DEVELOPMENT OF BIO-CELLULOSE MASK CONTAINING PUNICA GRANATUM L. PEEL EXTRACT

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Thesis entitled “Development of bio-cellulose mask containing
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by Preeyawass Phimnuan
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for the Master of Science Program in Cosmetic sciences of Naresuan University

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ABSTRACT

The aim of this study was to develop the bio-cellulose mask containing *Punica granatum* (pomegranate) peel extract for application as anti-acne product. Pomegranate peel extract (PPE) with the extraction of 50% ethanol (PPE_{50E}) exhibited the highest %yield, total phenolic content (TPC) and free radical scavenging activity. Also, PPE_{50E} exerted the bactericidal activity against both of *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Propionibacterium acne*. Thereby, The PPE_{50E} was selected for formulating anti-acne bio-cellulose (BC) film. The production of bio-cellulose, by the incubation of *Acetobacter xylinum*. The characteristics of the combination of BC and PPE was appeared with yellowish pellicle, toughness, durability with a thickness of 0.1 mm, in accordance with visualization. For the mechanical properties, BCP presented the increasing tightness but decreasing flexibility compared with BC. The TPC released from the BCs was relatively constant after 1h. The anti-bacterial efficacy of BCP was performed by disc diffusion method which. The results demonstrate that BCP possessed a satisfactory inhibition effect closed to those of gentamycin and clindamycin. From the remarkable characteristic of BC in the capability of mechanism properties and the potential of PPE in the bacterial inhibition support the BCP, which could be the interestingly alternative way to use as the anti-acne product.

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ABBREVIATIONS

3D	=	three dimension
α	=	alpha
β	=	beta
γ	=	gamma
°C	=	degree celsius
μg	=	microgram
μL	=	microliter
μm	=	micrometer
%	=	percent
AMPs	=	2-Acrylamido-2-methylpropane sulfonic acid
ARE	=	anti-oxidant response element
BC	=	bio-cellulose
BCP	=	bacterial cellulose combining pomegranate peel extract
BHI	=	brain Heart Infusion
BMMs	=	Bemberg microporous membranes
c-di-GMP	=	cyclic diguanylmonophosphate
CFU	=	colony-forming units
cm^2	=	centimeter square
Cont.	=	continued
COX-2	=	cyclo-oxyginase
DPPH	=	2, 2-Diphenyl-1-picrylhydrazyl
DMEM	=	Dulbecco's Modified Eagle Medium
EC_{50}	=	Half maximal effective concentration
EtOH	=	Ethanol
EGCG	=	epigallocatechin gallate
EUCAST	=	The European Committee on Antimicrobial susceptibility Testing
FFA	=	Free fatty acid
FRAP	=	ferric ion reducing antioxidant power

ABBREVIATIONS (CONT.)

GAE	=	gallic acid equivalents
GCS	=	glutamyl cystein synthetase
GPa	=	gigapascal
GYE	=	Glucose Yeast extract
H	=	hour
H^+	=	proton
HO-1	=	heme oxygenase
HS	=	Hestrin and Shcramm
IC_{50}	=	Half maximal inhibitory concentration
IL	=	Interleukin
MBC	=	minimum bactericidal concentration
MC	=	Microbial cellulose
MEAs	=	membrane electrode assemblies
mg	=	milligram
MIC	=	minimum inhibitory concentration
min	=	minute
mL^{-1}	=	per milliliter
mL	=	milliliter
mm	=	millimeter
MPa	=	megapascal
MWNT	=	multi-walled carbon nanotubes
NCCLs	=	National Committee for Clinical Laboratory Standards
NLRs	=	NOD-like receptors
nm	=	nanometer
NOS^o	=	reactive nitrogen species
N_2O	=	Nitrous oxide
NO^-	=	nitroxyl anion
NOD	=	Nitric oxide synthase
No.	=	number

ABBREVIATIONS (CONT.)

O_2^{o-}	=	superoxide
OD	=	Optical densitty
OH^-	=	hydroxyl group
OH^o	=	hydroxyl radicals
$ONOO^-$	=	peroxide nitrite radical
PPE	=	pomegranate peel extracts
RSA	=	radical scavenging activity
ROS	=	reactive oxygen species
SBC	=	slurry bacterial cellulose
SC	=	Stratum corneum
SCD	=	soyabean Casein Digest
SD	=	Standard deviation
SEM	=	scanning Electron Microscope
SPRE	=	standardized pomegranate rind extract
SOD	=	superoxide dismutase
TC	=	terminal complexes
TLR	=	Toll-like receptor
TNF	=	Tumor necrosis factor
TEWL	=	Transepidermal water loss
TEAC	=	Trolox equivalent antioxidant capacity
w/w	=	weight by weight
w/v	=	weight by volume

CHAPTER I

INTRODUCTION

Rational of the study

Acne vulgaris is skin disease which results of pilosebaceous follicles obstruction. Normally, it is located primarily on face and upper body (Leyden, 1997). The main factor involved in the pathogenesis of acne is the abnormality in colonization of normal flora, including *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermidis* (*S. epidermidis*); gram-positive facultative aerobic organism. They are the opportunistic pathogens, usually involve in superficial infection within the sebaceous units (Desbois and Lawlor, 2013). Additionally, *Propionibacterium acnes* (*P. acnes*) is an immobile, gram-positive, lipophilic obligate anaerobe that colonizes in the follicular duct. It provokes an inflammatory response by its capability to activate the surrounding composition and by the bacterial enzyme (lipase) that metabolizes the sebaceous triglycerides into free fatty acids, which result in irritation of the follicular wall and keratinization (Patil, et al., 2012). Nevertheless, the common therapy in the treatment of acne includes oral and topical therapy employing comedolytics and antibiotics. These comedolytics and antibiotics have several adverse effects on skin irritation, dry skin, peeling, burning, photosensitization, abnormal skin pigmentation (Lalla, et al., 2001; Niyomkam, et al., 2007). Moreover, the antibiotics resistance has been increasing in prevalence within the dermatologic setting (Swanson, et al., 2003). Thus, the concept of applying the natural innovation to use as the anti-acne product could be considered as alternative that minimizes such problem.

Pomegranate (*Punica granatum* L.) is a fruit that is commonly found in various countries. All parts of this fruit can be used for remedial purposes in medical terms (Wang, et al., 2011). The phenolics in pomegranate peel extract (PPE) have been demonstrated the outstanding in the anti-bacterial activity. Especially, the potent bacteriostatic effect against *S. aureus, S. epidermidis* and *P. acnes* which are the cause of acne (Panichayupakaranant, et al., 2010). Moreover, the phenolic compounds in

PPE can reduce the causes of acne through 3 main mechanisms; anti-bacterial activity (Scalbert, 1991; Spencer, et al., 1998), anti-inflammation (Gange, et al., 2009) and anti-oxidation (Fridovich, 1999; Malago, et al., 2002). The inhibition of these pathways represses the production of substrates or synthesis of bacteria products, which thus reduced the burden of acne (Romier-Crouzet, et al., 2009). However, to enhance the efficacy of the PPE for acne treatment, the design of an effective delivery device is important. Patch mask is one of the dosage form that attracts our attention as it generally enhances the transport of active compounds by altering the skin barrier functions through increasing hydration of stratum corneum (SC) (Suksaeree, et al., 2015). In addition, it can attach on the skin well, while at the same time serving as a potential physical barrier against any external infection (Czaja, et al., 2006).

Nowadays, the bacterial cellulose or bio-cellulose (BC) has been become famous due to its exclusive ability such as high strength and flexibility, reformability, and biodegradable. BC is safe for its compatibility with the skin, non-toxic to cells and does not cause irritation to the skin (Phisalapong, et al., 2008). Moreover, BC can be applied purely or impregnated alternatively with active ingredients particularly plant extracts (Klemm, et al., 2006). BC is a 3D nano-sized structure, results in a large surface area that higher water holding capacity (Phisalapong and Jatupaiboon, 2008). From the remarkable characteristic of the BC that mentioned above, we are interested to apply the BC for acne treatment. In the present study, therefore, PPE was combined with BC by simple soaking. The chemically pure structure of the BC contains a plenty of a hydroxyl group, are hydrophilic which can bind to active ingredients in PPE, especially phenolic compounds comprising hydroxyl groups as well, by the strong hydrogen bonds (Klemm, et al., 2006). The BC combining extract was then observed its physical properties, stability and its efficacy on anti-bacterial activity. The results indicate the potential of BC combining the extract for application as acne treatment.

Purposes of the study

1. To develop the BC film containing PPE
2. To determine the physical, chemical and anti-bacterial properties of the developed BC film

Hypothesis

From the remarkable benefit of the BC and the interesting advantage of the PPE, the researcher has the point of view to apply the BC combining PPEs in the cosmeceutical for anti-bacterial activity.

Expected output of the study

BC combining PPE developed from this study has ability potential to possess the anti-bacterial activities against the bacteria which is the cause of acne.

CHAPTER II

REVIEWS OF RELATED LITERATURE AND RESEARCH

Acne vulgaris

Acne is one of the most common multifactorial chronic inflammatory diseases of pilosebaceous follicles. It affects to permanent scarring, deformity and also have an adverse effects on psychological development, impact to profound emotional scarring, and leading to social phobias, withdrawal form society or clinical depression (Özkan, et al., 2000). It affects all age group i.e. teenagers (85%), 25-34 year (8%) and 35-44 year (3%) (Leyden, 2001). Acne occurs in the pilosebaceous units within the epidermis and dermis, which consist of sebaceous gland, hair follicle and associated hair shaft. In response to increased testosterone (androgen) levels, the sebaceous gland produces sebum, a mixture of fats and waxes that protect the skin and hair by retarding water loss and forming a barrier against external agents. The hair follicle is lined with epithelial cells that become keratinized as they mature. During puberty the production of androgenic hormones increases in both genders and testosterone levels rise. If the sebaceous glands become oversensitive to testosterone, they produce excess oil and the skin becomes greasy (Leyden, 1995). At the same time, keratin in the follicular epithelial wall undergoes change (Cunliffe, et al., 2000). Prior to puberty, dead cells are shed smoothly out of the ductal opening but at puberty this process is disrupted and in patients with acne these cells develop abnormal cohesion and partially block the opening in the epidermis and effectively reduce sebum outflow. Over time the opening of the duct becomes blocked, trapping oil in the hair follicle. Oil blocks the follicle openings in the epidermis and causes them to dilate beneath the skin surface. If the orifice of the follicular canal opens sufficiently, the keratinous material extrudes through it and an open comedone results. This is known as a blackhead as the keratinous material darkens in contact with the air. Because this material can escape, the comedone does not become inflamed. If the follicular orifice does not open sufficiently, a closed comedone results, within which inflammation can occur. Most acne sufferers have a combination of both open comedones (blackhead) and closed comedones (whitehead) (Trewet, 2008). Also, abnormality of normal flora, colonization by *S aureus* and. *S. epidermidis*, Gram-positive facultative aerobic organism. They are the opportunistic pathogens, usually involve in superficial infection within the sebaceous unit (Desbois and Lawlor, 2013). Additionally, *P. acnes* is an immobile, Gram-positive, lipophilic obligate anaerobe that colonizes in the follicular duct. Furthermore, it provokes an inflammatory response by its capability to activate the surrounding composition and by the bacterial enzyme (lipase) that metabolizes the sebaceous triglycerides into free fatty acids (FFAs) and glycerol. FFAs directly compromise the integrity of the follicular environment resulting in the release of IL-1-α, which results in irritate the follicular wall and keratinization (Srinivasan, et al., 2001). In addition, *P. acnes* also innates the immunity, that is the first line of defense against infection illnesses, by identifying pathogen recognition patterns and activating innate chemotactically immune responses via Toll-like receptor (TLR) 2 and 4, Peroxisome-activated receptors, Intracellular NOD-like receptors (NLRs) 1-3, Retinoic acid inducible gene-like intracellular receptors and AMPs. Besides, it induces the synthesis of local pro-inflammatory factor, such as α-Tumor necrosis factor (TNF-α), Interleukin (IL) 1β, Prostaglandins, Leukotrienes and IL-8 to eliminate the wasted cell surrounding the acne (Suelen, 2010; Dréno, et al., 2015). *P. acnes* also generates mild local inflammation by producing neutrophil chemotactic factors. Accordingly, neutrophils get attached to the acne lesions and constantly released inflammatory mediators such as reactive oxygen species (ROS) (Leyden, 1997; Pratibha, et al., 2012). *P. acnes* or any other bacteria with keratinocytes in terms of ROS production leading to the production of ROS during tuberculosis infection. Furthermore, keratinocytes are known to produce ROS upon exposure to toxic compounds such as inorganic arsenic or to ultraviolet radiations. Whatever the mechanism implicated in the induction of skin inflammation by *P. acnes*, ROS are probably involved in that process since the production of hydrogen peroxide (H_2O_2) is increased in neutrophils from acne patients. Moreover, the decreasing in superoxide dismutase (SOD) activity in patients with acne lesions is correlated with the severity of acne (Grange, et al., 2009). The clinical manifestations of acne include seborrhea (excess grease), non-inflammatory lesions, inflammatory lesions, and various degrees of scarring due to cyst formation

(Leyden, 1997). The severity of this skin disorder generally increases with age and time. The distribution of acne relates to the highest density of pilosebaceous units; it is distributed over face, forehead, neck, upper chest, shoulders, and back. According to the lesion type, acne can be classified into non-inflammatory acne (purely comedonal) and inflammatory acne (mild papular, scarring papular, and nodular) (Figure1). Grading upon its severity, it can be categorized into mild, moderate, and severe acne. Mild acne comprised of open and closed comedones (<20), inflammatory lesions (<15) with total lesions not exceeding 30. Likewise, in moderate acne numerous papules and pustules are observed along with comedones (20–100), inflammatory lesions (15– 50) whereas total lesions in the range of 30–125. Severe acne is diagnosed with extensive lesions including nodules and scarring together with cysts (>5), total comedone count (>100), total inflammatory count (>50) and total number of lesions more than 125 (Layton, 2005; Truter, 2009).

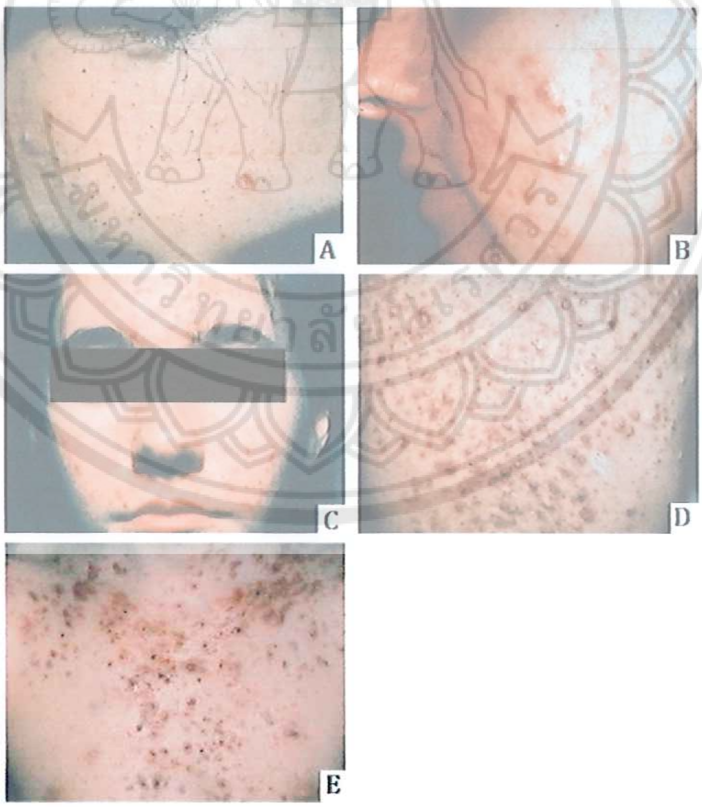

Figure 1 Photographs of patients with acne

Source: Layton, 2005

Cellulose

Background of cellulose

In the present day, the nano-scale cellulose fibers production and their application has achieved raising attention due to their ability, high mechanical strength and stiffness combined with low weight, biocompatibility, renewability and biodegradability. The cellulose application nanofibers in polymer encouragement are a relatively new research field. Although, there is increasing publication activity, the number of reports is still modest when compared to publication dealing with organic micro- and macrofillers or inorganic nanofillers (Figure2). The limited application of cellulose nanofibers to date may be partly because the reproducibility of BC fibers into likely in each batch has typically been a challenging process (Siró and Plackett, 2010). In this study we illustrated various approaches to the preparation of the nano-cellulosic from bacteria.

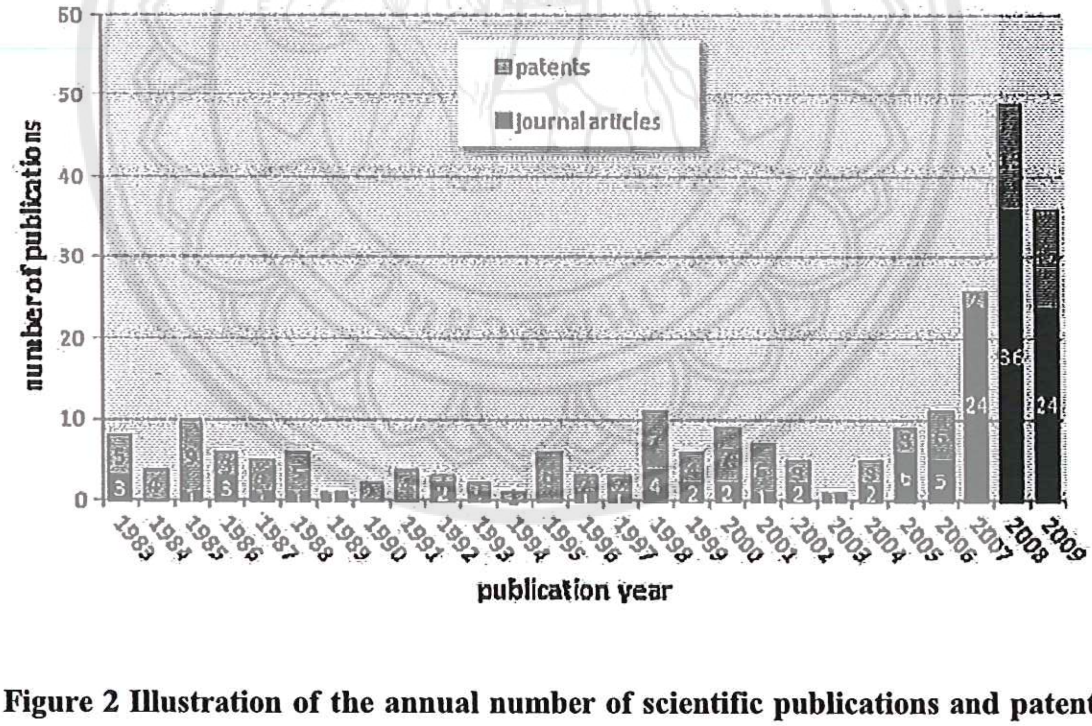

Figure 2 Illustration of the annual number of scientific publications and patents since 1983

Source: Siró and Plackett, 2010

There are several ways to produce the biopolymer cellulose. The first one consists in the biosynthesis of cellulose by different types of microorganisms. Algae (Vallonia), fungi (Saprolegnia, *Dictystelium discoideum*) or bacteria (Acetobacter, Archomobacter, Aerobacter, Agrobacterium, Pseudomonas, Rhizobium, Sarcina, Alcaligenes, Zoogloea) are known from the literature (Esa, et al., 2014). The second way is the most popular and industrial important isolation of cellulose from plants. The others are enzyme synthesis and chemosynthesis respectively (Klemm, et al., 2001) (Figure3). Cellulose is a biological substance that is found in nature especially in plants. In the cell walls of plants, cellulose is the main structure. It consists a large number of polysaccharide. Plant-derived cellulose is impure cellulose. It can be connected with other cellulose that depending on the plant species, such as lignin and hemicelluloses which is different from the BC is chemically pure and free of lignin and hemicellulose. BC has high crystallinity and a high degree of polymerization. Plant-derived cellulose and BC have the same chemical composition but different structures and physical properties. Cellulose which from the synthesis of bacteria in dormancy (stationary phase) by bacteria. Firstly, the bacteria cultured on agar plate characteristic like a jelly or a thin membrane. Then, moved to cultivate in liquid medium in the specific container to synthesize the nano-sized fibril. In the photo-synthesis of cellulose, using sugar (glucose) is the main reactant (Czaja, et al., 2006; Phisalapong, et al., 2008; Phisalapong and Jatupaiboon, 2008; Torres, et al., 2012).

