\text{An initial amount of drug}} \times 100$$

4) Wound healing assay/Cell migration assay

The wound-healing assay, also known as the scratch assay, is a well-established two-dimensional (2D) technique that can be used to study collective migration and wound healing *in vitro*. A scratch is made between cells in monolayer cultures to generate callus areas, which are then monitored. This phenomenon of peri-wound cell migration toward the scratch is wound healing. It can be successfully applied to model

and study cell movement in controlled in vitro environments. The technique replicates the wound by creating a gap in the confluent cell monolayer and consists of four main steps: culture preparation, scratch-making, data acquisition, and data analysis [40].

The first step in this assay is to grow a confluent cell monolayer. This monolayer represents the in vivo condition of the tissue prior to injury, such as intact epithelial cells. The next step is to create cell-free spaces in the monolayer. The most common method is to wind a single layer by mechanical scraping (scratching) or punching. Alternative methods of mechanical damage include thermal damage, electrical damage, and optical damage. Once the intercellular space is prepared, cells migrating into the wound area can be observed using a light microscope and a series of time-lapse images can be acquired. Finally, automated data analysis software, such as TScratch and ImageJ, can be used to quantify the rate of cell migration by calculating the ratio of the occupied area in the gap to the total area of the initial gap as a percentage of relative wound density over time. Hao Y et al (2022) investigated cell migration in benzaldehyde-terminated 4-arm PEG (4-arm-PEG-CHO)/carboxymethyl chitosan (CMCS)/basic fibroblast growth factor (bFGF) hydrogels (BP/CS-bFGF) conditioned medium by wound healing assay. The hydrogels were incubated in DMEM without FBS at 37 °C for 24 h. Fibroblasts were seeded in 6-well plates at a concentration of 2×10^6 cells/well. After 24 hours of cell attachment, 3 microscratch marks were made with a 20 μL pipette tip, then washed 3 times with PBS, and the hydrogel-conditioned medium was added to each well [62]. In addition, DMEM without FBS was used as a control group. Wound closures were periodically captured using an inverted microscope after 0, 6, 12, and 24 hours of incubation. Wound area and migration rate were quantified using ImageJ software. The results showed that the wound area percentages of the blank and BP/CS-bFGF hydrogels were 39.30% and 12.41%, respectively. Therefore, the

hydrogel based on BP/CS-bFGF significantly promoted the migration of fibroblasts and was beneficial to the repair of diabetic wounds.

CHAPTER 3

MATERIALS & METHODS

3.1 Chemicals and reagents

Three crude extracts of phycocyanins (PC) were prepared at the Institute of Agricultural Technology, Suranaree University of Technology. Herein, the crude extracts PC were prepared from *Arthrospira platensis* strain C005H (wild type spirulina, helical trichomes), *Arthrospira platensi*s strain C005L (straight trichomes or developed strain), and *Chlorella sp* strain CH by ultrasonic-assisted extraction method (Chaiyasitdhi et al., 2018). C005H strain was generously provided by Applied Algal Research Laboratory, Faculty of Science, Chiang Mai University. After a prolong period of time, straight trichomes were found immerged from the C005H culture. A single trichrome was then reisolated and renamed as C005L. Bovine serum albumin (BSA), phosphate buffer saline (PBS: tablet form), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and triton X-100 were obtained from Amresco (Washington, USA). Pluronic F-127 and Folin & Ciocalteu's phenol reagent were obtained from Sigma-Aldrich (Missouri, USA). Chitosan powder was obtained from Ta Ming Enterprises CO. LTD (Samut Sakon, Thailand). Acetic acid and sodium chloride (NaCl) were obtained from Merck (New Jersey, USA). Sodium carbonate (Na_2CO_3) was obtained from Ajax Finechem (Sydney, Australia). Kanolone® was obtained from L.B.S. Laboratory LTD., PART. (Bangkok, Thailand).

3.2 Hydrogels preparation and encapsulation of the crude extract PC

3.2.1 Chitosan solution with pluronic F-127 solution

Chitosan (1 g) was dissolved in 100 ml of acetic acid solution (2% v/v). The solution was then dialyzed in DI water for 2 days and normal saline for another day to normalize the pH at the natural condition. Dialyzed chitosan solution (10 ml) was mixed with pluronic F-127 solution (10%, 15%, 17.5%,

20% in PBS). The solution was mixed and kept at 4ºC overnight and at room temperature for 1 day.

3.2.2 Chitosan solution with pluronic F-127 powder

Chitosan solution was prepared following the previous section. Dialyzed chitosan solution (10 ml) was mixed with 1 g, 1.5 g, 1.75 g, 2 g of pluronic F-127 powder in a different ratio. The solution was mixed and kept at 4ºC overnight at room temperature for 1 day. After formulation, the hydrogels were kept at room temperature for up to 8 weeks with weekly observation for a stability investigation.

To prepare the crude extract of PC-loaded hydrogel, the crude extract of PC (5 mg) was dissolved in 1 ml DMSO solution (0.1% (v/v)). This DMSO solution was added to the chitosan solution. Pluronic F-127 powder was then added and prepared the hydrogel from the previous section.

3.3 *In vitro* crude extracted characterizations

3.3.1 Total phenolic compound and purity of the crude extract of PC

The total phenolic compound (TPC) of the crude extracts of PC (C005H, C005L, and CH) was determined using the Folin-Ciocalteu reagent [22]. Briefly, 100 µl (50 µg/ml) of the crude extract of PC was dissolved in deionized (DI) water and mixed with 100 µl of 1N of Folin-Ciocalteu reagent. The mixture was then left for 5 min at room temperature, followed by adding 80 µl of 5% (w/v) Na_2CO_3 solution. The reaction was plated in the dark for 1 hour. Absorbance was measured using a microplate reader spectrophotometer (HiPo MPP-96, BioSan, Latvia) at a wavelength of 568 nm. The standard gallic acid solutions (10, 25, 50, 100, 175, 250 µg/ml) were prepared and used to express all PCs in the unit of gram gallic acid equivalent (µg GAE/100g) using the following equation:

$$\mathrm{PC} = \frac{\text{Concentration from the standard curve} \times \text{Volume of the extract}}{\text{Mass of the extract}}$$

The purity PC through the extraction and purification steps were determined by measuring the OD_{620} and OD_{652}. Briefly, 1 mg of the crude extract of PC was dissolved in 20 ml of deionized (DI) water. Absorbance was measured using a microplate reader spectrophotometer (HiPo MPP-96, BioSan, Latvia) at 620 nm and 652 nm. The purity was calculated using the following equation [63]:

$$\text{Finally concentration of PC} = \frac{OD_{620} - 4.74 \times OD_{652}}{5.34}$$

$$\text{Purity (\%)} = \frac{\text{Finally concentration of PC}}{\text{The initial concentration of PC crude extract solution}}$$

3.3.2 Protein denaturation inhibition

1) Protocol 1

The crude extract of PCs at different concentrations (50 µg/ml) was mixed with 50 mg/ml of bovine serum albumin (BSA). The mixture at 1 ml was incubated at 37°C for 15 min, then heated at 54°C for 15 min [50]. After cooling down, the clear solution of the sample was measured the absorbance at 660 nm using a UV/VIS spectrometer (Genesys™ 10s UV-Vis, Thermo Scientific, USA). PBS was used as the control group. The inhibition of protein denaturation was calculated by using the following equation.

$$\text{Protein denaturation inhibition (\%)} = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2) Protocol 2

The crude extract of PCs at different concentrations (50 µg/ml) was mixed with 50 mg/ml of bovine serum albumin (BSA). The mixture at 1 ml was incubated at 37°C for 15 min, then heated at 70°C for 5 min [54]. After cooling down, the clear solution of the sample was measured the absorbance at 660 nm using a UV/VIS spectrometer (Genesys™ 10s

UV-Vis, Thermo Scientific, USA). PBS was used as the control group. The inhibition of protein denaturation was calculated by the same equation as shown in section 3.2.2.1.

3.3.3 LOX inhibition activity

The crude extract of PCs was investigated their LOX inhibitory potential by dissolving in DMSO at 50 µg/ml. The crude extract of PCs (25 µl) solution was mixed with 975 µl of LOX enzyme, 400 U/ml in 0.2 M, pH 9 borate buffer. One milliliter of the substrate (linoleic acid) at 250 µM was then added to the mixture [64]. The absorbance of the reaction mixture was measured at 234 nm at 3 min and 5 min after adding the substrate using a microplate reader (Tecan, Infinite^{TM} M200 Pro, Männedorf, Switzerland). DMSO was used as a control group, whereas quercetin and gallic acid were used as reference active compounds. The percentage of LOX inhibition activity was calculated by using the following equation:

$$\text{LOX inhibition (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

3.4 Characterization of PC-loaded hydrogels

3.4.1 Release profile

The PC-loaded hydrogels (C005H-, C005L-, and CH-loaded hydrogels) at 0.1 g was plated on a membrane filter (Whatman®) and left on the top of a 24-well plate containing 2 ml of artificial saliva (0.75 g KCl, 0.07 g $MgCl_2$, 0.199 g $CaCl_2$, 0.965 g K_2HPO_4, 0.435 g KH_2PO_4, 36 g Sorbitol, 2.4 g Sodium Benzoate, and 1,200 ml DI water, pH=7.4). At determination time points (30 min, 1, 2, 3, and 6 hours), the artificial saliva solutions were collected and refreshed with equal volume [29, 45]. The harvested solutions were measured the absorbance at 620 nm and calculated the release profile using the following equation.

$$\text{Cumulative PC release}(\%) = \frac{\text{Amount of the crude extract of the PC release}}{\text{An initial amount of the loaded crude extract of PC}} \times 100$$

The release curve was investigated and fitted with different mathematical models including Zero order, First order, Higuchi, and Hixson-Crowell model [65]. The equations of the mathematical models were shown below:

Zero order: $Q_t = Q_0 + k_0 t$

Q_t = Cumulative amount of drug, Q_0 = Initial amount of drug, K_0 = zero order release constant, t = time

First order equation: $Q_t = Q_0 e^{-kt}$

Q_t = cumulative amount of drug release, Q_0 = initial amount of drug, K = First order release constant, t = time

Higuchi equation: $Q = k_H \sqrt{t}$

Q = cumulative amount of drug release, K_H = Higuchi release constant, t = time

Hixson-crowell equation: $\sqrt[3]{Q_0} - \sqrt[3]{Q_t} = k_{HC} t$

Q_t = cumulative amount of drug release, Q_0 = initial amount of drug, K_{HC} = Hixson-Crowell release constant, t = time

Moreover, the released samples at 3 and 6 hours-incubation were then collected by centrifuge. The supernatants were used to investigate the protein denaturation inhibition and the LOX inhibition activity using artificial saliva solution as a control group. The procedures were mentioned in the previous section.

3.4.2 *In vitro* biocompatibility testing

L929 (Mouse fibroblast cell) cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA). The medium was supplemented with 10% (v/v) of fetal bovine serum (FBS, Gibco, USA) and 100 U/ml of penicillin/100 µg/ml of streptomycin (Gibco, USA). For gingival fibroblast cells (GF), cells were isolated from healthy patients using the approved protocol by the Human Research Ethics Committee, Faculty of Dentistry, Chulalongkorn University (No. 049/2020). The GF cells were cultured with the same culture medium as the L929 cell. Those cells were incubated and grown at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

1) Cytotoxicity of the crude extracts of PC

Cells (L929 or GF) were plated into 96 well plates with a density of 10,000 cells per well at least 1 day before the experiment. Serum-free culture medium was used to prepare the series dilution/various concentrations of the crude extracts of PC in the range of 0 – 1000 µg/ml. Cells were treated with several concentrations of the extracts at different periods, including 1 and 3 days at 37 ^{o}C [66]. Fresh serum-free DMEM and 1% Triton X-100 were used as negative control and positive control conditions, respectively. The cell viability of the study was evaluated by MTT assay.

2) Cytotoxicity of Chitosan/Pluronic F-127 hydrogels

The chitosan/pluronic F-127 hydrogels (with or without the crude extracts) were prepared by adding 1 ml of serum-free DMEM medium to 0.1 g of the hydrogels (following ISO 10993 part 12). The extracted medium was collected after 24 hour-incubation at 37 ^{o}C. The extracted medium was prepared at 100% and 50% by diluting with the fresh culture medium. These test media were plated to the cells (L929 and GF) which were plated into 96 well plates with a density of 10,000 cells

per well at least 1 day before the experiment [67]. All experiments were evaluated the number of cells by MTT assay after 1 day of incubation. In this study, fresh serum-free DMEM and 1% Triton X-100 were used as the control and negative control group.

3) Wound healing assay

GF cells were plated into 12 well plates with a density of 50,000 cells per well and incubated for at least 1 day to fulfill cell confluence in the well. The culture media were refreshed with the serum-free DMEM before the experiment for at least another 1 day. The cells were scraped in a perpendicular straight line using a 10 µl pipette tip (Schwitalla and Muller 2013). The migration of the GF cells was investigated by taking a series of bright-field images at the initial time and 1 day of incubation. All images were exported as 16-bit TIFF files and processed with the ImageJ (Version 1.53) program (National Institutes of Health) using a plugin "MRI_Wound_Healing_Tool.ijm" as a calculation tool.

