PREPARATION OF GREEN COSMETIC INGREDIENTS AND ITS UTILIZATION IN TOPICAL EMULSION

SIRIRAT MOO-KRIANG

MASTER OF SCIENCE
IN
COSMETIC SCIENCE

SCHOOL OF COSMETIC SCIENCE
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ABSTRACT

In the global efforts to reduce generated hazardous waste green chemistry is progressively integrating with modern developments in science and industry. Cosmetic chemists are combining natural ingredients with synthetic ones, providing a larger set of ingredients with which to formulate. The result is that products formulated with natural ingredients represent one of the fastest-growing cosmetic market segments. Natural cosmetics, 100% cosmetic ingredients and organic makeup are the example of recently cosmetic market. In this thesis, the research has focused on the preparation of new cosmetic ingredients by using natural base material to generate green cosmetic ingredients. The study is divided into two sections: the synthesis of silver nanoparticles using natural reducing agents and the preparation of aluminium (Al) complex of phycocyanin (PC) from *Spirulina platensis*. Silver nanoparticles were successfully prepared by an eco-friendly synthesis method using *A. concinna* pods and *P. emblica* extracts as reducing agent. The obtained silver-Soap pod nanoparticles have average particle size of 20.9 nm and silver-Emblica is 41.2 nm. They showed the antimicrobial activities against gram positive (*S. aureus*) and gram negative (*E. coli*) bacterial. Moreover the cosmetic emulsion product was successful developed using silver nanoparticles as antimicrobials. The Al-PC complex was successfully prepared. The complex is dark greenish-blue and it helps

maintaining and improving stability of PC. The utilization of Al-PC complex as colorant in cosmetic products was studied and it showed promising potential to be used as novel, natural derived blue color for cosmetics.

Keywords: Green cosmetic ingredient/Silver nanoparticle/Natural reducing agent/ Aluminium complex/Phycocyanin

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

CHAPTER 1

INTRODUCTION

In the global efforts to reduce generated hazardous waste green chemistry is progressively integrating with modern developments in science and industry. The new cosmetic categories was produced and expanded such as cosmeceuticals, this is being complemented by a simultaneous increase in the scientific understanding of botanicals. The researchers identify with greater precision the individual chemical constituents of botanicals, more specifically targeted and sophisticated natural ingredients are being incorporated into cosmetics. In addition, cosmetic chemists are combining natural ingredients with synthetic ones, providing a larger set of ingredients with which to formulate. The result is that products formulated with natural ingredients represent one of the fastest-growing cosmetic market segments. Natural cosmetics, 100% cosmetic ingredients and organic makeup are the example of recently cosmetic market. This thesis has focused on the preparation of new cosmetic ingredients by using natural base material to generate green cosmetic ingredients. The study is divided into two sections: the synthesis of silver nanoparticle using natural reducing agents and the preparation of aluminium complex of phycocyanin from *Spirulina platensis*.

1.1 The Synthesis of Silver Nanoparticle Using Natural Reducing Agents

1.1.1 Background

Nanoparticles are the simplest form of structures with sizes in the nanometers range. The application of nanoscale materials and structures, usually ranging from 1 to 100 nanometers (nm), is an emerging area of nanoscience and nanotechnology (Aswathanarayan & Vittal, 2011). Nanoparticles have a very high surface area to volume ratio. The large surface area of nanoparticles also results in a lot of interactions between the intermixed materials in nanocomposites, leading to special properties (increased strength, increased chemical, heat resistance). Many techniques of silver nanoparticles synthesis are extremely expensive and also involve the use of toxic, hazardous chemicals, which may pose potential environmental and biological risks (Guzmán, Dille & Godet, 2008; Solomon, Bahadory, Jeyarajasingam, Rutkowsky & Boritz, 2007). In the global efforts to reduce generated hazardous waste green chemistry and chemical processes are progressively integrating with modern developments in science and industry.

Many previous studies about green synthesis of metal nanoparticles are successed (Forough & Farhadi, 2010; Mallikarjuna et al., 2011; Safaepour, Shahverdi, Shahverdi, Khorramizadeh & Gohari, 2009) and the search for simple, environmentally benign, and quite efficient process is still in focus. Thus, this work attempts to develop green processes by using Soap Pod (*Acacia concinna*) and Emblica (*Phyllanthus emblica*) as reducing agent for silver nanoparticle synthesis. The preparation of green silver nanoparticles at different conditions (time, temperature and concentration of silver nitrate and reducing agent) were investigated. The nanoparticles were characterized by UV-Vis spectrophotometer, FT-IR, XRD, SEM and TEM technique. The antimicrobial properties of silver nanoparticles were studied. Moreover, the potential application of silver nanoparticles in cosmetic emulsion was reported.

1.1.2 Objectives

1.1.2.1 To develop eco-friendly processes by using natural reducing agent for silver nanoparticle synthesis.

1.1.2.2 To characterize the synthesized silver nanoparticles using UV-Visspectroscopy, FT-IR, SEM and TEM technique.

1.1.2.3 To study the properties, i.e. stability, antimicrobial and cytotoxicity of silver nanoparticles.

1.1.2.4 To study the potential application of silver nanoparticles in cosmetic emulsion.

1.1.3 Scope of research

1.1.3.1 Soap Pod and Emblica extract were prepared for use as reducing agent.

1.1.3.2 The physical properties of natural reducing agents were investigated.

1.1.3.3 The preparation of eco-friendly silver nanoparticles at different conditions (time, temperature and concentration of silver nitrate and reducing agent) were investigated.

1.1.3.4 The eco-friendly silver nanoparticles were characterized using UV-Vis spectroscopy, FT-IR, XRD, SEM and TEM technique.

1.1.3.5 The antimicrobial properties of silver nanoparticles were studied.

1.1.3.6 The cytotoxicity of silver nanoparticles was investigated.

1.1.3.7 The cosmetic emulsion containing silver nanoparticles was developed.

1.1.4 Expected outcome

1.1.4.1 The successful green synthesis of silver nanoparticles with shown antimicrobial properties.

1.1.4.2 The silver nanoparticles were applied as antimicrobial substances in cosmetic preparation.

1.2 The Preparation of Aluminium Complex of Phycocyanin From *Spirulina platensis*

1.2.1 Background

Phycocyanin (PC) is a blue, light-harvesting pigment in cyanobacterium *Spirulina (Arthrospira) platensis*. *S. platensis* has 62% amino acid content and is the world's richest natural source of vitamin B_{12} and contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments (Estrada, Bescos & Fresno, 2001). PC is composed of two relatively homologous subunits: the α-chain with one phycocyanobilin attached at cysteine 84 and the β-chain with two phycocyanobilins attached at cysteines 84 and 155. Both chains are rich in conserved α-helices, and the overall three-dimensional subunit structures are similar in different organisms. The two subunits form αβ monomers, which aggregate into α_3 β_3 trimers and further into disc-shaped α_6 β_6 hexamers, the functional unit of C- phycocyanin (Eriksen, 2008). PC is used in eye shadow, eye liner, lipsticks, candy ice cream, dairy products and soft drinks in Japan (Fatma & Venkataraman, 1999). It is not only used as nutrient ingredient and natural color for food and cosmetic but also used as potential therapeutic agent in oxidative stress-induced diseases (Patil & Raghavarao, 2007). Several studies have reported that *spirulina* can prevent or inhibit cancers in animals, antiviral effects, powerful stimulant for the immune system by increasing the phagocytic and the natural killer activities (Bermejo, Pinero & Villar, 2008). Moreover, PC showed antioxidants, anti-inflammatory and neuroprotective effects (Romay et al., 1998; Romay, Gonzalez, Ledon, Remirez & Rimbau, 2003). In water, PC gives brilliant blue colour with faint reddish fluorescence. But, it is poorly light stable. Phycocyanin is stable at pH 5-7.5, whereas temperature beyond 40 °C leads to instability (Sarada, Pillai & Ravishankar, 1999).

It has been previously reported that the irons ion can maintain the stability of phycocyanin exposed to thermal stress as it can prevent the decrease of visible light absorption and fluorescence intensity of phycocyanin (Gelagutashvili, 2007). Thus, it is of interest to study the preparation of phycocyanin in the form of metal complex and investigate the ability of metal ion on maintaining stability of phycocyanin. In this

work, the phycocyanin was extracted from *S. platensis* powder. The aluminium-phycocyanin complex (Al-PC complex) was prepared and its stability properties were studied at different conditions. Additionally, the use of Al-PC complex as coloring ingredient in cosmetic formulation was investigated.

1.2.2 Objectives

1.2.2.1 To extract phycocyanin from *Spirulina platensis.*

1.2.2.2 To prepare the Al-PC complex.

1.2.2.3 To characterize the Al-PC complex using FT-IR, XRD and SEM technique.

1.2.2.4 To study the stability of Al-PC complex.

1.2.2.5 To study the cytotoxicity of Al-PC complex.

1.2.2.6 To study the utilization of Al-PC complex in cosmetic products.

1.2.3 Scope of research

1.2.3.1 The phycocyanin was extracted from *Spirulina platensis* using DI water and phosphate buffer as solvents.

1.2.3.2 The quantity of phycocyanin was investigated using UV-vis spectroscopy technique.

1.2.3.3 Preparation of Al-PC complex using aluminium potassium sulphate (alum) at different conditions (time, temperature, pH and concentration of alum)

1.2.3.4 The properties of Al-PC complex was studied (solubility, surface morphology and cytotoxicity)

1.2.3.5 The stability of Al-PC complex was studied at different condition as follow:

1.2.3.5.1 Temperature (ambient temperature, 4 °C, 45 °C and 50 °C)

1.2.3.5.2 pH condition (acid, base)

1.2.3.5.3 Light condition (UV, fluorescent)

1.2.3.6 The cosmetic products were prepared using Al-PC complex.

1.2.3.6.1 Powder base

1.2.3.6.2 Anhydrous base

1.2.3.6.3 Emulsion base

1.2.4 Expected outcome

The Al-PC complex from *Spirulina platensis* for use as cosmetic colorant.

CHAPTER 2

LITERATURE REVIEW

2.1 Silver Nanoparticles

In biology and biochemistry nanoparticles have attracted much attention. Nanoparticles are defined as particulate dispersions or solid particles with a size in the range of 1-100 nm. They possess a very high surface to volume ratio and this can be utilized in areas where high surface areas are critical for success.

Silver nanoparticles have been synthesized using various techniques resulting in different shapes and sizes for use in numerous applications. The synthesis techniques are categorized into top-down and bottom-up approaches. The top-down techniques use silver metal in its bulk form, then, mechanically reduce its size to the nanoscale via specialized methodologies such as lithography and laser ablation. The bottom-up (also known as self-assembly) technique involves the dissolution of silver salt into a solvent and the subsequent addition of a reducing agent, with the supplemental use of stabilizing agents, if warranted, to prevent agglomeration of nanoparticles. Indeed, the solvents and reducing agents used in these processes affect the physical and morphological characteristics of manufactured silver nanoparticles. In turn, these specific characteristics will influence the fate, transport and toxicity of nanoparticles in the environment. For example, the use of sodium citrate as a reducing agent generates a negatively charged silver nanoparticle which may behave differently than a positively charged silver nanoparticle generated via branched polyethyleneimine. Intermediates or by-products are often generated in these

techniques, thereby playing a critical role in nanoparticles synthesis (Tolaymat et al., 2010).

Many methods aiming at the formation of silver nanoparticles, including the green ones, make use of an organic molecule. The latter interacts with the particles and provides them with stability against oxidation and agglomeration, or it can even act as a matrix only. In this sense, polymer molecules have been widely employed because their long chain offers many binding sites in which nanoparticles can be stabilized. Moreover, natural polymers are extremely important because many of them are biocompatible and non-toxic (Guidelli, Ramos, Zaniquelli & Baffa, 2011). Among such biomolecules are sucrose, maltose, chitosan, arabic gum and plant extracts such as the ones obtained from *Artocarpus heterophyllus* (Jagtap & Bapat, 2013). *Euphorbia hirta* (Elumalai, Prasad, Kambala, Nagajyothi & David, 2010) and *Ocimum sanctum* (Mallikarjuna et al., 2011). The successful silver nanoparticles synthesis using *Artocarpus heterophyllus* Lam. seed extract is an example of eco-friendly synthesis. The particles synthesized with 2% *A. heterophyllus* seed powder concentration gave a plasmon resonance band at 410 nm and the concentration of $AgNO_3$ increased, the intensity of the color also increased from yellow to deep red. The synthesis of silver nanoparticles using *A. heterophyllus* were irregular in shapes and different sizes ranging from 3 to 25 nm with 10.78 nm average size (Jagtap & Bapat, 2013).

2.2 Reducing Agents

2.2.1 *Phyllanthus emblica*

Phyllanthus emblica L. (*Emblica officinalis* Gaertn) (Figure 2.1.) is a medium to large deciduous tree belonging to a small subgenus of trees of the Euphorbiaceae. Emblica grows wild and is cultivated up to 1400 m. The name, *Phyllanthus emblica* L., was attributed by Linneaus with reference to a peculiar characteristic of this plant. Emblica is one of the most important plants of Ayurved, the Indian traditional medicine. For many years, the therapeutic potential of the fruits was attributed to their high ascorbic acid content about 1 g vitamin C per 100 mL of fresh juice. Because of the presence of tannins, the ascorbic acid does not oxidise even in dried fruit, thus

maintaining its antiscurvy property unchanged. Many studies have been published on the comparison between the effects of ascorbic acid and Emblica. The fruit extract has many pharmacological activities: it inhibits micronuclei formation, sister chromatid exchanges, clastogenicity and mutagenicity induced by metals such as lead, aluminum, cadmium, nickel, and caesium; it preserves against radiations, possesses antidiabetic activity, inhibits clastogenicity of benzopyrene and cyclophosphamide, is gastroprotective, cytoprotective, and immunomodulating (Scartezzini, Antognoni, Raggi, Poli & Sabbioni, 2006). In recent years, new pharmacological activities have been found for Emblica: it has cytoprotective activity against chromium, protects against oxidative stress in ischemic-reperfusion injury, shows antivenom capacity, ameliorates hyperthyroidism and hepatic lipid peroxidation, displays antiproliferative activity on MCF7 and MDA-MB-231 breast cancer cell lines, shows antitussive activity, and induces apoptosis in Dalton's Lymphoma Ascites and CeHa cell lines (Bhattacharya, Bhattacharya, Sairam & Ghosal, 2002). Determination of total phenol content as free and bound phenolics in *E. officinalis*, a major fraction was contributed from free phenolic acids which is constituted by gallic and tannic acid, as revealed by HPLC (Kumara, Nayaka, Dharmesha & Salimath 2006).

Figure 2.1 Emblica fruits

2.2.2 *Acacia concinna*

Acacia concinna is a tree native of Asia. Its parts used as bark, leaves and pods. It is a common, prickly, scandent shrub, occurring in tropical jungles. An infusion of the leaves is used in malarial fever (Raja & Sivaraj, 2012). A decoction of

the pods relieves biliousness and acts as a purgative. The pods are reported to be used in north Bengal for poisoning fish (Raja & Sama, 2012). The decoction of the pods of *A. concinna* is used in hair wash in lieu of soap (Natarajan & Natarajan, 2009). The pods ground up and formed into an ointment make a good application in skin diseases. The major compound of pod (Figure 2.2) of *A. concinna* is saponin which is detergent in nature may be the reason for its popular use. The saponin is reported to possess the cell wall toxicity and may be the reason for fungicidal activity. The phytochemical analysis of soap pod showed the presence of protein, fat and oil, flavonoids, saponin, phenol, tannin and quinine. The biochemical analysis of the soap pod showed maximum protein content compared to carbohydrates and phenol components (Setti, Ramakrishnan, Jaykar & Dadi, 2011).