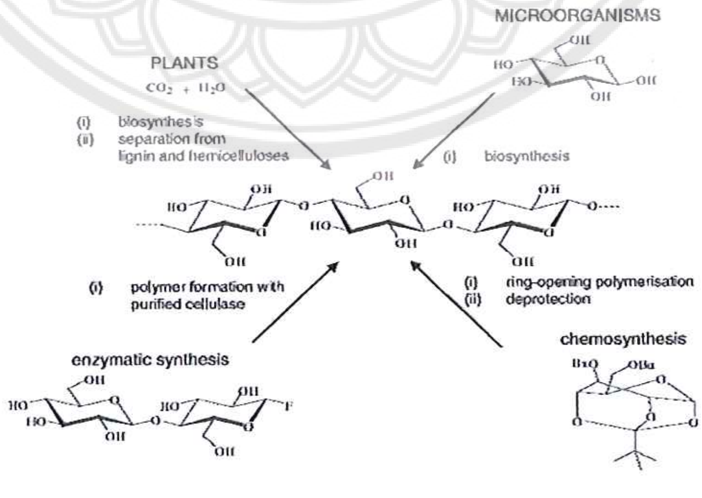

Figure 3 Pathways to synthesize the cellulose

Source: Klemm, et al., 200

The sites of synthesis of cellulose

Bacteria *Acetobacter xylinum* synthesizes the cellulose between outer and the cytoplasma membrane, which is in associated with pores at the surface of the bacteria. The synthesis of cellulose complexes or the triplet subunits are further termed as a terminal complexes (TC) are linearly arranged in the major axis of the cell (Figure4a). The single subunit extrudes a sub-elementary fibril, which is composed of spontaneously assembled cellulose molecular chains synthesized by each catalytic site to form a conformation (Figure4b). Firstly, cellulose formation glucan chain aggregates consisting of approximately 6-8 glucan chains are elongated from the complex. Then, the sub-elementary fibrils are assembled to form microfibrils followed by their tight assembly to form a ribbon as third step. The cellulose synthase is considered to be the most important enzyme in this process. Cyclic diguanylmonophosphate (c-di-GMP) was identified as activator of cellulose synthase. The proposal of principal biochemical pathway from glucose to cellulose (Figure5), which should be linked to cell growth as well as to cellulose formation. A self-assembly process should be responsible for the fibrils was completed (Klemm, et al., 2001; Tomita and Kondo, 2009; Jonas and Farah, 1998).

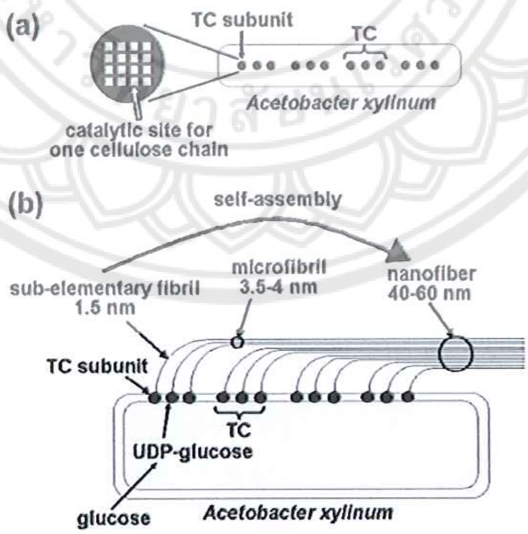

Figure 4 Schematic figures of the geometry of the Terminal complexes and its subunits in relation to the formation of cellulose nanofiber

Source: Tomita and Kondo, 2009

Glucokinase Phosphoglucomutase
Glucose-------------------> Glucose -6- Phosphate--------------------------> Glucose -1-

UDP-Glucose Pyrophosphorylase Cellulose Synthase
Phosphate--------------------------------------> UDP-Glucose----------------------->Cellulose

Figure 5 Proposed biochemical pathway for cellulose synthesis in *A. xylinum*

Source: Jonas and Farah, 1998

The properties of cellulose fiber

The characteristic structure of the cellulose fiber is a 3D nano-sized fibril shapes weave together that vary depending on the container which cultivate the bacteria (Figure6), resulting in formation of hydrogel sheet with high surface area and porosity. BC consists of sets of parallel chains of β-1, 4-D-glucopyranose units interlinked by intermolecular hydrogen bonds with molecular formula $(C_6H_{10}O_5)n$ (Esa, et al., 2014). As formation as well as super- and supra-structure of BC fibers and mask can be controlled by the variation of the nutrient medium components and the cultivation conditions (Klemm, et al., 2001). (Figure7) Fibers with a diameter of 100 nm, a length of about 100 μm. Cellulose fibers have a feature to swell in water to keep the porous structure, the research of Grande CJ et al. have studied the mechanical properties of cellulose fibers reported that cellulose fibers with tensile strength in the range of 219.56 - 263.28 MPa and the elongation in the range of 5.2 to 11.22% (Grande, et al., 2008), which is a distinctive feature that shows that fiber cellulose has strength and high flexibility. Young's modulus of BC is approximately 4 times greater than that of general organic fibers (Phisalapong, et al., 2008). Also the high modulus of microfibrils, estimated to be around 140 GPa in the longitudinal direction (Retegi, et al., 2008; Nakagaito, et al., 2005). Thus, the chemical composition with high stability and easily spreadibility. The cellulose fibers also have the crystallinity properties. BC is a gel containing 99% of water by weight, mainly due to its amorphous structure (Lina, et al., 2009). Affected to it has the absorption ability well (Phisalapong and Jatupaiboon, 2008). Also, it can be dry as a thin membrane which is suitable for constructing membrane electrode assemblies (MEAs) because it contains the equivalent amount of water to at least 200 times the dry weight of cellulose (Chen, et al., 2010).

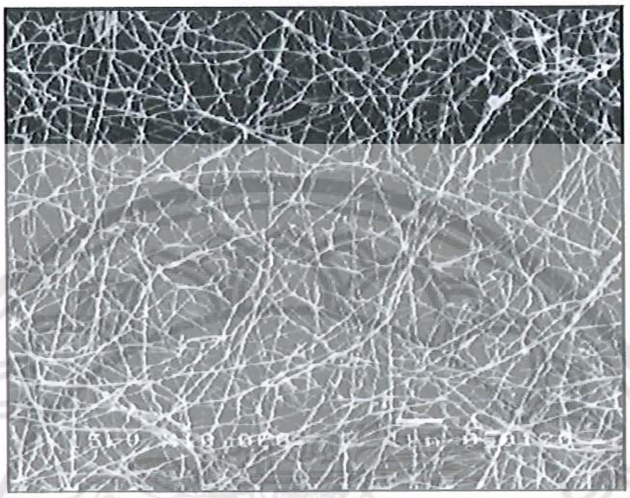

Figure 6 Characteristic of cellulose fiber from BC under SEM

Source: Phisalapong and Jatupaiboon, 2008

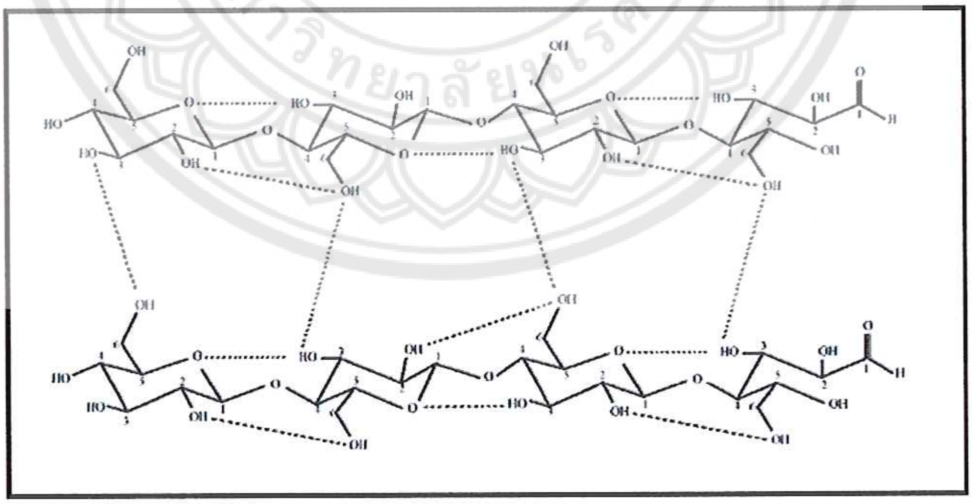

Figure 7 Inter- and intra-hydrogen bonding of BC

Source: Esa, et al., 2014

Biocompatibility of cellulose fiber

BC shows non-allergenic and can be safely sterilized without any change to its characteristics. Sanchavanakit N, et al. found that BC film supported the growth, spreading and migration of human keratinocytes but not those of human fibroblasts (Sanchavanakit, et al., 2006). Saska S, et al. reported that *in vitro* assays demonstrated no cytotoxic, genotoxic or mutagenic effects for any of the studied BC membranes (Saska, et al., 2012). Jeong S, et al. studied the toxicity evaluation of bacterial synthesized cellulose in endothelial cells and mice (*in vivo*) reported that BC nanofibers did not produce any adverse effects in body or organ weight, food consumption or gross findings (Jeong, et al., 2010). In clinical trial evaluation found that microbial cellulose (MC) wound dressing was superior to control group, pain control, ease of use and patient and nursing staff satisfaction (Solway, et al., 2010). In case of Solway Dr, et al. reported that the application of an MC wound dressing to a diabetic ulcer may enhance the rate of wound healing and shorten the time course of epithelization (Solway, et al., 2011). Namely both of the case the BC is biocompatible. Also, the great conformability of this cellulose material has been proven during clinical trials on a large number of patients. The BC was adhered to the wound sites very well, and its elastic properties allowed an excellent molding to all facial contours, displaying a high degree of adherence even to the moving parts like hands (Figure8), torso, face (Figure9) and so on (Lina, et al., 2009; Czaja, et al., 2006).

Figure 8 MC wound dressing applied on a wounded hand

Source: Lina, et al., 2009

Figure 9 BC dressing applied on wounded torso and face

Source: Czaja, et al., 2007

The application of BC in various fields

The unique physical and mechanical properties of BC as well as its purity and uniformity can be determined in various applications (Figure10) (Czaja, et al., 2006), which may take in form of a dry or wet form. It can also be combined with other materials. Ul-Islam M, et al. reported that BC conductive composite films combine the electronic characteristics of conducting polymers and nanomaterials with the excellent mechanical properties of the BC matrix. These can be used in optoelectronics, including flexible electrodes, flexible displays, and other electronic devices (Figure11) (UI-Islam, et al., 2015). From the properties that consists of extremely small clusters of cellulose microfibrils, the BC has been applied as a binder in papers to enhance the strength and durability of pulp when integrated into paper (Keshk, 2014). The microporous structure of the membrane is important as it is closely associated with plasma filtration rate and sieving properties for macromolecules (Zhou, et al., 2002). Also, there are some report related to the filtration with a Bemberg microporous membranes (BMMs) having mean pore size of 50, 30, 20, 10 and 9 nm caused a completed elimination of HIV infectivity in the filtrates, illustrating that HIV cannot through the BMM by all layers (Hamamoto, et al., 1989). The high mechanical strength in the wet state, substantial permeability for liquids and gases, and low irritation of skin demonstrated that membranous of bacterial was capable as an artificial skin for temporary covering of wounds (Klemm, et al., 2001). As superior mechanical and electrical properties of multi-walled carbon nanotubes (MWNT) are almost one-dimensional nanomaterials with a high aspect ratio. The MWNT and BC fibrils, which are nanofibers, could be used as biomaterials such as artificial muscles or artificial blood vessels (Yan, et al., 2008). In order to obtain a composite material with suitable properties for use in the field itself. In addition, the sterile, it can be done safely without causing properties changes. Also, in the biological properties Sanchavankit et al. reported that the films are made from cellulose fibers can support the growth, distribution and migration of human keratinocyte cells as well.

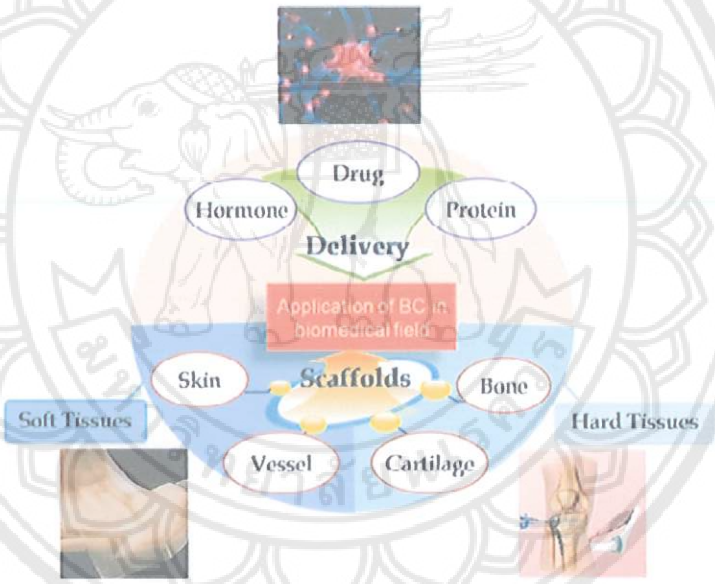

Figure 10 Biomedical applications of BC-based biomaterials

Source: Lina, et al., 2009

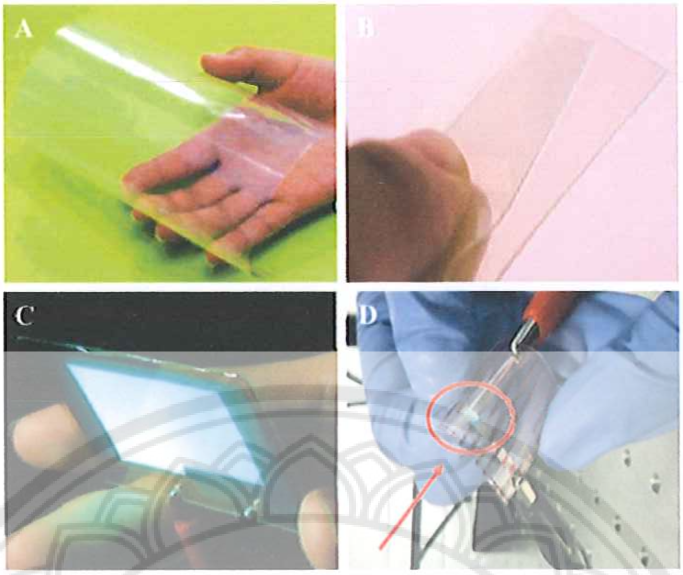

Figure 11 Transparent BC nanocomposite films and their application as substrate for OLED fabrication

Source: Ul-Islam, et al., 2015

The trend of using cellulose from bacteria in cosmetics

Cellulose mask from bacterial, the product produced by vinegar bacteria of the genus *A. xylinum* is a fine cellulose fibers in the form of a gel called cellulosic microfiber by the appearance of jelly that has a mucous membrane with white or cream. There are many names such as Nata de coco, lookprao, orange juice jelly, heaven jelly, Russian mushroom and red tea mushroom. Also, other bacterial genera that can make cellulose such as *Rhizobium sp.*, *Alcaligenes sp.*, *Agrobacterium sp.*, *Pseudomonas sp.*, and *Trichodermareesei* etc. The production of cellulose can use many kinds of raw material such as coconut water, pineapple, watermelon, coconut milk, skim milk. These raw materials used for production of cellulose sheets are similar, but different in smell of raw materials and the physical characteristics (Sheykhnazari, et al., 2011; Simmons, et al., 2008; Toda, et al., 1997; Yang, et al., 1998; Anant, 2010).

Adaptation of cellulose to patch mask

Transdermal patch or patch mask are made from different sources e.g. polymer, fabric, tissue, plant and especially bacteria. They are consisted of at least an adhesive layer and a backing membrane containing the active ingredient. These patches may be affected by influencing physicochemical properties, water vapor permeation, especially by water absorption and transdermal durability of patches considerably. Furthermore, evaporation of water from the skin is interrupted, potentially leading to occlusion and strong hydration of the skin along with an increase in skin permeability (Fokuhl and Müller-Goymann, 2013). In term of the active ingredients, they must be absorbed through the skin which is comprised of dermis and epidermis, especially the SC barrier including sweat glands, sebaceous glands, and hair follicles and penetrate into deeper dermis layers (Ranade, 1991). The main function of the SC as the outer layer of the skin is prevention of the water evaporation from the underlying viable tissue and protection the organism to against harmful substances from the environment. The combination of the intercellular lamellar lipids along with the highly keratinized intracellular environment in the dead and flattened corneocytes make the SC very effective barrier in this context (Esa, et al., 2014; Ranade, 1991). Under occlusive effects to composition inside the SC swells which leading to an enhanced permeability for many active ingredients or any additives. Hence, the permeation behavior of the active ingredient might be influenced. In addition, the water vapor permeability of transdermal patches is crucial for their tolerability on the skin, especially when applied continually for several days on the same area. When applied to the skin the active ingredient is released at a constant rate (Suksaeree, et al., 2015; Fokuhl and Müller-Goymann, 2013; Treffel, et al., 1992; Qiao, et al., 1993; Mint, et al., 1994). The total amount of water loss through the skin is generally determined *in vivo* by measuring the transepidermal water loss (TEWL), is correlated with the state of hydration and it is a well-established method for evaluation of epidermal barrier function (Pinnagoda, et al., 1990). TEWL is generally considered a passive diffusion phenomenon and expressed by steady state flow of the water vapor per unit area of surface in unit time, at a specified humidity and temperature. Changing in TEWL may be caused by physical trauma or induced by chemical treatment or by occlusion (Casiraghi, et al., 2002). Moreover, Proksch E, et al. reported that occlusive treatment of irritated skin resulted in a reduction of barrier repair activities in hairless mice (Proksch, et al., 1991). Moreover, both the mechanical and electrical properties of SC are markedly influenced by its water content. Consequently, skin permeability to substances contained in the patch, in addition to xenobiotics, could be markedly altered. For these reasons it is important to determine the degree of occlusion resulting from the prolonged application of adhesive patches (Treffel, et al., 1992; Casiraghi, et al., 2002; Bucks and Maibach, 2002; Zhai and Maibach, 2002).