3.5 Statistical analysis

All studies were investigated at least triplicate and presented as the mean ± standard deviation for all assays tested. A student t-test was performed to compare all data. The differences were considered to be significant at a level of $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Chitosan/pluronic F-127 hydrogel preparation

4.1.1 Chitosan solution and pluronic F-127 solution

Results of chitosan/pluronic F-127 hydrogel preparation were shown in Table 4.1. The mixture of chitosan solution (1 % w/v) with 10, 15, 17.5, and 20% (w/v) of pluronic F-127 solution did not form as gels. Although the mixture started to form the hydrogels, they still provided as low viscosity solutions. At the highest concentration of pluronic F-127 (20%) could not generate the hydrogel or act as a cross-networking molecular. It should be noted that pluronic F-127 can not be dissolved at over 20% (w/v) .

Table 4.1 Hydrogel formulation: chitosan solution (1% w/v) and various concentrations of pluronic F-127.

Pluronic F-127 (% (w/v))	Chitosan solution (1% w/v)	
	Pluronic F-127 solution	Pluronic F-127 powder
10	No	P
15	No	P
17.5	P	Yes
20	P	Yes

No: No hydrogel formulation; P: precipitation of chitosan/pluronic F-127; Yes: formulation of the hydrogel

Figure 4.1 Chitosan/pluronic F-127 hydrogel prepared by mixing 1% (w/v) of chitosan solution with (a) 20% (w/v) of pluronic F-127 solution, (b) 20% pluronic F-127 powder.

4.1.2 Chitosan solution and pluronic F-127 powder

One milliliter of chitosan solution (1% w/v) and pluronic F-127 powder (10%, 15%, /17.5%, and 20% (w/v)) were used to prepare chitosan/pluronic F-127 hydrogel in triplicate. Our finding has shown that over 15% (w/v) of pluronic F-127 in the mixture could formulate the hydrogel. At 17.5% and 20% (w/v) conditions successfully became hydrogels: 1 ml of chitosan solution required 0.175 g and 0.2 g of pluronic F-127. Blank hydrogel showed colorless and transparent, and it was stable at room temperature. Consistent with the results by García-Couce J et al. (2022), increasing the concentration of pluronic F-127 helped in gel formation, and increasing the concentration also reduces the time for gel formation [68]. Chitosan had a positive charge, while pluronic F-127 had a negative surface charge, the opposite charges caused an attractive force and pulled them towards each other, electrostatically interacting and resulting in a hydrogel formulation. Unlike the curcumin-QCS/PF1.0 hydrogel by Qu J et al. (2018), our hydrogels did not provide a bond like a methyl bond by glycidytrimethylammonium chloride (GTMAC) to formulate the hydrogel [69]. Therefore, the gelation time of our hydrogel would be increased up to a day.

4.2 Stability test of chitosan/pluronic F-127 hydrogel

Chitosan/pluronic F-127 hydrogels prepared in the previous section were kept at room temperature for 8 weeks for the stability study, as shown in Figure 4.2. The result figures

showed that the hydrogel did not have any significant difference after 8 weeks. The overall visible structure was the same as the initial time. In addition, the higher pluronic F-127 contained in the mixture could form the hydrogel for up to 2 months at room temperature as mentioned in PF127-TMC/DTX hydrogels by Turabee MH et al. (2019), the statement is reliable with our stability study results [32].

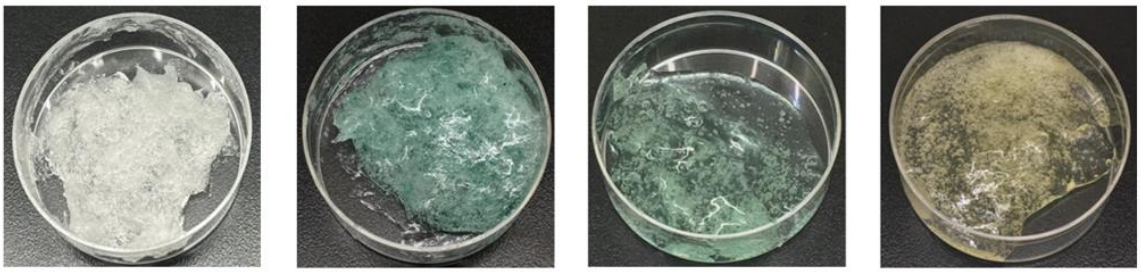
Figure 4.2. Stability study for chitosan/pluronic F-127 hydrogel prepared by 1% (w/v) chitosan solution with 17.5% or 20% pluronic F-127 powder.

4.3 Preparation of the crude extract PC-loaded Hydrogel

The mixture of chitosan solution (1% (w/v)) with 20% (w/v) of pluronic F-127 and the crude extract was successfully formed as gels. Due to the crude extract PC was hardly dissolve in water, PC was dissolved in 0.1% (v/v) DMSO solution at the highest concentration at 5 mg. PC could not be perfectly dissolved over this number. The increasing of PC solution also affected the gelation process such as precipitation of the PC or separation of the formulated hydrogel. Thus, 1 g of the hydrogel contained 0.38 mg of the encapsulated PC. The mass ratio of the the PC-loaded hydrogel was shown in Table 4.2. The blank hydrogel and the crude extract or PC-loaded hydrogel images were shown in Figure 4.3. The blank hydrogels appeared as a white matrix, Figure 4.3(a). The addition of the crude extracts resulted in a different color of these pigments in the matrix (green or yellow). C005H-, C005L-, and CH-loaded hydrogel images were shown in Figures 4.3(b), 4.3(c), and 4.3(d), respectively.

Table 4.2 Mass ratio of the prepared PC-loaded hydrogel.

Components	Weight/Volume	Weight (mg)
Chitosan	1% (w/v): 10 ml	100
Pluronic F-127	2 g	2,000
PC solution (C005H, C005L or CH)	5 mg in 1 ml DMSO (0.1% v/v)	5
Water/solvent	10 ml: chitosan solution 1 ml: PC solution	11,000
Total		13,105



(a) (b) (c) (d)

Figure 4.3 Photos of (a) blank hydrogel (b) C005H-loaded hydrogel (c) C005L-loaded hydrogel, and (d) CH-loaded hydrogel.

4.4 Total phenolic compound (TPC) and purity

Results of the total phenolic compound and purity were shown in Figure 4.4. The total phenolic compound (TPC) of the crude extracts of PC from C005H, C005L, and CH was evaluated using the Folin-Ciocalteu assay. In this assay, the concentration of the 3 PC crude extracts was fixed at 50 µg/ml using gallic acid as a standard. The TPC values of C005H, C005L, and CH were 4.9 ± 0.6, 3.6 ± 0.9, and 6.1 ± 1.1 µg GAE/100g, the purity of C005H,C0005L, and CH were 4.1 ± 0.9, 2.2 ± 0.4, and 3.9 ± 0.8, respectively. For this study, the wild-type strain of spirulina (C005H), straight trichomes strain (C005L), and *Chlorella sp* strain did not show any different numbers of the TPC. Both of the spirulina (C005H) and CH showed relatively close values of purity, which were significantly higher than straight trichomes strain (C005L). The lower purity of PC crude extract might become a limitation and interfere with subsequent studies.

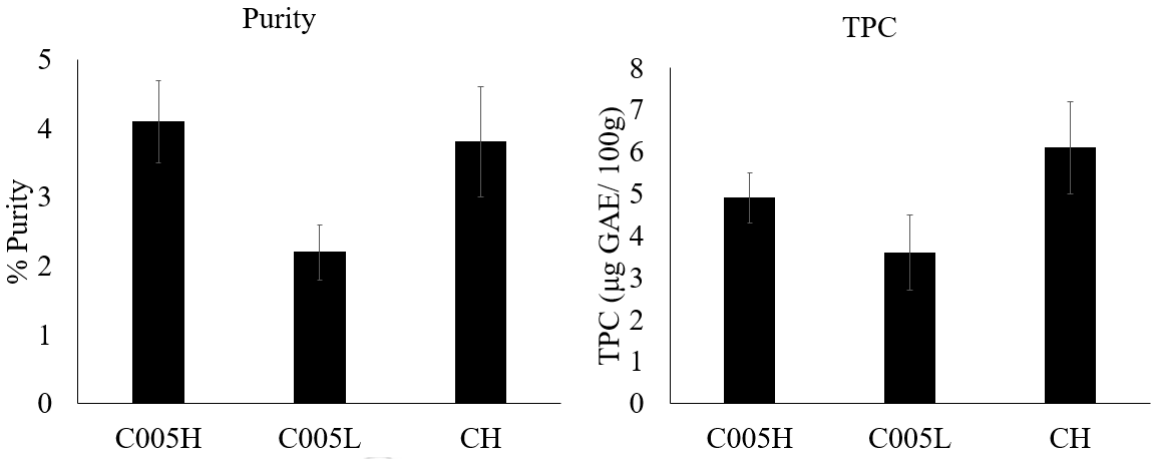


Figure 4.4 Total phenolic compound and purity value of C005H, C005L, and CH crude extract at concentration = 50 µg/ml.

4.5 Protein denaturation inhibition activity

Results of protein denaturation inhibition activity were shown in Figure 4.5. The protein denaturation of the crude extracts (C005H, C005L, and CH) was investigated using the heat shock (54 ^{o}C for 15 min)with the mock protein, bovine serum albumin (BSA). In this study, the crude extracts were prepared at 50 µg/ml with 50 mg/ml of BSA. The result showed that the extract from C005H, C005L, and CH referred significantly higher than the control group at 31.3 $\pm$ 6.3, 28.5 $\pm$ 5.8, and 37.5 $\pm$ 8.29 % inhibition, whereas the control group (PBS) was 23.6 $\pm$ 3.4 %. But the values of % inhibition were too low, From the literature reviews, we believed that the PC extract could present a higher anti-inflammatory potential which is higher than 70 %. The protocol had been adjusted and modified some steps such as temperature and heating time [70].

After a literature review, another protocol of protein inhibition assay was decided to use. The same steps were used except for the reaction temperature and induce time, we changed 51 ^{o}C and 15 min to 70 ^{o}C and 5 min for inducing the protein denaturation reaction. The result showed that the extract from C005H, C005L, and CH strain referred significantly higher than the control group at 91.0 $\pm$ 5.2, 86.4 $\pm$ 9.8, and 96.0 $\pm$ 7.2 % inhibition, whereas the control group (PBS) was 64.8 $\pm$ 13.0 %. Compared with the case without the crude extract (64.8%), the addition of 3 crude extracts from C005H, C005L, and CH significantly increased the percentage of the inhibition of the protein (BSA) degradation at high temperature, while the result of the previous group heated at 54 ^{o}C showed 23%-45% inhibition. Experiments had shown that different temperatures and different incubation time leaded to different values of results, maybe because of the higher temperature leads to faster degradation of protein, so that our crude extracts of PC provide a better exert the anti-inflammatory ability.

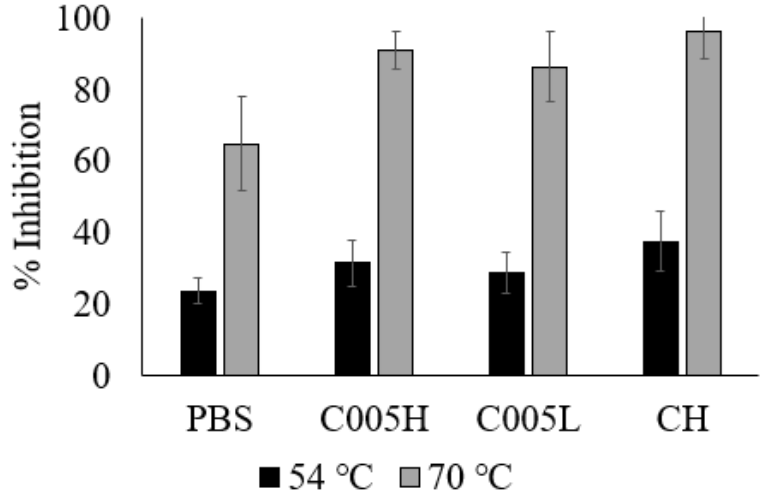


Figure 4.5 Inhibition value (%) from protein denaturation study of blank, C005H, C005L, and CH crude extract (heat at 54 ºC and 70ºC) at concentration = 50 µg/ml.

4.6 LOX inhibition activity

LOX inhibitory activity was used to explain the anti-inflammatory potential. For lipoxygenase enzyme or LOX, this enzyme plays a role in the synthesis of mediators like leukotrienes. LOX inhibition activity results were shown in Figure 4.6. In this test, quercetin (48.6 $\pm$ 3.1 % at 3 min, 50.5 $\pm$ 2.8 % at 5 min) and gallic acid (42.6 $\pm$ 2.4% at 3 min, 37.5 $\pm$ 2.5 % at 5 min) were used as reference activities compound. The extracts of C005H, C005L, and CH were 75.0 $\pm$ 2.5 %, 86.7 $\pm$ 1.0 %, and 97.1 $\pm$ 0.2 % LOX inhibition activity at 3 min. The percent inhibition of C005H, C005L, and CH extracts were 72.0 $\pm$ 2.5 %, 83.4 $\pm$ 1.2 %, and 95.8 $\pm$ 0.5 % within 5 minutes, respectively,. The results showed that the PC crude extracts of the three strains all had a strong anti-inflammatory response-ability, which was more than two times higher than that of the active compounds quercetin and gallic acid.