Figure 2.2 *Acacia concinna* pod

2.3 Optical Properties

One of the most fascinating and useful aspects of nanomaterials is their optical properties. Applications based on optical properties of nanomaterials include optical detector, laser, sensor, imaging, phosphor, display, solar cell, photocatalysis, photoelectrochemistry and biomedicine. The optical properties of nanomaterials depend on parameters such as feature size, shape, surface characteristics, and other variables including doping and interaction with the surrounding environment or other nanostructures. The simplest example is the well-known blue-shift of absorption and photoluminescence spectra of semiconductor nanoparticles with decreasing particle

size, particularly when the size is small enough (Zhang, 2009). The specific optical properties of metal nanoparticles are mostly associated to electron driven local enhancement of the electromagnetic field. The spectral signature of this effect, known as the Surface Plasmon Resonance is directly connected to the nanoparticle geometry (size, shape, structure) and local environment (adsorbed molecules, matrix). These parameters fluctuate from particle to particle severely limiting the information that can be extracted from ensemble optical studies (Billaud et al., 2007). The distinctive colors of colloidal gold and silver are due to a phenomenon known as plasmon absorbance. Incident light creates oscillations in conduction electrons on the surface of the nanoparticles and electromagnetic radiation is absorbed. The wavelength of the plasmon absorption maximum in a given solvent can be used to indicate particle size (Mallikarjuna et al., 2011). Previous studies of silver nanoparticle using *Euphorbia hirta* as reducing agent showed that the absorption spectra of silver nanoparticles formed in the reaction media at 430 nm have diameter in range of 40 to 50 nm (Elumalai et al., 2010).

2.4 Antibacterial Activity of Silver Nanoparticles

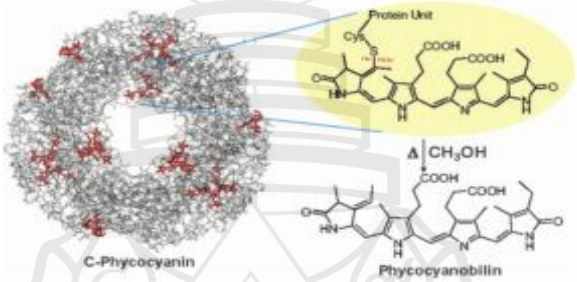
It is well known that silver ions and silver-based compounds have strong antimicrobial effects, and many investigators are interested in using other inorganic nanoparticles as antibacterial agents (Vijayakumar, Priya, Nancy, Noorlidah & Ahmed, 2013). These inorganic nanoparticles have a distinct advantage over conventional chemical antimicrobial agents. The most important problem caused by the chemical antimicrobial agents is multidrug resistance. Generally, the antimicrobial mechanism of chemical agents depends on the specific binding with surface and metabolism of agents into the microorganism. Various microorganisms have evolved drug resistance over many generations. Therefore, an alternative way to overcome the drug resistance of various microorganisms was needed. siver ions and silver salts have been used for decades as antimicrobial agents in various fields because of their growth-inhibitory capacity against microorganisms. Also, many other researchers have tried to measure the activity of metal ions against microorganisms. However, to

use silver in various fields against microorganisms, it is essential to prepare the silver with cost-effective methods and to know the mechanism of the antimicrobial effect. Besides, it is important to enhance the antimicrobial effect. The mechanism of the inhibitory effects of silver ions on microorganisms is partially known. Some studies have reported that the positive charge on the silver ion is crucial for its antimicrobial activity through the electrostatic attraction between negative charged cell membrane of microorganism and positive charged of silver ion. In another study, the antimicrobial activity of silver nanoparticles on gram-negative bacteria was dependent on the concentration of Ag nanoparticle, and was closely associated with the formation of "pits" in the cell wall of bacteria. Then, silver nanoparticles accumulated in the bacterial membrane caused the permeability, resulting in cell death (Kim et al., 2007). Previous study on green synthesis of silver nanoparticles was shown antibacterial activity against Gram positive (*Bacillus cereus*, *Bacillus subtilis* and *Staphyloccocus aureus*) and Gram negative (*Pseudomonas aeruginosa*) microorganisms (Jagtap & Bapat, 2013).

2.5 Phycocyanin

Phycocyanin (PC, Figure 2.3) is a blue, light-harvesting pigment in cyanobacterium *Spirulina (Arthrospira) platensis* and in the two eukaryote algal genera, Rhodophyta and Cryptophyta. It is phycocyanin that gives many cyanobacteria their bluish color and why these cyanobacteria are also known as blue-green algae. It is water soluble, strongly fluorescent and has antioxidant properties (Romay et al., 1998). Phycocyanin and related phycobiliproteins are utilized in a number of applications in foods and cosmetics, biotechnology, diagnostics and medicine (Patil & Raghavarao, 2007). *Spirulina* can prevent or inhibit cancers in animals, antiviral effects, powerful stimulant for the immune system by increasing the phagocytic and the natural killer activities (Bermejo, Pinero & Villar, 2008). Moreover, PC showed antioxidants, anti-inflammatory and neuroprotective effects (Romay et al., 1998; Romay et al., 2003). Phycocyanin is composed of two relatively homologous subunits: the α-chain with one phycocyanobilin attached at cysteine 84

and the β-chain with two phycocyanobilins attached at cysteines 84 and 155. Both chains are rich in conserved α-helices, and the overall three-dimensional subunit structures are similar in different organisms. The two subunits form αβ monomers, which aggregate into α_3 β_3 trimers and further into disc-shaped α_6 β_6 hexamers, the functional unit of C- phycocyanin. Crystal structures of C-phycocyanin from a number of cyanobacteria and rhodophytes have been elucidated. They all show this overall organisation of C-phycocyanin complexes with only minor species-dependent variations (Eriksen, 2008).



Sources Suresh, Mishra, Mishra & Das (2009).

Figure 2.3 Stucture of phycocyanin

In the last 15 years, there has been increasing interest in the potential uses of phycocyanin in fluorescent applications, nutraceuticals and pharmaceuticals. Phycocyanin has been stabilized chemically and by protein engineering, and new purification procedures allow very pure Phycocyanin to be obtained at high yields. Positive health effects have been related to intake of purified Phycocyanin (Eriksen, 2008). Phycocyanin is used in eye shadow, eye liner, lipsticks, candy ice cream, dairy products and soft drinks in Japan. For the use in cosmetic industry, it is treated with organic solvent for denaturation and precipitation of the pigment. To get more concentrated extracts of blue-red chromophores, enzymic and acid hydrolysis is also possible. The chromatographic separation of phycocyanin from other proteins is very complicated for commercial application (Fatma & Venkataraman, 1999). Moreover,

phycocyanin is high organized globular protein (hexamer). Monomer of phycocyanin consists from α- and β- polypeptide chains with 3 covalently binding chromophores. Whereas tetrapyrrol group (phycocyanobilin) in C-phycocyanin is chromophore, the possibility of complex formation between metal ions and chromophore of phycocyanin can be supposed. It is quite possible that such a distribution of the charge also plays an important role in the interaction with metal ions (Gelagutashvili, 2007). It has been previously reported that the irons ion can maintain the stability of phycocyanin exposed to thermal stress as it can prevent the decrease of visible light absorption and fluorescence intensity of phycocyanin (Jian-hong, Zi-hou & Tseng, 1998).

2.6 Lake Pigment

A lake, in the broadest sense of the term, can be described as a soluble dyestuff or coloring matter rendered insoluble by some suitable means and precipitated on a base or sub-stratum (Anstead, 1959). Apart from the dyestuff, by far the most common ingredient was alum, generally potash alum, potassium aluminium sulphate, $AlK(SO_4)_2 \cdot 12H_2O$. The use of alum as a mordant in textile dyeing is well known; in the case of lake pigments it was used as a reagent to form a substrate for the dyestuff, to make a pigment. The reaction between this and an alkali forms a type of hydrated alumina which precipitates together with the dyestuff, in solution with one or other of the reagents (Kirby, Spring & Higgitt, 2005). The alkali was commonly lye prepared form wood ash, but it could equally well be made from lime, or varieties of calcium carbonate, such as chalk, marble dust, egg shells or cuttlefish bone could have been used. These last are more usually found in recipes for yellow lake pigments, but also occur in recipes for red or rose-pink lake pigments from brazilwood, *Caesalpinia spp* (Kirby, Spring & Higgitt, 2005). The natural coloring matters are mostly used in lake form. The familiar Carmine which is obtained from the insect *Coccus cactii* (Cochineal) is mainly the aluminium salt of carminic acid formed in the presence of alumina hydrate (Anstead, 1959). Other natural red shade coloring matters such as Brazil Wood and Lima Wood, which make Rose Pink, and

Quercitron Bark and Persian Berries, which make Dutch Pink, are similar compounds. These are mentioned as interest is being shown in them arising from recent regulations governing the use of the natural colors in foodstuffs (Anstead, 1959). Rose madder lake, originally from the root of the madder plant, is also known as alizarin crimson in its synthetic form. Since rose madder is fugitive when exposed to light, its use has been largely superseded, even in synthetic form, by quinacridone pigments (Wikipedia, 2013). For a dark purplish blue-indigo lake was originally produced from the leaves of woad, and was known in ancient Egypt. In the late middle ages, a fashion for woad as a textile dye led to overplanting and soil exhaustion in many parts of Europe. After trade routes opened to the east, indigo was imported from India as a substitute for woad, and the cultivation of woad became uneconomical in Europe. Today, the dark blue dye known as indigo once produced from woad and *Indigofera tinctoria* is largely of synthetic origin (Wikipedia, 2013). Finally, lac natural dye is a red color product obtained from female insect *Coccus laccae* (*Laccifer lacca* Kerr). The lake pigment from Thai lac dye is the one of the example color used in lake form. Lake pigments of four metal salts $AlK(SO_4)_2.12H_2O$, $AlCl_3$, $CaCO_3$, and $Ca[CH_3COO]_2.H_2O$ were successfully prepared. The lac lake was prepared at different conditions such as pH, temperature, reaction time and metal salt concentration. The different metal salts complexes were given the different colors (Wongwad, Jimtaisong, Saewan & Krisadaphong, 2012). The shade of lakes was varies with the pure dye content and natural source (Table 2.1).

Table 2.1 Natural lake pigment in cosmetics

Name	Color shade
Indigo lake	dark purplish blue
Rose madder lake	reddish pink
Carmine lake	red

2.7 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectroscopy has proved to be a powerful tool to comply with the purpose of comprehensive characterisation. The unique characteristic of the material presented by the spectrum sheds light on material properties, its behaviour as well as on specific components represented by their functional groups. The substantial progress regarding the infrared spectroscopic measurement is achieved by the recording of the interferogram, the fast detection systems and the Fourier transformation. This combination provides many advantages and has caused a high interest in this method for process and quality control in many areas. Infrared spectroscopy is based on interactions of infrared radiation with matter. Infrared light causes functional groups to vibrate. The uptake of energy is indicated by absorption bands in the spectrum. Measured band intensities depend on the content of the substance to be determined and on the individual interaction of the functional group with infrared radiation at a distinct energy level. The near-infrared region from 14,000 cm^{-1}–4000 cm^{-1} and the mid-infrared region from 4000 cm^{-1}– 400 cm^{-1} are commonly applied for process and product control in many industrial fields (Smidt, Bohm & Schwanninger, 2013). Mid-infrared spectroscopy measures the absorption of radiation in the frequency range from about 4000 to 400 cm^{-1}. The absorption involves transitions between vibrational energy states and rotational substates of the molecule. A selection rule applies to these transitions, and absorption of infrared light can occur only if the vibration causes a change in the dipole moment of molecule. It is possible to assign absorptions to specific functional groups, making IR spectroscopy very useful in structural elucidation. Because the intensity of the absorption is proportional to the concentration of the absorbing species, quantitative analysis is also possible (Kacurakova & Wilson, 2001). The FT-IR spectrum suggests that the biological molecules could possibly perform dual functions of formation and stabilization of silver nanoparticles in the aqueous medium (Mallikarjuna et al., 2011).

2.8 Scanning Electron Microscopy (SEM)

The operation of the Scanning electron microscopy (SEM) consists of applying a voltage between a conductive sample and filament, resulting in electron emission from the filament to the sample (Russell, Batchelor & Thornton, 2013). It is a powerful and popular technique for imaging the surfaces of almost any material with a resolution down to about 1 nm. The image resolution offered by SEM depends not only on the property of the electron probe, but also on the interaction of the electron probe with the specimen. The interaction of an incident electron beam with the specimen produces secondary electrons, with energies typically smaller than 50 eV, the emission efficiency of which sensitively depends on surface geometry, surface chemical characteristics and bulk chemical composition. SEM can thus provide information about the surface topology, morphology and chemical composition. The high resolution capability afforded by SEM make it convenient for probing nanomaterials of which the structural features on the nanoscale are critical to their properties and functionalities (Zhang, 2009). To gain further insight into the features of the silver nanoparticles, analysis of the sample was performed using SEM and EDX techniques. The element analysis of the silver nanoparticles was performed using EDX on the SEM. SEM provided further insight into the morphology and size details of the silver nanoparticles (Forough & Farhadi, 2010).

2.9 X-Ray Diffraction (XRD)

X-Ray Diffraction (XRD) has long been used to determine the atomic-scale structure of materials. This technique is based on the fact that the wavelength of X-rays is comparable to the distances between atoms in condensed matter. When a material exhibiting a long-range (i.e. at least micrometers), periodic atomic order, such as a crystal, is irradiated with X-rays it acts as an extended, well-defined grating and produces a diffraction pattern showing numerous sharp spots, called Bragg diffraction peaks. By measuring and analyzing the positions and intensities of these peaks it is possible to determine the spatial characteristics of the grating i.e. to

determine the three-dimensional (3-D) arrangement of atoms in the crystalline material being studied. This is the essence of ‘crystal structure’ determination by XRD. Over the years the technique has been refined and applied successfully to a variety of crystalline materials from simple solids to complex proteins. XRD has also been successfully applied to study the structure of materials where atoms are ordered only at short distances (i.e., less than a nanometer), such as glasses and liquids (Flohr, 2013).

2.10 Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is almost always the first method used to determine the size and size distribution of nanoparticle samples. It is a high spatial resolution structural and chemical characterization tool. A modern TEM has the capability to directly image atoms in crystalline specimens at resolutions close to 0.1 nm, smaller than interatomic distance. An electron beam can also be focused to a diameter smaller than 0.3 nm, allowing quantitative chemical analysis from a single nanocrystal. This type of analysis is extremely important for characterizing materials at a length scale from atoms to hundreds of nanometers. TEM can be used to characterize nanomaterials to gain information about particle size, shape, crystallinity, and interparticle interaction. The TEM components have a similar to SEM, a stream of monochromatic electrons produced by an electron gun is focused into a small, thin, coherent beam by two condenser lenses. The electron beam is restricted by the condenser aperture to remove high angle electrons before it reaches the specimen. It is important in this case that the specimen is thin enough to allow some electrons to transmit through the sample. Interaction between the electron beam and specimen generates elastically and inelastically scattered electrons, along with some unscattered electrons, in the forward direction after the sample has been detected. The detected signal contains information about the sample. The detection involves several lenses to focus the electrons to be detected before they reach the detection phosphor screen. Also shown in the figure are optional objective and selected area metal aperture that can be used to restrict the beam, with the objective aperture enhancing contrast by

blocking out high-angle diffracted electrons and the selected area aperture enabling the user to examine the periodic diffraction of electrons by ordered arrangements of atoms in the sample examined (Zhang, 2009). From previous study, TEM image of silver nanoparticles derived from *Ocimum* leaf extract was determined. The obtained nanoparticles are in the range of sizes 3–20 nm and few particles are agglomerated (Mallikarjuna et al., 2011).

CHAPTER 3

THE SYNTHESIS OF SILVER NANOPARTCLE USING NATURAL REDUCING AGENTS

3.1 Materials

3.1.1 Materials and chemicals

Emblica fruit was collected from Chiangrai, Thailand during the month of November - December. *Acacia concinna* pod was collected from Chiangrai, Thailand during the month of March. Solvent for plant extraction and silver nanoparticles synthesis was deionized water. Silver nitrate was of A.R. grade and purchased from Merck, Germany. All ingredients for cosmetic emulsion formulation are cosmetic grade.

3.1.2 Equipment

Equipments	**Model, Maker, Country**
Hot plate stirrer	Lab Tech, LMS-100, USA
Balance	Sartorius, ED623S Milligram Scale, Germany
Homogenizer	Rose Scientific Ltd.,Ultra-Turrax® T25 basic, USA
Centrifuge	Hettich, Mikro 22 R, USA
Chromameter	Konica Minolta CO., Ltd., CR-400, Japan
Hot air oven	Memment, UNE/UFE, Germany
pH meter	Eutech, pH 510, USA

Equipments	Model, Maker, Country
UV-Vis Spectrophotometer	Biochroms, Libra S22, UK
Viscometer	Brookfield, RVDV-II+P, USA
FT-IR spectrometer	Perkin Elmer/FTIR Spectrum GX, USA
Scanning Electron Microscopy	JEOL model JSM-5910LV, Japan
Transmission Electron Microscopy	JEOL model JEM-2010, Japan

3.2 Methodology

3.2.1 Preparation of reducing agent

The Emblica pulp and DI water was blended by a blender and the juice was squeezed. The Soap Pod was thoroughly immersed into DI water and macerated at 50 °C for 4 h. The solution extract was filtrated.