In the past, the cellulose mask is interestingly treated as a new type of natural in using the cosmetic products with various features as mentioned above, might be used for applications in the cosmetic field. BC can be applied purely or impregnated alternatively with active ingredients applied in cosmetics such as essential oil, enriching solution, extract from algae and especially plant extracts. The additives are bound inside the cellulose matrix with hydrogen bond. These bonds are selective enough to localize the substances in the matrix as well as to allow their migration into the skin during apply on the skin. The patch mask can be considered as an alternative to improve in the skin. The cellulose fiber can attach on the skin as well. Thereby, making it possible to enhancing deliver the active ingredients into the skin, which show outstandingly absorption properties and do not dry in normal air. From research to get the suitable materials, and qualified as an ideal cosmetic products. Cellulose mask has properties similar but lacks some features such as flexibility and reproducibility. The use of extracts to combined with cellulose mask to enhance the effect of the extract longer and more efficiently. The use of BC as a carrier in cosmetic the following main conditions have to be considered (Nishio, 2006)

1. Endotoxin test

The homogeneity of BC is proved by an endotoxin test using an E-TOXATE®-Kit (Sigma Aldrich). The results illustrate that the biopolymer is free of endotoxins.

2. Washing and cleaning

The purified biopolymer which no treatment with bases (e.g., sodium or potassium hydroxide) should be carried out. These can accumulate inside the polymer and lead to irritation of the skin. Nishio Y, et al. has used solutions with an alkaline reaction but free of bases and list medical applications.

3. Biocompatibility

3.1 To investigate the biocompatibility of BC are accomplished using two methods: cell culture and clinical examination.

3.2 To determine the direct interaction of purified BC using human keratinocytes (HaCaT cells) in cell culture, result demonstrate good biocompatibility of BC with cells, with no any cytotoxicity effects.

3.3 Several randomized studies with humans have been realized: a human patch test for 72h and a repetitive epicutaneous test for six weeks, as well as tests on the influence of BC on the moisture of the skin after short-term treatment (20 min, measuring of moisture up to 12h after treatment). The results demonstrate that the applied BC will not cause any unwanted skin reactions due to irritating, sensitizing or early allergic effects and that the biopolymer is safe for this indication. An additional finding was that the moistness of 9the treated skin was significantly higher than in untreated areas (W V., 2004; W V., 2001; W V., 2004).

Based on these results, an application of materials based on purified BC. For instance, two cosmetic tissue products are successful on the commercial product; the mask basis material NanoMasque® and a series of masks based on BioCellulose (Figure12) (Klemm, et al., 2006).

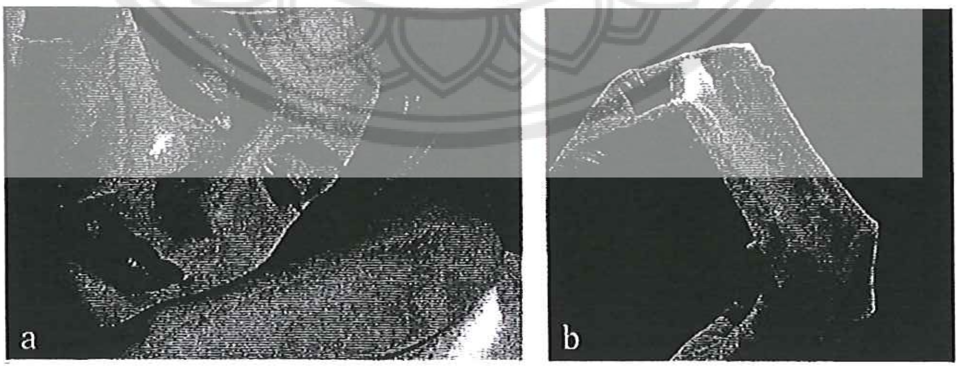

Figure 12 Example of cosmetic application of NanoMasque®, treatment of the skin (a), NanoMasque® material (b)

Source: Klemm, et al., 2006

Pomegranate (peel)

Background of pomegranate peel

Pomegranate (*Punica granatum* L.) is a fruit that is commonly found in various countries e.g. Africa, Iran, Afghanistan, India, China and Thailand. The growing popularity has especially in the north of Thailand. Pomegranate tree, typically grows 12-16 feet and has many branches, is a perennial or small shrub species leave the shell surface is gray. The leaves are oval shaped leaves at the end of a narrow, tapering short. The flower is orange, white or red with petals of about 6 cloves. The pulp is a relatively smooth, rounded surface cortical thickness when the ripe with yellow to red. The nature of the break-out, it will have a large amount of pink polygon seeds. All parts of the pomegranate (root, bark, flowers, fruits, peel, and leaves) (Figure13, 14) can be used for remedial purposes in medical terms. However, the name pomegranate originates from the genus 'Punica', which was the Roman name for Carthage, where the best pomegranates were known to grow. In the past, pomegranate has been used in the treatment for centuries (Zhenbin, et al., 2011; Bhandari, 2012). The pomegranate is one of the important dietary sources of anti-oxidant phenolics (Ozgen, et al., 2008). Pomegranate peel is recognized for its many health-promoting qualities and apparent wound-healing properties (Chidambara, et al., 2004), antimicrobial activity (Braga, et al., 2005), anticancer property (Jeune, et al., 2005), anti-inflammatory (Lansky and Newman, 2007), antiatherosclerotic and antioxidative capacities (Tzulker, et al., 2007) as shown in Figure15. This anti-oxidant activity has been mainly attributed to the water-soluble polyphenols, Anthocyanidins and hydrolysable tannins (Ibrahium, 2010). Guo C, et al. reported that pomegranate peel had the highest anti-oxidant activity among the peel, pulp and seed fractions of 28 kinds of fruits commonly consumed in China, as determined by Ferric reducing anti-oxidant power (FRAP) assay (Guo, et al., 2003). Also, the pomegranate peel with 50% ethanol extraction exerted the outstanding bioactivity among other extraction, as determined by DPPH radical scavenging assay, Trolox equivalent anti-oxidant capacity (TEAC) assay or ABTS assay, FRAP assay and Folin-Ciocalteu method, as shown in Table1-4 (Ingkaninant, 2557). From the evidence reported the investigation of the cytotoxicity of PPE with fibroblast in cell culture using XTT assay found that PPE at the concentration of 100 μg/ml exerted the highest concentration which presented the %viability significant nearly comparing with the Dulbecco's Modified Eagle Medium (DMEM) and 0.1% DMSO as control. It can be implied that PPE safe for its compatibility with the skin, non-toxic and changed any morphology to cells and does not cause irritation to the skin (Figure16) (Viyoch, 2557). Therefore, it seems that pomegranate peel may be a rich source of natural anti-oxidants (Li, et al., 2006). Additional by, the pomegranates which researchers intend to extract is an extraction of the peel, which is as much as 73% of all the pomegranates (Zhenbin, et al., 2011).

Figure13 Pomegranate tree parts

Source: http://en.wikipedia.org/wiki/Pomegranate.

Figure 14 Pomegranate peel, pulp and seed

Source: Bhandari, 2012

Figure 15 Major functional and medical effects of pomegranate

Source: Bhandari, 2012

Table 1 Determination of anti-oxidant activity at the concentration of 1 mg/ml and IC_{50} using DPPH radical scavenging assay (n=3)

Samples	%free radical scavenging (average ± SD)	IC_{50} (µg/ml) (average ± SD)
PPE_{50E}	90.14 ± 5.63	8.18 ± 0.41
PPE_{70E}	93.89 ± 0.13	8.98 ± 0.63
Trolox	90.80 ± 0.19	8.03 ± 0.48
L-ascorbic acid	93.22 ± 0.08	6.70 ± 0.31

Table 2 Determination of anti-oxidant activity at the concentration of 1 mg/ml using Trolox equivalent anti-oxidant capacity (TEAC) assay or ABTS assay (n=3)

Samples	mM TE/g (average ± SD)
PPE_{50E}	1,361.74 ± 231.28
PPE_{70E}	1,305.64 ± 80.58

Table 3 Determination of anti-oxidant activity at the concentration of 1 mg/ml using FRAP assay (n=3)

Samples	µmole Fe(II) /g (average ± SD)
PPE_{50E}	4,034.12 ± 45.82
PPE_{70E}	3,888.60 ± 106.09

Table 4 Determination of TPC using Folin-Ciocalteu method (n=3)

Samples	gGAE/kg (average ± SD)
PPE_{50E}	310.85 ± 5.78
PPE_{70E}	297.87 ± 5.26

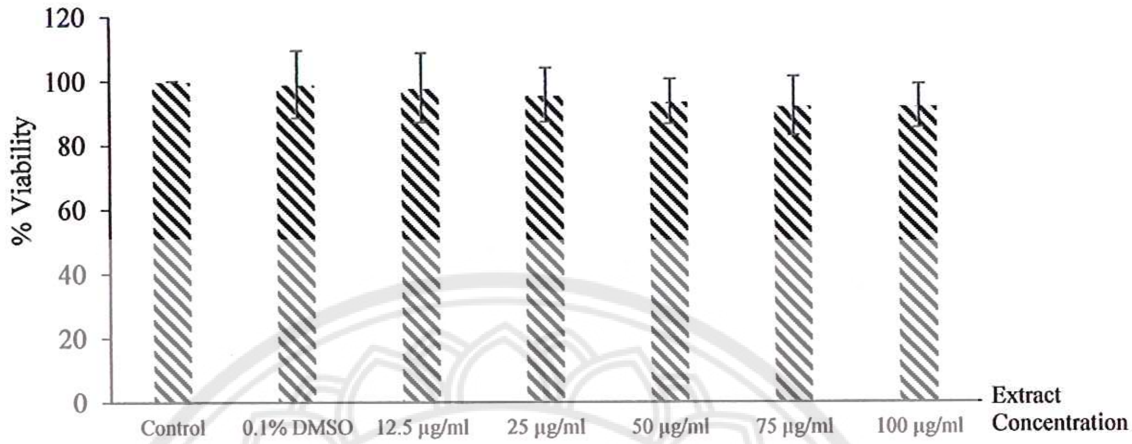

Figure 16 Determination of cytotoxicity of PPE in fibroblast using XTT assay

Source: Viyoch, 2014

The composition of pomegranate peel

The health benefits of PPE have been attributed to the polyphenol content and composition of this fruit (Basu and Penugonda, 2009). The main polyphenols have been proven to exert anti-oxidant and anti-bacterial bioactivities in pomegranate include the ellagitannins and anthocyanidins, which are concentrated in the peel of the pomegranate (Neyrinck, et al., 2013). From previous studies, they found that these parts of pomegranate have a lot of nutrients, as shown in Table5. Therefore, experts advise using pomegranate peel extract as a dietary supplement and nutritional properties to be active ingredients and used as anti-bacterial especially, etc (Bhandari, 2012).

Table 5 The active substance in each part of the pomegranate

Parts of pomegranate	Components
Juices	Anthocyanins, Glucose, Ascorbic acid, Ellagic acid, Gallic acid, Caffeic acid, Catechin, Epigallocatechin gallate (EGCG), Querticin, Rutin, Iron, and Amino acids
Seed oil	Sterols, Punicic acid
The pericarp (peel, rind)	Phenolics, Proanthocyanidins, Punicalgins, Flavonoids, Flavonones and other flavanols
Leaves	Tannins, Punicalin, Punicafolin, flavone glycosides like luteolin and apigenin
Flowers	Ursolic acid, Triterpinoids like maslinic acid, and Asiatic acid
Roots and barks	Ellagitannins and Piperidine alkaloids

Source: Bhandari, 2012

The anti-bacterial activity of pomegranate peel

From the remarkable bioactivities of the pomegranate peel, the researcher was interested in the anti-bacterial activity. Among constituents of plants, polyphenols have receive a great deal of attention, due to their diverse biological functions. The anti-bacterial activity of polyphenols, flavonoids and tannins, is well documented (Naz, et al., 2008; Ahmad and Beg, 2001; Machado, et al., 2003; Shan, et al., 2007). The consumption of tannins-containing beverages, mainly tea, and Thai herbs, could cure or prevent various illnesses. Since ancient civilizations such as those of Egypt, tannins have been used in tanning the making leather. Tannins are high molecular weight phenolics compounds which are present in many plants, including pomegranate (*Punica granatum* L.) fruit pericarp (Cowan, 1999). Tannins are water-soluble polyphenols that are commonly found in high herbaceous and woody plants (Spencer, et al., 1988). They can be classified into two categories: hydrolysable and non-hydrolysable (condensed). Hydrolysable tannins are ester of phenolic acids and a polyol, usually glucose. The phenolic acids are either gallic acid in gallotannins or hexahydroxydiphenic acid in ellagitannins. The hexahydroxydiphenic acid of ellagitannins undergoes lactonization to produce ellagic acid (Scalbert, 1991; Lansky and Newman, 2007). Tannins have been reported to be bacteriostatic or bactericidal against both Gram positive *S. aureus*, *B. subtitis*, *L. monocytogenes*, *S. mutans*, *L. acidophilus* and negative *P. aeruginosa*, *E. coli*, *K. pneumonia*, *Y. enterocolitica* bacteria as well as against pathogenic yeast, *C. albicans* (Khan and Hanee, 2011; Fawole, et al., 2012; Sadeghian, et al., 2011; Nikfallah, et al., 2014; Chung, et al., 1993; Endo, et al., 2010). Especially, the pomegranate peel can exhibited a potent bacteriostatic effect against *P. acnes*, a Gram-positive anaerobe bacteria as well (Panichayupakaranant, et al., 2010). The biological effect in reducing the causes of acne of the phenolic compounds or tannins is divided into 3 main mechanisms. The first, anti-bacterial activity, tannins are reacting to the complexation with peptide, which is the main component of the bacteria cell wall, by the hydrogen bonding affects to reducing the hydrophilic properties and resulting in precipitation (Scalbert, 1991). In addition, tannins, which compete with microbial ligands and enzymes, inhibit of extracellular enzymes involved in bacteria cell wall synthesizing complex. It may also deprive of substrates that directly affect the metabolism of the bacteria (Spencer, et al., 1988). The second, anti-oxidant activity, when the acne was occurred which caused by *P. acnes*, the immune response are activated locally to induce inflammatory cytokines such as TNF-α, IL-1β, IL-8. Afterward, the lysosomal enzyme are released by attached neutrophil during the phagocytosis of the bacteria results in producing the reactive oxygen species (°ROS) such as hydroxyl radicals (OH°) and superoxide ($O2^{o-}$) including Reactive nitrogen species (NOS°) such as nitrus oxide (N_2O) and (nitroxyl anion (NO^-) that can collapse the surrounding follicular epithelium (Grange, et al., 2009; Nand and Gupta, 2012). By the structure of tannins, consist of the hydroxyl group (-OH), which has the features to donate the proton (H^+) to free radical results as non-reaction. In addition, tannins (ellagic acid) affects in cellular as well, it encourages the transcription of Anti-oxidant Response Element (ARE) gene in nucleus cell, which activates the synthesis of enzyme such as Heme oxygenase (HO-1), Super Oxide Dismutase (SOD) and α-Glutamyl Cystein Synthetase (α-GCS) that involved in neutralization resulted decreasing the toxicity of free radicals. The third, anti-inflammatory activity, tannins can inhibit the enzyme Nitric oxide synthase (NOD), which has the effect of changing L-arginine into nitric oxide radical (NO°), resulting in the reducing of inflammatory genes expression. It also donate protons to Peroxide nitrite radical ($ONOO^-$), which have the effect of stimulating blood vessel growth, cellular toxicity and pain (Fridovich, 1999). In addition, it continues to affect the enzyme Cyclo-oxyginase (COX-2), which has the effect of changing Arachidonic acid into Prostaglandins, as plays an important role in inflammatory processes and causes pain finally (Malago, et al., 2002). The inhibition of these pathways of the reaction represses the production of substrates or synthesis of bacteria products, which thus regulates locally the causes of acne (Romier-Crouzet, et al., 2009). Besides, the chemically pure structure of the BC contains a plenty of a hydroxyl group, are hydrophilic, can bind to substances in pomegranate peel extract, especially phenolic compounds comprising hydroxyl groups as well, by the strong hydrogen bonds. Pomegranate peel extracts can be applied with BC for use as anti-acne products.

From the mention above the PPE shows the effects to inhibit or against *P. acnes*, which caused acne vulgaris. Sukatta U, et al. reported that pomegranate peel the highly anti-bacterial activity against *P. acnes*, with antimicrobial index of 1.00 ± 0.11 and clear zone (mm) with 12.00 ± 0.63 (Sukatta, et al., 2010). Also, Panichayupakaranant P, et al. showed the results that standardized pomegranate rind extract exhibited a potent bacteriostatic effect against *P. acnes*, a gram-positive anaerobe, with a MIC of 15.6 µg/ml (Panichayupakaranant, et al., 2010). The strong anti-bacterial of the PPE suggests its potential as a therapeutic agent for acne vulgaris.