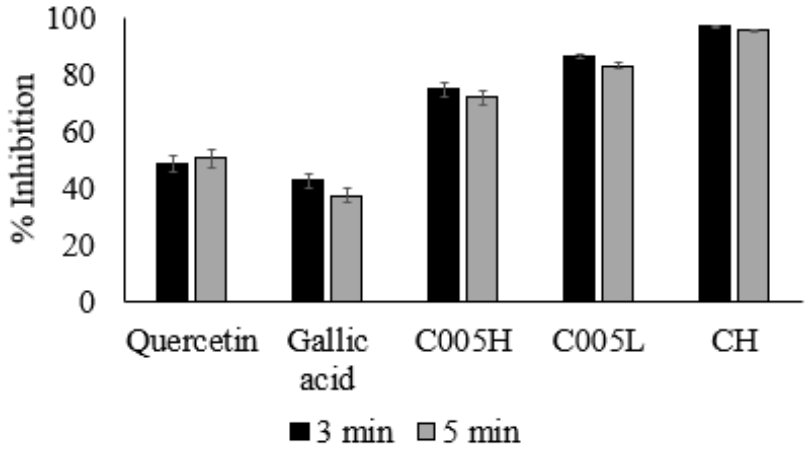


Figure 4.6 LOX inhibition value (%) of quercetin, gallic acid, C005H, C005L CH crude extract at concentration = 50 µg/ml.

4.7 Release profile

As shown in Figure 4.7, the cumulative release *vs* time of C005H-loaded hydrogel, C005L-loaded hydrogel, and CH-loaded hydrogel were compared. Figure 4.8 showed the photo of the release study for PC-loaded hydrogel. All of the hydrogels were set in artificial saliva and the release samples were collected by centrifuge. The supernatants were used to investigate the protein denaturation inhibition and the LOX inhibition activity using an artificial saliva solution as a control group. In this study, the cumulative release was gradually increasing over time. The % cumulative release of crude PC extracts from C005H-loaded hydrogels, C005L-loaded hydrogels, and CH-loaded hydrogels in 6 hours were 81.5%, 72.6%, and 70.2%, respectively. There were no significant different release profiles between these loaded hydrogels.

Although the C005H, C005L, and CH compounds were not attached to the backbone of the hydrogel matrix, the release of PC crude extracts (C005H, C005L, and CH) was controllable and did not provide burst release within the first 2 hours. Since the electrostatic and gelation processes are random, it is a simple process to capture crude extracts of PC into gels using pluronic F-127. As expected, the amount of PC crude extract resulted in almost 100% encapsulation. According to the calculation, the loading efficiency of PC crude extracts (C005H, C005L, and CH) in chitosan/pluronic F-127 was about 384.9 µg per 1 gram of the hydrogel. The weight of released crude PC extracts from

C005H-loaded hydrogels, C005L-loaded hydrogels, and CH-loaded hydrogels at 6 hours were 0.41 mg, 0.36 mg, and 0.35 mg in a gram of the hydrogel, respectively.

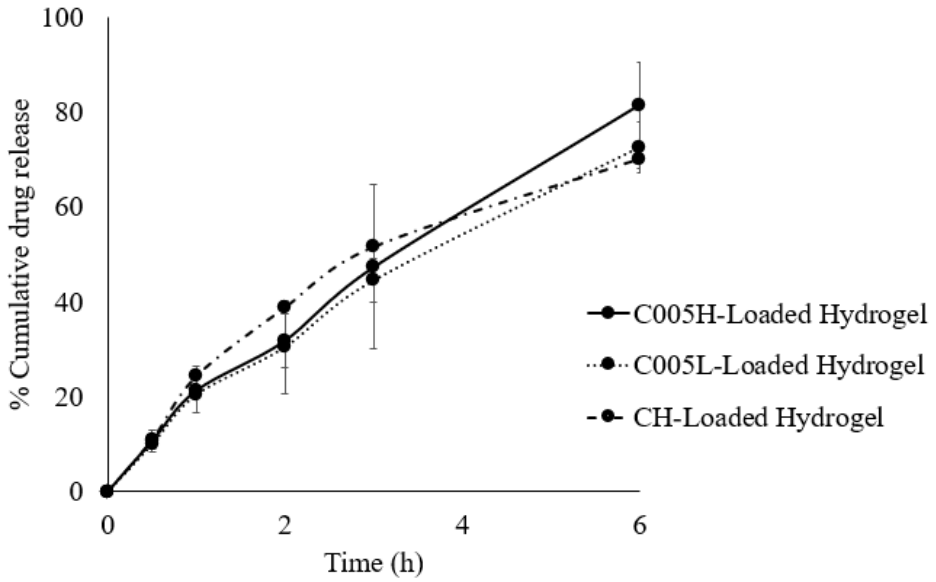


Figure 4.7 The release profile of the crude extract of phycocyanin (PC) from the hydrogels.

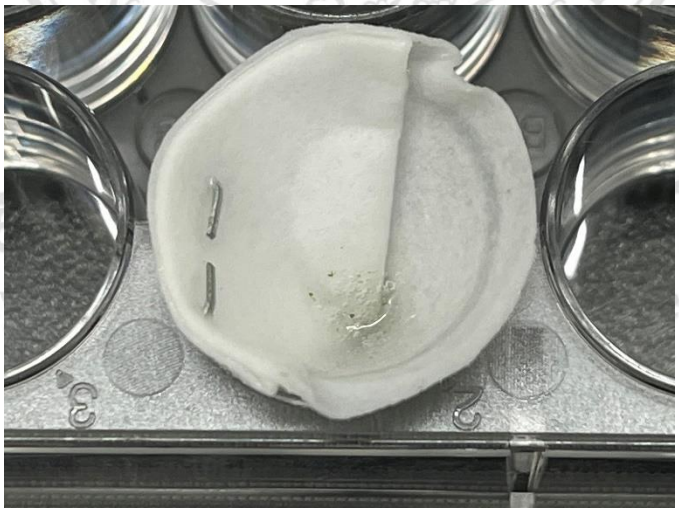


Figure 4.8 PC-loaded hydrogel on filter paper for release study.

To describe the PC release mechanism from the hydrogels, different mathematicl models were used to explain including Zero order, First order, Higuchi, and Hixson-Crowell. The coefficient of determination or R squared (r^2) from relationship of the release profile curves using different mathematical models were shown in Table 4.3. Overall results showed that the models that provided a good fitting were Zero order and Higuchi mathematical models. This results suggested that the PC released through matrix (hydrogels) at a constant rate. For Higuchi model, the r^2 value of CH-loaded hydrogel was higher than C005H-loaded hydrogel and C005L-loaded hydrogel. The result indicated the CH diffusion from the hydrogel very well. Here, CH sample was easy to prepare as a solution from the previous study. Here, the First-order and Hixon-Crowell did not fit to the release curved due to the First-order generally describes the soluble compound in a porous matrix. The Hixson-Crowell is used to mention the carrier with the decreasing surface area (proportionally over time). The hydrogel made from chitosan and pluronic F-127 in this study did aim to generate pore or deform in function of time.

Table 4.3 R squared (r^2) from the release profile curves using different mathematical models.

Hydrogels	R squared (r^2) values			
	Zero order	First order	Higuchi	Hixson-Crowell
C005H-loaded hydrogel	0.9745	-4.4800	0.8833	-2.9130
C005L-loaded hydrogel	0.9571	-4.5660	0.9062	-3.3080
CH-loaded hydrogel	0.8492	-6.0980	0.9506	-4.5720

Results of protein inhibition and LOX inhibition value of released samples at 3 and 6 hours-incubation were shown in Figure 4.9. To ensure the anti-inflammatory capacity of PC-loaded hydrogels, here, released samples at 3 and 6 hours incubation were collected and their anti-inflammatory potential was investigated. The results for percent inhibition

of protein denaturation showed that released samples prepared from C005H-loaded hydrogel at 3 and 6 hours-incubation were increased from 11.8 ± 1.1 % and 20.0 ± 10.4 %. The LOX activity were increasing from 29.6 ± 4.3 to 36.3 ± 3.1 % at 3 min, and 26.4 ± 4.0 to 32.5 ± 2.7 % at 5 min, respectively. The results of protein denaturation from C005L-loaded hydrogel were not significantly different from the C005H-loaded hydrogel samples at 10.7 ± 6.0 and 15.5 ± 4.7% (3- and 6 hours-incubation samples). But the LOX inhibition activity from C005L-loaded hydrogel showed significantly higher than C005H-loaded hydrogel samples at 43.1 ± 3.0 to 51.9 ± 1.8% from 3 and 6 hours-incubation at 3 min, 39.6 ± 2.7 and 47.5 ± 1.5 % at 5 min. Percent protein denaturation inhibition from CH-loaded hydrogel showed significantly higher than C005H-loaded hydrogel samples and C005L-loaded hydrogel samples at 24.1 ± 14.8 % to 49.5 ± 23.7 %. However, the results of % inhibition of LOX activity from CH-loaded hydrogel did not showed inhibition at 3-hours incubation time for both 3 min and 5 min at 25.0 ± 4.6 % and 20.7 ± 2.4 %, and for 6-hours incubation the result showed significantly lower than C005H-loaded hydrogel and C005L-loaded hydrogel samples at 15.9 ± 5.7 to 9.0 ± 3.7 % from 3-5 min. There were no significantly different for the LOX inhibition values between 3 min and 5 min. It showed lower LOX inhibition values than the results of crude extract of PC, since the PC concentration of 6 hours-released samples was about 26 µg/ml , which was lower than the PC solution used in LOX of PC extract section. At the same time, the solvent of released samples was artificial saliva, different from the DI water solvent used in the pervious part. The anti-inflammatory activity of the released samples showed the potential of the use of these hydrogels for gingivitis treatment.

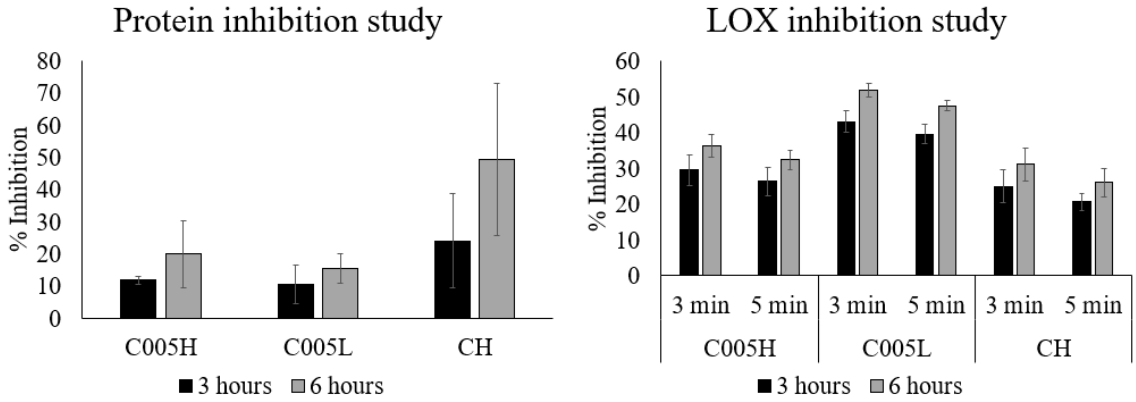


Figure 4.9 Percent protein inhibition and LOX inhibition of released samples at 3 and 6 hours-incubation.

4.8 *In vitro* biocompatibility testing

4.8.1 Cytotoxicity of the PC crude extracted against L929 and GF

Cytotoxicity results and morphology of L929 and GF cells treated by the extracted PC were shown in Figure 4.10 - Figure 4.12. For L929 cells, the MTT test results on the first day showed that the % relative survival of cells for all PC species at different concentrations was basically over 70%. Increasing the incubation time to 3 days, the % survival was reduced in all species but still greater than 70%, except C005L which showed toxic above 194 µg/ml. Meanwhile, the cytotoxicity results of GF cells showed that the % survival for all of the 3 species of PC was above 80% on the first day, C005H had a higher % relative cell survival, and the cell viability is about 100% between the concentration of 0.01 µg/ml and 1,000 µg/ml. On day 3, although the cell viability deceased, it was still around 80%.