3.2.2 Determination of reducing power

The different concentrations of reducing agents (100, 300 and 500 ppm) in 1 ml of DI water were mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.66) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min, after that added 2.5 ml of 10% trichloroacetic acid to follow by centrifuging at 5000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm. The increase in absorbance of reaction mixture indicated the reducing power of the natural reducing agent. Reducing power was measured by direct reduction of $[Fe(CN)_6]^{3-}$ to $[Fe(CN)_6]^{4-}$ and was determined by measuring absorbance resulted from the formation of blue complex following by the addition of excess Fe^{3+} (ferricyanide) in stoichiometric excess relative to reducing power. The reducing power of Emblica and Soap Pod were indicated by absorbance measured at 700 nm and vitamin C and BHT were used as standards. The increasing in absorbance of the reaction mixture indicates greater reducing capability (Muhammad et al., 2007).

3.2.3 Preparation of silver nanoparticles

3.2.3.1 The effect of natural reducing agent

The effect of natural reducing agent on silver nanoparticle preparation was studied by adding different quantities of natural reducing agent, i.e. 0.1-5ml of extracts into 50ml of 0.5mM silver nitrate and stirred for 24 h at ambient temperature. Peaks located of silver nanoparticles were investigated by UV-vis spectrophotometer.

3.2.3.2 The effect of concentration of silver nitrate

The effect of concentration of silver nitrate on silver nanoparticles synthesis was investigated by adding the extract into 50ml of different silver nitrate concentrations and stirred for 24 h at ambient temperature. The peaks located of silver nanoparticles were investigated by UV-vis spectrophotometer.

3.2.3.3 The effect of time and temperature

The effect of time and temperature on the reaction was studied by adding the suitable quantity of reducing agents into 50ml of silver nitrate 0.5 mM and stir and heat with hotplate stirrer at ambient temperature, 60 and 80 °C for different reaction time. The peaks located of silver nanoparticles were investigated by UV-vis spectrophotometer.

3.2.4 The characterization of silver nanoparticles

3.2.4.1 UV-Vis spectrophotometry

The colorless $AgNO_3$ solution turned yellow to brown or reddish yellow to deep red, indicated the formation of silver nanoparticles. The appearance of the brown color was due to the excitation of the surface plasmon vibrations, typical of silver nanoparticles having λ_{max} values which were reported earlier in the visible range of 400–500 nm (Jagtap & Bapat, 2013). Synthesized silver nanoparticles were confirmed. The silver nanoparticles were scanned by UV-Vis spectrophotometer at the wavelength of 350–700 nm on spectrophotometer.

3.2.4.2 Fourier transforms infrared spectroscopy (FT-IR)

The FT-IR spectra were obtained by using FT-IR spectrophotometer (Perkin Elmer/FT-IR Spectrum GX). The silver nanoparticles were mixed uniformly with potassium bromide (KBr) and incubated at 110 °C, 8 h. The KBr discs were

prepared by compressing the powders in a hydraulic press. The discs were scanned in the range of 4000-400 cm^{-1} to obtain FT-IR spectra.

3.2.4.3 Scanning electron microscopy (SEM)

The morphology of the silver nanoparticles were investigated by scanning electron microscopy (SEM, JEOL model JSM-5910LV, Japan). The surface of samples was coated with gold as a conductive material.

3.2.4.4 Transmission electron microscopy (TEM)

The size of silver nanoparticles was investigated by transmission electron microscopy (TEM, JEOL model JEM-2010, Japan). A drop of silver nanoparicle solution was placed on Cu grid. The size distribution was calculated from the measurement of particle size for more than 300 particles in the TEM picture of silver nanoparticles.

3.2.4.5 Antimicrobial activity of silver nanoparticles

The antimicrobial activity of AgNPs was evaluated against gram negative bacterial; E. *coli* (TISTR 780), and gram positive bacterial; *S. aureus* (TISTR 1466) by the agar disk diffusion method of the National Committee for Clinical Laboratory Standards (NCCLS; now renamed as Clinical and Laboratory Standards Institute, CLSI, 2000) with some modification (Kim et al., 2007). Approximately 10^7 colony forming units of each microorganism were inoculated on potato dextrose agar (PDA) plates, and then paper disc with different concentration of silver nanoparticles was put on. The Chloramphenicol was used as positive controls. The plates were incubated for 24 h at 37 °C. To evaluate the growth inhibition of silver nanoparticles, after 24 h incubation, each plate was measured the clear zone of microorganism. The results on the three plates corresponding to a particular sample were averaged (Kim et al., 2007).

3.2.5 Cytotoxicity test

The toxic effects of silver nanoparticles were evaluated using MTT cytotoxicity test on HDFn cell lines (human dermal fibroblast, neonatal). The method was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). The MTT assay (Plumb, Milroy & Kaye, 1989) is a tetrazolium-dye based colorimetric microtitration assay. Briefly, 50 µl of MTT in PBS at 5 mg/ml was added to the medium in each

well and the cells were incubated for 4 h. Medium and MTT were then aspirated from the wells, and formazan solubilized with 200 μL of DMSO and 25 μl of Sorensen's Glycine buffer, pH10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The experiments were done 3 times to get the values and standard deviation. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the IC_{50} for each toxin sample. A dose-response curve was derived from 8 concentrations in the test range using 4 wells per concentration. Results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells compared to controls. Based on the % cell survival of each test concentration, the toxicity of sample can be indicated as; "non-cytotoxic effect" if cell survived >50% and "cytotoxic effect" if cell survived <50%.

3.2.6 Preparation of cosmetic emulsion containing silver nanoparticles

The development of cosmetic emulsion containing silver nanoparticles as antimicrobials was formulated. Mikrocount® combi test kit was used to determine the microbial contamination of the product. The physical properties of the developed products were evaluated by both visual observation and equipments.

3.2.7 Stability study

The preliminary stability test was studied by centrifugation at 6000 rpm for 30 minutes. Then, the stable products were chosen for accelerated test at different storage conditions. The products were kept in different conditions for 1 month, i.e., at 4 °C in refrigerator, ambient temperature, 45°C in hot air oven and heating-cooling cycle (45°C 24 h, 4°C 24 h). The samples were monitored every week with respect to changes in appearance, color, pH and viscosity.

3.3 Results and Discussions

3.3.1 Preparation of reducing agent

The Emblica pulp (100 g) and 60 ml DI water was blended by a blender and the juice was squeezed. The Soap Pod (10 g) was thoroughly immersed into 30 ml DI water and macerated at 50 °C for 4 hr. The solution extract was filtrated. Emblica solution is yellowish green color with pH value of 2.9 and Soap Pod solution is light-brown color with pH value of 2.5 as can be seen in Figure 3.1.

Figure 3.1 Emblica extract (left) and Soap Pod extract (right)

3.3.2 Determination of reducing power

The different concentrations of reducing agents (100, 300 and 500ppm) in 1 ml of DI water were mixed with 2.5 ml of phosphate buffer (0.2M, pH 6.66) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min, after that added 2.5 ml of 10% trichloroacetic acid to follow by centrifuging at 5000 rpm for 10 min. The upper layer (2.5 ml) was mixed with 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700 nm. The increase in absorbance of reaction mixture indicated the reducing power of the natural reducing agent. Reducing power was measured by direct reduction of $[Fe(CN)_6]^{3-}$ to $[Fe(CN)_6]^{4-}$ and was determined by measuring absorbance resulted from the formation of blue complex following by the addition of excess Fe^{3+} (ferricyanide) in stoichiometric excess relative to reducing power. The reducing power of Emblica and

Soap Pod were indicated by absorbance measured at 700 nm and vitamin C and BHT were used as standards. The increasing in absorbance of the reaction mixture indicates greater reducing capability (Muhammad et al., 2007). The Emblica has reduction capacity relatively close to BHT but lower when compared with Vitamin C as can be seen from Figure 3.2. The Emblica solution is a strong reducing agent when compared with Soap Pod solution. The Soap Pod showed lower absorbance indicates poor reducing capability or a weaker reductant.

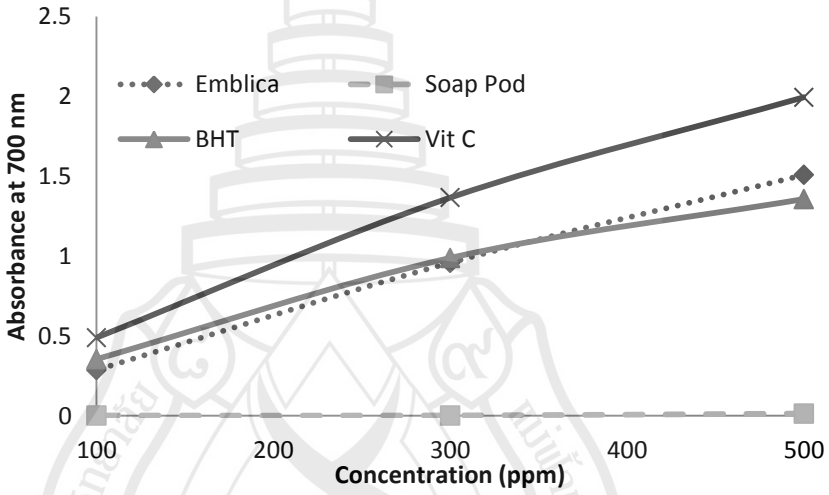


Figure 3.2 The reducing power of Emblica and Soap Pod

3.3.3 Preparation of silver nanoparticles

3.3.3.1 The effect of natural reducing agent

It is well known that silver nanoparticles exhibit a yellowish-brown color in aqueous solution due to excitation of surface plasmon vibrations in silver nanoparticles. Reduction of silver ions to silver nanoparticles could be followed by a color change and UV-Vis spectroscopy (Forough & Farhadi, 2010). The effect of reducing agent was studied by adding different quantities of Emblica solution (0.2-0.5 ml) and Soap Pod solution (2-5ml) into 50ml of 0.5 mM silver nitrate and stirred for 24 h. The result showed local peak of silver-Emblica peaks at 430 nm and silver-Soap Pod peaks at 422 nm (Figure 3.3). The quantity of reducing agent has an influence on AgNPs synthesis; the higher absorbance indicated the higher yield. The

plasmon resonance peak of Ag-Emblica is longer wavelengths and broadens compared with Ag-Soap Pod which suggested that the size of Ag-Emblica nanoparticles is bigger than the Ag-Soap Pod nanoparticles (Khalil, Ismail, EL-Baghdady & Mohamed, 2013; El-Sherbiny, Salih & Reicha, 2013). Moreover, the using small quantity of Emblica solution showed the higher absorbance peak comparing with Soap Pod solution. It indicated that Emblica solution is strong reducing agent when compared with Soap Pod solution.

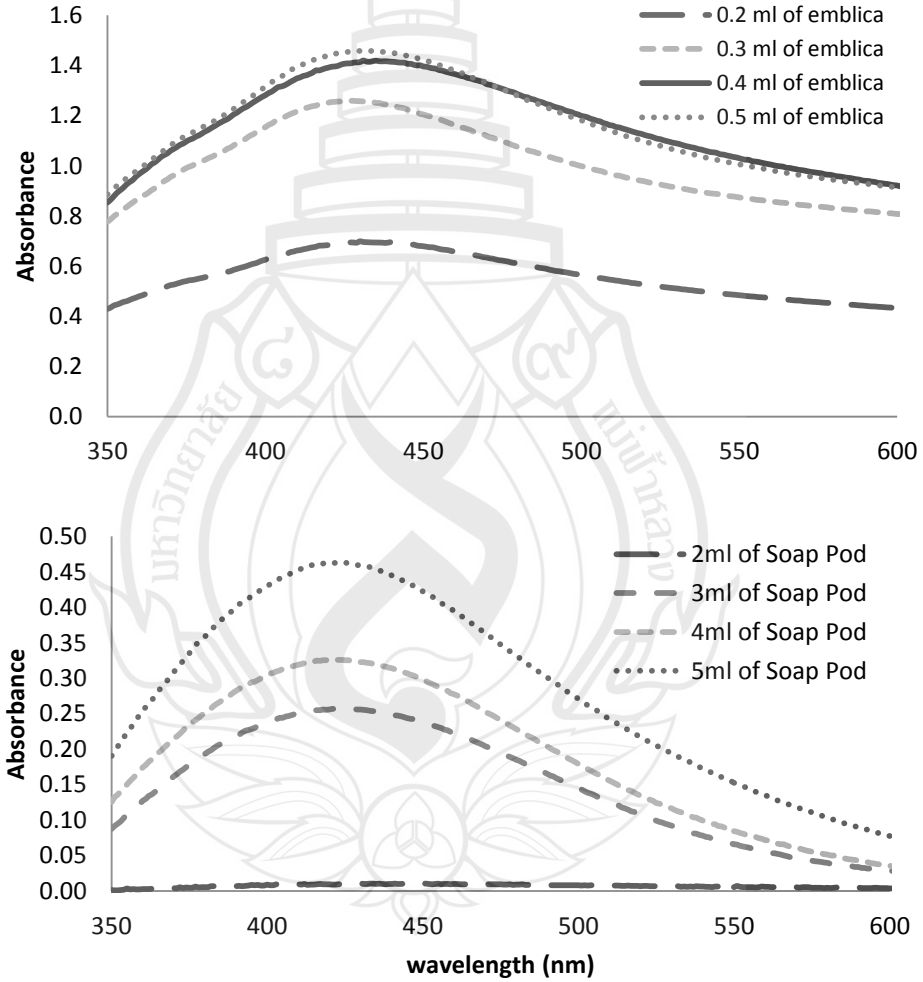


Figure 3.3 The absorbance peak of AgNPs at different quantities of natural reducing agent

3.3.3.2 The effect of concentration of silver nitrate

The effect of concentration of silver nitrate on silver nanoparticles synthesis were investigated by adding the extract (Soap Pod 3 ml and Emblica 0.4 ml) into 50ml of different silver nitrate concentrations and stirred for 24 h at ambient temperature. The different concentrations of silver nitrate displayed difference in absorbance height of AgNPs, Figure 3.4. The higher concentration of silver nitrate showed the higher absorbance, thus higher yield. Moreover, the plasmon resonance peak shifts to longer wavelengths and broadens, when the concentration of silver nitrate is increased which suggested that the size of silver nanoparticles is increased (Khalil, Ismail, EL-Baghdady & Mohamed, 2013; El-Sherbiny, Salih & Reicha, 2013).