CHAPTER III

RESEARCH METHODOLOGY

Chemicals and Media

1. Ethanol (analytical grade, MERCK, Darmstadt, Germany)
2. Sodium Hydroxide (NaOH, analytical grade, Ajax Finechem, Auckland, New Zealand)
3. D-glucose anhydrous ($C_6H_{12}O_6$, analytical grade, Ajax Finechem, Auckland, New Zealand)
4. Peptone, Bacteriological (HiMedia, Mumbai, India)
5. Yeast Extract Powder (HiMedia, Mumbai, India)
6. Di-Sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, analytical grade, AJAX FINECHEM, Auckland, New Zealand)
7. Citric acid ($C_6H_8O_7$), analytical grade, Ajax Finechem, Auckland, New Zealand)
8. Agarose (Molecular biology grade, Ultraclean™, San diego, U.S.A.)
9. Calcium carbonate ($CaCO_3$, analytical grade, Ajax Finechem, Auckland, New Zealand)
10. Barium chloride ($BaCl_2 \cdot 2H_2O$, analytical grade, Ajax Finechem, Auckland, New Zealand)
11. Sulfuric acid (H_2SO_4, analytical grade, Ajax Finechem, Auckland, New Zealand)
12. Mueller Hinton Agar HiMedia, Dindori, Mumbai, India)
13. Soyabean Casein Digest Medium (Tryptone Soya Broth, HiMedia Laboratories Pvt Ltd., Mumbai, India)
14. Soyabean Casein Digest Agar (Tryptone Soya Broth/Casein Soyabean Digest Agar, HiMedia, Mumbai, India)
15. Brain Heart Infusion Broth (HiMedia Laboratories Pvt Ltd., Mumbai, India)

16. Brain Heart Infusion Agar (Special Infusion Agar, HiMedia Laboratories Pvt Ltd., Mumbai, India)

17. 2, 2-Diphenyl-1-picrylhydrazyl, freie Radikal ($C_{18}H_{12}N_5O_8$, Fluka, Sigma-Aldrich, St. Louis, Missouri, U.S.A.)

18. Gallic acid ($C_7H_6O_5$, Sigma-Aldrich, Chemie Gmbh, Munich, Germany)

19. Fulin Ciocaltue's reagent (analytical grade, MERCK, Darmstadt, Germany)

20. Sodium Bicarbonate (Na_2CO_3, analytical grade, Ajax Finechem, Auckland, New Zealand)

21. L(+)-Ascobic acid ($C_6H_{12}O_6$, POCH S.A., Gliwice, Poland)

22. Methanol (analytical grade, MERCK, Darmstadt, Germany)

23. Gentamicin (CT0024B, OXOID, Hampshire, U.K.)

24. Clindamycin (CT0064B, OXOID, Hampshire, U.K.)

Plants

Punica granatum L. (Pomegranate), purchased in purchased from market in Phitsanulok province, Thailand during June to August

Instruments

1. Magnetic stirrer (Heidolph, MR3001, ITS group, Bangkok, Thailand)
2. Hot air oven (UFP800DW, MEMMERT, Schwabach, Germany)
3. Blender (HR2020, PHILIPS, Amsterdam, Netherlands)
4. Rotary shaker (SK3PO, CTL, California, U.S.A.)
5. Freeze dryer (FTS systems Dura dry type FD 95C12, LabX, Ontario, Canada)
6. Rotary evaporator (R-200, BUCHI, Postfach, Switzerland)
7. Microplate reader (Eon™, BioTek instrument, Vermont, U.S.A.)
8. Laminar flow (ClassII-A/B3 Biological Safety Cabinet, BEC THAI, Bangkok, Thailand)
9. Anaerobic jar (OXOID, Hampshire, U.K.)
10. Incubator (VO400cool, MEMMERT, Schwabach, Germany)

11. Texture analyzer (TA.XT Plus, Stable Micro Systems, Ltd, Godalming, U.K.)

12. Rotary-Pumped Sputter Coater/Carbon Coater (Q150RS, Quorum, Laughton, U.K.)

13. Scanning electron microscopy (EDAX®, LEO1455VP, New Jersey, U.S.A.)

14. Thickness gage (Mitutoyo, Kawazakmi, Japan

15. pH meter (PL-700, Gondo, Nangang, Taiwan)

16. Antibiotic Assay Discs (Whatman®, Sigma-Aldrich, St. Louis, Missouri, U.S.A.)

Methodology

1. Preparation of pomegranate peel extract (PPE)

Pomegranates ('Indian' variety) were purchased from market in Phitsanulok province, Thailand during June to August, 2015. Their peels were separated and dried in the hot air oven at a temperature of 45°C for 72h. The dried peels were then blended into fine powder, sieved through 80 mesh and macerated into various solvents: water (PPE_{water}), 50% (v/v) (PPE_{50E}), 70% (v/v) (PPE_{70E}), or 95% (v/v) (PPE_{95E}) ethanol in the ratio of 1:15 (w/v) of powder of dried peels/solvent at 30°C for 24h using rotary shaker (light protection). The mixtures were filtrated through Whatman No. 1 filter paper. The PPE_{water} was dry by lyophilization, whilst the PPE_{50E}, PPE_{70E} and PPE_{95E} were initially evaporated the ethanol using rotary evaporator, followed by lyophilization to get the dry powder form. The PPEs powder was stored at 4°C and protected from the light (Wang, et al., 2011) until used. The dry powder of PPEs was weighed for calculation of the percentage of total extract yield.

1.1 Determination of total phenolic content (TPC) of the extracts

The TPC in PPEs was performed using Folin-Ciocalteu method (Li, et al., 2006). Gallic acid in methanol at various concentrations was prepared for standard curve construction. PPEs were prepared in concentration of 1 mg/ml in methanol. Ten μL of gallic acid at various concentrations or PPEs were added into 96-well plate, and then 130 μL of water and 10 μL of Folin-Ciocalteu's mixture solution. After shaken for 5 minutes, 100 μL of 7% (w/v) Na_2CO_3 solution was added with mixing. The solution mixture were incubated in dark for 30 minutes and measured the absorbance at 750 nm with Microplate Spectrophotometer. The amount of total phenolics was calculated as gallic acid equivalents (GAE) from the calibration curve. The experiment was performed in triplicate.

1.2 Determination of free radical scavenging activity of the extracts

The screening of the free radical scavenging activity of the PPEs was accomplished by using DPPH assay (Singh, et al., 2002), comparing with L-ascorbic acid. PPEs and L-ascorbic acid were dissolved at various concentrations in methanol. 75 µL of these dissolved sample were added into 96-well plate, followed by adding 150 µL of DPPH solution (78.8 µg/ml). The mixture was left standing in dark for 30 minutes at room temperature. Then, the absorbance of the remaining of DPPH was measured by microplate spectrophotometer at wavelength of 515 nm. Radical scavenging activity of the extracts or L-ascobic acid was expressed as the inhibition percentage (%Rs) and calculated using the following formula:

$$\%Rs = [(A_c - A_s)/A_c] \times 100\%$$

Where: Ac is the absorbance of the DPPH without the sample.

As is the absorbance of the DPPH with the sample.

The test was run in triplicate and percent inhibition was expressed as mean ± SD. IC_{50}, the equivalent concentration to give the 50% effect, was determined by log-probit analysis of the samples. The experiment was performed in triplicate. Additionally, the PPE with highest TPC and anti-oxidant activity was selected to further determine the anti-bacterial efficacy and combined with the BC.

2. Determination of anti-bacterial efficacy test of the extracts

Microorganism and media

Staphylococcus aureus (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and *Propionibacterium acnes* (DMST 14916) were obtained from the Department of Medical Sciences, Ministry of Public Health, National Institute of Health of Thailand. Fresh cultures of the isolated bacteria were maintained on soyabean casein digest (SCD) agar for *S. aureus* and *S. epidermidis*, and brain heart infusion (BHI) agar for *P. acnes*.

Minimum inhibitory concentration (MIC)

MIC of the selected PPE were determined by the microdilution assay (Cunliffe, et al., 2000). The final inocula of bacteria; *S. aureus*, *S. epidermidis* and *P. acnes* were prepared in 1.5x10^5 CFU/ml, using SCD broth and BHI broth medium, respectively. PPE was prepared in aqueous solution with the final concentration in range of 1.9 to 1,000 μg/ml. 100 μL of PPE was added into 96-well plate. Consequently, 100 μL of the inocula was added into each well, comparing with the control solution which consisted of 100 μl of broth medium and 100 μl of inoculum and 100 μL of inocula. Blank solution was the prepared mixture of 100 μl medium and 100 μl of sterile water by without the inoculum. The cultures of *S. aureus* and *S. epidermidis* were incubated at 37°C for 24h whereas *P. acnes* was incubated at 37°C for 72h in anaerobic condition using anaerobic jar. Then, the samples were brought to measure the OD value at wavelength 600 nm using microplate spectrophotometer. All wells will be performed in triplicate and MIC will be evaluated by calculating the percentage of inhibition (%I) as followed:

$$\%I = \frac{((T_F\text{Growth} - T_0\text{Growth}) - (T_F\text{Blank} - T_0\text{Blank}))}{((T_F\text{PPE} - T_0\text{PPE}) - (T_F\text{Blank} - T_0\text{Blank}))}$$

Where: $T_{Fsample}$ is the absorbance of sample after incubation.

$T_{0sample}$ is the absorbance of sample before incubation.

T_{Fblank} is the absorbance of blank after incubation.

T_{0blank} is the absorbance of blank before incubation.

$T_{Fgrowth}$ is the absorbance of positive control after incubation.

$T_{0growth}$ is the absorbance of positive control before incubation.

The determination will be followed by EUCAST (The European Committee on Antimicrobial Susceptibility Testing) which specify the standard the percentage of the inhibition of the sample that at least 80% has the potential to inhibit against the bacteria (EUCAST, 2003).

Minimum bactericidal concentration (MBC)

MBC of PPE activity was further determined continually to the MIC values by selecting the well, exhibited the percentage of inhibition (>80%) against test bacteria from MIC plate. Then, transferred using the cotton stick spread on specific agar and incubated at 37°C for 24h for *S. aureus* and *S. epidermidis* whereas *P. acnes* were incubated at 37°C for 72h in anaerobic condition. MBC value was defined as the dilution that yielded no single bacterial colony on the agar plates.

3. Bio-cellulose (BC) preparation

BC film was prepared from culture production bacteria *A. xylinum* (TISTR107) obtained from Thailand Institute of Scientific and Technological Research. The cultures of bacteria was separated into an individual colony by cross streak on the glucose yeast extract (GYE) agar containing 10% (w/v) glucose, 1% (w/v) yeast extract, 1% (w/v) calcium carbonate ($CaCO_3$) and 2% (w/v) agar and incubated at 30°C for 5-7 days. Then, working cultures were regularly prepared on GYE and store at 4°C until use. The individual colonies of culture were transferred into Erlenmeyer flask containing GYE broth consisting of 10% (w/v) glucose, 1% (w/v) yeast extract and shaken using magnetic stirrer at 30°C for 48h to produce the slurry bacterial cellulose (SBC). The SBC were added into specific sterile container containing Hestrin and Shcramm (HS) liquid medium containing 2% (w/v) glucose, 0.5% (w/v) yeast extract, 0.5% (w/v) bacto-peptone, 0.27% (w/v) disodium phosphate and 0.115% (w/v) citric acid and incubated at 30°C for 14 days in the static condition. After incubation the BC pellicle, that floating between the surface and air of the medium, was collected and soaked in 0.25M NaOH for 24h to remove the excess bacteria and the BC pellicle was washed thoroughly with distilled water to neutralize pH. The worked BC pellicle was dried by lyophilization and protected from the moisture in desiccator before further used. The thickness of each pellicle was controlled approximately at 0.1 mm.

4. Combination of BC with PPE

The prepared BC pellicles were cut in round-shaped in the diameter of 6-mm and soaked in the 1 ml of saturated solution of the selected PPE aqueous solution (5 or 10 mg/ml) at 25-30°C for 1h. Afterward, the soaked BC pellicles were dry at 37°C for 2h in hot air oven, The BC pellicles combined with PPE (BCP) were kept away from the moisture in desiccator before used for further evaluation.

5. Characteristics of the BCP

5.1 Surface morphology

Morphology of the surface of BCs and BCPs was coated with Au^+ particles by cathodic spreading in a Rotary-Pumped Sputter Coater/Carbon Coater and examined under a SEM with the operating at an accelerating voltage of 15 kV.

5.2 Mechanical properties

Texture analyzer was performed for measuring tensile strength and elongation at break of BCs and BCPs. They were cut into rectangular shape (10 mm x 70 mm), thickness was approximately 0.1 mm. The samples were clamped and adhered tape on the top and end using 30kg load cell. The crosshead rate set in the test was 1.00 mm/sec, and the distance between grips used 50 mm. At least three samples of BCs and BCPs in dried and wet (soak in water for 1h) states were tested for each set being average values reported. The tensile strength and elongation at break were evaluated by calculating followed:

$$\text{Tensile strength (MPa)} = \frac{\text{Breaking force}}{\text{Cross-section area of sample}}$$

and

$$\text{Elongation at break (\%)} = \frac{\text{Difference in length at breaking x 100}}{\text{Original length}}$$

5.3 Determination of anti-bacterial susceptibility test

The anti-bacterial susceptibility activity, was performed by disc diffusion method (Wilkins, et al., 1972), was conducted to evaluate the inhibitory spectrum of PPEs and BCPs against tested bacteria. The suspension of tested bacteria (1.5×10^8 CFU/ml) was spread with the sterilized cotton stick on petri disc containing SCD agar for *S. aureus* and *S. epidermidis* while BHI agar for *P. acnes*. Consequently, 20 µl of the selected PPE that was prepared in an aqueous solution at concentrations of 1.25, 2.5, 5 and 10 mg/ml (equivalent to 11.19 ± 0.11, 22.74 ± 0.52, 28.75 ± 0.78 and 32.10 ± 0.33 µg of TPC/disc) were pipetted on the sterilized disc with the diameter of 6-mm whereas the BCP was placed on the agar, according to determination of TPC in the BCP, we found that the TPC containing in the 6-mm BCP prepared from soaking the BC pellicle in 5 and 10 mg/ml of the PPE aqueous solution was 89.65 ± 27.53 and 178.95 ± 15.35 μg/film, respectively. Gentamicin (2 μg/disc) was used as positive control for *S. aureus* and *S. epidermidis* and Clindamycin (2 μg/disc) was used as positive control for *P. acnes* to determine the sensitivity of each tested bacterial species. The inoculated plates were incubated at 37°C for 24h for *S. aureus* and *S. epidermidis* while *P. acnes* at 37°C for 72h under anaerobic conditions.

All disc diffusion tests will be performed in triplicate and anti-bacterial susceptibility activity will be evaluated by the measuring the diameter of clear zone of the test bacteria expressed in millimeters.

5.4 Release study

The release study was performed by determination of the TPC in BCs. The BCs were cut into square-shapes size 6 mm and soaked in 1 ml of PPE at the concentration of 5 and 10 mg/ml, which concentration were performed by stock solution of PPE, at 24°C for 1h. Then, saturated BCs were impregnated in 1 ml of distilled water at 24°C in various times (1, 5, 10, 15, 30, 60 and 120 minute). Then, the mixed solution were collected to determine TPC by Folin-Ciocalteu method as described aboved. The study was performed in triplicate.

CHAPTER IV

RESULTS AND DISCUSSION

Pomegranate peel extracts

The yield, TPC and free radical scavenging activity

Table 6 The effect of different solvents on %yield, TPC and free radical scavenging activity (DPPH assay) of the pomegranate peel extract

Samples	Yield (%, w/w)	TPC (gGAE/100g dry fruit)	DPPH assay (IC_{50}, µg/ml)
PPE_{water}	19.62	52.50 ± 1.79	29.65 ± 1.00
PPE_{50E}	34.09	55.95 ± 1.44	18.77 ± 1.73
PPE_{70E}	15.04	48.00 ± 1.17	33.80 ± 2.17
PPE_{95E}	7.54	45.04 ± 1.16	75.80 ± 5.00
L-Ascorbic acid	-	-	4.61 ± 0.44

The first evaluation in chemical based assay of PPEs was to select the operating solvent which used in extraction to achieve the optimized PPE. The total extract yield was shown in Table 6. The yields were exhibited as percentage of g of extract per 100g dry pomegranate peel indicated that PPE_{50E} gave the maximum yield (34.09%), followed by PPE_{water} (19.62%), PPE_{70E} (15.04%) and PPE_{95E} (7.54%), respectively when extracted with the ratio of 1:15 (w/v) of powder/solvent at 30ºC for 24h. It might be resulted from the polarity in the differences mixture of the solvents, the solubility of the substrate in solvent are anticipated to be deferent. The TPC of the PPEs, explicated as g of GAE per 100g dry peel. At the concentration of 1 mg/ml, PPE_{50E} was highest amount of phenolic compounds (55.95 ± 3.40 gGAE/100g dry peel), followed by PPE_{water} (52.05 ± 17.95 gGAE/100g dry peel), PPE_{70E} (48.00 ± 1.17 gGAE/100g dry peel) and PPE_{95E} (45.04 ± 11.64 gGAE/100g dry peel), respectively. Free radical scavenging activity of PPEs were tested by the DPPH assay which elucidated as IC_{50} (μg/mL) and the results shown PPE_{50E} gave the lowest IC_{50} (18.77 ± 1.73 μg/ml), whereas PPE_{95E} gave the highest IC_{50} (75.80 ± 5.00 μg/ml). The IC_{50} in PPE_{water} and PPE_{70E} were found to be 29.65 ± 1.00 and 33.80 ± 2.17 μg/ml, when comparing to L-ascorbic acid as the positive control exerted the IC_{50} with 4.61 ± 0.44 μg/ml. The level of anti-oxidant activity could be attributed to the level of TPC (Wang, et al., 2011; Gil, et al., 2000). It can be considered that the variation in the anti-oxidant activity of solvents may be attributed to differences in their phenolic contents because it may differ in their solubility in different solvents (Li, et al., 2006; Negi, et al., 2003). Interestingly, the results of the anti-oxidant activity may be directly correlated to the TPC of various extracts. In addition, Shan B, et al. reported that a highly positive link between the concentrations of phenolic contents in the extracts and anti-bacterial activity (Shan, et al., 2007). Thus, the PPE_{50E} could be the suitable extract to determine the anti-bacterial activity because it presented highest anti-oxidant activity and the TPCs as compared to the other extracts because the TPC are considered to their relatively toxicity to microorganisms as the anti-bacterial activity (Dréno, et al., 2015).

The determination of anti-bacterial efficacy test of the extracts

Table 7 MIC and MBC of PPE against *S. aureus*, *S. epidermidis* and *P. acnes*

Microorganism	MIC (μg/ml)	MBC (μg/ml)
S. aureus (ATCC 25923)	500	1,000
S. epidermidis (ATCC 12228)	500	1,000
P. acnes (DMST 14916)	1,000	>1,000

The anti-bacterial efficacy test of PPE, against *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228) and *P. acnes* (DMST 14916) was performed by broth dilution method with MIC and MBC valued (μg/ml) as shown in Table7. It exhibited that PPE was more effectively against *S. aureus* and *S. epidermidis* with the MIC value of 500 μg/ml than *P. acnes* with the MIC value of 1,000 μg/ml. In addition, PPE gave the MBC against *S. aureus* and *S. epidermidis* with the value of 1,000 μg/ml, whilst PPE exhibits >1,000 μg/ml against *P. acnes*. This was agreement with

Panichayupakaranant P, et al. that accomplished with the same trend (Panichayupakaranant, et al., 2010). Nevertheless, the limitation of the solubility between the phenolic compounds which consisted of PPE and the mineral substances in broth medium formed complex and led to precipitation. Thus, in this experimental the highest concentration of PPE which can be exhausted solute in broth medium was 1,000 µg/ml. The MICs used for quantitatively measuring the *in vitro* anti-bacterial activity against the bacteria, which used to determine the efficacy of the substances (EUCAST, 2003). From the results demonstrated that *P. acnes* possessed the longer rate of growth or metabolism than *S. aureus* and *S. epidermidis* resulting in stressing condition. Therefore, *P. acnes* might display more resistant to the inhibition of PPE. It implied that PPE exerted the activity of bactericidal against *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228) and *P. acnes* (DMST 14916). Bactericidal activity has been defined as a ratio of MIC to MBC<4 (Pankey, et al., 2004).