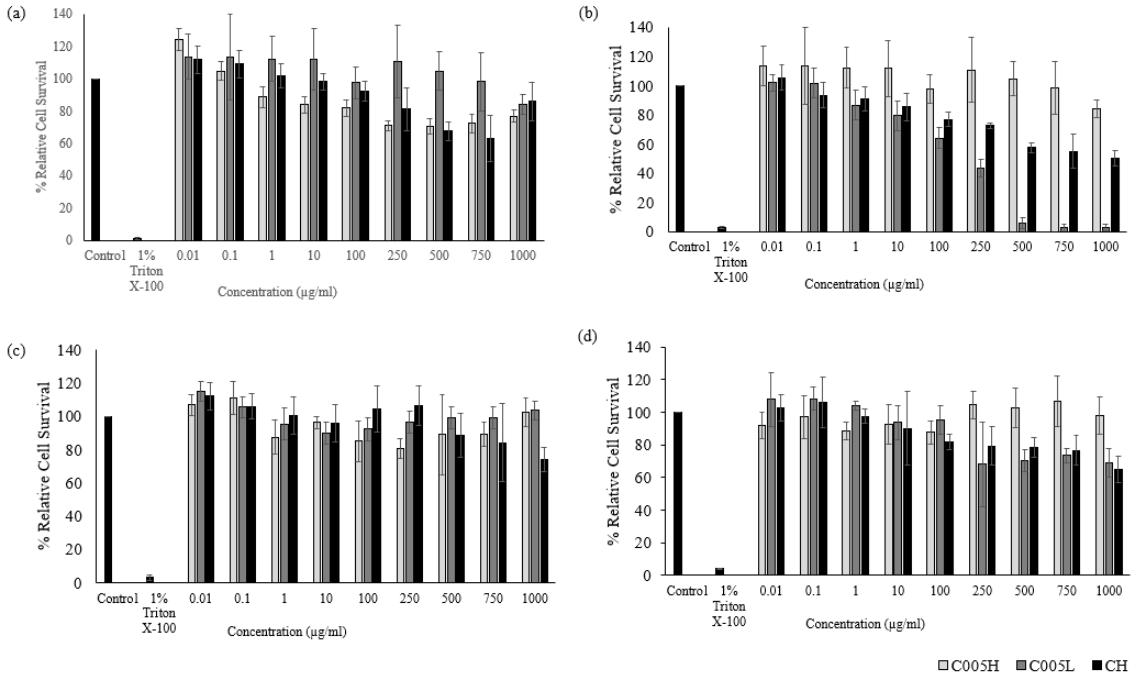


Figure 4.10 *In vitro* cytotoxicity of the crude extract from 3 strains: *Arthrospira platensis* strain C005H, *Arthrospira platensis* strain C005L, and *Chlorella sp* (CH). Graphs showed the relationship between cell viability (%) and concentration of the crude extract: (a) 1-day incubation with L929 cells day, (b) 3-day incubation with L929 cells, (c) 1-day incubation with GF cells, and (d) 3-day incubation with GF cells. (n = 6)

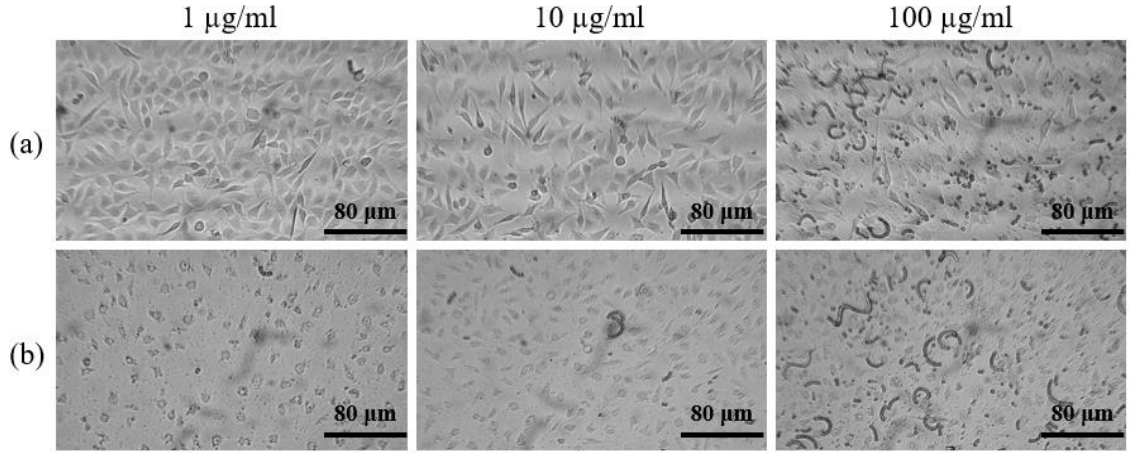


Figure 4.11 Morphology of L929 treated with the C005L-PC crude extract (a) 1 day and (b) 3 days.

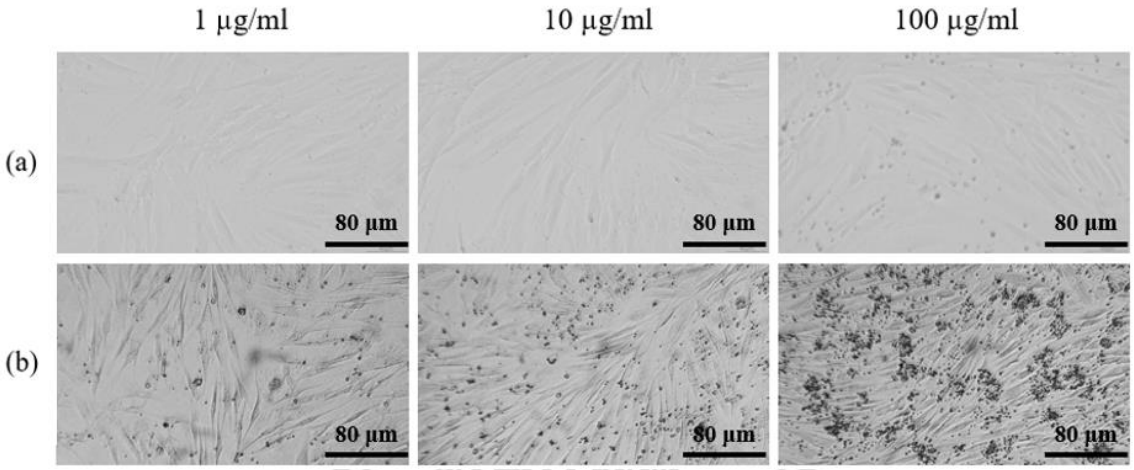


Figure 4.12 Morphology of GF treated with the CH-crude extract (a) 1 day and (b) 3 days.

4.8.2 Cytotoxicity of Chitosan/Pluronic F-127 hydrogel

Cytotoxicity results of the hydrogels and cell morphological observation were shown in Figures 4.13 - Figure 4.18. Both L929 and GF cells were treated with the extracted medium prepared from the blank hydrogel, C005H-, C005L-, and CH-loaded hydrogels. Overall results of the blank and extracted PC-loaded hydrogel showed over 90% relative cell survival. There were no significantly toxic to L929 and GF cells after treating those cells with the extracted medium, Figure 4.13 - Figure 4.18. Meanwhile, treated cells with 1% (v/v) Triton X-100 had altered cell shapes and below 3% cell viability. All of the PC-loaded hydrogel groups present over 99% relative cell survival. 50% C005L shows the highest % relative cell survival which is 109.59%. From the cell morphology in Figure 4.13 - Figure 4.18, all of the conditions were still alive in DMEM.

Moreover, a commercial product (Kanolone®), which is widely used for treating gingivitis was used to compare. Cell morphological observation of GF cells treated with the extracted medium prepared from 100% and 50% of Kanolone® were shown in Figure 4.17. 50 % Kanolone® extracted medium presents 84.35% relative cell survival and 100% Kanolone® extracted medium presents 87.87% relative cell survival. There was no significant toxicity after treating L929 and GF cells using the same procedure as mentioned above.

The prepared PC-loaded hydrogels were not toxic to L929 and GF cells, and showed less toxic due to the low concentrations of PC and good biocompatibility of chitosan and pluronic F-127. Photos shows that L929 cells are still alive after treating cells with extracted medium for 1 day. Attending to its nontoxic, we believe that PC-loaded hydrogel can be a second choice for treating gingivitis.

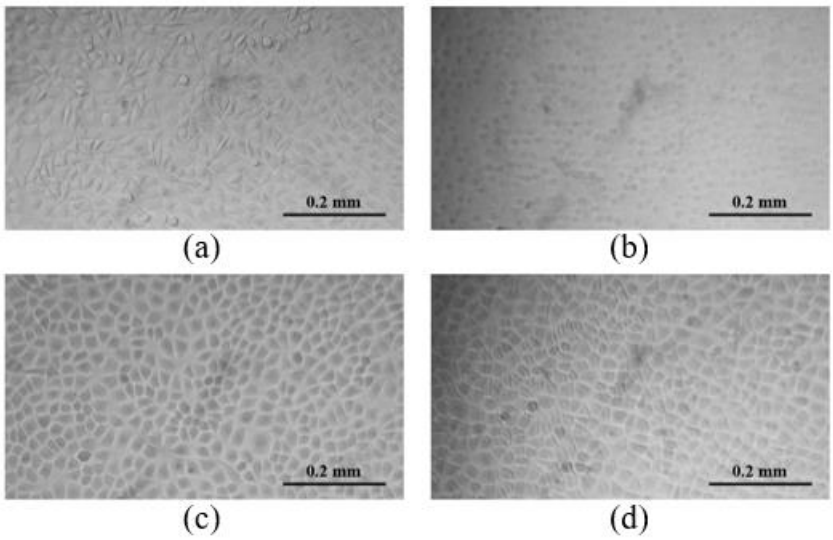


Figure 4.13 Morphology of L929 cells treated with (a) fresh culture medium (control), (b) 1% Triton X-100, (c) 50% extracted medium prepared from blank hydrogel, and (d) 100% extracted medium prepared from the blank hydrogel.

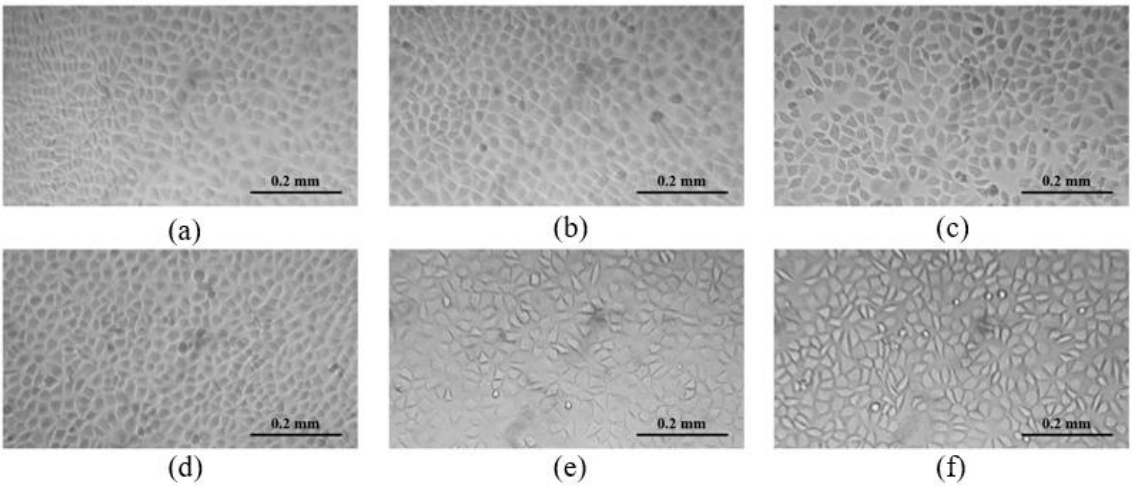


Figure 4.14 Morphology of L929 cells were treated with 50% (a) and 100% (b) extracted medium prepared from C005H-loaded hydrogel, 50% (c) and 100% (d) extracted medium prepared from C005L-loaded hydrogel, and 50% (e) and 100% (f) extracted medium prepared from CH-loaded hydrogel.

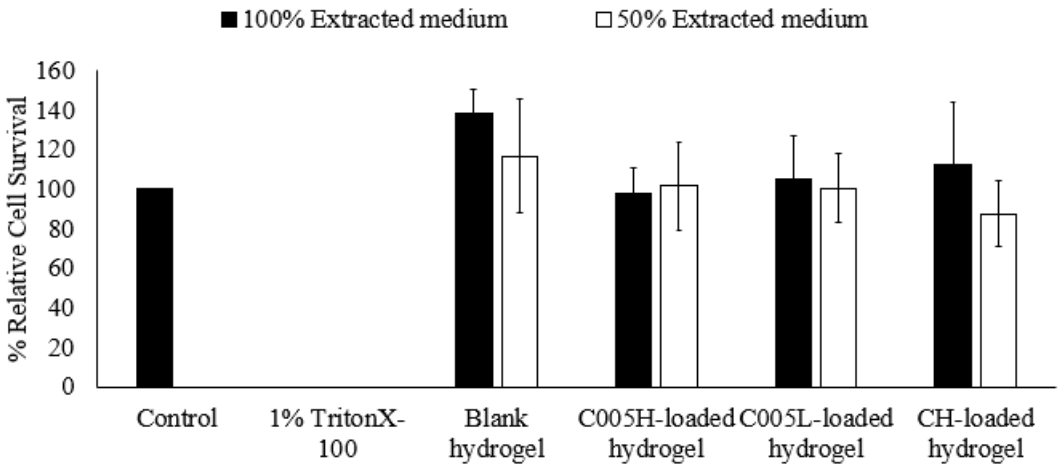


Figure 4.15 L929 cell viability after 1 day treated with extracted culture medium (100% and 50%) prepared from chitosan/pluronic F-127 hydrogel and crude extract PC-loaded hydrogel on L929 cells.