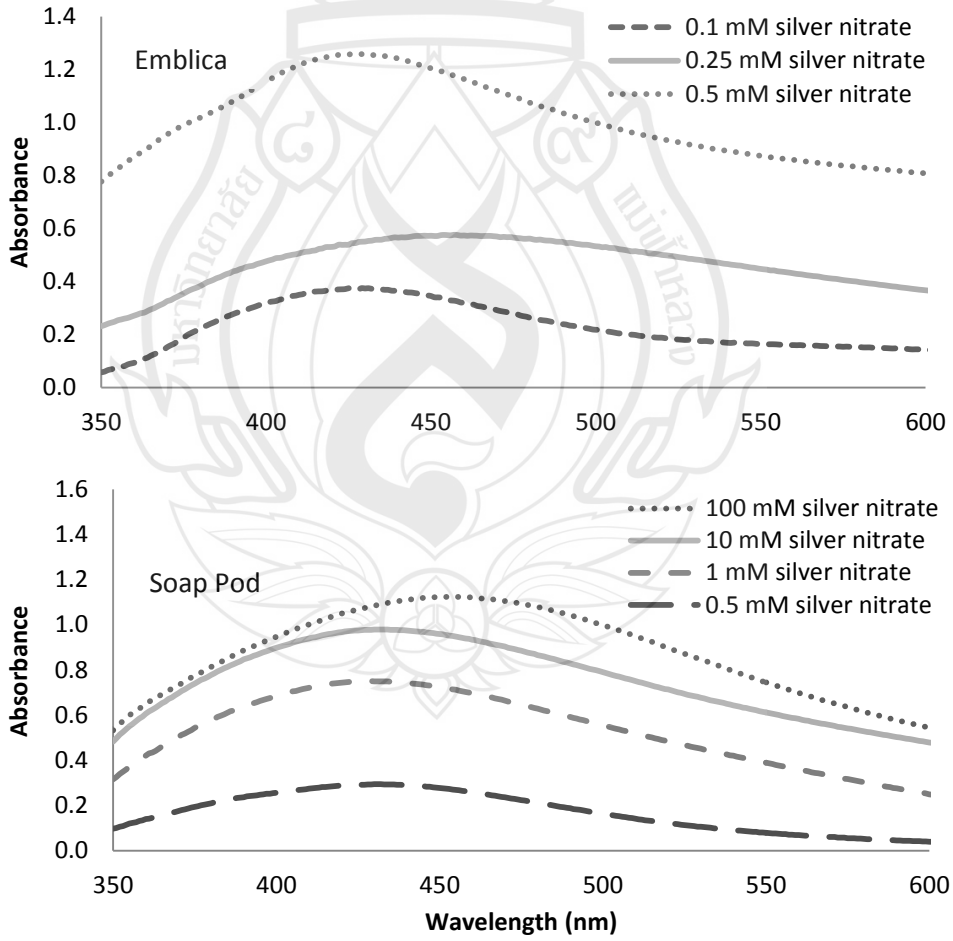


Figure 3.4 Effect of concentration of silver nitrate

3.3.3.3 The effects of time and temperature

The effects of time and temperature on the synthesis of silver nanoparticles were studied at ambient temperature, 60 and 80 °C. Silver-Emblica nanoparticle, at ambient temperature the absorbance increased from 2 h to 16 h and stayed constant when the reaction time increased up to 20 h. At 60 °C and 80 °C, the absorbance peaks are higher than ambient temperature. The reaction at 60 °C produced more of nanoparticles at longer period and showed a slow increase after 10h. At 80 °C the absorbance peak is not substantial raised after 4h reaction time as shown in Figure 3.5. Silver-Soap Pod, at ambient temperature the absorbance peak increased from 1 h to 3 h and after that stayed constant over 5 h. However, reducing power of Soap pods extract was destroyed when heated. The absorbance peaks decreased when heating reaction at 60 °C and 80 °C (Figure 3.6).

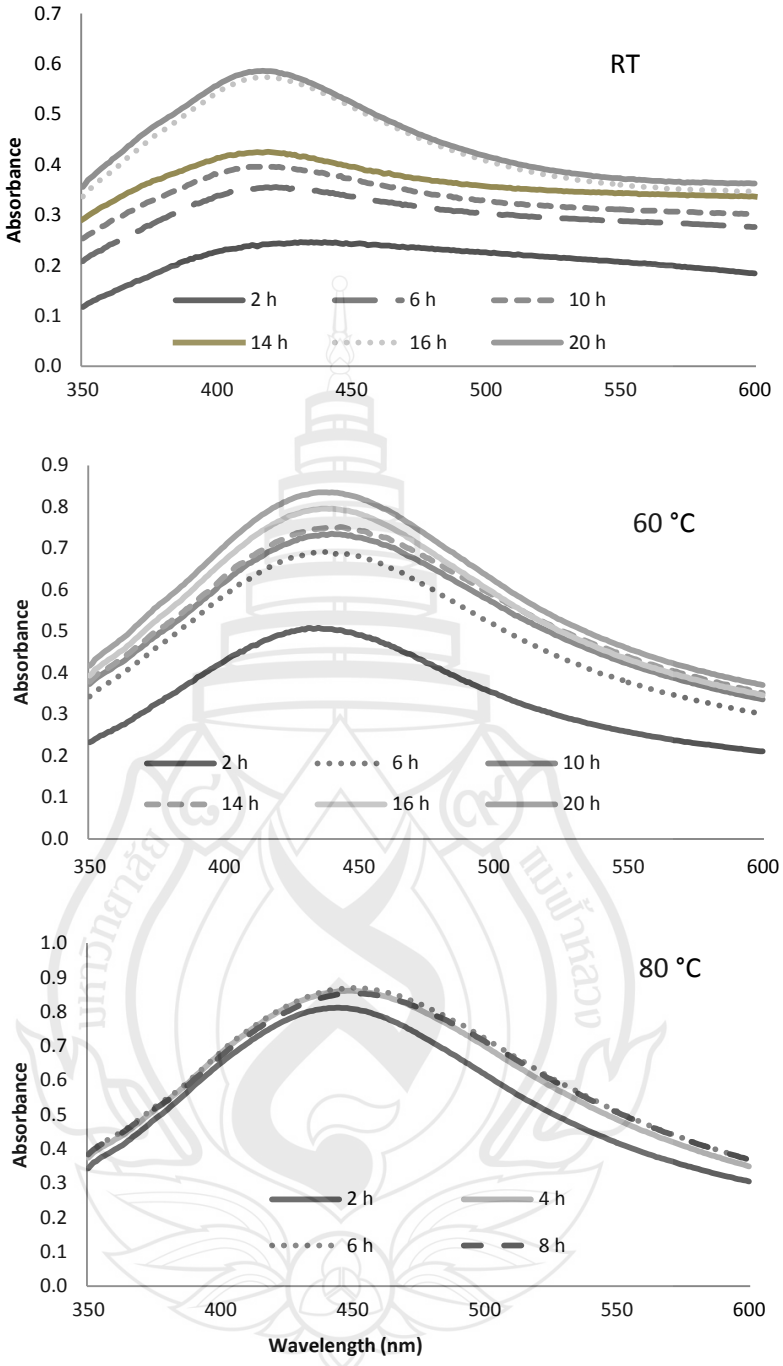


Figure 3.5 The absorbance peak of Ag-Emblica nanoparticles at different temperature

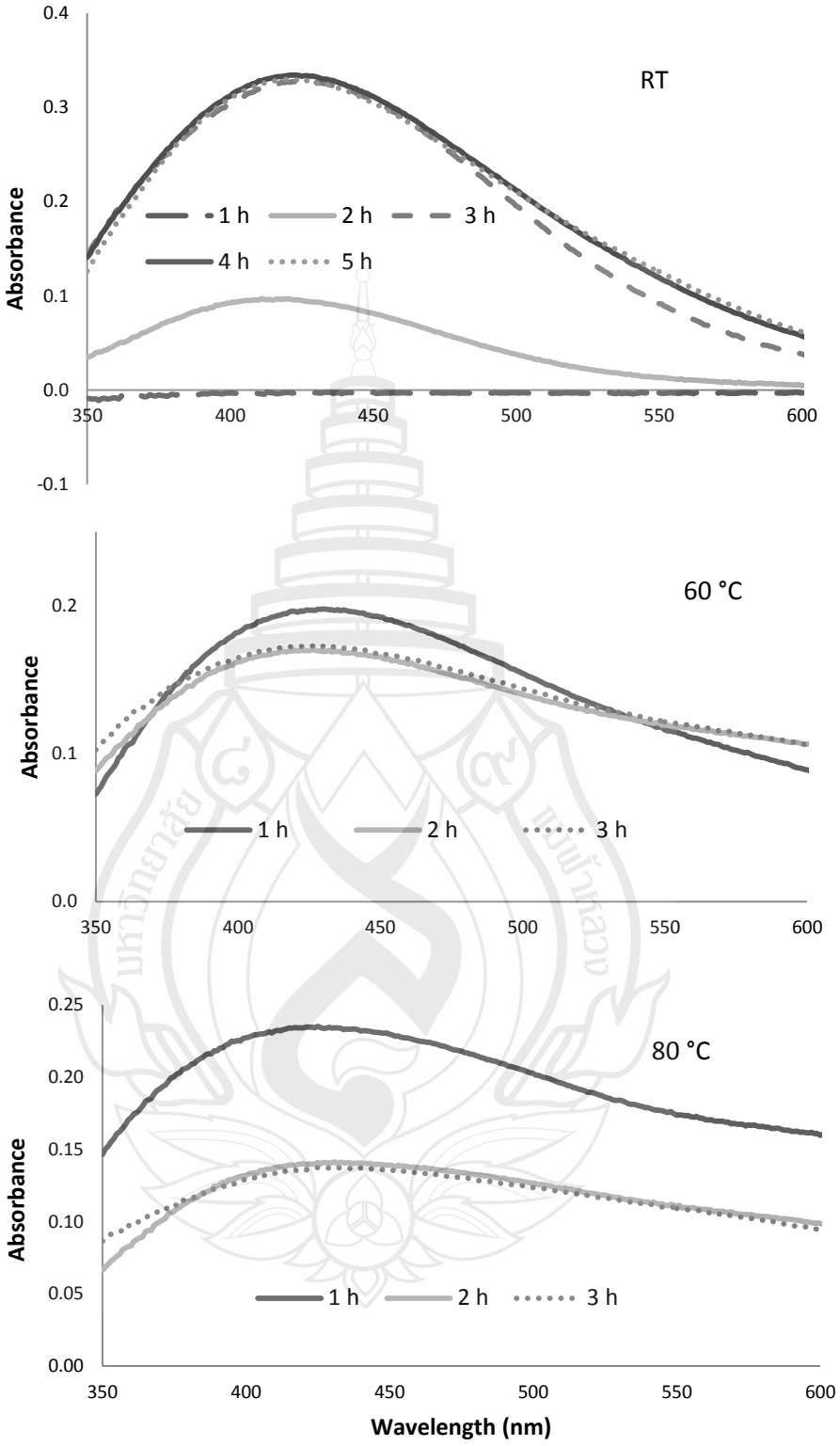


Figure 3.6 The absorbance peak of Ag-Soap Pod nanoparticles at different temperature

3.3.4 Stability of AgNPs

The stability of the AgNPs solutions was studied for a period of 6 months using UV-vis spectroscopy technique. The results showed the absorbance peak of Ag-Soap Pod nanoparticles was decreased slightly from 0 month to 3 month and decreased rapidly in 6 month after preparation. The absorbance peak of Ag-Emblica nanoparticle decreased slightly from 0 month to 6 compared with Ag-Soap Pod nanoparticle (Figure 3.7). The plasmon resonance peak shifts to longer wavelengths and broadens, which suggested that the size of silver nanoparticles is increased (Khalil, Ismail, EL-Baghdady & Mohamed, 2013; El-Sherbiny, Salih & Reicha, 2013). After 2 months of preparation, the particles size was also determined by transmission electron microscopy. From TEM image, the particle size of the as prepared Ag-Emblica nanoparticle is 35.7 nm at 0 month and after 2 months of preparation the particle size is increased to 58.0 nm (Figure 3.8). The TEM also shows that the resultant particles are aggregated which is formation of clusters in a colloidal suspension.

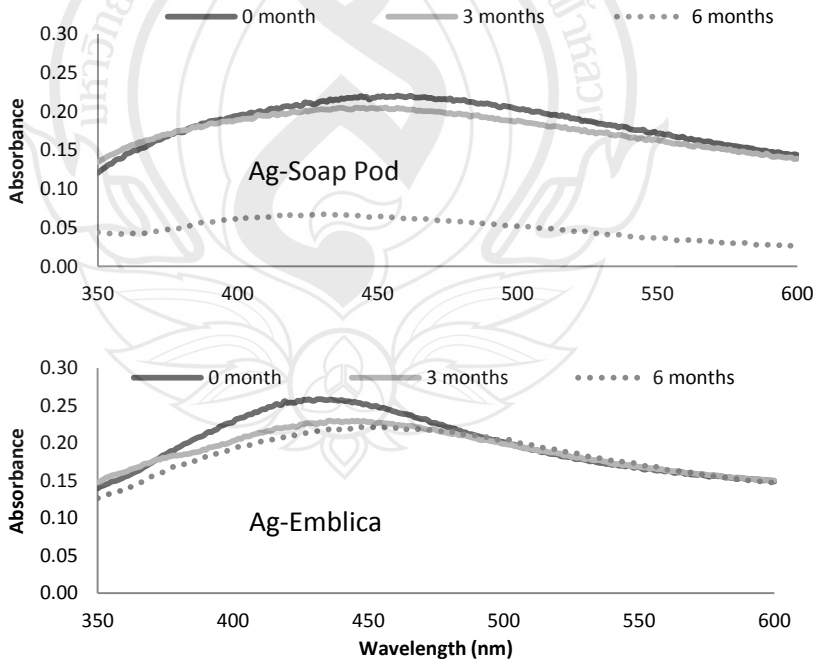


Figure 3.7 The absorbance peak of AgNPs storage at ambient temperature

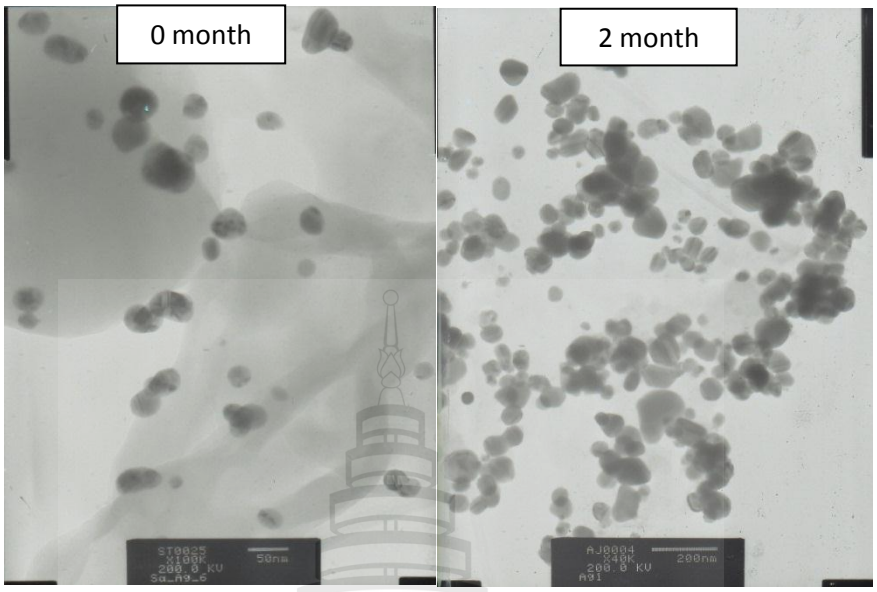


Figure 3.8 TEM images of AgNPs at 0 and 2 month after preparation

3.3.5 Fourier transforms infrared spectroscopy (FT-IR)

The FT-IR spectrum is obtained by using FT-IR spectrophotometer (Perkin Elmer/FT-IR Spectrum GX, USA). The analysis was carried out to identify the possible biomolecules responsible for capping of AgNPs synthesized by reducing agents (Figure 3.9). The FT-IR spectra of silver-Soap Pod and silver-Emblica nanoparticles showed peak of nitrate band at 1384 cm^{-1} of both AgNPs and silver nitrate (Smidt, Böhm & Schwanninger, 2011). The FT-IR spectra of silver-Soap Pod showed peak near 1789-1749 cm^{-1} assigned to carbonyl groups (C=O stretch) in Soap Pod extract found shifted to 1630 cm^{-1} in silver-Soap Pod. Peak at 3458 cm^{-1} assigned to hydroxyl groups (O-H stretch) which shifted from peak near 3399 cm^{-1} of Soap Pod. Moreover, the FT-IR spectra of silver-Emblica nanoparticle showed peak of hydroxyl groups (O-H stretch) at 3297 cm^{-1}. Peak at 2922 cm^{-1} and 2851 cm^{-1} assigned to CH_3 and CH_2. Peak at 1784 to 1617 cm^{-1} assigned to carbonyl groups (C=O stretch) in Emblica (Leslie, Sev & John 2008). These changes may indicate that the carbonyl and hydroxyl groups of the extract are associated with the silver clusters and it suggested that the obtained AgNPs were capped with reducing agent (Mallikarjuna et al., 2011).