Production and characteristics of BC and BCP

The incubation of *A. xylinum* in the volume of 50 ml of inoculum per 400 ml of culture medium in a beaker for 14 days in static condition. The characteristics of BC were white round pellicle like a jelly, tough, flexible and has approximately thickness 0.5 mm as shown in Figure 17A. After, the lyophilization of BC was a dry, soft white pellicle, uniformity with a thickness of 0.1 mm as shown in Figure 17B. In addition, the BCP was appeared with yellowish pellicle which according to the color of PPE, toughness, durable as shown in Figure 17C, in accordance with visualization.

Figure 17 Appearance of BC pellicle in wet form (A), BC pellicle in dried form (B) and BCP (C)

Surface morphology

The morphology of pellicles were examined using SEM. Figure18A demonstrated the surface morphology of BC which assembled as the 3D nano-sized fibril shapes with approximately 50 nm in diameter and length about 100 µm. Moreover, the assemble fibril structure of the BC affected to the mechanical properties, resulting the physical strength and absorption ability to various substances such as PPE which attached inside the cellulose fiber as shown in Figure18B. Additionally, Figure18C and 18D illustrated the cross-sectional view of BC and BCP which possessed multiple layers structure. The layered structure was the formation of nano-fibril which curled up and intertwined into a cross-linked 3D porous network (Yan, et al., 2008).

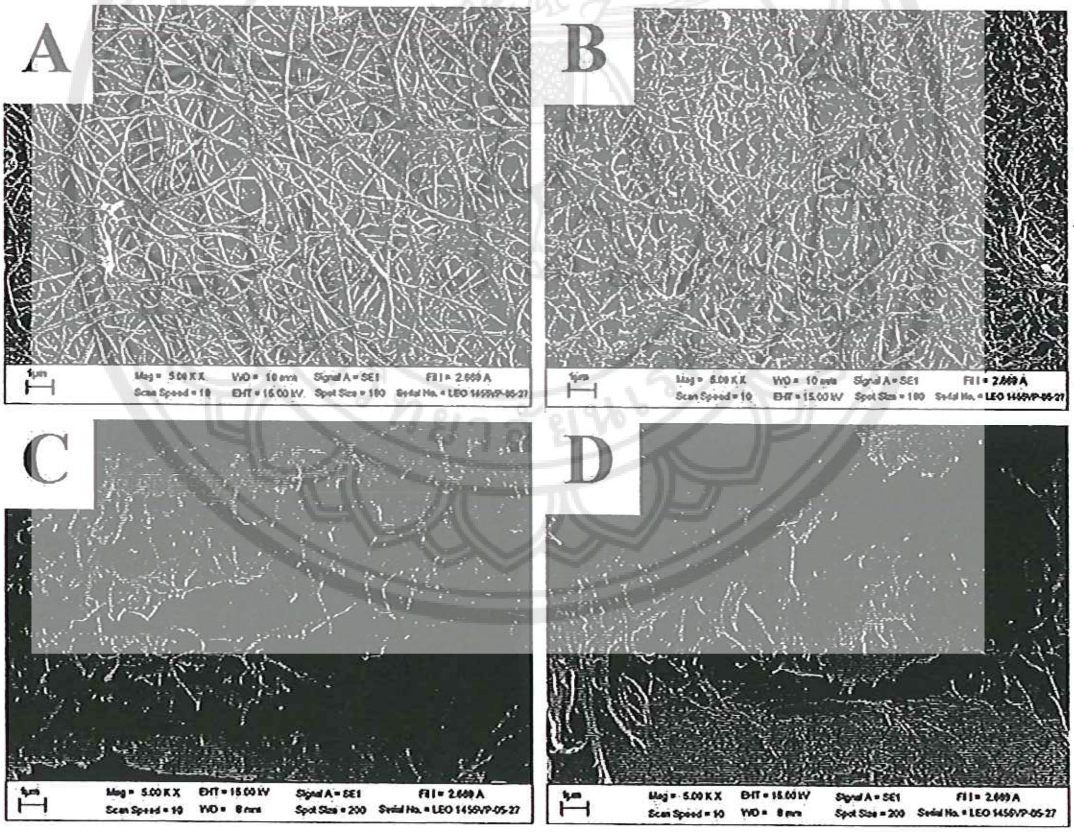

Figure 18 Scanning electron micrographs of BC (A: surface morphology; C: cross-section morphology) and BCP (B: surface morphology; D: cross section morphology)

Mechanical properties

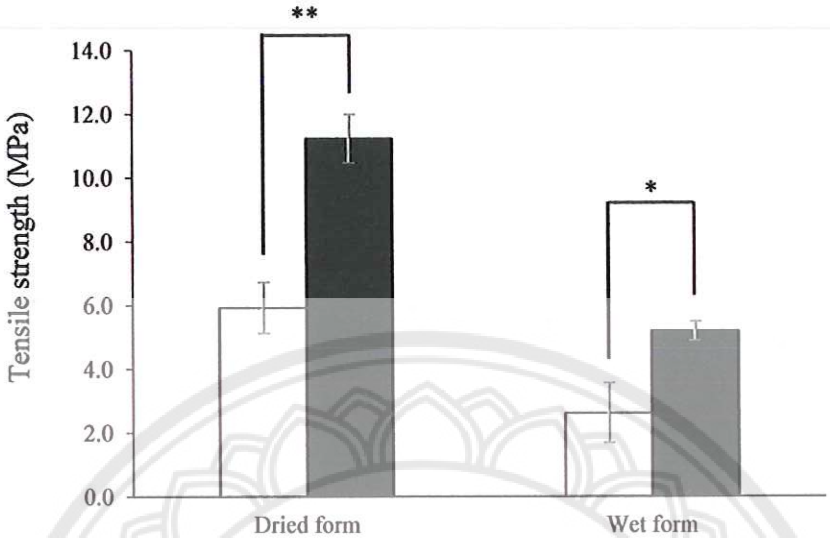

Figure 19 Tensile strength of the BC (light bar) and BCP (dark bar) at dried and wet form. Each bar represents mean ± SD of triplicate study. *p<0.05, **p<0.01, when compared between each group (n = 3, Student's t-test).

Figure 20 Elongation at break of the BC (light bar) and BCP (dark bar) at dried and wet form. Each bar represents mean ± SD of triplicate study. *p<0.05, **p<0.01, when compared between each group (n = 3, Student's t-test).

The tensile strength of the BCs and BCPs in the dry and wet form was exhibited in Figure19. For comparison in dry and wet form, the tensile strength of BC with thickness of 0.098 mm in dry form was higher than wet form because the wet form has the maximum swelling property in the cellulose fiber which similar to the BCP. Moreover, tensile strength of the BCP in both of dry and wet form (11.22 ± 0.75 and 5.18 ± 0.29 MPa) was increased significantly when compare with the BC (2.61 ± 0.93 and 5.91 ± 0.79 MPa), it was noticeable that after combination with the PPE might be interact with the chemical interaction with the cellulose fiber. Figure20 showed the parameter of elongation at break. For comparison in dry and wet form, BC in dry form was less than the wet form, it might from the molecule of the water which attached inside the cellulose fiber affected to the swelling property could give more flexible, resulting in wet form exerted the elongation at break value higher than dry form which as same as to the BCP. In dry form of BC presented the elongation at break with 4.95 ± 0.58% which significantly higher than BCP was 2.16 ± 0.68%, it probably from the decreasing of porosity in cellulose fiber. In the other hand, the wet form of BC and BCP was 14.47 ± 1.74 and 13.58 ± 2.63%, respectively which no significant difference. It was noticeable that the maximum swelling property could provide the maximum elongation at break. In addition, during the soaking process the PPE which attached inside the cellulose fiber might release out to external solution. So, the elongation at break of BC slightly higher than and BCP in the wet form. From the results can be implied the mechanism that the increasing tightness but decreasing flexibility of dry and wet BC compared with both form of BCP. As we have known, the chemically pure structure of the BC contains a plenty of a hydroxyl group, are hydrophilic which can bind to substances in PPE, such as phenolic compounds comprising hydroxyl groups as well (Klemm, et al., 2006). Complexation of PPE which are attracted inside the cellulose fiber, resulting from the interaction between strong hydrogen bond. Resulting in PPE possibly reinforced with suitable degree and accordingly influences a tightness of the nanoscale fibers woven network. Thus, The BPC of appropriate toughness and flexibility were achieved in this study.

Determination of anti-bacterial susceptibility test

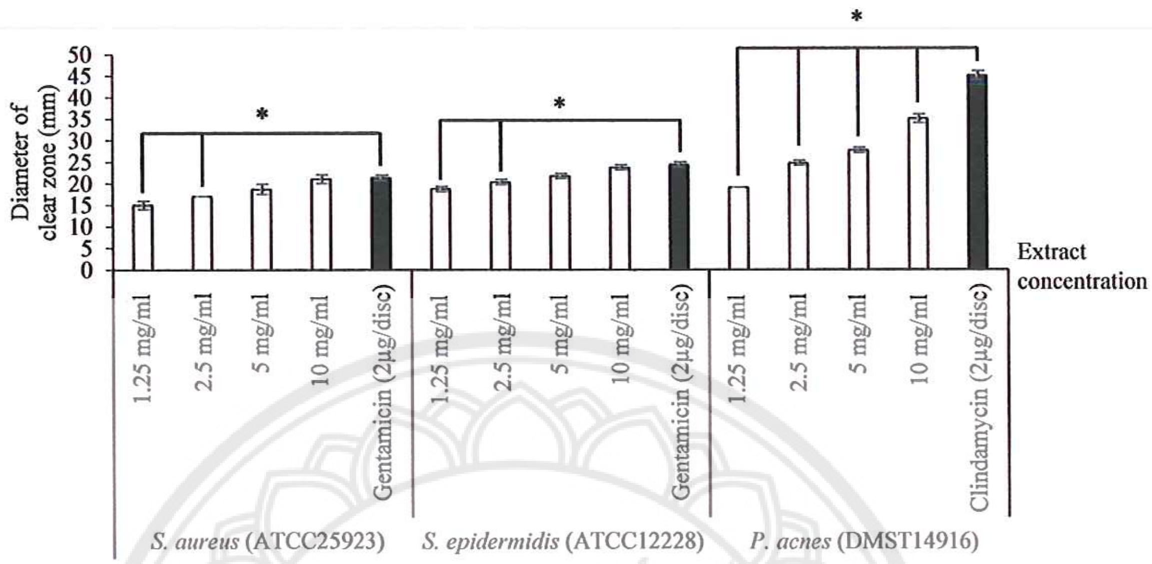

Figure 21 The diameter of clear zone of various concentrations of PPE against *S. aureus*, *S. epidermidis* and *P. acnes*. Each bar represents mean ± SD *p<0.05, when compared between each group

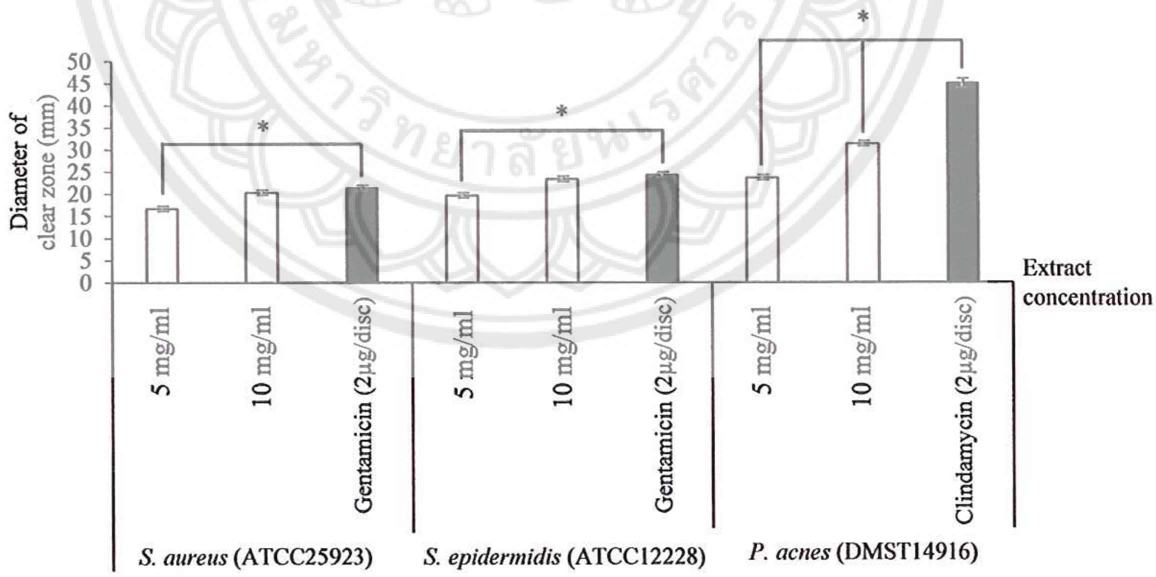

Figure 22 The diameter of clear zone of various concentrations of BCP against *S. aureus*, *S. epidermidis* and *P. acnes*. Each bar represents mean ± SD *p<0.05, when compared between each group

Anti-bacterial susceptibility determination was performed by disc diffusion method. The diameter of clear zone (mm.) was expressed to evaluate PPE_{50E} and BCPs against *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228) and *P. acnes* (DMST 14916), respectively as shown in figure21 and 22. The results elucidated that PPE_{50E} at the concentration of 5 and 10 mg/ml presented the potential to inhibit *S. aureus* (18.7 ± 1.00 and 21.0 ± 1.00 mm) and *S. epidermidis* (21.7 ± 0.58 and 23.7 ± 0.58 mm), respectively which nearly by Gentamicin (2µg/disc) as positive control significantly against *S. aureus* and *S. epidermidis* with the diameter of 21.3 ± 0.58 and 24.3 ± 0.58 mm, respectively. While, PPE_{50E} at the same concentration presented inhibitory activity against *P. acne* (27.7 ± 0.58 and 35.0 ± 1.00 mm) as compare to Clindamycin (2 µg/disc) as positive control exerted the diameter of clear zone with 45.0 ± 1.00 mm. Thus, PPE_{50E} at the concentration of 5 and 10 mg/ml were selected to combine with the BC. The diameter of clear zone of BCP at the concentration of 10 mg/ml possessed efficiently to against the *S. aureus* (20.3 ± 0.58 mm) and *S. epidermidis* (23.3 ± 0.58 mm) which nearly by Gentamicin (2µg/disc) as positive control with the diameter similar to the PPE_{50E}. In the other hand, BCP at the concentration of 5 and 10 mg/ml showed inhibitory activity against *P. acnes*, but significantly different to the Clindamycin (2 µg/disc) exerted the diameter similar to the PPE. The bacteriostatic effects of tannins which was the composition of PPE were reacting to the complexation with peptide, which was the main component of the bacteria cell wall, by the hydrogen bonding affects to reducing the hydrophilic properties and resulting in precipitation (Scalbert, 1991). In addition, tannins, which compete with microbial ligands and enzymes, inhibit of extracellular enzymes involved in bacteria cell wall synthesizing complex. It may also deprive of substrates that directly affect the metabolism of the bacteria (Spencer, et al., 1988).

Release study

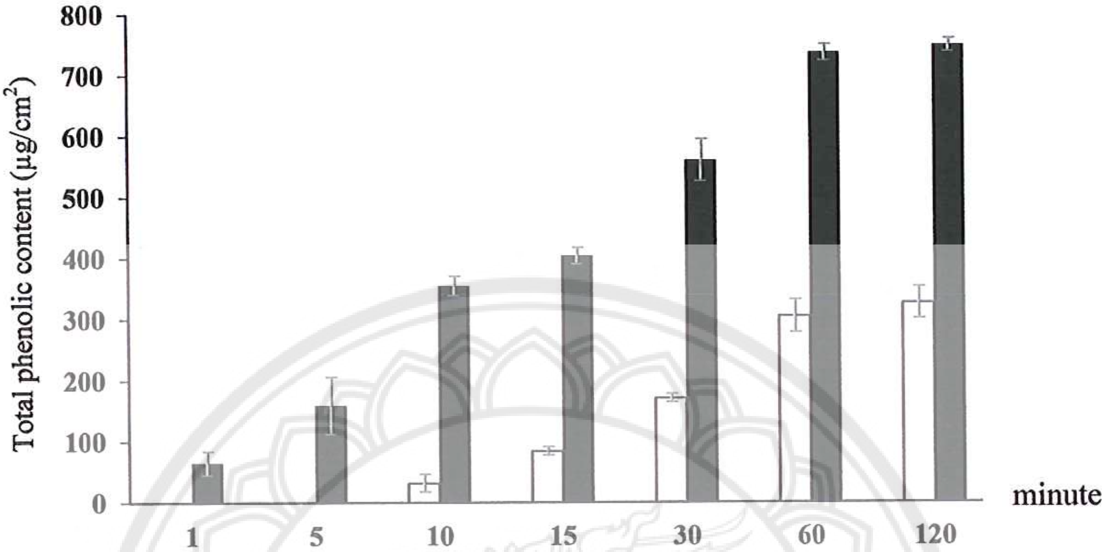

Figure 23 Release study of TPC of the BC soaked with PPE at the concentration of 5 mg/ml (light bar) and 10 mg/ml (dark bar) in various times. Each bar represents mean ± SD

The release study was evaluated the determination of total phenolic released from the BCs which performed by Folin-Ciocalteu method which calculated which expressed as µg/cm^2 as shown in Figure23. These concentrations of PPE_{50E} at the concentration of 5 and 10 mg/ml. The TPC was detected initially at 10 minutes at the concentration of 5 mg/ml with the value of 0.03 ± 1.45 mg/ml, whereas at the concentration of 10 mg/ml can be detected at 1 min with the value of 0.06 ± 1.93 mg/ml. After 60 minutes the phenolic content was quite stable, it probably exhausted released form the BC pellicle. The releasing of TPC was the time dependent. The appropriate time to apply the BCP at the concentration of 5 mg/ml was 30 minutes, whereas the BCP at the concentration of 10 mg/ml was 10 minutes because this period presented the amount of total phenolic which possessed the effective inhibitory activity against the bacteria. In additional, it mimicked for the realistic application. In order to, enhanced substance can be penetrated fully and effectively into the skin.