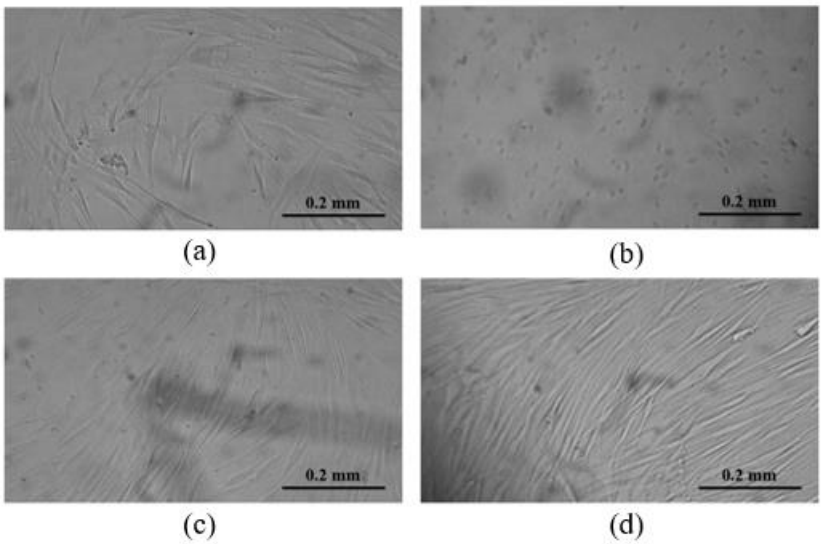


Figure 4.16 Morphology of GF cells treated with (a) fresh culture medium (control), (b) 1% Triton X-100, (c) 50% extracted medium prepared from blank hydrogel, and (d) 100% extracted medium prepared from blank hydrogel.

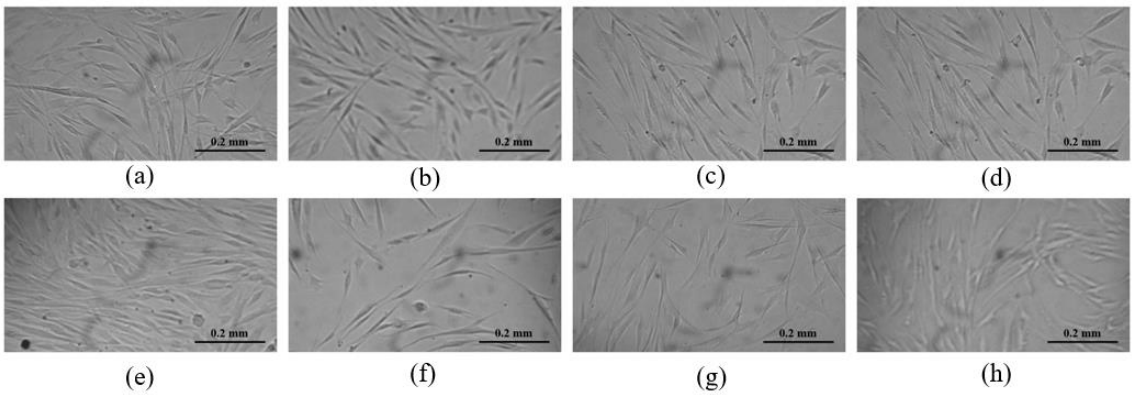


Figure 4.17 Morphology of GF cells treated with 50% (a) and 100% (b) extracted medium prepared from C005H-loaded hydrogel, 50% (c) and 100% (d) extracted medium prepared from C005L-loaded hydrogel, 50% (e) and 100% (f) extracted medium prepared from CH-loaded hydrogel, and 50% (g) and 100% (h) extracted medium prepared from Kanolone®.

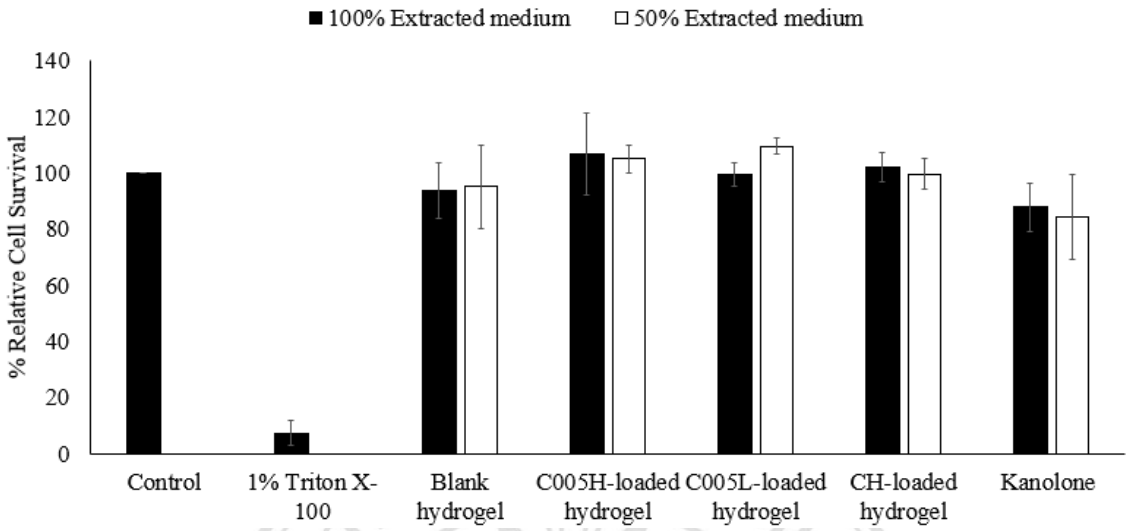


Figure 4.18 GF cell viability after 1 day treated with extracted culture medium (100% and 50%) prepared from chitosan/pluronic F-127 hydrogel and crude extract PC-loaded hydrogel on GF cells.

4.8.3 Wound healing assay

The wound healing assay of GF cells was determined by measuring the recovery area after 24 hours of scratching the monolayer of GF cells. The GF cells were cultured under different conditions of the culture medium as shown in Figure 4.19. Recovery area of GF cells. The recovery area of the GF cells treated with serum-free medium and the extracted medium prepared from Kanolone® were 38.5% and 53.1%, respectively. Differently, the testing medium prepared from blank hydrogel, C005H-, C005L-, and CH-loaded hydrogels were 0.2%, 20.5%, 13.4%, and 19.6% respectively.

Since the present study used the 50% concentration of extraction medium and exposed to GF cells for 24 hours, the wound healing effect of PC-loaded hydrogel was much lower than that of Kanolone®. The lower purity of PC may reveal an ineffective compound as a limitation of this study. Q. M. To et al. (2020) found their PC-loaded hydrogel presented significant wound coverage over 90 % after 24 hours when the PC at a concentration above 12.5 ug/ml. Their PC concentration was 1.13 ± 0.05 mg/ml, and the purity index was 2.79 ± 0.25, which is significantly higher than our crude extract of PC. It was demonstrated that our low-purity PC showed as an ineffective compound

and became a limitation in this work [71]. Compared to our PC-loaded hydrogel, Kanolone® has the limition that it contains triamcinolone acetonide which is a synthetic corticosteroid drug. Those who are allergic to adrenal corticosteroids and those with liver and kidney insufficiency are not suitable for this drug. At this point, PC-loaded hydrogel made from chitosan may lead to the presence of allergnic protein in patients. Thus, we believe that the hydrogels can be an alternative choise that fills the gap where Kanolone® is not suitable for steroid-allergic patients..

Furthermore, this study was not performed for more than 24 hours due to a previous report by Afrasiabi S et al. (2021). Chitosan hydrogels were found to be toxic to human gingival fibroblasts (HGF) if the hydrogel concentration exceeded 156.2 μg/ml and incubated for 72 hours. [72]. Therefore, we found that extraction media prepared from blank hydrogels did not increase the recovered area as much as media extracted from PC-loaded hydrogels. Duvnjak Romić M et al. (2019) showed that chitosan/pluronic F-127 microspheres showed improvement in wound healing rate after treating at pH 7.4, but not found significant. It appeared to be consistent with our experimental results [73]. Different from ur Rehman SR, we cultured GF cells and used in wound healing assay instead of endothelial [74]. And after 24 h, their study showed that the hydrogel accelerated wound healing of endothelial cells when compared to the control in the 24-hours period after cell treatment.

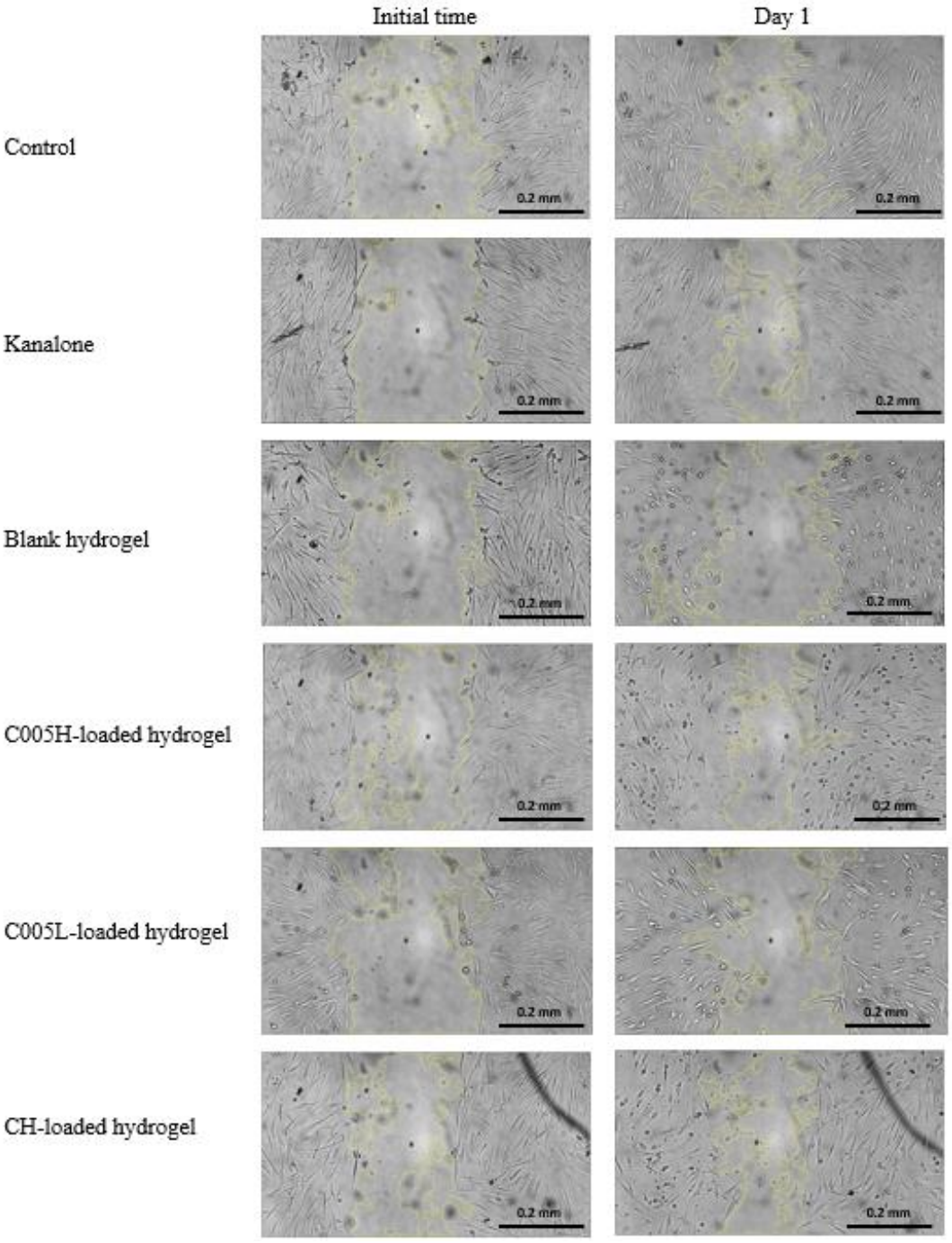


Figure 4.19 The wound-healing assay area of the GF cells was analyzed by using the Image J software after scratching. The recovery area was presented after 24 hours of incubation.