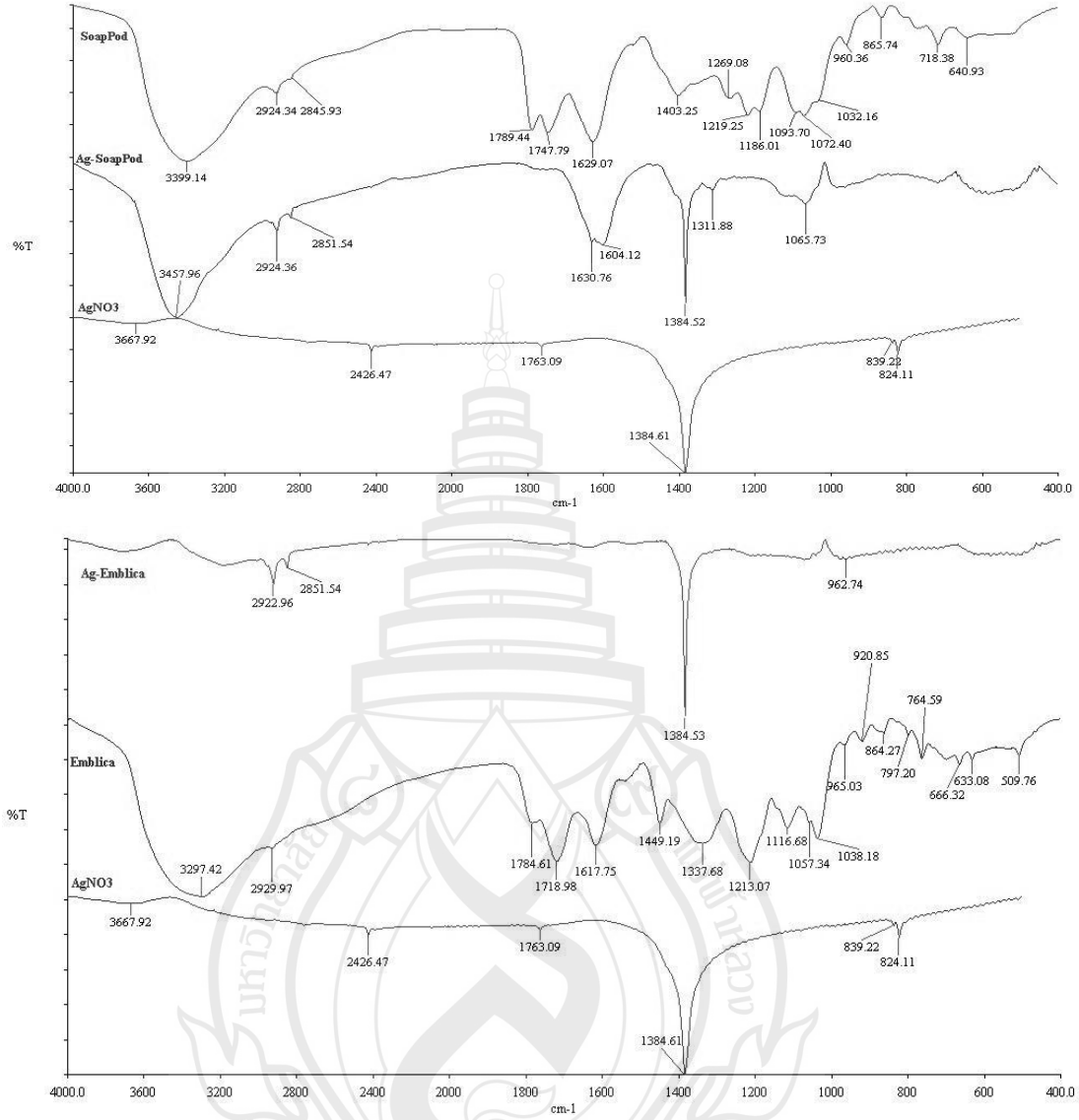


Figure 3.9 FT-IR pattern of Ag-Soap Pod and Ag-Emblica nanoparticles

3.3.6 Scanning electron microscopy (SEM)

The morphology of the silver nanoparticles were investigated by scanning electron microscopy (SEM, JEOL model JSM-5910LV, Japan). The morphology of the silver-Soap Pod nanoparticles showed that the silver particles were dispersed on the plant extract and the size is normally rounded and the silver-Emblica particles exhibited small size and agglomerated on the plant extract. The size of silver-Soap pod is smaller than silver-Emblica as can be seen in Figure 3.10. In the analysis of silver nanoparticles by Energy Dispersive Spectroscopy (EDS), the presence of

elemental silver signal was confirmed the formation of high content of silver nanoparticles.

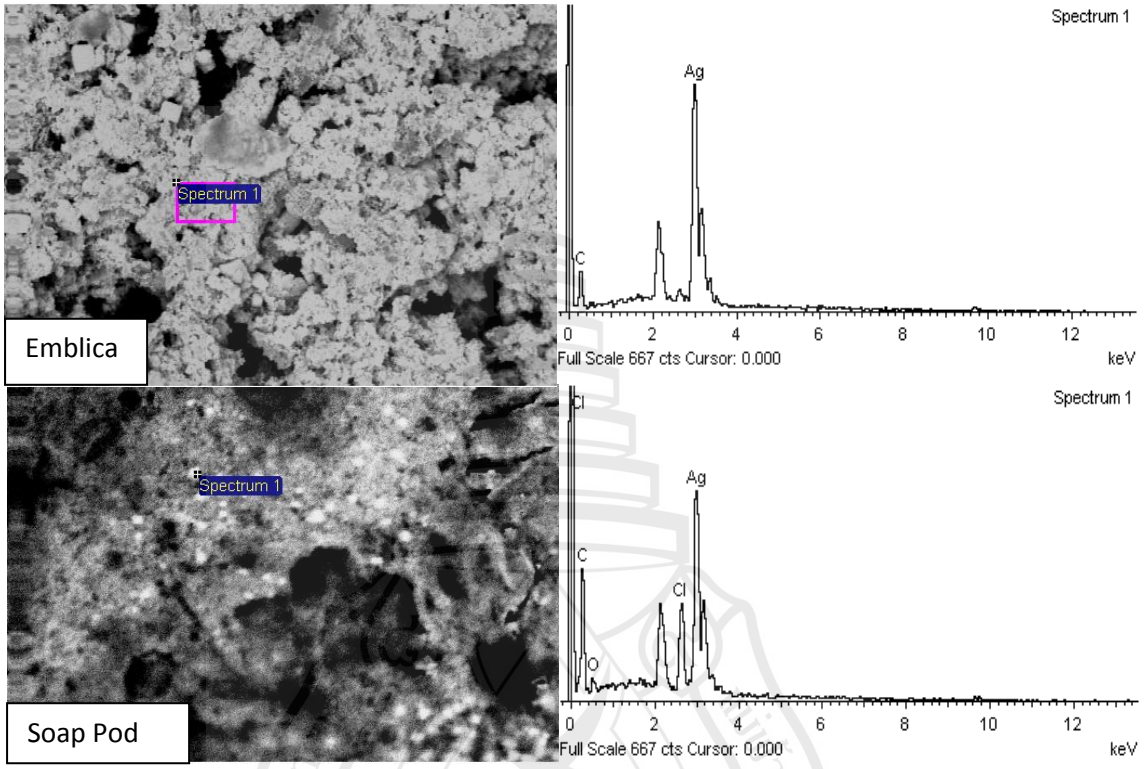


Figure 3.10 The SEM picture and EDS analysis of AgNPs

3.3.7 Transmission electron microscopy (TEM)

The size of silver nanoparticle was investigated by transmission electron microscopy (TEM, JEOL model JEM-2010, Japan). TEM analysis revealed that the particle size of silver-Soap Pod nanoparticles is 20.9 nm with narrow size distribution and shows that the resultant particles are all spherical and monodispersed particles. The particle size of silver-Emblica is about 41.2 nm in average diameter and showed an irregular form of particles (Figure 3.11). The previous studies showed that strong reducing agent resulted in smaller monodisperse particles and the generation of larger particles was a problem. Use of a weaker reductant, resulted in a lower reduction rate but polydispersity is significantly higher (Kaler, Patel & Banerjee, 2010).

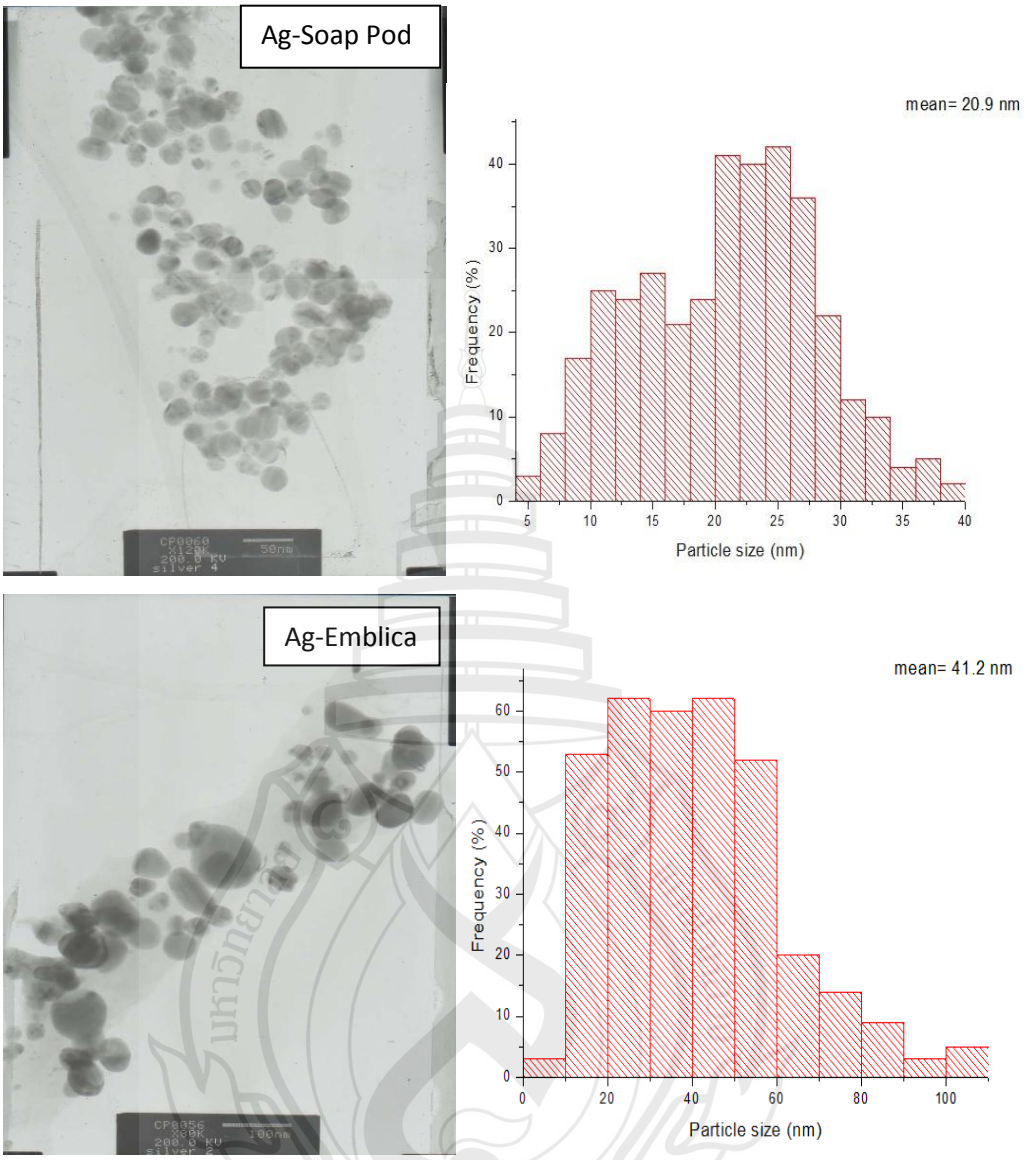


Figure 3.11 TEM picture and size distributions determined from the TEM image

3.3.8 Antimicrobial activity of silver nanoparticles

The antimicrobial activity of AgNPs was evaluated against gram negative bacterial; *E. coli* (TISTR 780), and gram positive bacterial; *S. aureus* (TISTR 1466) by the agar disk diffusion method of the National Committee for Clinical Laboratory Standards (NCCLS; now renamed as Clinical and Laboratory Standards Institute, CLSI, 2000) with some modification (Kim et al., 2007). The antimicrobial activity of Ag-Soap Pod nanoparticle as shown in Figure 3.12, comparing with the silver nitrate

solution (500µg/ml), showed a similar growth inhibition effect against *E.coli*. When increasing concentration of Ag-Soap Pod nanoparticle from 100 to 500 µg/ml, the growth inhibition effect is higher. The results were correlated to those found from silver nanoparticles using olive leaf extract (Khalil, Ismail, EL-Baghdady & Mohamed, 2013) and bamboo leaves extract (Yasin, Liu & Yao, 2013). The antimicrobial activity of Ag-Emblica nanoparticle showed no inhibition zone in *S. aureus* at all concentration (Table 3.1). When increasing concentration of Ag-Emblica nanoparticle to 1000 µg/ml, the growth inhibition effect against *E.coli* is poor. It was reported that the antibacterial activity of silver nanoparticles was found to be dependent on the size of silver particles. So, the controlled synthesis of silver colloid particles was attempted using a two-step reduction process in order to control the particle size. In this process, a strong reducing agent was first applied to produce small silver particles, which were then enlarged in a secondary step using a weaker reducing agent (Panacek et al., 2006).

Table 3.1 Antimicrobial activities of AgNPs against *E.coli* and *S.aureus*

Sample	**Inhibition zone diameter (in cm)**	
	E.coli	***S.aureus***
Ag-Emblica NPs		
- 1000 ppm	None	None
- 500 ppm	0.83	None
- 320 ppm	0.75	None
- Chloramphenicol (positive)0.15%	2.76	2.86
- DI water (negative)	None	None
Ag-Soap Pod NPs		
- 500 ppm	0.86	0.81
- 100 ppm	0.70	0.68
- Solution	0.98	0.85
- Chloramphenicol (positive) 500 ppm	2.30	2.20
- Silver nitrate 500 ppm	0.96	0.95
- DI water (negative	None	None

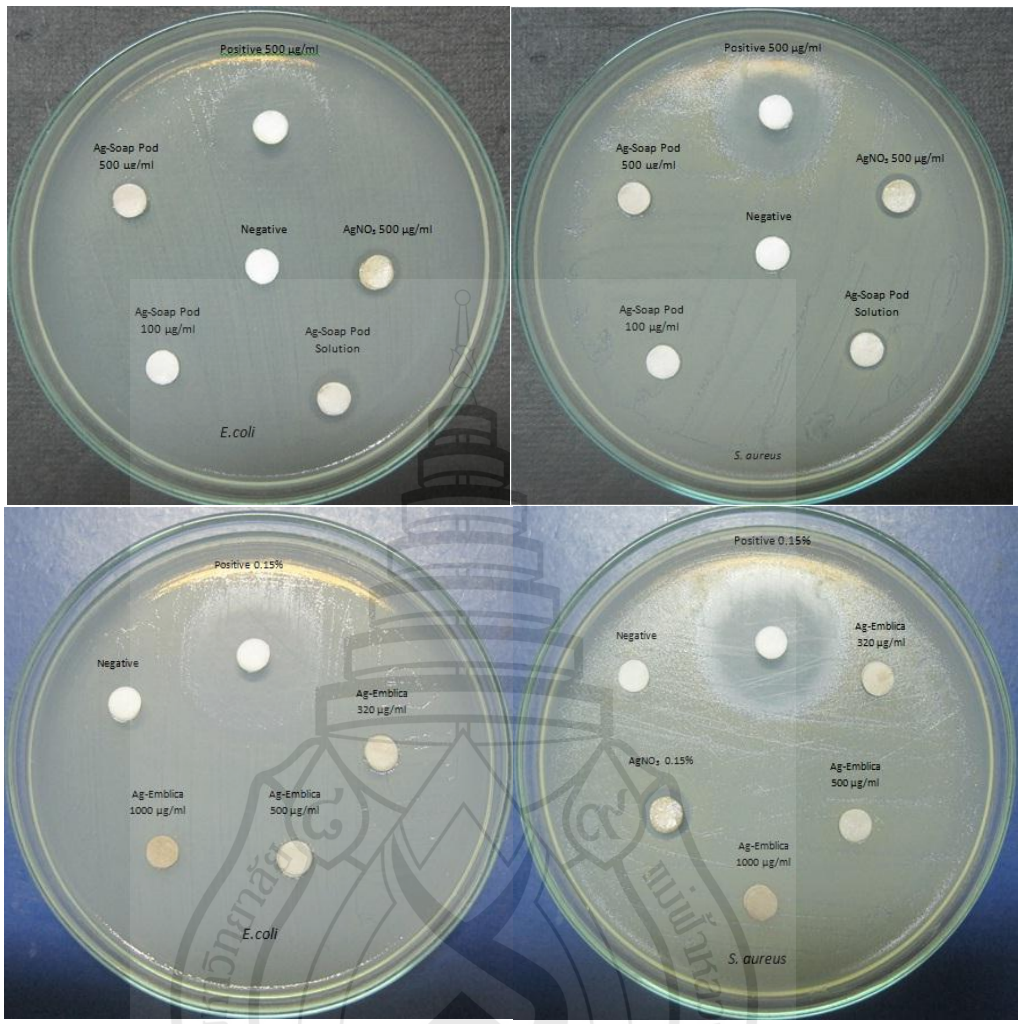


Figure 3.12 Antimicrobial activities of AgNPs against *E.coli* and *S.aureus*

3.3.9 Cytotoxicity test

The toxic effects of silver nanoparticles were evaluated using MTT cytotoxicity test on HDFn cell lines (human dermal fibroblast, neonatal). The % survival of the cells cultured with silver nanoparticles at various concentrations (compared to control) and the minimum inhibitory concentration (IC_{50}) value was summarized in Table 3.2. The results showed that Ag-Soap Pod and Ag-Emblica nanoparticles were not cytotoxic to human dermal fibroblast over the test concentration. Thus, it indicated that the prepared silver nanoparticles may be competent for application in the cosmetic and pharmaceutical industry. However, more studies are needed to better understand the mechanisms, as well as the

correlations between silver nanoparticles and their impact on the human health which would lead to the possible applications of these nanomaterials in a safe manner (Lima, Seabra & Durán, 2012).