CHAPTER V

CONCLUSIONS

In this study, PPE_{50E} exhibited the highest %yield, anti-oxidant activity and the highest of TPC. Thereby, the PPE_{50E} could be the suitable extract to further experimental, it presented highest activity as compared to the other extracts. Also, PPE_{50E} exerted the activity of bactericidal against *S. aureus*, *S. epidermidis* and *P. acne*.

The characteristics BC film prepared from the culture production of bacteria *A. xylinum* were wet round white pellicle like a jelly, tough, flexible and has approximately thickness 0.5 mm. After, the lyophilization of BC was white soft pellicle, tough, durable, uniformity with a thickness of 0.1 mm. In addition, the combination of BC and PPE was appeared with yellowish pellicle, toughness, durability with the same thickness of the dried BC, in accordance with visualization. The morphology of pellicles were examined using SEM, demonstrated the surface morphology of BC and BCP which assembled as the 3D nano-sized fibril shapes which affected to the mechanical properties, resulting the physical strength and absorption ability to various substances. Also, cross-sectional view which possesses multiple layers structure. The layered structure is the formation of nanofibril which curl up and intertwined into a cross-linked 3-dimentional porous network. The tensile strength and Elongation of the BCs and BCPs in the dried and wet form, presented the increasing tightness but decreasing flexibility of BC as compare to BCP. Thus, the BPC of appropriate toughness and flexibility were achieved in this study. The determination of anti-bacterial susceptibility test of PPE_{50E} at the concentration of 5 and 10 mg/ml, presented highest potential to inhibit *S. aureus*, *S. epidermidis* and *P. acne*. Additionally, both BCP formula exerted anti-bacterial activity against *S. aureus*, *S. epidermidis* and *P. acne*. The inhibition zone of the BCP prepared from 10 mg/ml extract was larger when compared to that of the BCP prepared from 5 mg/ml extract. The release study of the TPC released from the BC was the time dependent.

From the remarkable characteristic of BC in the capability of mechanism properties and the potential of PPE in the bacterial inhibition support the BCP, which could be the interestingly alternative way to use as the anti-acne product.

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APPENDIX

APPENDIX A POMEGRANATE PEEL EXTRACT

Appearance of PPE in various solvent

Figure 24 PPE with water (PPE_{water})

Figure 25 PPE with 50% EtOH (PPE_{50E})

Figure 26 PPE with 70% EtOH (PPE_{70E})

Figure 27 PPE with 95% EtOH (PPE_{95E})

APPENDIX B CALCULATION OF ANTI-OXIDANT ACTIVITY OF EXTRACTS

Anti-oxidant activity of pomegranate peel extract

Table 8 The percentage of radical scavenging of pomegranate peel in various extracts (I)

Concentration	/3	Log concentration	% Radical Scavenging				
			Ascorbic acid	PPE_{water}	PPE_{50E}	PPE_{70E}	PPE_{95E}
0.0005	0.0002	-3.778	1.26	-1.84	-1.23	1.89	3.29
0.001	0.0003	-3.477	3.74	-1.11	1.89	1.91	4.85
0.005	0.0017	-2.778	11.83	-2.53	3.09	1.91	6.63
0.01	0.0033	-2.477	21.05	4.13	6.38	4.21	11.65
0.05	0.0167	-1.778	91.06	23.46	37.90	24.75	12.11
0.1	0.0333	-1.477	92.32	55.99	71.27	54.30	41.47
0.5	0.1667	-0.778	92.76	90.57	91.33	91.54	63.23
1	0.3333	-0.477	92.61	90.81	91.21	91.73	90.30
1.25	0.4167	-0.380	92.23	91.42	90.86	91.83	91.77
2.5	0.8333	-0.079	92.32	91.52	90.57	91.97	91.22
5	1.6667	0.222	92.54	91.94	89.63	91.92	90.61

Table 9 The percentage of radical scavenging of pomegranate peel in various extracts (II)

Concentration	/3	Log	% Radical Scavenging				
		concentration	Ascorbic acid	PPE_{water}	PPE_{50E}	PPE_{70E}	PPE_{95E}
0.0005	0.0002	-3.778	11.56	-1.84	-8.95	-0.32	3.56
0.001	0.0003	-3.477	10.37	2.44	-6.47	7.96	4.85
0.005	0.0017	-2.778	18.59	3.63	-1.58	15.63	9.37
0.01	0.0033	-2.477	39.19	14.79	3.05	38.67	10.59
0.05	0.0167	-1.778	92.32	25.46	28.92	91.33	12.11
0.1	0.0333	-1.477	92.55	53.99	63.15	91.47	37.47
0.5	0.1667	-0.778	92.32	89.24	91.54	91.52	65.60
1	0.3333	-0.477	92.43	90.57	91.52	91.89	90.30
1.25	0.4167	-0.380	92.35	91.33	91.50	91.47	91.77
2.5	0.8333	-0.079	92.35	92.65	91.24	91.47	90.10
5	1.6667	0.222	92.51	92.53	90.93	90.75	91.06

Table 10 The percentage of radical scavenging of pomegranate peel in various extracts (III)

Concentration	/3	Log	% Radical Scavenging				
		concentration	Ascorbic acid	PPE_{water}	PPE_{50E}	PPE_{70E}	PPE_{95E}
0.0005	0.0002	-3.778	1.26	-1.85	3.29	2.4	-3.29
0.001	0.0003	-3.477	3.74	1.13	4.85	2.405	4.85
0.005	0.0017	-2.778	16.83	2.19	6.63	1.249	6.63
0.01	0.0033	-2.477	24.85	4.95	11.65	6.887	12.11
0.05	0.0167	-1.778	92.06	29.65	44.76	8.511	20.47
0.1	0.0333	-1.477	92.21	50.26	62.42	61.286	34.60
0.5	0.1667	-0.778	92.18	89.60	91.77	85.43	55.15
1	0.3333	-0.477	92.21	90.55	91.25	88.159	90.42
1.25	0.4167	-0.380	92.21	90.74	91.65	87.188	91.77
2.5	0.8333	-0.079	92.06	90.99	91.22	89.202	91.22
5	1.6667	0.222	92.60	91.23	90.61	89.167	90.61

APPENDIX C CALCULATION OF TOTAL PHENOLIC CONTENT OF EXTRACTS

Total phenolic content of pomegranate peel extract

Table 11 Mean absorbance, concentration of mg/mL and gGAE/100g of dry fruit of pomegranate peel in various extracts

Samples	Absorbance			mean	mg/ml			mean	(gGAE/100g of dry fruit)			mean	SD
	1	2	3		1	2	3		1	2	3		
PPE_{water}	1.23	1.23	1.17	1.21	0.54	0.54	0.50	0.53	53.56	53.58	50.46	52.53	1.79
PPE_{50E}	1.28	1.28	1.24	1.27	0.57	0.57	0.54	0.56	56.60	56.96	54.30	55.95	1.44
PPE_{70E}	1.11	1.15	1.14	1.13	0.47	0.49	0.48	0.48	46.68	48.92	48.39	48.00	1.17
PPE_{95E}	1.10	1.09	1.06	1.09	0.46	0.45	0.44	0.45	45.98	45.40	43.75	45.04	1.16
Blank	0.06	0.06	0.06	0.06									

***All extracts were tested at the concentration of 1 mg/ml**

APPENDIX D ANTI-BACTERIAL EFFICACY TEST OF EXTRACTS

Anti-bacterial efficacy test of pomegranate peel extract

Table 12 The percentage of inhibition of pomegranate peel extract against *S. aureus*, *S. epidermidis* and *P. acnes*

Concentration (µg/ml)	1000	500	250	125	62.5	31.250	15.625	7.813	3.906	1.953
	90.18	82.30	72.39	65.86	61.19	58.90	57.20	54.06	53.56	50.42
S. aureus	84.53	79.05	66.83	53.57	53.35	53.83	54.58	53.81	50.99	48.14
(ATCC 25923)	85.74	82.36	71.68	62.33	60.24	56.92	55.49	54.73	46.79	42.34
mean	86.817	81.236	70.298	60.585	58.261	56.551	55.757	54.201	50.445	46.966
SD	2.973	1.896	3.027	6.329	4.275	2.551	1.331	0.477	3.419	4.167
	96.45	87.18	72.26	61.41	53.31	39.22	35.86	34.08	24.88	18.20
S. epidermidis	90.11	84.60	70.03	66.34	61.36	59.99	57.75	54.81	53.49	48.51
(ATCC 12228)	96.21	89.32	78.08	63.69	53.56	38.67	31.04	25.53	20.40	18.04
mean	94.256	87.033	73.455	63.815	56.076	45.959	41.548	38.140	32.922	28.248
SD	3.593	2.359	4.154	2.469	4.578	12.154	14.235	15.054	17.951	17.543
	1000	500	250	125	62.5	31.250	15.625	7.813	3.906	1.953

Table 12 (Cont.)

Concentration (µg/ml)	1000	500	250	125	62.5	31.250	15.625	7.813	3.906	1.953
	87.60	80.34	73.63	66.14	56.87	49.27	49.39	50.17	48.83	47.04
P. acnes	82.01	75.00	67.68	59.96	51.34	42.80	39.57	31.46	25.98	24.45
(DMST 14916)	83.21	73.46	69.62	58.47	49.13	44.03	42.93	37.73	37.18	29.14
mean	84.273	76.266	70.310	61.524	52.446	45.369	43.962	39.787	37.328	33.542
SD	0.846	1.087	1.366	1.059	1.567	0.865	2.373	4.432	7.924	11.921

APPENDIX E PRODUCTION OF BIO-CELLULOSE

Variation of volume of bacteria and medium (v/v) using stainless container

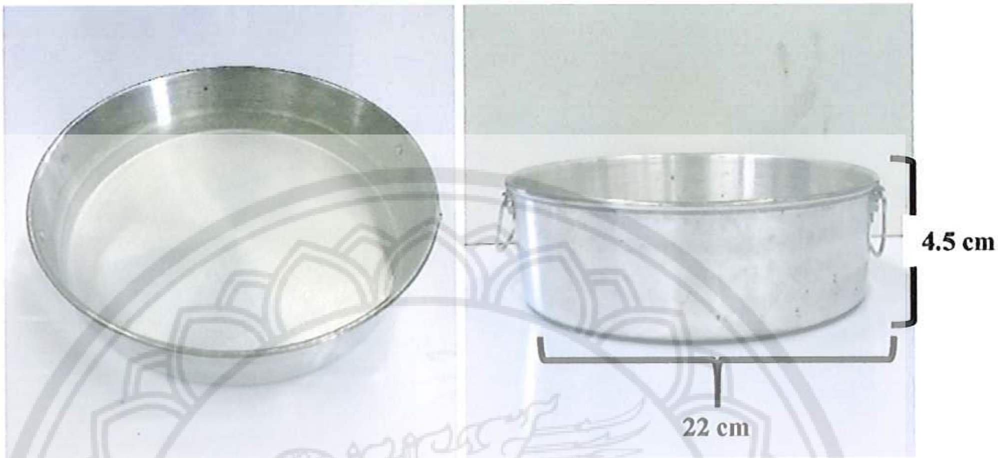

Figure 28 The cultivation container (stainless) for incubate the *A. xylinum*

$$\text{Volume} = \pi r^2 \times h$$
$$= \left(\frac{22}{7}\right) 11^2 \times 4.5$$
$$= 1{,}711.28\ \text{cm}^3$$

Table 13 Variation of volume of bacteria and medium (v/v) using stainless container

	Medium (ml)		
Bacteria (ml)	**250**	**500**	**1000**
25	25/250	25/500	25/1000
50	50/250	50/500	50/1000
100	100/250	100/500	100/1000

Appearance of Bio-cellulose

Figure 29 Freeze-dried BC at the volume of 25/250 (v/v) using stainless container

Figure 30 Freeze-dried BC at the volume of 50/250 (v/v) using stainless container

Figure 31 Freeze-dried BC at the volume of 100/250 (v/v) using stainless container

Figure 32 Freeze-dried BC at the volume of 25/500 (v/v) using stainless container

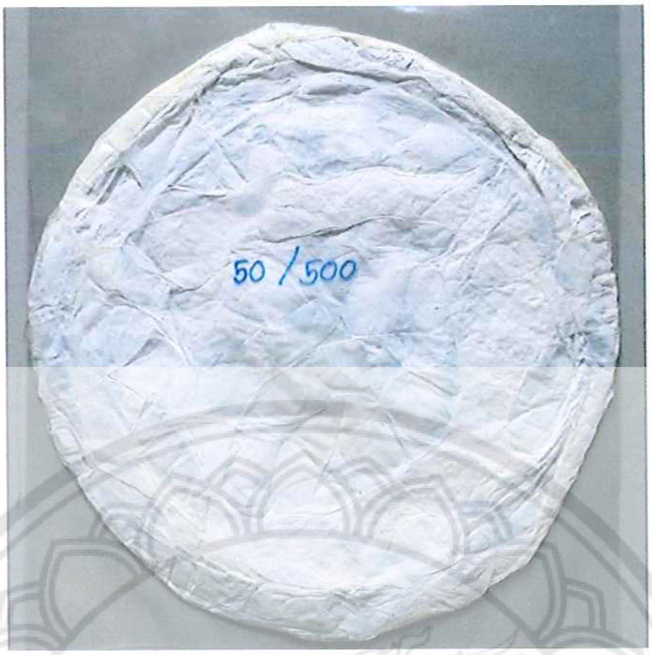

Figure 33 Freeze-dried BC at the volume of 50/500 (v/v) using stainless container

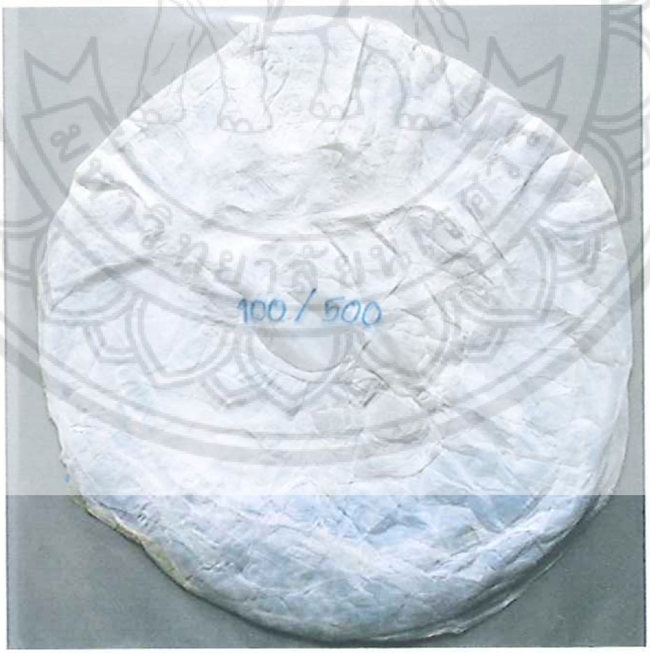

Figure 34 Freeze-dried BC at the volume of 100/500 (v/v) using stainless container

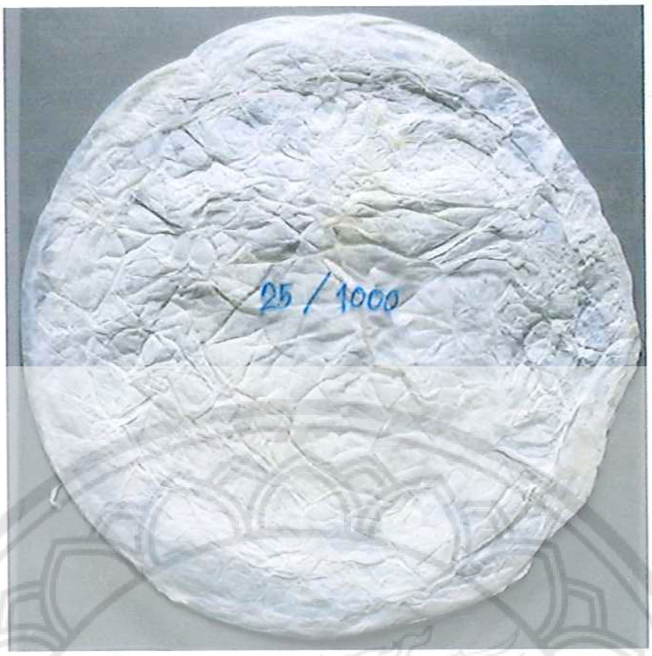

Figure 35 Freeze-dried BC at the volume of 25/1000 (v/v) using stainless container

Figure 36 Freeze-dried BC at the volume of 50/1000 (v/v) using stainless container

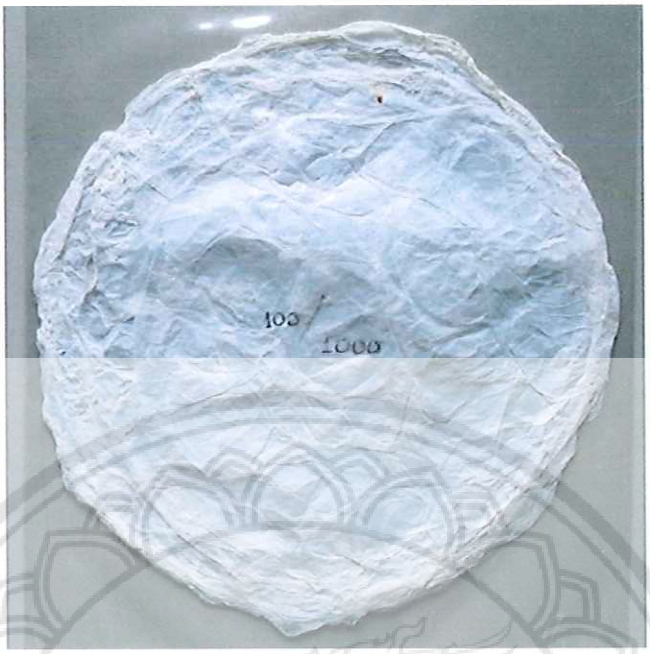

Figure 37 Freeze-dried BC at the volume of 100/1000 using (v/v) stainless container

Variation of time for incubation using stainless container for the *A. xylinum*

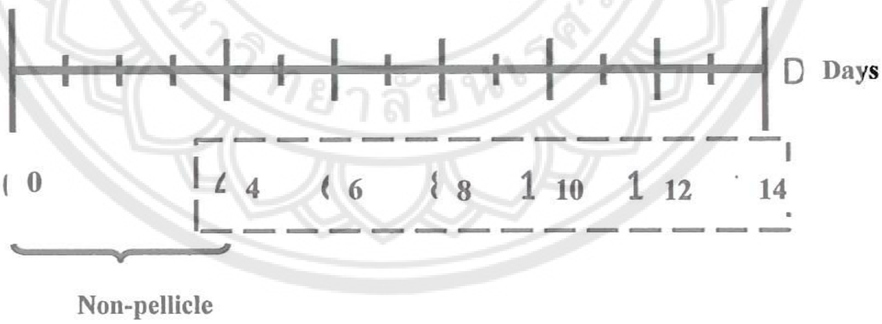

Figure 38 Variation of time using stainless container

Figure 39 Freeze-dried BC at the volume of 50/500 (v/v) at 4th day using stainless container