CHAPTER 5

CONCLUSION

This study described the anti-inflammatory capacity, purity, and cytotoxicity of PC, as well as the hydrogel preparation, hydrogel stability, PC release profile, and cytotoxicity of PC-containing chitosan/pluronic F-127 hydrogel. Crude extract of PC from all strains showed the potential to inhibit protein degradation and LOX inhibitory activity. All PCs showed no cytotoxic at concentrations below 100 μg/ml. The total phenolic content (TPC) value of the crude extracts was in the range of 2 to 7 μg GAE/100g, the purity was in the range of 2~5%. Chitosan/Pluronic F-127 hydrogels were successfully fabricated, and stability tests showed that the blank hydrogels did not present significant change when stored at room temperature up to 8 weeks. C hitosan/pluronic F-127 hydrogels containing PC crude extracts were also successfully prepared, these PC crude extracts were completely encapsulated in the chitosan/pluronic F-127 hydrogels. In addition, the system did not contain cross-linking agents. As hydrogel materials, chitosan and pluronic F-127 were linked together by electrostatic statics, and it increased the biocompatibility of the hydrogel for there were no possible toxic by-products. The hydrogel could be easily degraded in the presence of water, and the drug delivery system was found to prolong the release time of the crude extract within 6 hours with an effective anti-inflammatory activity which showed more than 85% inhibition in the protein denaturation inhibition assay, and over 70 % inhibition tested using lipoxygenase (LOX) assay. At the same time, the lower concentration of PC crude extract became a limitation for the anti-inflammatory ability of PC crude extracts and PC-containing chitosan/Pluronic F-127 hydrogel, which is expected to be higher in the case of higher purity, the better anti-inflammatory ability could play a better therapeutic effect on gingivitis.

There are some commercial products on the market today with more or less side effects. For example, Kanolone® is not suitable for steroid-allergic patients. Non-steriod compound like PC would be able to apply, but an additional labeling term as proprietary

blend: chitosan from shrimp, crab or lobster must be presented. At least, the PC-loaded chitosan/Pluronic F-127 hydrogel from this study may be an alternative treatment for human gingivitis.

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APPENDICES

APPENDIX A

Study protocol and consent form approval

No. 049/2020

Study Protocol and Consent Form Approval

The Human Research Ethics Committee of the Faculty of Dentistry, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol and patient/participant information sheet dated and/or amended as follows in compliance with the **ICH/GCP**

Study Title	:	Dental stem cells derived extracellular matrix as a novel source for biomaterials in regenerative dentistry
Study Code	:	HREC-DCU **2020-036**
Study Center	:	Chulalongkorn University
Principle Investigator	:	Professor Dr. Thanaphum Osathanon
Protocol Date	:	April 9, 2020
Date of Approval	:	June 10, 2020
Date of Expiration	:	June 9, 2022

.....

(Assistant Professor Dr. Kanokporn Bhalang)
Chairman of Ethics Committee
Associate Dean for Research

*A list of the Ethics Committee members (names and positions) present at the Ethics Committee meeting on the date of approval of this study has been attached (upon requested). This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Approval is granted subject to the following conditions: (see back of the approval)

APPENDIX B

Editor:
Wasu Pathum-Aree,
Chiang Mai University, Thailand

Article history:
Received: May 10, 2022;
Revised: June 2, 2022;
Accepted: July 6, 2022;
https://doi.org/10.12982/CMUJNS.2022.052

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Research article

In Vitro Preparation and Evaluation of Chitosan/Pluronic F-127 Hydrogel as a Local Delivery of Crude Extract of Phycocyanin for Treating Gingivitis

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Abstract The phycocyanin (PC) in the crude extract is one of the main active compounds that has significant anti-inflammatory properties and the potential to treat gingival inflammation, the common oral disease. This work reports the preparation and characterization of chitosan/pluronic F-127 hydrogels entrapping the crude extracts of PC from *Arthrospira platensis* (C005H and C005L) as a local drug delivery system aiming to prolong PC release for the treatment of gingivitis. The results showed that the total phenolic content (TPC) values of the crude extracts were in the range of 2 to 5 µg GAE/100g and presented above 85% inhibition in the protein denaturation test and over 65% using a lipoxygenase (LOX) inhibition test. The hydrogels incorporating the crude extracts from C005H and C005L were perfectly prepared via the electrostatic interaction between chitosan and pluronic F-127 with very high encapsulation efficiency. The crude extracts of PC C005H and C005L were released over 70% from the loaded hydrogel within 6 hours under artificial saliva conditions. The anti-inflammatory activity of the released supernatant from the hydrogels after 6 hours was around 32-47% by LOX inhibition. The hydrogel vehicles and loaded-hydrogels did not show any cytotoxic effects against mouse fibroblast cell lines (L929) and human gingival fibroblast cells. Our current work shows that the crude extract PC-loaded chitosan/pluronic F-127 hydrogel is biocompatibility with human cells and shows prolonged crude extract release properties, suggesting it as an alternative treatment approach for gingivitis.

Keywords: Phycocyanin, Gingivitis, Hydrogel, Chitosan, Pluronic F-127

Funding: This work was supported by The Junior Researcher Fund, Chiang Mai University (R0000022723). TP is supported by Health Systems Research Institute (65-139) and National Research Council of Thailand (NRCT)(N42A650229).

Citation: Li, M., Kamdenlek,P., Kuntanawat, P., Eawsakul, K., Porntaveetus, T., Osathanon, T., and Manaspon, C. 2022. *In vitro* preparation and evaluation of chitosan/pluronic F-127 hydrogel as a local delivery of crude extract of phycocyanin for treating gingivitis. CMUJ. Nat. Sci. 21(4): e2022052.

INTRODUCTION

Gingivitis is one of the common oral diseases which mainly caused by the accumulation of dental plaque, leading to gingival inflammation. The progress of this symptom is caused by periodontal bacteria (gram negative), which is involved to the surface of the tooth surface and gums. According to the statistical data, around 8.52% of adults aged 20 to 64 face gingivitis (Eke et al., 2018). The traditional treatment for gingivitis is the removal of dental plaque and tartars together with the infected bacteria from the tooth surfaces and gums. Local application of antibiotic agents such as chlorhexidine mouthwash is applied as adjuvant therapy.

Phycocyanin (PC) is one of the major pigment constituents of cyanobacteria, it is nutritional and therapeutic values have been very well documented around the world. PC can be directly extracted from the cyanobacteria using different techniques such as the acid extraction method and ultrasonic-associated extraction method (Pagels et al., 2021). It shows an excellent anti-inflammatory potential, which is a series of multi-site effects such as removing various reactive oxygen species (ROS) and inhibiting the protein denaturation or enzyme (Prabakaran et al., 2020), resulting in the recovery of the tissue. Moreover, the toxicity of the PC is low or nontoxic as an orally active compound. Its lethal dose (LD50) in rats and mice has been reported at concentrations greater than 2 g/kg (Grover et al., 2021).

The local drug delivery system, which makes the drug or compound reach its site of action with a higher concentration than the original dosage form, is an emerging research field with minimal to no side-effect vehicles for drugs or compounds used in humans (Rajeshwari et al., 2019). Using delivery systems, the drug or compound is protected by the carrier from biological environments such as saliva washout, temperature, or other secreted biological solutions. Therefore, the local drug delivery system optimizes the therapeutic properties of the drug in terms of safety, effectiveness, and reliability.

Chitosan is a cationic polysaccharide obtained by deacetylation of chitin. It is a low-toxic, inexpensive, hydrophilic, biodegradable, & biocompatible biopolymer. Thus, chitosan polymer is widely used in tissue engineering and drug delivery application research (Mohebbi et al., 2019). Liu et al. (2022) reported the various preparation of chitosan-based hydrogels such as cross-linked networks ionic complexes or self-assembling vehicles for drug delivery of a therapeutic payload. Here, the developed chitosan hydrogels could control its release to high bioavailability at the site of action (Liu et al., 2022). Wei et al. (2022) developed a crosslinking-based hydrogel from carboxymethyl chitosan (CMCS) and polyethylene glycol (PEG) as a wound dressing. Based on CMCS backbone of the hydrogel, antioxidant and antibacterial agents were also confirmed the grafting using Fourier Transform Infrared Spectroscopy (FTIR) and ^{1}H NMR. Pluronic F-127 also called Poloxamer 407, is a hydrophilic polymer. Pluronic F-127 has been approved by the US Food and Drug Administration (FDA) for use in humans and has been widely used as a drug carrier and tissue engineering (PJ Jaquilin et al., 2022). Turabee et al. (2019) fabricated docetaxel-loaded hydrogels from pluronic F127 and *N,N,N*-trimethyl chitosan to treat brain tumors. They performed the hydrogels under different pH to sustain the release of docetaxel. The released docetaxel significantly inhibited the glioblastoma (U87MG) xenograft tumor growth in mice (Turabee et al., 2019). García-Couce et al. (2022) developed chitosan/pluronic F-127 hydrogels-based using TPP (triphosphate) as a crosslinking agent. This hydrogel was used to entrap dexamethasone (DMT) for osteoarthritis treatment. The mixture was transformed into the gel by raising the temperature above 37°C. This hydrogel enhanced drug permeation and prolonged the retention, especially the formulation with TPP. The cumulative DMT release was around 65% after 170 hours. Curcumin-loaded quaternized chitosan/benzaldehyde-terminated pluronic® F127 (QCS/PF1.0) hydrogel was prepared and investigated its gelation, antibacterial activity, and antioxidant property by Qu et al. 2018. The results showed excellent biocompatibility with L929 cell line, over 90% killing ratio with *E. coli* and *S. aureus*,

and over 80% scavenging efficiency by DPPH antioxidant test. Curcumin-QCS/PF1.0 hydrogels also promoted fibroblast migration and 4-folds upregulated vascular endothelial growth factor (VEGF) resulting in an assisting as a wound dressing in Kunming mouse.

In this work, we reported hydrogel preparation from chitosan and pluronic F-127. The hydrogel was used to encapsulate the crude extracts of phycocyanin (PC) from 2 strains of cyanobacterial (*Arthrospira platensis* strain C005H and strain C005L). Several essential properties such as protein denaturation inhibition, lipoxygenase (LOX) inhibition activity, total phenolic compound (TPC), and *in vitro* release studies were carried out. The characteristic properties of the hydrogel carrier were evaluated as well as its biocompatibility using fibroblast cell line (L929) and gingival fibroblast cells (GF).

MATERIAL AND METHODS

Materials

Two crude extracts of phycocyanins (PC) were prepared at the Institute of Agricultural Technology, Suranaree University of Technology. Herein, the crude extracts PC were prepared from *Arthrospira platensis* stain C005H (wild-type spirulina, helical trichomes) and *Arthrospira platensis* stain C005L (straight trichomes) by ultrasonic-assisted extraction method (Chaiyasitdhi et al., 2018). C005H strain was generously provided by Applied Algal Research Laboratory, Faculty of Science, Chiang Mai University. After a prolong period of time, straight trichomes were found immerged from the C005H culture. A single trichrome was then reisolated and renamed as C005L. Bovine serum albumin (BSA), phosphate buffer saline (PBS: tablet form), 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and triton X-100 were obtained from Amresco (Washington, USA). Pluronic F-127, Folin & Ciocalteu's phenol reagent, lipoxygenase (LOX) enzyme, linoleic acid, and quercetin were obtained from Sigma-Aldrich (Missouri, USA). Chitosan powder was obtained from Ta Ming Enterprises CO. LTD (Samut Sakon, Thailand). Acetic acid and sodium chloride (NaCl) were obtained from Merck (New Jersey, USA). Sodium carbonate (Na_2CO_3) was obtained from Ajax Finechem (Sydney, Australia). Kanolone® was obtained from L.B.S. Laboratory LTD., PART. (Bangkok, Thailand). Boric acid was obtained from RCI-Labscan LTD (Bangkok, Thailand).

In vitro crude extracted characterizations

Total phenolic compound (TPC) of the crude extract of PC.

The total phenolic compound (TPC) of the crude extracts of PC (C005H and C005L) was determined using the Folin-Ciocalteu reagent (Hidayati et al., 2020). Briefly, 100 µl of the crude extract of PC was dissolved in deionized (DI) water and mixed with 100 µl of 1N of Folin-Ciocalteu reagent. The mixture was then left for 5 min at room temperature, followed by adding 80 µl of 5% (w/v) Na_2CO_3 solution. The reaction was plated in the dark for 1 hour. Absorbance was measured using a microplate reader spectrophotometer (HiPo MPP-96, BioSan, Latvia) at a wavelength of 568 nm. The standard gallic acid solutions (10, 25, 50, 100, 175, 250 µg/ml) were prepared and used to express all PCs in the unit of gram gallic acid equivalent (µg GAE/100g) using the following equation:

$$\mathrm{PC} = \frac{\text{Concentration from the standard curve} \times \text{Volume of the extract}}{\text{Mass of the extract}}$$

Protein denaturation inhibition

The crude extract of PCs at 50 µg/ml was mixed with 1% (w/v) of bovine serum albumin (BSA). The mixture at 1 ml was incubated at 37°C for 15 min, then heated at 70°C for 5 min (Gunathilake et al., 2018; Leelaprakash and Dass 2011).