Table 3.2 The % survival of HDFn cell lines (human dermal fibroblast, neonatal)

Concentration (µg/ml)	Ag-Soap Pod solution			Ag-Emblica solution		
	Mean %Survival	Mean SD	IC_{50} (µg/ml)	Mean %Survival	Mean SD	IC_{50} (µg/ml)
8.49	95	2		97	1	
4.25	97	4		104	6	
2.12	106	4		103	7	
1.06	100	1		96	5	
0.53	99	1	>8.49	99	6	>8.49
0.27	97	1		99	2	
0.13	96	1		95	1	
0.07	101	3		99	3	

3.3.10 Preparation of cosmetic emulsion containing silver nanoparticles

The development of cosmetic emulsion containing silver nanoparticles was formulated. The product was developed using silver nanoparticles as antimicrobials. Mikrocount® combi test kit was used to determine the microbial contamination of the product. The physical property of the developed product was evaluated by both visual observation and equipments. First, base formulation was developed under the concept of smooth, light, non-greasy. Oil phase ingredients consisted of dimethicone (5%), jojoba oil (5%), cetyl alcohol (1.5%), beeswax (1.5%) and span-80 (3%). The water phase ingredients are DI water, xanthan gum (1%), propylene glycol (5%) and tween-80 (2%). The product was prepared as oil in water (O/W) emulsion with containing 1% AgNPs solution. The product is white cream gel with pH of 5.03.

3.3.10.1 Microbial contamination test

Mikrocount® combi test kit was used to determine the microbial contamination of the product. It is a plastic slide coated on one side with TTC agar (bacterial growth) and on the other side with Rose-bengal-agar (yeast and mould growth). The results showed that base cream gel formula showed mould growth

which is different from cream gel containing AgNPs. It can confirm that the AgNPs had potential to use as antimicrobials in emulsion (Figure 3.13).

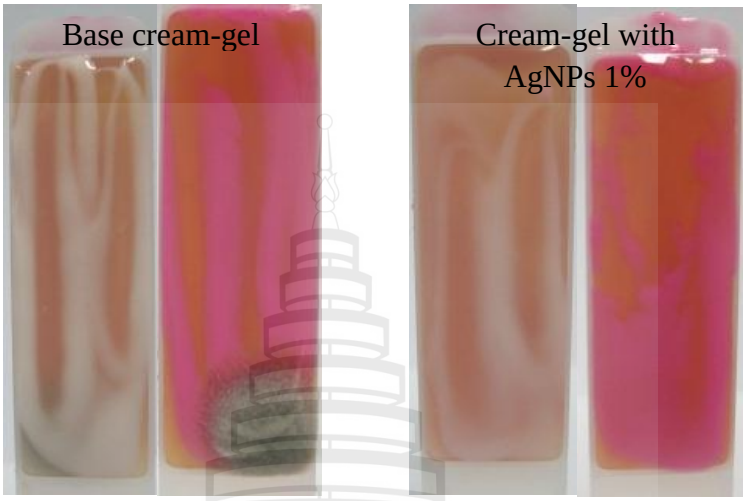


Figure 3.13 Microbial contamination test of emulsion cream gel

3.3.10.2 Stability study

The preliminary stability test was studied by centrifugation at 6000 rpm for 30 minutes and the products showed no phase separation. Therefore, the products were chosen for accelerated test at different storage conditions. The products were kept in different conditions for 1 month, i.e., at 4 °C in refrigerator, ambient temperature, 45°C in hot air oven and heating-cooling cycle (45°C 24 h, 4°C 24 h). The samples were monitored every week with respect to changes in appearance, color, pH and viscosity.

The viscosity of products was measured using a viscometer and results are graphically shown in Figure 3.14. As can be seen from the Figure, the viscosity of cream gel product was decreased rapidly from week 0 to week 1 and fairly constant from week1 to week 4. It is change about 58-60% in viscosity values from the initial. The textures showed only little changes compared with the initial for all conditions.

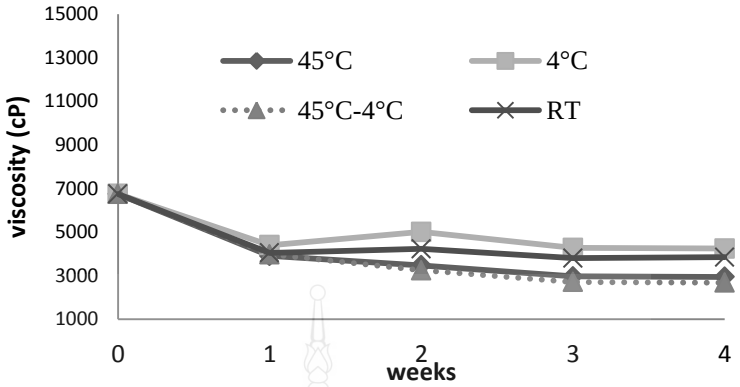


Figure 3.14 Viscosity values of cosmetic cream gel containing 1% AgNPs solution

The color of the product was visually observed and also measured by a chromameter (Minolta CR-400) every week and the results were recorded in L*a*b* values, as shown in Figure 3.15.

The L* describes the lightness of the product, the high values show more lightness. It was found that after 4 weeks, the L* values of all storage conditions showed slightly change about 7-9%. The change in L* values showed no different compared with the initial.

The a* is the color correlation between green and red where the negative values indicate green while positive values indicate red. After 4 weeks, the a* values of all storage conditions showed slightly change and all of products are no different visually observed compared with the initial.

The b* refers to blue and yellow color where negative values indicate blue and positive values indicate yellow. The b* values of all storage conditions showed slightly change about8-16% which showed no different color from the initial as visually observed.

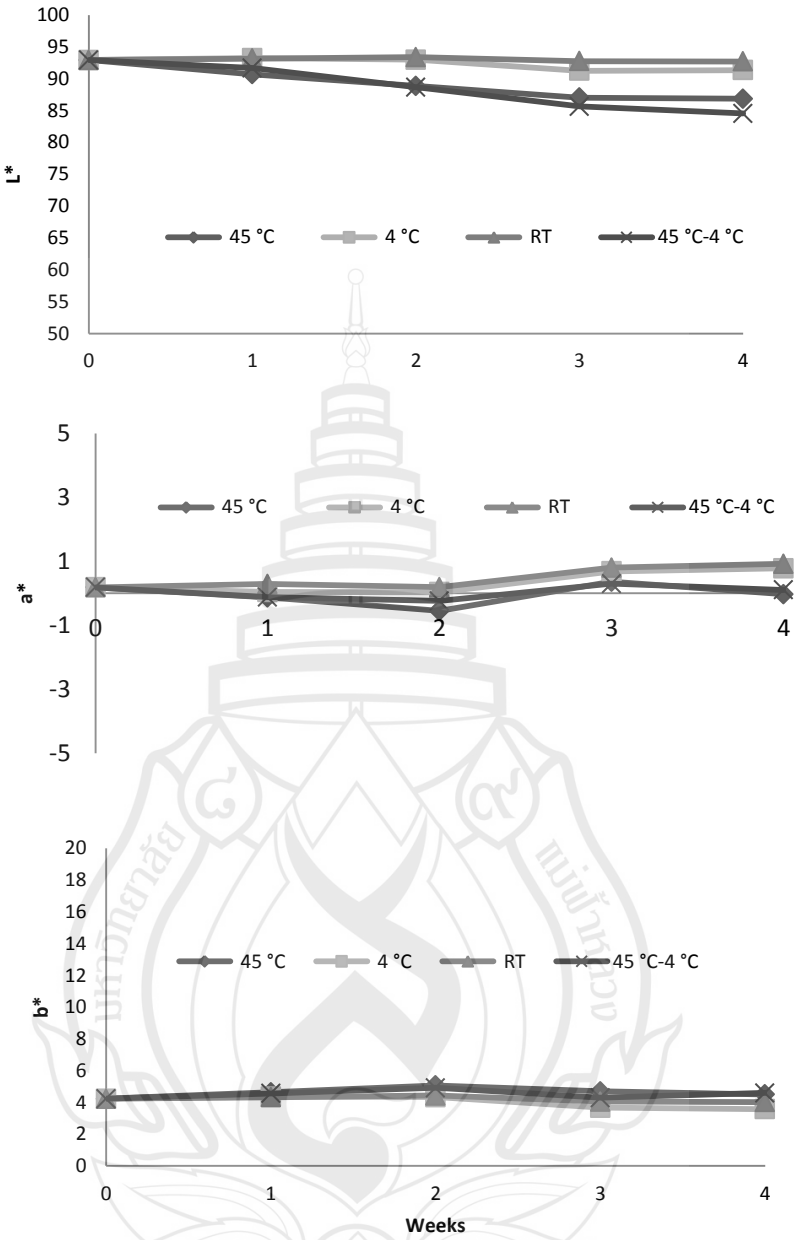


Figure 3.15 The L*a*b* values of cream gel containing 1% AgNPs solution

CHAPTER 4

THE PREPARATION OF ALUMINIUM COMPLEX OF PHYCOCYANIN FROM *Spirulina platensis*

4.1 Materials

4.1.1 Materials and chemicals

Spirulina platensis dry powder is of Effective Marketing International Co., Ltd, Thailand. The solvent for extraction is sodium phosphate buffer and deionized water. The chemicals for extraction and preparation of Al-PC complex were of A.R. grade. All ingredients for cosmetic formulation are cosmetic grade.

4.1.2 Equipments

Equipments	**Model, Maker, Country**
Hot plate stirrer	Lab Tech, LMS-100, USA
Balance	Sartorius, ED623S Milligram Scale, Germany
Homogenizer	Rose Scientific Ltd.,Ultra-Turrax® T25 basic, USA
Centrifuge	Hettich, Mikro 22 R, USA
Chromameter	Konica Minolta CO., Ltd., CR-400, Japan
Hot air oven	Memment, UNE/UFE, Germany
pH meter	Eutech, pH 510, USA
UV-Vis Spectrophotometer	Biochroms, Libra S22, UK
FT-IR spectrometer	Perkin Elmer/FTIR Spectrum GX, USA

Equipments	Model, Maker, Country
Scanning Electron Microscopy	LEO/1450 VP LEO, Germany
X-Ray diffractometer	PANalytical/X'Pert Pro MPD, Netherlands
Water bath	Memmert, WNB22, Germany
Ultrasonic washer	Crest, 690D, USA

4.2 Methodology

4.2.1 Extraction of phycocyanin

Phycocyanin was extracted from *Spirulina platensis* powder using the following methods (Sarada et al., 1999).

4.2.1.1 Water extraction: *S. platensis* powder (10 g) was suspended in distilled water (100 ml) and the phycocyanin leached out was estimated spectrophotometrically.

4.2.1.2 Buffer extraction: *S. platensis* powder (10 g) was suspended in 50 mM sodium phosphate buffer pH 6.8 (100 ml). The extract was centrifuged and the supernatant contained phycocyanin.

4.2.2 The characterization of phycocyanin solution

4.2.2.1 UV-vis spectroscopy

The purity of phycocyanin (PC) preparations is evaluated based on the ratio between absorbencies from phycocyanobilin at 620 nm, A_{620} and aromatic amino acids in all proteins in the preparation at 280 nm, A_{280}. PC preparations with A_{620}/A_{280} greater than 0.7 was considered food grade, while A_{620}/A_{280} of 3.9 was considered reactive grade and A_{620}/A_{280} greater than 4.0 analytical grade (Eriksen, 2008).

4.2.3 Preparation of Al-PC complex

The Al-PC complex was prepared according to the reported method (Wongwad et al., 2012) with some modifications. The reaction mixture was

centrifuged at 8000 rpm, 10 min and the precipitates were incubated at 40 °C overnight. The complex was prepared at different conditions as follows.

4.2.3.1 Effect of quantity of aluminium potassium sulphate (alum)

The PC extract 25 ml was mixed with alum (10% w/v solution) 2.5-15 ml and stirred 1 h at ambient temperature.

4.2.3.2 Effect of pH

The PC extract 25 ml were mixed with alum (10% w/v solution) 5 ml and the pH of reaction mixture was adjusted in the range of 2 to 12 and stirred for 1 h at ambient temperature.

4.2.3.3 Effect of reaction time

The PC extract 25 ml was mixed with alum (10% w/v solution) 5 ml. The reaction mixture was stirred at ambient temperature for 30, 60 and 120 min.

4.2.3.4 Effect of temperature

The PC extract 25 ml was mixed with alum (10% w/v solution) 5 ml. The reaction mixture was stirred for 30 min at ambient temperature, 60 °C and 80 °C.

4.2.4 Characterization of Al-PC complex

4.2.4.1 Fourier transforms infrared spectroscopy (FT-IR)

The FT-IR spectra were obtained by using FT-IR spectrophotometer (Perkin Elmer/FT-IR Spectrum GX, USA). The sample was mixed uniformly with potassium bromide (KBr) and incubated at 110 °C, 8 h. The samples were scanned in the range of 4000-400 cm^{-1} to obtain FT-IR spectra.

4.2.4.2 X-Ray diffractometry (XRD)

Powder XRD patterns was obtained using XRD diffratometer (PANalytical/X'Pert Pro MPD, Netherlands) with Cu Kα target tube, NaI detector, variable slits, a 0.010-0.020 step size, operated at a voltage of 30 kV, 15 mA current, at 2 theta (2θ)/min scanning speed, and scanning angles ranged from 0 to 80°.

4.2.4.3 Scanning electron microscopy (SEM)

The surface morphology of the samples was investigated by scanning electron microscopy (LEO/1450 VP LEO, Germany). The surface of samples was coated with gold as a conductive material.

4.2.5 Stability of Al-PC complex

The stability of Al-PC complex was investigated at ambient temperature, 4°C, 45°C, 50°C, UV light and fluorescent light condition. The sample was mixed with talcum at 50% w/w and 20% w/w in castor oil. The physical properties of Al-PC complex were evaluated every week for 1 month by both visual observation and measurement by using Chromameter (Konica Minolta/CR-400, Japan).

4.2.6 Cytotoxicity test

The cytotoxicity of phycocyanin extract and Al-PC complex was tested against human dermal fibroblast, neonatal (HDFn) C-004-5C cell using resazurin microplate assay (REMA). This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). The cells were seeded in a 96-well plate at various densities of cell per well, and incubated for 48 hours. The sample at various concentrations were added to the cells and incubated for 24 hours. After 24 incubation period, 25 µl of 125 µg/ml resazurin solution is added to each well, and the plates are then incubated at 37oC for 4-6 hours. Fluorescence signal is measured using SpectraMax M5 multidetection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm.