Figure 40 Freeze-dried BC at the volume of 50/500 (v/v) at 6th day using stainless container

Figure 41 Freeze-dried BC at the volume of 50/500 (v/v) at 8th day using stainless Container

Figure 42 Freeze-dried BC at the volume of 50/500 (v/v) at 10th day using stainless container

Figure 43 Freeze-dried BC at the volume of 50/500 (v/v) at 12th day using stainless Container

Figure 44 Freeze-dried BC at the volume of 50/500 (v/v) at 14th day using stainless container

Variation of volume of bacteria and medium (v/v) using beaker container

Figure 45 The cultivation container (beaker) for incubate the *A. xylinum*

$$\text{Volume} = \pi r^2 \times h$$
$$= \left(\frac{22}{7}\right) 11^2 \times 4.5$$
$$= 1{,}711.28 \text{ cm}^3$$

Table 14 Variation of volume of bacteria and medium (v/v) using beaker container (I)

Bacteria (ml)	Medium (ml)		
	200	400	800
25	25/200	25/400	25/800
50	50/200	50/400	50/800
100	100/200	100/400	100/800

Figure 46 Freeze-dried BC at the volume of 25/200 (v/v) using beaker container

Figure 47 Freeze-dried BC at the volume of 50/200 (v/v) using beaker container

Figure 48 Freeze-dried BC at the volume of 100/200 (v/v) using beaker container

Figure 49 Freeze-dried BC at the volume of 25/400 (v/v) using beaker container

Figure 50 Freeze-dried BC at the volume of 50/400 (v/v) using beaker container

Figure 51 Freeze-dried BC at the volume of 100400 (v/v) using beaker container

Figure 52 Freeze-dried BC at the volume of 25/800 (v/v) using beaker container

Figure 53 Freeze-dried BC at the volume of 50/800 (v/v) using beaker container

Figure 54 Freeze-dried BC at the volume of 100/800 (v/v) using beaker container

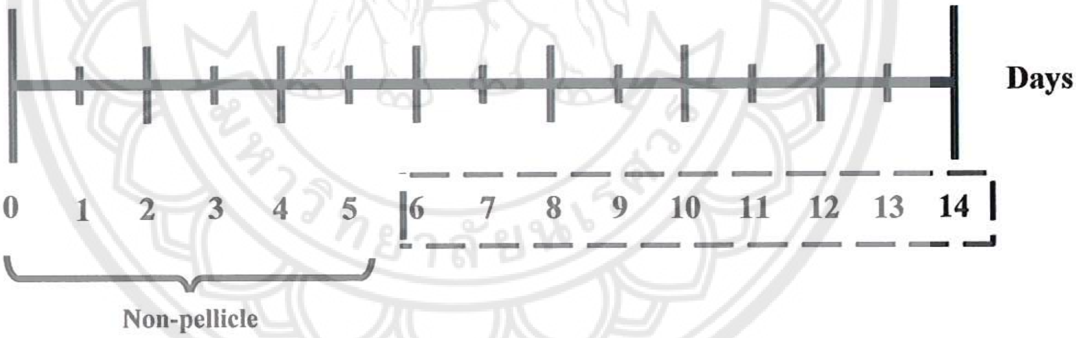

Figure 55 Variation of time using beaker container

Figure 56 Cultivation of BC at the volume of 50/400 (v/v) at 1st day using beaker container

Figure 57 Cultivation of BC at the volume of 50/400 (v/v) at 2nd day using beaker container

Figure 58 Cultivation of BC at the volume of 50/400 (v/v) at 3rd day using beaker container

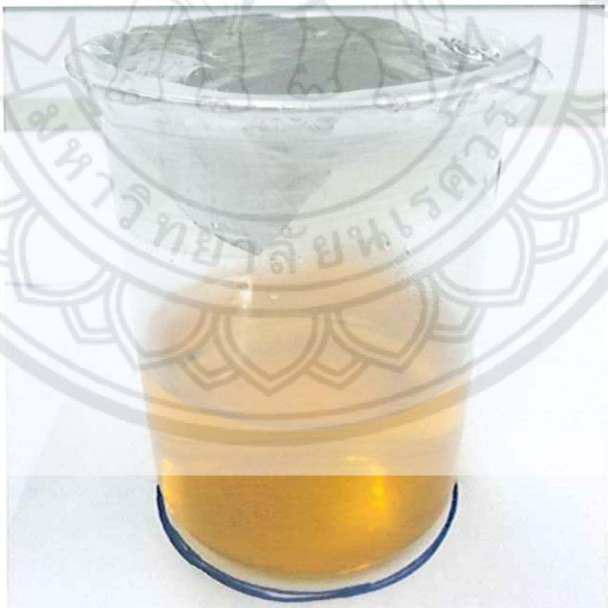

Figure 59 Cultivation of BC at the volume of 50/400 (v/v) at 4th day using beaker container

Figure 60 Cultivation of BC at the volume of 50/400 (v/v) at 5th day using beaker container

Figure 61 Cultivation of BC at the volume of 50/400 (v/v) at 6th day using beaker container

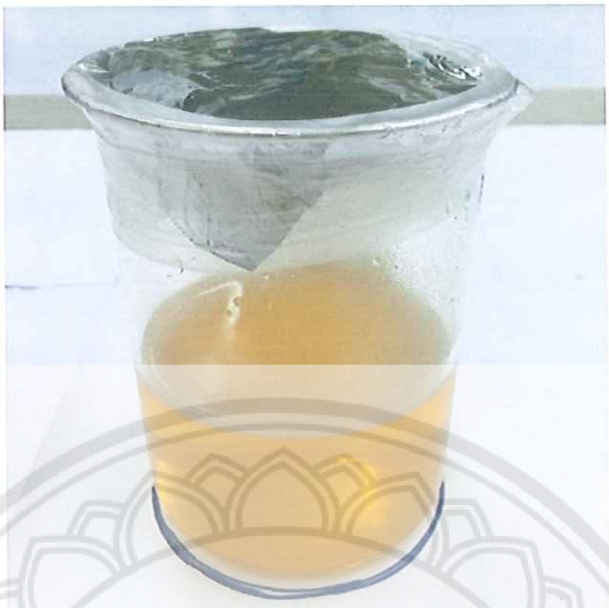

Figure 62 Cultivation of BC at the volume of 50/400 (v/v) at 7th day using beaker container

Figure 63 Cultivation of BC at the volume of 50/400 (v/v) at 8th day using beaker container

Figure 64 Cultivation of BC at the volume of 50/400 (v/v) at 9th day using beaker container

Figure 65 Cultivation of BC at the volume of 50/400 (v/v) at 10th day using beaker container

Figure 66 Cultivation of BC at the volume of 50/400 (v/v) at 11th day using beaker container

Figure 67 Cultivation of BC at the volume of 50/400 (v/v) at 12th day using beaker container

Figure 68 Cultivation of BC at the volume of 50/400 (v/v) at 13th day using beaker container

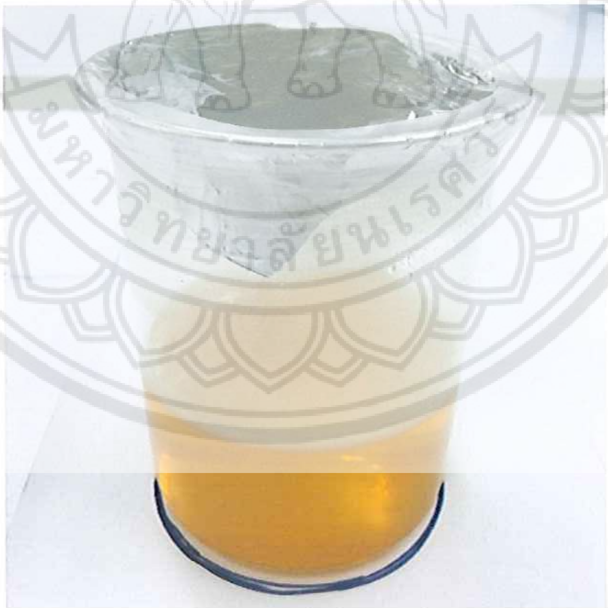

Figure 69 Cultivation of BC at the volume of 50/400 (v/v) at 14th day using beaker container

Table 15 Variation of volume of bacteria and medium (v/v) using beaker container (II)

Bacteria (ml)	Medium (ml)			
	300	**400**	**500**	**700**
40	40/300	40/400	40/500	40/700
50	50/300	50/400	50/500	50/700
60	60/300	60/400	60/500	60/700
80	80/300	80/400	80/500	80/700

Figure 70 Freeze-dried BC at the volume of 40/300 (v/v) using beaker container

Figure 71 Freeze-dried BC at the volume of 50/300 (v/v) using beaker container

Figure 72 Freeze-dried BC at the volume of 60/300 (v/v) using beaker container

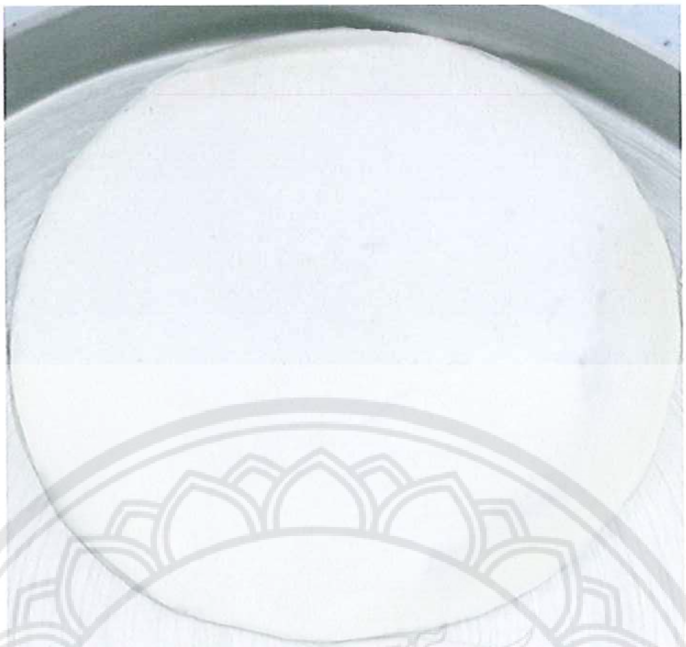

Figure 73 Freeze-dried BC at the volume of 80/300 (v/v) using beaker container

Figure 74 Freeze-dried BC at the volume of 40/400 (v/v) using beaker container

Figure 75 Freeze-dried BC at the volume of 50/400 (v/v) using beaker container

Figure 76 Freeze-dried BC at the volume of 60/400 (v/v) using beaker container

Figure 77 Freeze-dried BC at the volume of 80/400 (v/v) using beaker container

Figure 78 Freeze-dried BC at the volume of 40/500 (v/v) using beaker container

Figure 79 Freeze-dried BC at the volume of 50/500 (v/v) using beaker container

Figure 80 Freeze-dried BC at the volume of 60/500 (v/v) using beaker container

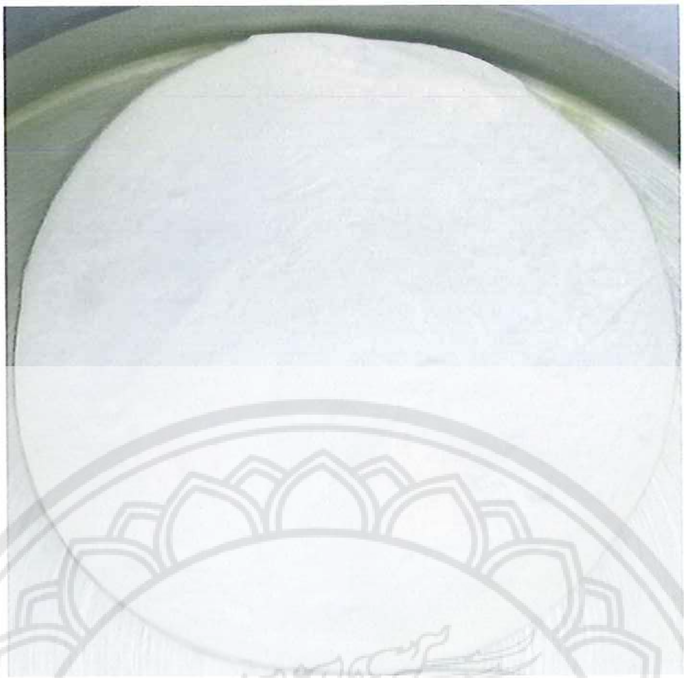

Figure 81 Freeze-dried BC at the volume of 80/500 (v/v) using beaker container

Figure 82 Freeze-dried BC at the volume of 40/700 (v/v) using beaker container

Figure 83 Freeze-dried BC at the volume of 50/700 (v/v) using beaker container

Figure 84 Freeze-dried BC at the volume of 60/700 (v/v) using beaker container

Figure 85 Freeze-dried BC at the volume of 80/700 (v/v) using beaker container

APPENDIX F ABSORPTION ABILITY OF BIO-CELLULOSE

The absorption ability of Bio-cellulose

Table 16 Absorption ability of BC

Time	1 min	5 mins	10 mins	15 mins	30 mins	1h	2h	4h	8h	16h	24h	48h
Before (g)	1.21	1.84	1.3	0.9	1.08	1.17	1.03	1.02	0.7	1.02	1.36	0.82
After (g)	2.44	3.15	3.3	3.17	3.89	4.23	4.04	4.08	3.79	4.09	4.31	3.76
Difference (g)	1.23	1.31	2	2.27	2.81	3.06	3.01	3.06	3.09	3.07	2.95	2.94

APPENDIX G THICKNESS OF BIO-CELLULOSE

The thickness of Bio-cellulose

Table 17 Thickness of variation of time of BC in dried form using stainless container

Day		4	6	8	10	12	14
	1	0.01	0.03	0.05	0.16	0.47	0.55
	2	0.02	0.02	0.07	0.08	0.14	0.31
Thickness	3	0.03	0.06	0.07	0.15	0.19	0.29
(mm)	4	0.06	0.05	0.13	0.38	0.22	0.12
	5	0.08	0.11	0.22	0.32	0.31	0.44
Average		0.04	0.054	0.108	0.218	0.266	0.342
SD		0.029	0.035	0.069	0.126	0.129	0.162

Table 18 Thickness of variation of volume of BC in dried form using stainless container

Volume		25/250	50/250	100/250	25/500	50/500	100/500	25/1000	50/1000	100/1000
	1	0.04	0.08	0.05	0.06	0.47	0.37	0.03	0.16	0.54
	2	0.03	0.03	0.19	0.05	0.25	0.19	0.07	0.19	0.62
Thickness	3	0.09	0.06	0.21	0.14	0.14	0.34	0.06	0.56	0.57
(mm)	4	0.05	0.17	0.18	0.09	0.49	0.28	0.11	0.2	0.56
	5	0.03	0.07	0.07	0.18	0.42	0.22	0.14	0.51	0.65
Average		0.05	0.08	0.14	0.10	0.35	0.28	0.08	0.32	0.59
SD		0.025	0.053	0.074	0.055	0.152	0.076	0.043	0.194	0.045

Table 19 Thickness of variation of volume of BC in dried form using beaker container (I)

Volume		25/200	50/200	100/200	25/400	50/400	100/400	25/800	50/800	100/800
	1	0.03	0.10	1.51	0.08	0.08	2.07	0.09	0.63	2.02
	2	0.05	0.08	1.43	0.08	0.09	2.01	0.02	0.55	2.89
Thickness	3	0.08	0.09	1.69	0.12	0.09	2.03	0.17	0.53	2.84
(mm)	4	0.04	0.01	2.14	0.09	0.10	2.09	0.13	0.51	2.92
	5	0.04	0.06	1.04	0.10	0.11	2.08	0.07	0.61	2.85
Average		0.048	0.068	1.522	0.094	0.094	2.056	0.096	0.566	2.704
SD		0.019	0.036	0.401	0.017	0.011	0.034	0.057	0.052	0.384

Table 20 Thickness of variation of volume of BC in dried form using beaker container (II)

Volume		40/300	50/300	60/300	80/300	40/400	50/400	60/400	80/400
	1	0.09	0.04	0.18	0.71	0.09	0.09	0.34	0.73
	2	0.08	0.17	0.22	0.47	0.12	0.11	0.02	0.68
Thickness	3	0.06	0.07	0.13	0.19	0.04	0.12	0.54	0.28
(mm)	4	0.10	0.08	0.18	0.51	0.13	0.08	0.05	0.39
	5	0.09	0.04	0.16	1.25	0.06	0.09	0.17	0.51
Average		0.084	0.080	0.174	0.626	0.088	0.098	0.224	0.518
SD		0.015	0.053	0.033	0.395	0.038	0.016	0.217	0.190
Volume		40/500	50/500	60/500	80/700	40/700	50/700	60/700	80/700
	1	0.08	0.14	0.38	0.58	0.11	0.21	0.72	0.99
	2	0.09	0.09	0.24	0.71	0.14	0.54	0.15	0.86
Thickness	3	0.12	0.12	0.31	0.84	0.12	0.25	0.74	0.72
(mm)	4	0.07	0.12	0.28	0.77	0.16	0.26	0.06	0.86
	5	0.04	0.09	0.31	0.05	0.17	0.27	0.68	0.67
Average		0.080	0.112	0.304	0.590	0.140	0.306	0.470	0.820
SD		0.029	0.022	0.051	0.317	0.025	0.133	0.335	0.127

APPENDIX H MECHANICAL PROPERTIES OF BIO-CELLULOSE

The mechanical properties of Bio-cellulose

Table 21 Tensile strength of variation of time of BC in dried form using stainless container

Day			4	6	8	10	12	14
Breaking force	g	1	383.73	372.32	480.44	1066.02	1391.49	1553.35
		2	352.09	518.97	311.22	1121.09	1177.89	1328.66
		3	502.01	379.25	531.71	1073.24	1275.95	1689.13
		Average	412.61	423.51	441.12	1086.78	1281.78	1523.71
		SD	79.02	82.743	115.38	29.92	106.91	182.05
		N	4.04	4.15	4.32	10.66	12.57	14.948
Tensile strength (MPa)			0.80	0.83	0.86	2.13	2.51	2.98

Table 22 Elongation at break of variation of time of BC in dried form using stainless container

Day		4	6	8	10	12	14
Difference in length at breaking point	1	0.34	1.09	1.79	0.98	1.89	1.17
	2	1.02	1.66	1.38	1.37	1.27	0.69
	3	0.73	1.58	1.57	1.33	1.69	0.77
	Average	0.70	1.45	1.58	1.23	1.62	0.88
	SD	0.34	0.31	0.20	0.21	0.31	0.25
Elongation at break (%)		1.41	2.90	3.17	2.46	3.24	1.76