After cooling down, the clear solution of the sample was measured the absorbance at 660 nm using a UV/VIS spectrometer (Genesys™ 10s UV-Vis, Thermo Scientific, USA). PBS was used as the control group. The inhibition of protein denaturation was calculated by using the following equation.

$$\text{Protein denaturation inhibition } (\%) = \frac{1 - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

LOX inhibition activity

The crude extract of PCs was investigated its LOX inhibitory potential by dissolving in DMSO at 50 µg/ml. The crude extract of PCs (25 µl) solution was mixed with 975 µl of LOX enzyme, 400 U/ml in 0.2 M, pH 9 borate buffer. One milliliter of the substrate (linoleic acid) at 250 µM was then added to the mixture (Marathe SJ et al., 2022). The absorbance of the reaction mixture was measured at 234 nm at 5 min after adding the substrate using a microplate reader (TECAN, Infinite 200 Pro M Plex, Männedorf, Switzerland). DMSO was used as a control group, whereas quercetin and gallic acid were used as reference active compounds. The percentage of LOX inhibition activity was calculated by using the following equation.

$$\text{LOX inhibition activity } (\%) = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Hydrogels preparation and encapsulation of the crude extract PC

Chitosan (1 g) was dissolved in 100 ml of acetic acid solution (2% v/v). The solution was then dialyzed in DI water for 2 days and normal saline for another day to normalize the pH at the natural condition. Dialyzed chitosan solution (10 ml) was mixed with 2 g of pluronic F-127. The solution was mixed and kept at 4°C overnight and at room temperature for 1 day. After formulation, the hydrogels were kept at room temperature for up to 8 weeks with weekly observation for a stability investigation.

To prepare the crude extract of PC-loaded hydrogel, the crude extract of PC (5 mg) was dissolved in 1 ml DMSO solution (0.1% (v/v)). This DMSO solution was added to the chitosan solution. Pluronic F-127 powder was then added and prepared the hydrogel from the previous section.

Characterization of PC-loaded hydrogels

Release profile

The crude extract of PC-loaded hydrogels (C005H- and C005L-loaded hydrogels) at 0.1 g was plated on a membrane filter (Whatman®) and left on the top of a 24-well plate containing 2 ml of artificial saliva (0.75 g KCl, 0.07 g $MgCl_2$, 0.199 g $CaCl_2$, 0.965 g K_2HPO_4, 0.435 g KH_2PO_4, 36 g sorbitol, 2.4 g sodium Benzoate, and 1,200 ml DI water, pH 7.4). At determination time points (30 min, 1, 2, 3, and 6 hours), the artificial saliva solutions were collected and refreshed with equal volume (Gupta et al., 2014; Pelegrino et al., 2018). The harvested solutions were then measured the absorbance at 620 nm and calculated the release profile using the following equation.

$$\text{Cumulative PC release} (\%) = \frac{\text{Amount of the crude extract of the PC release}}{\text{An initial amount of the loaded crude extract of PC}} \times 100$$

Hence, the released samples at 3 and 6 hours-incubation were then collected by centrifuge. The supernatants were used to investigate the protein denaturation inhibition and the LOX inhibition activity using artificial saliva solution as a control group. The procedures were mentioned in the previous section.

***In vitro* biocompatibility testing**

L929 (Mouse fibroblast cell) cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank). Cells were grown

in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA). The medium was supplemented with 10% (v/v) of fetal bovine serum (FBS, Gibco, USA) and 100 U/ml of penicillin/100 µg/ml of streptomycin (Gibco, USA). For gingival fibroblast cells (GF), cells were isolated from healthy patients using the approved protocol by the Human Research Ethics Committee, Faculty of Dentistry, Chulalongkorn University (No. 049/2020). The GF cells were cultured with the same culture medium as the L929 cell. Those cells were incubated and grown at 37°C in a humidified atmosphere with 5% CO_2.

Cytotoxicity of the crude extracts of PC

Cells (L929 or GF) were plated into 96 well plates with a density of 10,000 cells per well at least 1 day before the experiment. Serum-free culture medium was used to prepare the series dilution/various concentrations of the crude extracts of PC in the range of 0 – 1,000 µg/ml. Cells were treated with several concentrations of the extracts at different periods, including 1 and 3 days at 37°C (Hernandez et al., 2017). Fresh serum-free DMEM and 1% Triton X-100 were used as negative control and positive control conditions, respectively. The cell viability of the study was evaluated by MTT assay.

Cytotoxicity of Chitosan/Pluronic F-127 hydrogels

The chitosan/pluronic F-127 hydrogels (with or without the crude extracts) were prepared by adding 1 ml of serum-free DMEM medium to 0.1 g of the hydrogels (following ISO 10993 Part 12). The extracted medium was collected after 24 hours-incubation at 37°C. The extracted medium was prepared at 100% and 50% by diluting with the fresh culture medium. These test media were plated to the cells (L929 and GF) which were plated into 96 well plates with a density of 10,000 cells per well at least 1 day before the experiment. All experiments were evaluated the number of cells by MTT assay after 1 day of incubation. In this study, fresh serum-free DMEM and 1% Triton X-100 were used as control and negative control group.

Wound healing assay

GF cells were plated into 12 well plates with a density of 50,000 cells per well and incubated for at least 1 day to fulfil cell confluence in the well. The culture media were refreshed with the serum-free DMEM before the experiment for at least another 1 day. The cells were scraped in a perpendicular straight line using a 10 µl pipette tip (Kominato et al., 2022). The migration of the GF cells was investigated by taking a series of bright-field images at the initial time and 1 day of incubation. All images were exported as 16-bit TIFF files and processed with the ImageJ (Version 1.53) program (National Institutes of Health) using a plugin "MRI_Wound_Healing_Tool.ijm" as a calculation tool.

Statistical analysis

All studies were investigated at least triplicate and presented as the mean ± standard deviation for all assays tested. A student t-test was performed to compare all data. The differences were considered to be significant at a level of $P < 0.05$.

RESULTS

Total phenolic compound (TPC), protein denaturation inhibition, and LOX inhibition activity

The total phenolic content (TPC) of the crude extracts of PC from *Arthrospira platensis* strain C005H and Arthrospira platensis strain C005L was evaluated using the Folin-Ciocalteu assay. The investigated concentration of the crude from both strains was 50 µg/ml. The TPC value of C005H and C005L were 4.9 ± 0.6 and 3.6 ± 0.9 µg GAE/100g, respectively. Moreover, the protein denaturation of the crude extracts (C005H and C005L) was investigated using the heat shock with the mock protein, bovine serum albumin (BSA). In this study, the crude extracts were prepared at 50 µg/ml with 1% (w/v) of BSA. The result showed that the extract from C005H and C005L strain referred significantly higher than the control group at

91.0 ± 5.2 and 86.4 ± 9.8% inhibition, whereas the control group (PBS) was 64.8 ± 13.0%. For lipoxygenase enzyme or LOX, this enzyme plays a role in the synthesis of mediators like leukotrienes. The study of inhibition of LOX could refer to the anti-inflammatory effect. In this test, the crude extract samples at the same concentration as the protein denaturation study (50 µg/ml) were prepared and investigated using quercetin (49.7 ± 2.7%) and gallic acid (37.5 ± 2.5%) as reference active compounds. The extract from C005H was 75.0 ± 2.5%, and 86.4 ± 9.8% for the crude from C005L.

Preparation and Characterization of the crude extract PC-loaded Hydrogel

The mixture of chitosan solution (1% w/v) with 20% (w/v) of pluronic F-127 was successfully formed as a gel. The blank hydrogel and the crude extract of PC-loaded hydrogels images were shown in Figure 1. The visible result of the blank hydrogels appeared as a white matrix, Figure 1a. Interestingly, there slightly changed by losing their humidity during the two months at room temperature. The addition of the crude extract resulted in a different color of the mixture. C005H- and C005L-loaded hydrogels were cleanly fabricated and shown in Figures 1b and 1c.

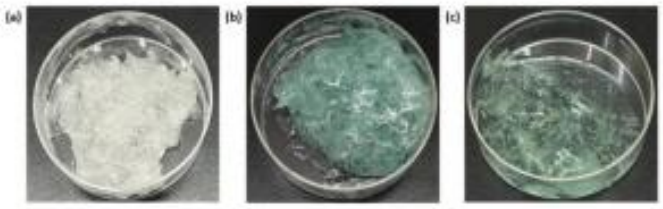

Figure 1. Photos of (a) blank hydrogel (b) C005H-loaded hydrogel (c) C005L-loaded hydrogel.

The relationship between the cumulative release vs time for C005H-loaded hydrogel and C005L-loaded hydrogel was compared as shown in Figure 2. In this study, the cumulative release was increasing gradually over time. The cumulative release of the crude extracts of PC from C005H-loaded hydrogel and C005L-loaded hydrogel at 6 h was 81.5% and 72.6%, respectively. There was no different release profile between those loaded hydrogels.

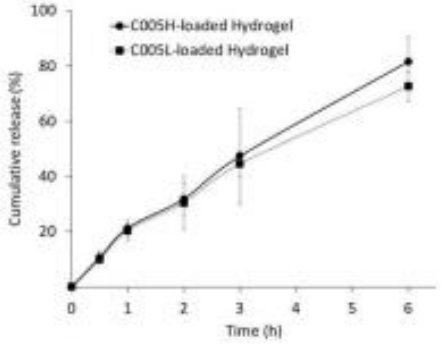

Figure 2. The release profile of the crude extract of phycocyanin (PC) from the hydrogels.

Herein, the released samples at 3 and 6 hours-incubation were collected and investigated their anti-inflammatory potential. The results of % inhibition of LOX activity showed that released samples prepared from C005H-loaded hydrogel at 3

and 6 hours-incubation were increasing from 26.4 + 4.0 to 32.5 + 2.7%. The inhibitions of protein denaturation were noted as 11.2 + 1.1 and 20.0 + 10.4% at 3- and 6-hours incubation time, respectively. The LOX inhibition activity of the samples prepared from C005L-loaded hydrogel showed significantly higher C005H-loaded hydrogel samples at 39.6 + 2.7 and 47.5 + 1.5% at 3 and 6 hours-incubation. But, the results from protein denaturation were not significantly different from the C005H-loaded hydrogel samples at 10.7 + 6.0 and 15.5 + 4.7% (3- and 6 hours-incubation samples).

In vitro biocompatibility testing

Cytotoxicity of the extracted against L929 and GF

Cytotoxicity results of the extracted PC on L929 and GF cells were shown in Figure 3. For both L929 and GF cells, the MTT results from 1 day-incubation showed that the relative cell survival of C005H and C005L at different concentrations was basically above 70%. Increasing the incubation time to 3 days, overall cell survival was still greater than 80%, except the crude extract of PC from C005L showed the toxic above 100 µg/ml. Herein, the IC_{50} (Inhibitory Concentration) of the C005L crude extract was approximately 194 µg/ml.

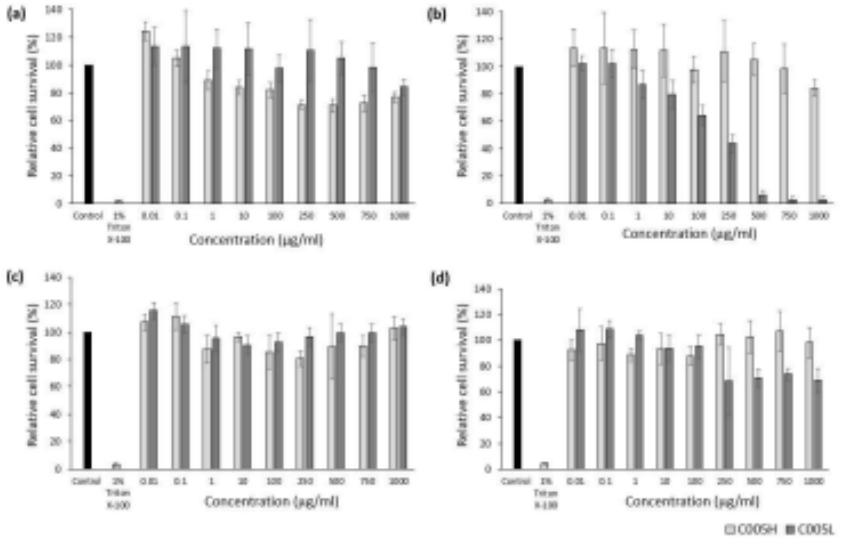

Figure 3. ***In vitro*** **cytotoxicity of the crude extract from 2 strains:** ***Arthrospira platensis*** **strain C005H and** ***Arthrospira platensis*** **stain C005L.** Graphs showed the relationship between cell viability (%) and concentration of the crude extract: (a) 1-day incubation with L929 cells day, (b) 3-day incubation with L929 cells, (c) 1-day incubation with GF cells, and (d) 3-day incubation with GF cells. (n = 6).

Cytotoxicity of Chitosan/Pluronic F-127 hydrogel

Cytotoxicity results of the hydrogels and cell morphological observation were shown in Figures 4 and 5. Both L929 and GF cells were treated with the extracted medium prepared from the blank hydrogel, C005H-, and C005L-loaded hydrogels. Overall results of the blank and extracted PC-loaded hydrogel showed over 90% cell survival, Figures 4e and 5e. There were no significantly toxic to L929 and GF cells after treating those cells with 100% extracted medium, Figure 4c-d, and Figure 5c-d. Meanwhile, treated cells with 1% (v/v) Triton X-100 had altered cell shapes and below 3% cell viability, Figure 4b and Figure 5b. Herein, a commercial product (Kanolone®) was used to compare. There was no cellular toxicity after treated L929 and GF cells using the same procedure as mentioned above.