4.2.7 Preparation of cosmetic product containing Al-PC complex

The cosmetic products containing Al-PC complex was prepared. Base formulas of cosmetics, i.e., powder, emulsion and anhydrous base were first developed. Then, the cosmetic products containing Al-PC complex was prepared. The characteristics of cosmetic products were evaluated by both visual observation and equipment.

4.3 Results and Discussions

4.3.1 Extraction of phycocyanin

4.3.1.1 The effects of solvents and extraction methods

The conditions for extraction were studied to get the higher quantity and purity of PC. *S. platensis* powder (10 g) was soaked in 100 ml deionized water or sodium phosphate buffer (50 mM, pH 6.8) with stirred, sonicated or 4 °C refrigerated conditions for 4 h. The extracts were centrifuged and the supernatant contained PC. The purity of PC was evaluated based on the ratio between absorbencies from phycocyanobilin at 620 nm, and aromatic amino acids in all proteins in the preparation at 280 nm (Eriksen, 2008). The results showed slight difference of the purity of PC in all conditions as shown in Table 4.1. The PC extract using stirring condition is higher in both of phycocyanobilin and aromatic amino acids spectrum (Figure 4.1). In *Spirulina* cells, carotenoids, chlorophyll, and phycocyanin are major pigments amounting to 0.4, 1.0 and 14% dry weight, respectively (Belay, 1997). The green color may indicate the contamination of chlorophyll which is due to disintegration of cells (Sarada et al., 1999).

Table 4.1 The purity of phycocyanin extract with different condition

No.	Condition	Solvent	pH	A_{280}	A_{620}	Purity
A		Buffer	6.2	1.186	1.149	0.97
B	Sonication	DI water	6.2	1.189	1.206	1.01
C		Buffer	6.7	0.895	1.010	1.13
D	4°C	DI water	6.8	0.876	0.849	0.97
E		Buffer	6.3	1.337	1.352	1.01
F	Stir	DI water	6.2	1.565	1.588	1.01

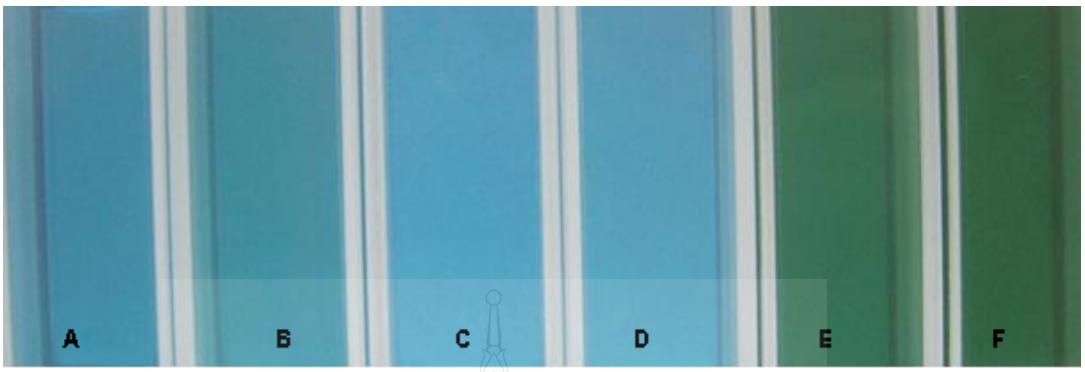


Figure 4.1 The supernatant of phycocyanin extract

4.3.1.2 The effects of extraction times

The PC was extracted from *S. platensis* powder using deionized water and refrigerated at 4 °C for 4, 6, 8, 16 and 24 h. The results showed that the purity of PC was increased from 4 h to 8 h and constants at 16 h as can be seen from Table 4.2 and Figure 4.2. Moreover, the absorbencies of phycocyanobilin at 620 nm clearly increased compared with that of proteins at 8 h and the purity showed no difference after 8 h.

Table 4.2 The purity of phycocyanin at different time

Extraction time (h)	A_{280}	A_{620}	Purity
4	0.876	0.849	0.97
6	1.054	1.066	1.01
8	1.154	1.358	1.18
16	1.198	1.350	1.13
24	1.185	1.370	1.16

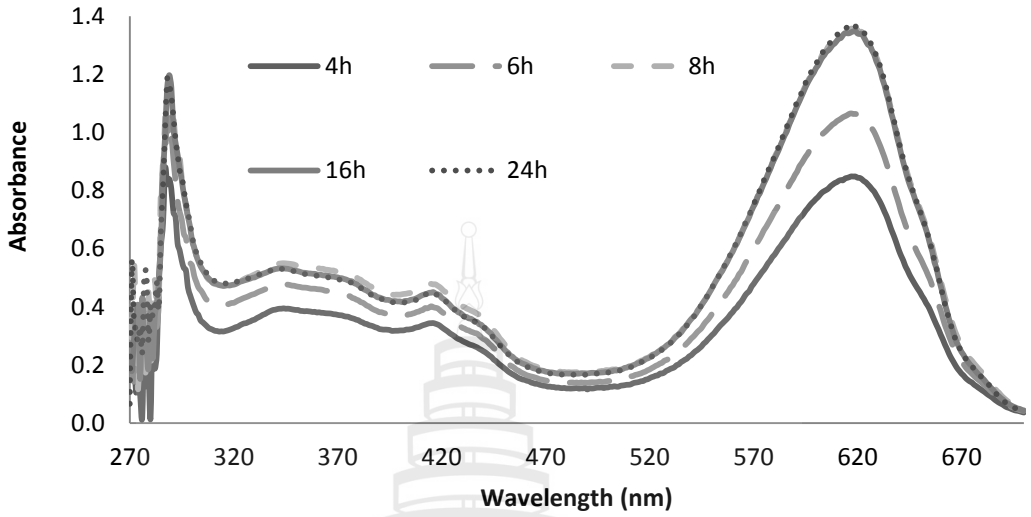


Figure 4.2 The UV-Vis spectra of phycocyanin extracted at different time

4.3.2 The purity of phycocyanin extract

The purity of PC extracts is evaluated based on the ratio between absorbencies from phycocyanobilin at 620 nm, A620 and aromatic amino acids in all proteins in the preparation at 280 nm, A280. PC preparations with A620/A280 greater than 0.7 was considered food grade, while A620/A280 of 3.9 was considered reactive grade and A620/A280 greater than 4.0 analytical grade (Eriksen, 2008). The purity of PC extract was increased using ammonium sulfate precipitation method (Burgess, 2009). The optimal ammonium sulfate precipitation condition was determined only two centrifugation steps. The solid ammonium sulfate 3 g was added into 25 ml of extract with continuous stirred until homogeneous and centrifuged at 8000 rpm, 10 min to obtain the supernatants. The volumes of the supernatants were determined and again solid ammonium sulfate is added and centrifuged to get pellets. The pellets were re-dissolved with deionized water and then measure the absorbance of phycocyanin by using UV-vis spectrophotometer. The clear blue phycocyanin was obtained after protein precipitation with increased the purity from 1.18 to 1.52 (Figure 4.3).

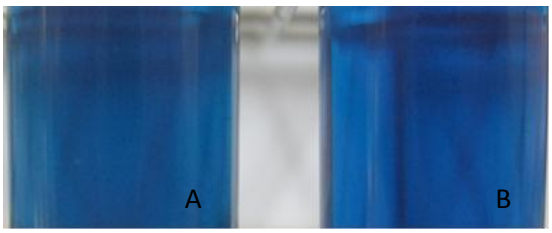


Figure 4.3 The color of phycocyanin extract before (A) and after (B) protein precipitation

4.3.3 Preparation of aluminium-phycocyanin (Al-PC) complex

The Al-PC complex was prepared according to the reported method (Wongwad et al., 2012) with some modifications. The Al-PC complex was prepared at different conditions as follows.

4.3.3.1 Effect of quantity of aluminium potassium sulphate (alum)

The PC extracts 25 ml was mixed with alum (10%w/v solution) at 2.5, 5, 10 and 15 ml. The weights of Al-PC complex when using alum solution up to 10 ml were relatively constants and at 15 ml alum solution has highest dry weights (0.24, 0.26, 0.25 and 0.31g, respectively). Each condition showed the difference in color of complex as can be seen in Figure 4.4. The Al-PC complex using 5 ml of alum solution possessed dark blue, homogenous color which suitable for further use.

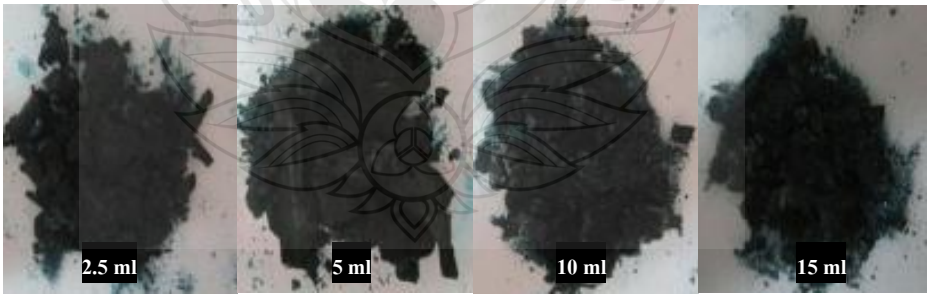


Figure 4.4 The Al-PC complexes prepared at different quantity of alum

4.3.3.2 Effect of pH

The PC extract 25 ml (pH 6.1) were mixed with 10% alum solution 5 ml (pH 3.0) and the pH of reaction mixture (pH 3.7) was adjusted to 2, 4, 6, 8, 10 and 12. The results showed that the reaction mixture at pH 6 has the highest dry weight of the complex and the weight decreased at high and low pH value (0.22, 0.27, 0.42, 0.30 and 0.22g, respectively). The reaction mixture at pH 12 showed no precipitation of Al-PC complex. All conditions showed difference in color of pellets. The color of the complex of highest weight is not relative bright which is less preferable for use as colorant, Figure 4.5. So, the Al-PC was prepared with no adjust pH of reaction.

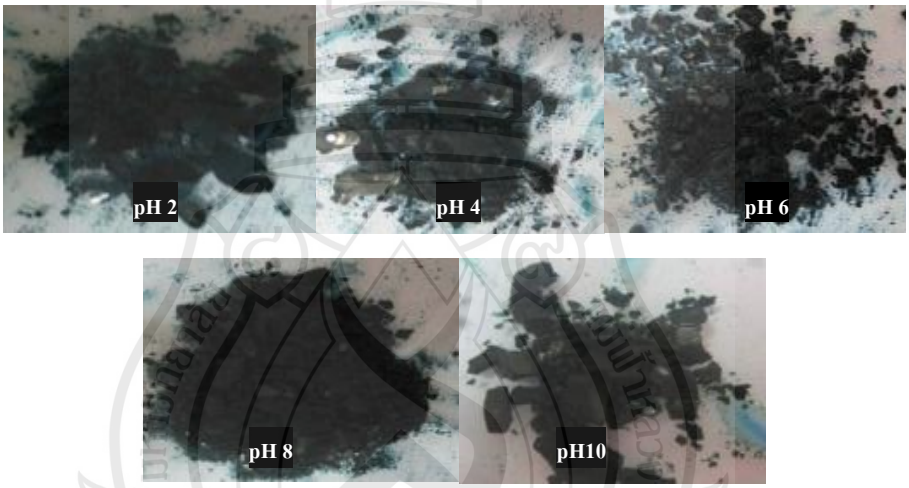


Figure 4.5 The Al-PC complexes at different pH values

4.3.3.3 Effect of reaction time

The PC extract 25 ml was mixed with alum (10% w/v solution) 5 ml. The reaction mixture was stirred at ambient temperature for 30, 60 and 120 min. The obtained mass of the complexes did not affect by reaction time (0.26, 0.28 and 0.27g, respectively) and the color of the complex was not different as can be seen in Figure 4.6. So, the suitable time for reaction is 30 min.

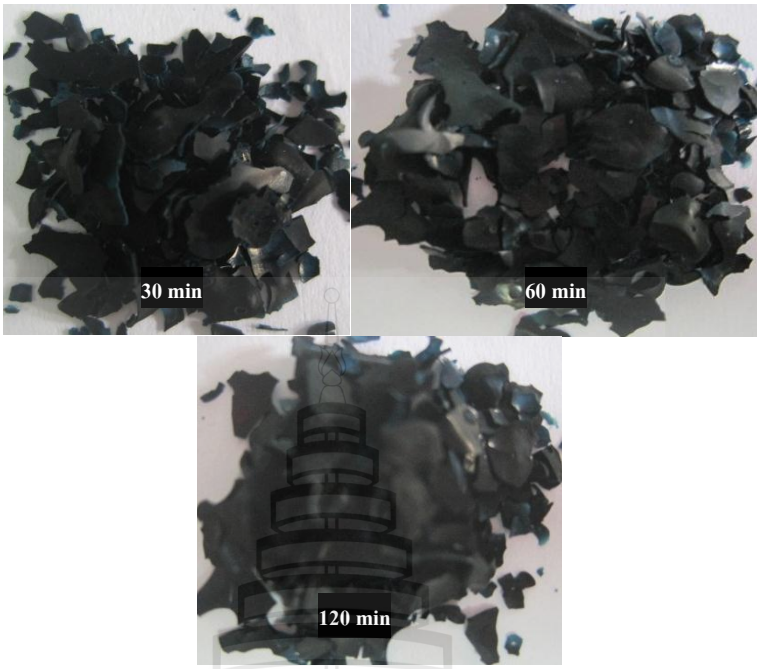


Figure 4.6 Effect of reaction time on the preparation of Al-PC complex

4.3.3.4 Effect of temperature

The PC extract 25 ml was mixed with alum (10% w/v solution) 5 ml. The reaction mixture was stirred for 30 min at ambient temperature, 60 °C and 80 °C. Dry weight of Al-PC complex increased when increased the temperature (0.26, 0.30 and 0.37g, respectively) but the color of complex is not bright and not homogenous as can be seen in Figure 4.7. This may result from the instability of PC at high temperatures (Sarada et al., 1999).

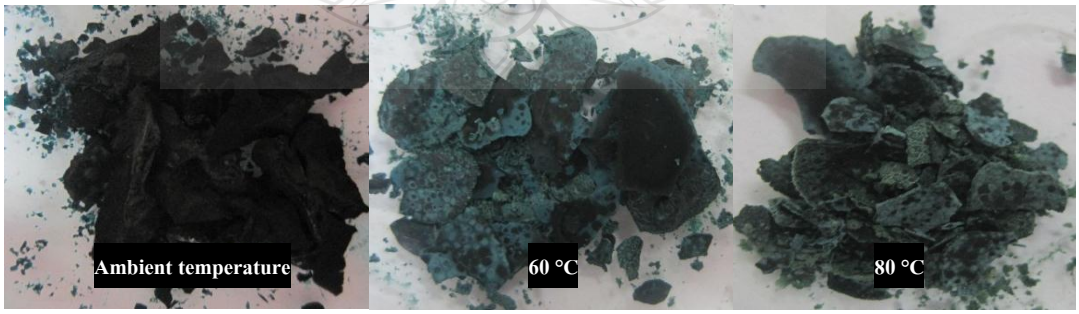


Figure 4.7 The Al-PC complex prepared at different reaction temperature

4.3.4 Characterization of Al-PC complex

4.3.4.1 Fourier transforms infrared spectroscopy (FT-IR)

The FT-IR spectrum was obtained using FT-IR spectrophotometer (Perkin Elmer/FT-IR Spectrum GX, USA). The spectrum of PC extract was changed after purification using ammonium sulfate precipitation method. The new absorption band at 611 cm^{-1} of purified PC assigned to C-S in sulfides and it showed peak of ammonium sulfate at 1410 cm^{-1} ("Infrared Spectra Study Booklet", 2014). The spectrum of Al-PC complex shows the band of OH-group or NH-group at the wide peak at 3252 cm^{-1} which shifted from 3202 cm^{-1} of PC spectrum (Leslie et al., 2008). The absorption band at 1658 cm^{-1} of Al-PC complex assigned to C=O group in amide which shifted from 1672 cm^{-1} of PC extract. The absorption band at 1438 cm^{-1} and 1404 cm^{-1} of complex assigned to OH in carboxylic group which shifted from 1443 cm^{-1} and 1409 cm^{-1} of PC extract. The absorption band at 1236 cm^{-1} and 1082 cm^{-1} of complex assigned to C-N group which shifted from 1230 cm^{-1} and 1109 cm^{-1} of PC extract and the absorption band at 616 cm^{-1} assigned to C-S in sulfides (Ramchandra & Vandana, 2011). These changes may indicate that the carboxyl and amide groups of the PC extract are coordinated with the metal salt (Wongwad et al., 2012) (Figure 4.8).