Table 23 Tensile strength of variation of volume of BC in dried form using stainless container

Volume		25/250	50/250	100/250	25/500	50/500	100/500	25/1000	50/1000	100/1000
Breaking g force	1	1767.78	1239.44	2238.68	2552.37	3093.10	2302.27	706.09	1499.81	980.30
	2	679.851	1998.25	2015.21	2730.73	3320.22	2054.99	1470.14	1406.40	1008.82
	3	998.022	2038.10	1967.29	3451.35	3717.19	2220.88	918.46	1057.73	1336.12
	Average	1148.55	1758.60	2073.73	2911.48	3376.84	2192.71	1031.56	1321.31	1108.41
	SD	559.36	450.04	144.85	475.97	315.87	126.02	394.38	232.99	197.71
	N	11.26	17.25	20.34	28.56	33.12	21.51	10.12	12.96	10.87
Tensile strength (MPa)		2.25	3.45	4.06	5.71	6.62	4.30	2.02	2.59	2.17

Table 24 Elongation at break of variation of volume of BC in dried form using stainless container

Volume		25/250	50/250	100/250	25/500	50/500	100/500	25/1000	50/1000	100/1000
Difference in length at breaking point	1	1.42	0.87	1.79	1.11	1.72	4.25	0.73	1.17	1.81
	2	0.11	1.61	1.07	0.61	2.02	3.51	1.63	1.04	1.59
	3	0.72	1.36	1.11	1.28	2.59	4.44	1.00	1.06	1.85
	Average	0.75	1.28	1.33	1.00	2.11	4.07	1.12	1.09	1.75
	SD	0.66	0.38	0.41	0.35	0.44	0.49	0.46	0.07	0.14
Elongation at break (%)		1.51	2.56	2.65	2.00	4.22	8.14	2.24	2.18	3.50

Table 25 Tensile strength of variation of time of BC in dried form using beaker container

Day		6	7	8	9	10	11	12	13	14
Breaking g force	1	2741.79	2275.50	2521.64	2795.77	2819.96	3377.43	3002.36	3310.49	3307.06
	2	1822.55	2453.41	2022.29	2679.32	3018.94	3078.65	3650.57	3289.12	3482.45
	3	2291.78	2188.87	2541.57	2654.29	2923.98	2855.33	2398.88	3505.90	3520.30
	Average	2285.37	2305.93	2361.83	2709.79	2920.96	3103.80	3017.27	3368.50	3436.60
	SD	459.66	134.87	294.22	75.50	99.52	261.96	625.98	119.47	113.77
	N	22.42	22.62	23.17	26.58	28.65	30.45	29.60	33.04	33.71
Tensile strength (MPa)		4.48	4.52	4.63	5.32	5.73	6.09	5.92	6.61	6.74

Table 26 Elongation at break of variation of time of BC in dried form using beaker container

Day		6	7	8	9	10	11	12	13	14
Difference in	1	0.89	0.79	0.79	1.09	1.21	1.17	0.95	1.34	1.69
length at	2	0.75	0.64	0.85	1.07	1.26	1.11	1.27	1.31	1.37
breaking point	3	0.59	0.83	0.86	0.79	1.08	1.07	1.16	1.41	1.49
	Average	0.75	0.76	0.83	0.98	1.19	1.12	1.13	1.35	1.52
	SD	0.15	0.10	0.04	0.17	0.09	0.05	0.17	0.05	0.16
Elongation at break (%)		1.49	1.51	1.67	1.97	2.37	2.24	2.26	2.71	3.03

Table 27 Tensile strength of variation of volume of BC in dried form using beaker container (I)

Volume		25/200	50/200	100/200	25/400	50/400	100/400	25/800	50/800	100/1000
Breaking g force	1	1841.79	2275.50	3102.23	2971.78	3194.57	2721.23	5648.21	5228.19	6811.75
	2	2822.55	2453.41	3758.50	3137.22	3082.45	3124.74	5583.18	7231.99	6806.95
	3	2291.78	3188.87	3108.69	2967.67	3262.34	3030.12	5020.77	5800.79	6993.23
	Average	2318.70	2639.26	3323.14	3025.56	3179.79	2958.70	5417.38	6086.99	6870.64
	SD	490.93	484.21	377.05	96.72	90.85	211.03	345.02	1032.10	106.19
	N	22.75	25.89	32.60	29.68	31.19	29.02	53.14	59.71	67.40
Tensile strength (MPa)		4.55	5.18	6.52	5.94	6.24	5.80	10.63	11.94	13.48

Table 28 Tensile strength of variation of volume of BC in dried form using beaker container (II)

Volume			40/300	50/300	60/300	80/300	40/400	50/400	60/400	80/400
Breaking force	g	1	1610.41	2521.64	2795.77	2819.96	2140.87	3607.06	3310.49	4226.14
		2	1654.45	2022.29	2679.32	3018.94	2768.69	3082.45	3289.12	2974.44
		3	1711.19	2541.57	2654.29	3423.98	2578.69	3320.30	3505.90	3161.10
		Average	1658.68	2361.83	2709.79	3087.62	2496.08	3336.60	3368.50	3453.89
		SD	50.52	294.22	75.50	307.81	321.96	262.68	119.47	675.26
		N	16.27	23.17	26.58	30.29	24.49	32.73	33.04	33.88
Tensile strength (MPa)			3.25	4.63	5.32	6.06	4.90	6.55	6.61	6.78
Volume			40/500	50/500	60/500	80/700	40/700	50/700	60/700	80/700
Breaking force	g	1	2615.35	3002.36	3377.43	3312.39	2806.34	3274.44	2975.66	3687.46
		2	1384.50	3650.57	3078.65	3309.27	2928.65	3359.70	4341.30	4339.93
		3	2378.64	2398.88	2855.33	3225.30	3086.94	3218.99	2961.82	3434.17
		Average	2126.16	3017.27	3103.80	3282.32	2940.65	3284.37	3426.26	3820.52
		SD	653.11	625.98	261.96	49.41	140.68	70.88	792.48	467.31
		N	20.86	29.60	30.45	32.20	28.85	32.22	33.61	37.48
Tensile strength (MPa)			4.17	5.92	6.09	6.44	5.77	6.44	6.72	7.50

Table 29 Elongation at break of variation of volume of BC in dried form using beaker container (I)

Volume		25/200	50/200	100/200	25/400	50/400	100/400	25/800	50/800	100/1000
Difference in	1	0.64	0.82	1.10	1.11	1.21	1.03	1.55	1.76	1.60
length at	2	0.83	1.07	1.18	1.02	1.87	1.97	1.27	1.99	1.79
breaking point	3	0.85	1.48	1.27	1.03	0.90	1.52	1.16	1.71	1.77
	Average	0.78	1.12	1.19	1.06	1.33	1.51	1.33	1.82	1.72
	SD	0.12	0.34	0.08	0.05	0.49	0.47	0.20	0.15	0.10
Elongation at break (%)		1.55	2.25	2.37	2.11	2.65	3.01	2.66	3.64	3.45

Table 30 Elongation at break of variation of volume of BC in dried form using beaker container (II)

Volume		40/300	50/300	60/300	80/300	40/400	50/400	60/400	80/400
Difference in	1	0.88	0.73	1.43	1.79	1.45	1.59	1.64	1.36
length at breaking	2	0.76	1.71	1.29	1.85	1.29	1.87	1.11	1.69
point	3	0.52	1.26	1.86	1.26	1.07	0.79	1.41	1.51
	Average	0.72	1.24	1.24	1.63	1.27	1.42	1.39	1.52
	SD	0.18	0.49	0.49	0.33	0.19	0.56	0.27	0.17
Elongation at break (%)		1.44	2.47	2.47	3.27	2.55	2.83	2.77	3.04
Volume		40/500	50/500	60/500	80/700	40/700	50/700	60/700	80/700
Difference in	1	1.79	1.44	1.71	2.11	1.21	1.31	1.77	2.79
length at breaking	2	1.45	1.32	1.83	1.79	1.17	1.51	2.22	1.06
point	3	1.48	1.89	1.21	1.08	1.09	1.07	1.79	1.69
	Average	1.58	1.55	1.58	1.66	1.16	1.30	1.93	1.85
	SD	0.19	0.30	0.33	0.53	0.06	0.22	0.26	0.88
Elongation at break (%)		3.15	3.10	3.17	3.33	2.31	2.59	3.86	3.70

Table 31 Tensile strength of BC and BCP in dried and wet form

Sample			BC		BCP	
			Dried	Wet	Dried	Wet
Breaking force		1	2926.12	1238.58	5808.77	2676.50
		2	3083.59	1327.96	5669.81	2619.22
		3	3041.15	1429.45	5682.59	2633.60
	g	Average	3016.95	1332.00	5720.39	2643.11
		SD	81.47	95.49	76.81	29.79
		N	29.59	13.06	56.11	25.92
Tensile strength (MPa)		Average	5.91	2.61	11.22	5.18
		SD	0.79	0.93	0.75	0.29

Table 32 Elongation at break of BC and BCP in dried and wet form

Sample		BC		BCP	
		Dried	Wet	Dried	Wet
Difference in length at breaking point	1	2.52	8.01	1.06	5.28
	2	2.16	6.29	0.74	7.74
	3	2.74	7.39	1.43	7.34
	Average	2.47	7.23	1.08	6.79
	SD	0.29	0.87	0.34	1.31
Elongation at break (%)		4.95	14.47	2.16	13.58

APPENDIX I ANTI-BACTERIAL SUSCEPTIBILITY TEST OF BIO-CELLULOSE AND BIO-CELLULOSE COMBINED WITH POMEGRANATE PEEL EXTRACT

The anti-bacterial susceptibility test of BC and BCP

Table 33 The diameter of clear zone of PPE using disc diffusion method

Concentrations of extract		Bacteria		
		S. aureus (ATCC 25923)	*S. epidermidis* (ATCC 12228)	*P. acnes* (ATCC 14916)
1.25 mg/ml	Clear zone (mm)	15.00	18.7	19.0
	SD	1.00	0.58	0.00
2.5 mg/ml	Clear zone (mm)	17.00	20.3	24.7
	SD	0.00	0.58	0.58
5 mg/ml	Clear zone (mm)	18.7	21.7	27.7
	SD	1.15	0.58	0.58
10 mg/ml	Clear zone (mm)	21.0	23.7	35.0
	SD	1.00	0.58	1.00
Positive control	Clear zone (mm)	21.3	24.3	45.0
	SD	0.58	0.58	1.00

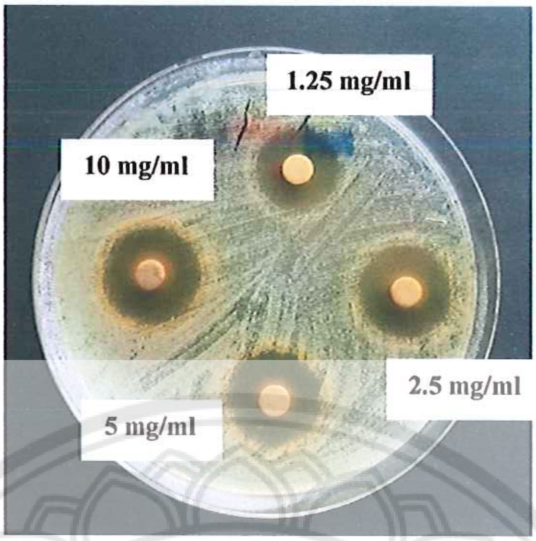

Figure 86 The diameter of clear zone of PPE at the concentration of 1.25, 1.5, 5 and 10 mg/ml against *S. aureus*

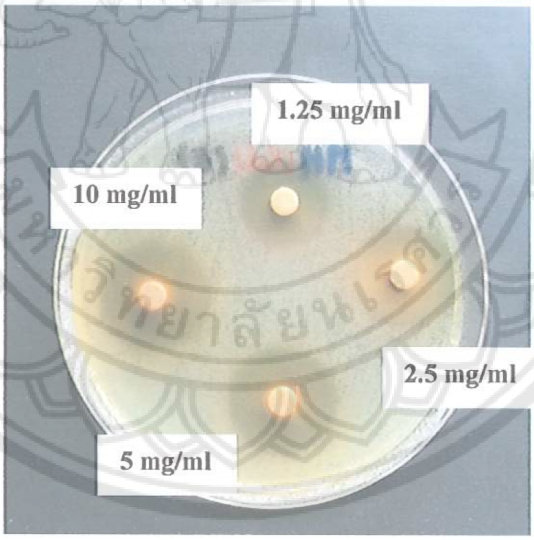

Figure 87 The diameter of clear zone of PPE at the concentration of 1.25, 1.5, 5 and 10 mg/ml against *S. epidermidis*

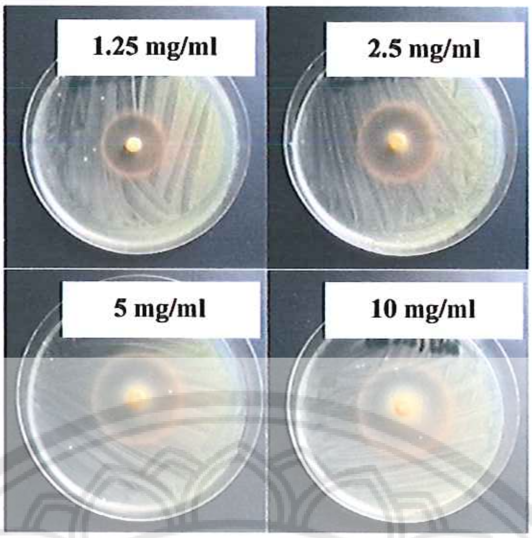

Figure 88 The diameter of clear zone of PPE at the concentration of 1.25, 1.5, 5 and 10 mg/ml against *P. acnes*

Table 34 The diameter of clear zone of BCP using disc diffusion method

Concentrations of BCP		Bacteria		
		S. aureus **(ATCC 25923)**	***S. epidermidis*** **(ATCC 12228)**	***P. acnes*** **(ATCC 14916)**
5 mg/ml	**Clear zone (mm)**	16.7	19.7	23.7
	SD	0.58	0.58	0.58
10 mg/ml	**Clear zone (mm)**	20.3	23.3	31.33
	SD	0.58	0.58	0.58
Positive control	**Clear zone (mm)**	21.3	24.3	45.0
	SD	0.58	0.58	1.00

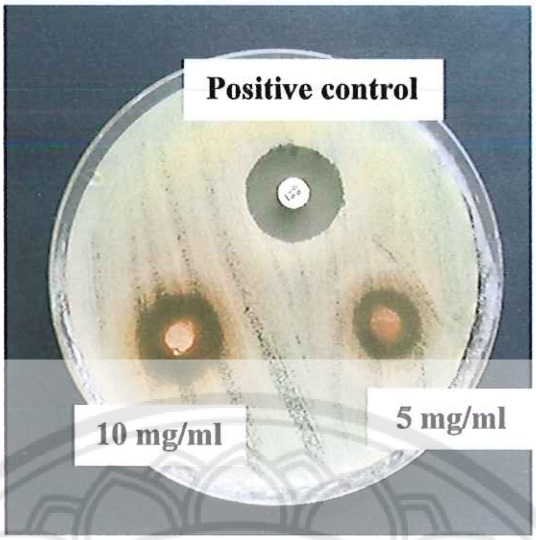

Figure 89 The diameter of clear zone of BCP at the concentration of 5 and 10 mg/ml and positive control against *S. aureus*

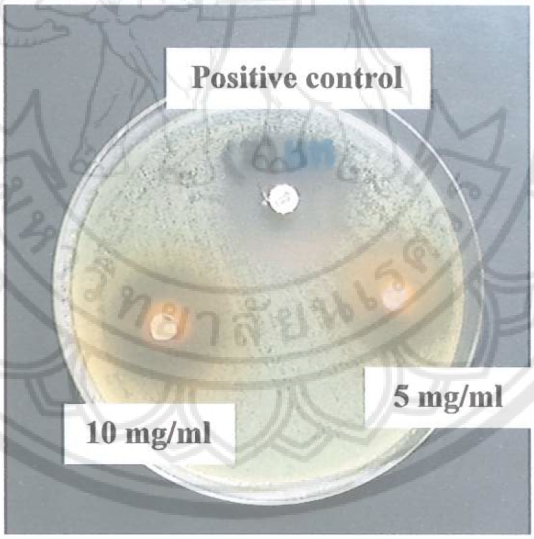

Figure 90 The diameter of clear zone of BCP at the concentration of 5 and 10 mg/ml and positive control against *S. epidermidis*

Figure 91 The diameter of clear zone of BCP at the concentration of 5 and 10 mg/ml and positive control against *P. acnes*

APPENDIX J RELEASE STUDY OF TOTAL PHENOLIC CONTENT OF THE BIO-CELLULOSE SOAKED WITH POMEGRANATE PEEL EXTRACT

The Release study of TPC of the BC soaked with PPEs

Table 35 The TPC released from the BC soaked in PPE at the concentration of 5 mg/mL

Minutes	Absorbance				mg/ml			Mean	SD
	1	2	3	Mean	1	2	3		
1	0.21	0.25	0.29	0.258	-0.072	-0.049	-0.025	-0.048	0.02
5	0.32	0.32	0.32	0.323	-0.007	-0.011	-0.009	-0.009	0.00
10	0.39	0.36	0.41	0.391	0.033	0.016	0.045	0.032	0.01
15	0.48	0.47	0.46	0.477	0.091	0.083	0.077	0.084	0.01
30	0.60	0.62	0.63	0.621	0.164	0.170	0.178	0.170	0.01
60	0.79	0.84	0.88	0.844	0.278	0.305	0.331	0.305	0.03
120	0.83	0.88	0.92	0.880	0.301	0.326	0.352	0.326	0.03
Blank	0.05	0.05	0.05	0.057					

Table 36 The TPC released from the BC soaked in PPE at the concentration of 10 mg/ml

Minutes	Absorbance				mg/ml				
	1	2	3	Mean	1	2	3	Mean	SD
1	0.48	0.45	0.41	0.446	0.085	0.064	0.046	0.065	0.02
5	0.68	0.53	0.60	0.602	0.206	0.114	0.157	0.159	0.05
10	0.92	0.96	0.90	0.926	0.351	0.372	0.342	0.355	0.02
15	1.02	1.02	0.98	1.008	0.410	0.412	0.389	0.404	0.01
30	1.22	1.33	1.25	1.267	0.534	0.599	0.548	0.560	0.03
60	1.57	1.57	1.53	1.559	0.745	0.743	0.722	0.736	0.01
120	1.594	1.561	1.581	1.579	0.758	0.737	0.750	0.748	0.01
Blank	0.056	0.058	0.056	0.057					