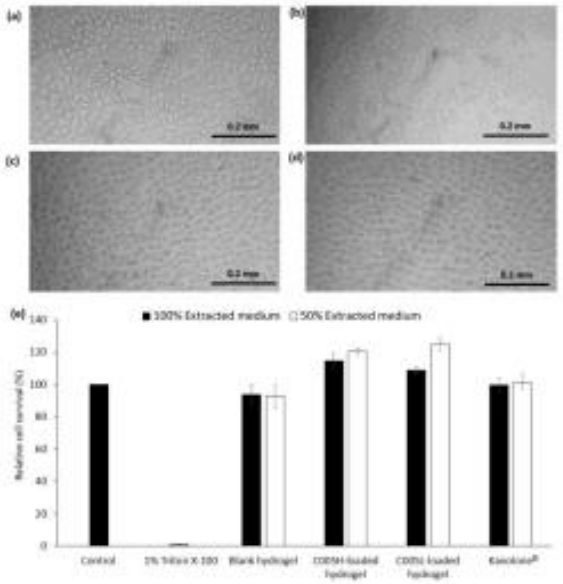

Figure 4. Morphology and viability of L929 cells: L929 cells were treated with (a) fresh culture medium (control), (b) 1% Triton X-100, 100% extracted medium prepared from (c) C005H-loaded hydrogel, and (d) C005L-loaded hydrogel. (e) L929 cell viability after 1 day treated with extracted culture medium (100% and 50%) prepared from chitosan/pluronic F-127 hydrogel and crude extract PC-loaded hydrogel on L929 cells. The scale bar is 0.2 mm.

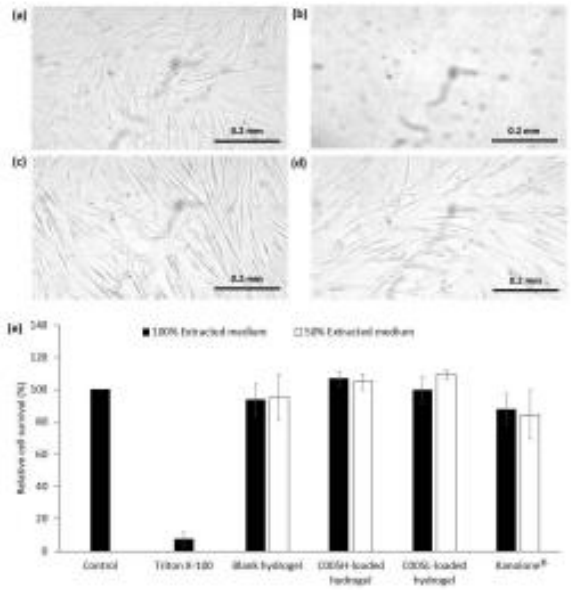

Figure 5. Morphology and viability of GF cells: GF cells were treated with (a) fresh culture medium (control), (b) 1% Triton X-100, 100% extracted medium prepared from (c) C005H-loaded hydrogel, and (d) C005L-loaded hydrogel. (e) GF cell viability after 1 day treated with extracted culture medium (100% and 50%) prepared from chitosan/pluronic F-127 hydrogel and crude extract PC-loaded hydrogel on GF cells. The scale bar is 0.2 mm.

Wound healing assay

The wound-healing assay of GF cells was evaluated by measuring the recovery area after 24 hours of scratching the monolayer of GF cells. The GF cells were cultured under different conditions of the culture medium as shown in Figure 6. The recovery area of the GF cells treated with serum-free medium and the extracted medium prepared from Kanolone® were 38.5% and 53.1%. In contrast, the testing medium prepared from blank hydrogel, C005H-, and C005L-loaded hydrogel were 0.2%, 20.5%, and 13.4%, respectively.

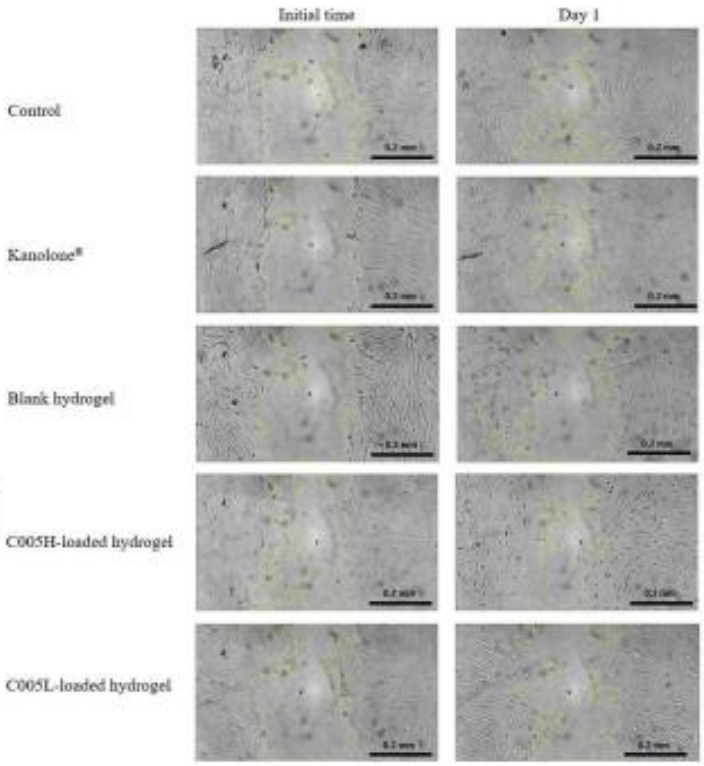

Figure 6. The wound-healing assay area of the GF cells was analyzed by using the Image J software after scratching. The recovery area was presented after 24 hours of incubation.

DISCUSSION

PC is one of the main phenolic compounds found in the crude extract from Arthrospira platensis. The phenolic compounds exhibit free radical inhibition, peroxide decomposition, and protection against oxidative disease burden in biological systems (Tayag et al., 2010). The phenolic compounds act as good electron donors because their hydroxyl groups can directly contribute to antioxidant effects. The free radical scavenging ability of the extract was related to its total phenolic content (TPC) concentration. Generally, the crude extract from spirulina has been used as food additives mainly in Asia-Pacific where the market is grown at a Compound Annual Growth Rate (CAGR) of 6.1% by 2026 reported by Mordor Intelligence on the website, May 2022.

For this study, the wild-type strain of spirulina (C005H) and the straight trichomes stain (C005L) did not show different numbers of the TPC value. However, the crude extracts had studied the antioxidant capacity like protein denaturation. Compared with the case without the crude extract (64.8%), the addition of 2 crude

extracts from both C005H and C005L significantly increased the percentage of the inhibition of the mock protein (BSA) degradation at high temperature. Herein, the LOX inhibition activity was used to explain the anti-inflammatory potential. The activity of the crude extracts of PCs were significantly greater than our reference active compound (quercetin and gallic acid) at least 2 times. These results indicated that the crude extract of PC from both stains had a strong resistance to inflammatory reactions. However, the limitations of this work were *in vitro* experiments and the low purity of the PC. The recovery and purification process of the PC might require cost and time. It might interrupt the activity level of the extracted compound. For biocompatibility testing, the cell viability results of the crude extract did not show any significant severe toxicity against L929 and GF cells. Both L929 and GF cells appeared normal under a microscope. Herein, we recommended using both crude extracts of PC below 100 µg/ml for future studies. This present study employed the benefit of the use of the direct crude extract of PC from the spirulina C005H and C005L stains for future development. The extract could be increased the value by incorporating the hydrogel carriers.

The chitosan/pluronic F-127 hydrogel was spontaneously formed when pluronic F-127 was added to the chitosan solution. Pluronic F-127 acted as a non-toxic cross-linker between chitosan cationic polymers using an electrostatic interaction between different polar of those polymers (Mohamed et al., 2022). Our finding has shown that over 15% (w/v) of pluronic F-127 in the mixture could form the hydrogel. In addition to stability improvement, higher pluronic F-127 contained in the mixture could form the hydrogel for up to 2 months at room temperature as mentioned in PF127-TMC/DTX hydrogels by Turabee et al. (2019). Unlike the curcumin-QCS/PF1.0 hydrogel by Qu et al. (2018), our hydrogels did not provide a bond like a methyl bond by glycidytrimethylammonium chloride (GTMAC). Thus, gelation time would be increased up to a day. Swelling and gel recovery were not studied by stretching/compression and releasing the hydrogels. Although C005H and C005L compound did not link to the backbone of the hydrogel matrix, the release of the crude extract of PC (C005H and C005L) could be controlled and did not provide as a burst release within the first 2 hours. Because the electrostatic and gelation processes are random, this was a simple process that could entrap the crude extracts of PC into the gels using pluronic F-127 as a challenging matter as the polyplex nanoparticles caused by the charges of dsRNA and chitosan (Lichtenberg et al., 2019). As expected, the amount of the crude extracts of PC resulted in almost 100% encapsulation efficiency. The photos of C005H- and C005L-loaded hydrogel showed in green color due to the presence of those pigments. Based on the calculation, the loading efficiency of the crude extract of PC (both C005H and C005L) into the chitosan/pluronic F-127 was approximately 0.5 %. This condition was selected for further studies.

In a real application, the non-strong cross-linker, pluronic F-127, can be easily disrupted by the biological solution. Over 70% of released-crude extract of PC showed a prolonged period (6 hours) at the site and kept the concentration of the PC within the therapeutic index. Compared with QCS/PF1.0 hydrogels, the release of curcumin was approximately 20% within 100 hours due to the conjugation bond requiring an acidic condition to cleave (Qu et al, 2018). Moreover, the anti-inflammatory activity of the released samples showed the potential of the use of these hydrogels for gingivitis treatment. It should be noted that all conditions of the PC-loaded hydrogels were no toxic effect on L929 and GF cells. In the part of wound healing, we compared the area of the cell treated with the extract medium from the PC-load hydrogels with the control and commercial product, Kanolone®. The effectiveness of wound healing of the PC-loaded hydrogels was rather lower than Kanolone® due to this study used a 50% concentration of the extracted medium and was exposed to the GF cells for 24 hours. The lower purity of PC might show an ineffective compound as a limitation of this study. Furthermore, this study did not perform over 24 hours due to the previous report by Afrasiabi et al. (2021). The toxicity of chitosan hydrogel to human gingival fibroblast cells (HGFs) was found if the concentration of the hydrogel was over 156.2 µg/ml with 72-hour-incubation (Afrasiabi et al., 2021). Thus, we found that the extracted medium prepared from

the blank hydrogel did not increase the recovery area as the extracted medium from PC-loaded hydrogels.

A major advantage of this preparation is simply because of a spontaneous method. Consequently, the hydrogels are likely to be non-toxic and biocompatible. The hydrogels can be easily degraded in the presence of water to release the crude extract of PC. We believe that this preparation is promising as an oral delivery vehicle for the treatment of gingivitis.

CONCLUSION

The present study described the anti-inflammatory capacity and cytotoxicity of PC, as well as the hydrogel preparation, PC release profile, and cytotoxicity of PC-containing chitosan/pluronic F-127 hydrogels. The crude extract of PC from both stains showed potential in inhibiting protein degradation and LOX inhibition activity with no cytotoxicity at concentrations below 100 µg/ml. These crude extracts of PC were completely encapsulated into the chitosan/pluronic F-127 hydrogels. In addition, the system was free of cross-linking agents and increased the biocompatibility of the hydrogel as there were no possible toxic by-products. The hydrogels were found to prolong-release the crude extracts within 6 hours with effective anti-inflammatory activity. Thus, PC-loaded chitosan/pluronic F-127 hydrogel from this study might be an alternative treatment for human gingivitis.

ACKNOWLEDGMENTS

The authors would like to acknowledge Applied Algal Research Laboratory, Chiang Mai University especially for providing the *Arthrospira platensis* starter stain (C005H). This study was supported by the Junior Researcher, Chiang Mai University (R000022723). TP was supported by Health Systems Research Institute (65-139) and National Research Council of Thailand (NRCT)(N42A650229).

AUTHOR CONTRIBUTIONS

ML and PK (Kamdenlek) contributed to experimental design, data acquisition, statistical analysis, data interpretation, and manuscript drafting. PK (Kuntanawat) provided the extract samples, information, and contributed to the experimental design and data interpretation. KE, TP, and TO contributed to experimental design and data interpretation. CM contributed to study conceptualization, experimental design, and data interpretation. All authors critically revised the manuscript and gave final approval for publication.

CONFLICT OF INTEREST

All authors declare no competing interests.

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OPEN access freely available online
Chiang Mai University Journal of Natural Sciences [ISSN 16851994]
Chiang Mai University, Thailand.
https://cmuj.cmu.ac.th

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