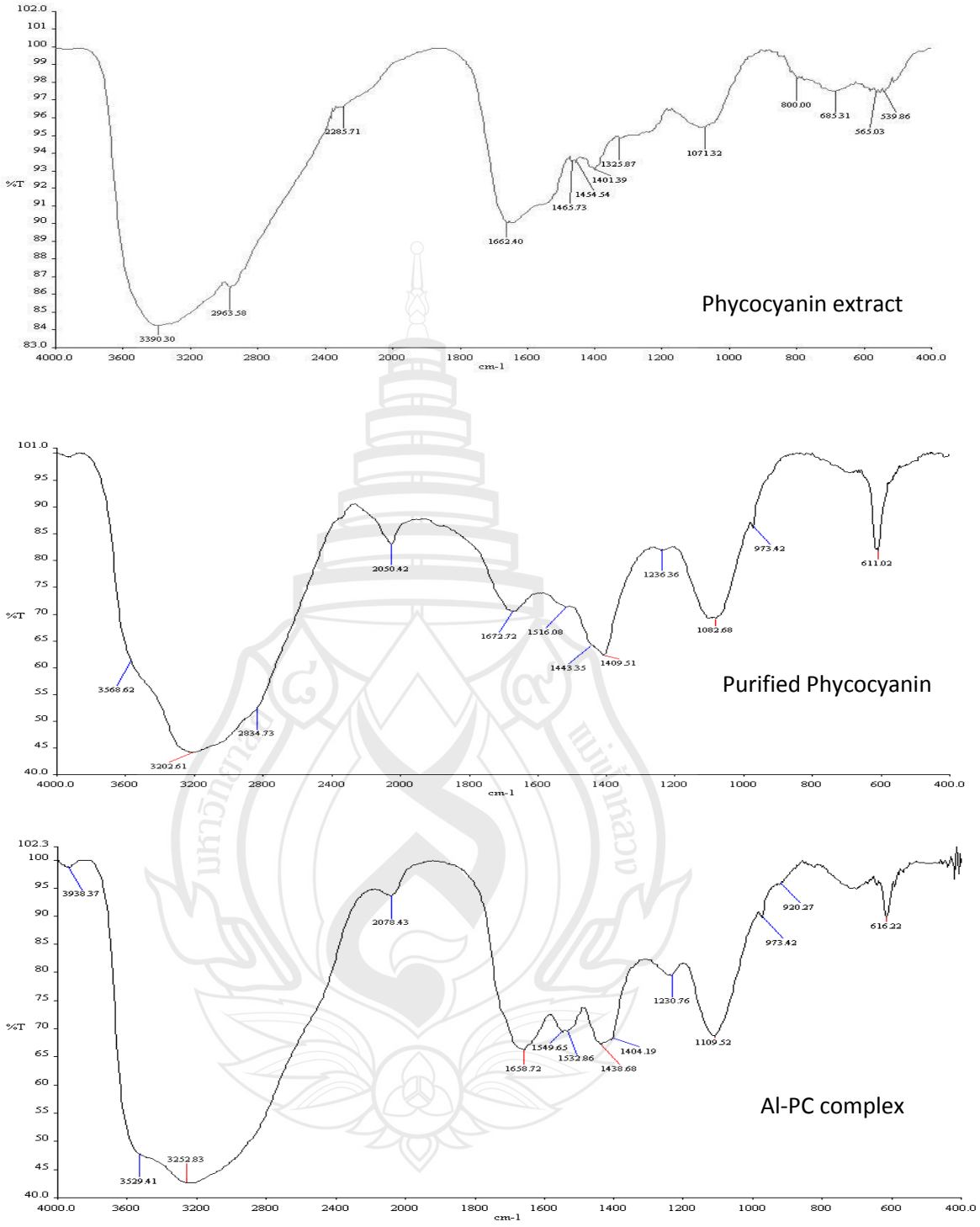


Figure 4.8 The FT-IR spectrums of PC extract, purified PC and Al-PC complex

4.3.4.2 X-Ray diffractometry (XRD)

Powder XRD patterns was obtained using XRD diffratometer (PANalytical/X'Pert Pro MPD, Netherlands) with Cu Kα target tube, NaI detector, variable slits, a 0.010-0.020 step size, operated at a voltage of 30 kV, 15 mA current, at 2 theta (2θ)/min scanning speed, and scanning angles ranged from 0 to 80°. The XRD pattern of PC extract exhibited the characteristic crystalline peak around 17°, 20°, 23°, 29°, 34°, 38°, 42°, 44°, 47°, 52°, 56° and 72° and for Al-PC complex the sharpness of peaks decreased and some diffraction peak were disappeared after complexion (Figure 4.9). It can be indicated that the structure of PC was changed after reacted with aluminium salts and new crystalline was formed (Ramchandra & Vandana, 2011; Xiaohui, Yumin, Lihong, Hui & Ying, 2005).

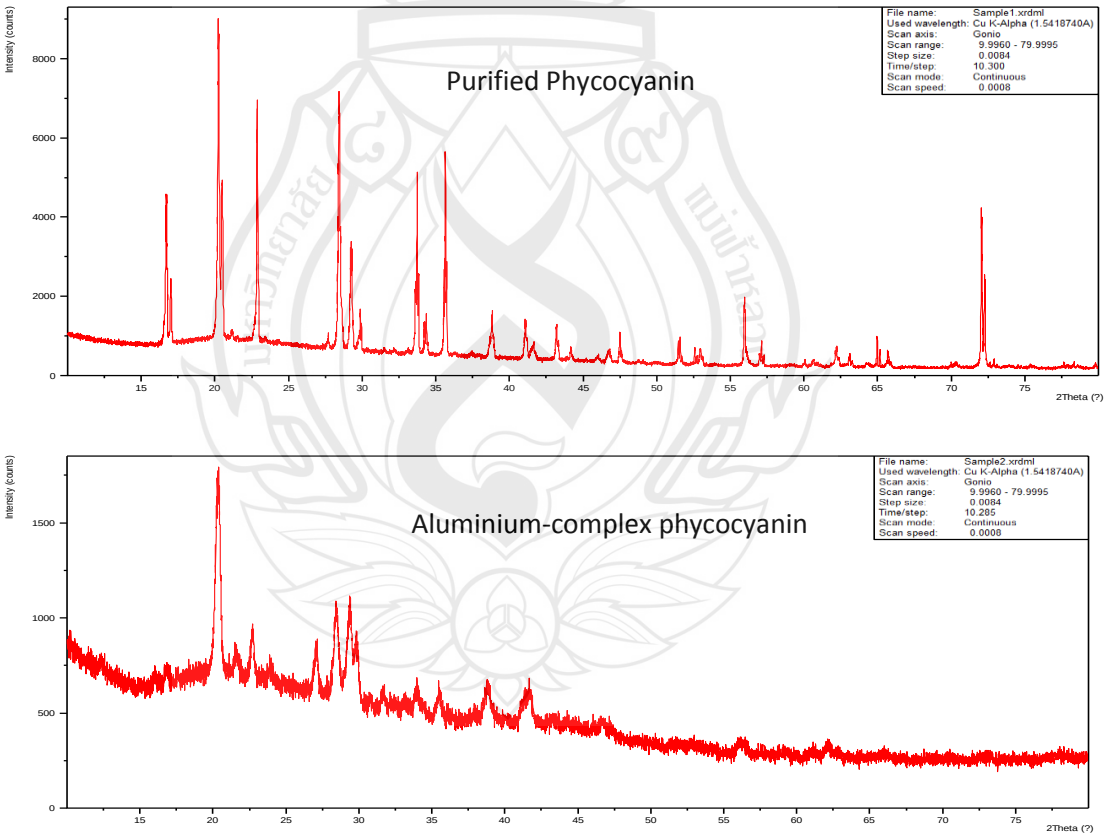


Figure 4.9 X-ray diffraction of purified PC extract and its complex

4.3.4.3 Scanning electron microscopy (SEM)

The morphology of the samples was investigated by scanning electron microscopy (LEO/1450 VP LEO, Germany). The results showed that the morphology of PC extract before purification rarely smooth surface. The purified PC exhibited small shape and agglomerated which is different from the complex (Figure 4.10). The analysis of Al-PC complex by Energy Dispersive Spectroscopy, the presence of elemental aluminium signal indicated that PC was complexed with aluminium salt.

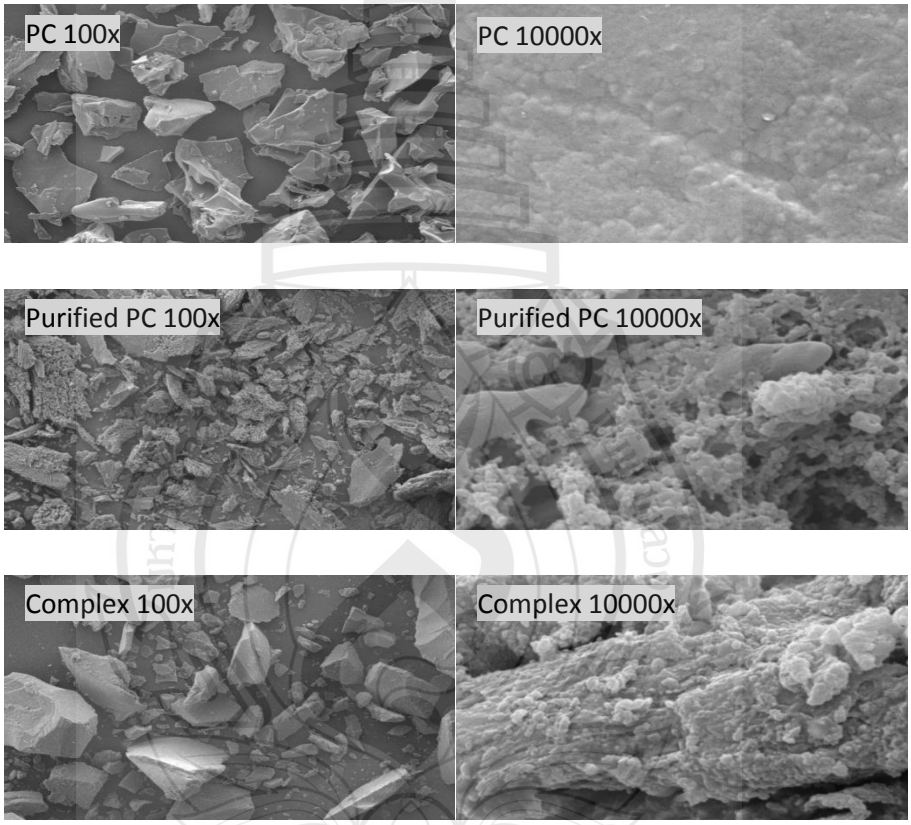


Figure 4.10 SEM images of purified PC extract, PC extract, Al-PC complex and aluminium salt in the magnification of 100 and 10000X

4.3.5 Stability study of Al-PC complex

The stability of the complex was investigated at ambient temperature, 4°C, 45°C, 50°C, UV light and fluorescent light condition for 4 weeks. The color of the samples was evaluated by both visual observation and equipment (Chromameter;

Konica Minolta/CR-400, Japan). The results showed that the color of samples stored at UV light condition clearly changed in both of PC and its complex compared with another conditions. Moreover, the color of the samples mixed with talcum clearly change compared with sample in castor oil. The change of ΔL* (lightness), Δa* (green and red) and Δb* (blue and yellow) of PC extract at week 4 is higher than that of the complex in all conditions which indicated that Al-PC complex is more stable than PC extract (Table 4.3).

Table 4.3 The color difference of the samples at different conditions

Color Difference	Phycocyanin in castor oil																	
	ΔL*						Δa*						Δb*					
	4°C	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**
W0/W1	0.72	0.23	-0.97	1.80	0.08	-0.25	-0.20	-0.63	-0.41	-0.74	0.16	0.03	0.54	-0.11	-0.37	-5.12	-0.57	0.25
W0/W2	-0.01	0.31	1.04	1.02	0.58	0.05	-0.20	-0.61	-0.59	-0.35	0.35	0.39	0.23	-0.33	-0.05	-6.08	-1.21	0.45
W0/W3	0.31	0.52	0.43	1.87	1.89	0.77	-0.61	-1.17	-1.34	-0.98	-0.35	-0.27	0.35	-0.82	-0.19	-5.45	-2.53	-0.20
W0/W4	1.07	1.72	1.82	2.37	4.10	3.30	-0.28	-0.83	-1.09	-0.44	-0.33	-0.32	0.85	0.19	0.19	-3.25	-3.26	1.35
Color Difference	Phycocyanin in talcum																	
	ΔL*						Δa*						Δb*					
	4°C	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**
W0/W1	-0.70	-2.90	4.20	0.33	17.93	3.51	-0.25	-0.15	-0.97	0.69	6.76	0.35	0.32	1.76	0.76	0.10	13.98	-1.39
W0/W2	1.81	-1.82	4.11	0.59	25.26	3.69	-0.87	-0.45	-0.92	0.36	6.73	0.69	-0.33	1.80	1.13	0.23	18.69	-1.79
W0/W3	2.63	-1.00	4.37	1.07	18.54	3.84	-0.91	-0.89	-1.32	0.05	6.04	1.23	-0.23	2.00	1.09	0.36	16.28	-2.05
W0/W4	4.31	0.90	6.09	2.43	25.88	4.82	-1.44	-1.38	-2.12	-0.49	5.76	0.50	-0.56	1.55	0.89	-0.35	18.25	-2.16
Color Difference	Complex in castor oil																	
	ΔL*						Δa*						Δb*					
	4°C	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**
W0/W1	-0.64	-0.10	-0.28	1.73	-0.20	0.42	-0.69	-0.16	-0.23	0.04	0.17	-0.68	-0.64	-0.48	-0.30	-0.48	-0.18	-1.01
W0/W2	0.22	0.11	-0.06	2.33	0.93	0.34	-0.72	-0.23	-0.36	-0.24	0.15	-0.16	-0.69	-0.62	-0.42	-0.27	-0.42	-0.60
W0/W3	-0.39	0.28	-0.11	0.74	1.38	0.57	-0.56	-1.69	-0.19	-0.66	0.09	-0.32	-0.40	-0.59	-0.07	-0.78	-0.38	-0.87
W0/W4	0.40	-0.07	-0.35	0.73	2.79	0.68	-1.14	-1.06	-0.80	-1.46	-1.38	-1.16	-0.71	-0.47	0.13	-0.95	-1.64	-0.97
Color Difference	Complex in talcum																	
	ΔL*						Δa*						Δb*					
	4°C	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**	**4°C**	**45°C**	**50°C**	**AT**	**UV**	**FC**
W0/W1	2.47	-0.70	0.97	1.13	3.83	-3.97	-0.04	1.31	1.29	0.52	5.77	0.94	-0.16	0.50	0.50	0.44	6.52	0.34
W0/W2	2.64	-0.39	1.42	1.86	12.12	-3.21	0.02	1.36	1.24	0.43	6.34	1.07	-0.12	0.57	0.63	0.67	10.16	0.48
W0/W3	1.90	-0.69	1.34	1.15	10.64	-2.93	-0.08	0.83	1.02	0.41	6.44	1.57	0.13	0.91	0.91	0.94	10.52	0.70
W0/W4	4.01	1.72	4.64	1.57	13.74	-1.16	-0.84	0.28	0.27	0.14	5.32	1.05	-0.11	0.74	0.72	0.96	11.29	0.94

Note. AT= ambient temperature, FC= fluorescent light, UV= ultraviolet light

The stability of PC and its complex at different pH value was studied. The PC extract solution (pH 6.4) was adjusted pH to 2, 4, 8, 10 and 11.5. The results showed that the PC color was changed at different pH. The Al-PC complex solution (pH 4.3) was adjusted pH to 2, 4, 6, 8, 10, 12 and 12.6. At the pH value of 2 to 4.3, the Al-PC complex showed greenish blue, at pH value of 6 to 10 it has blue shade and at pH 12 it showed black color. So, the Al-PC complex is more stable than PC solution extract at different pH as can be seen in Figure 4.11.

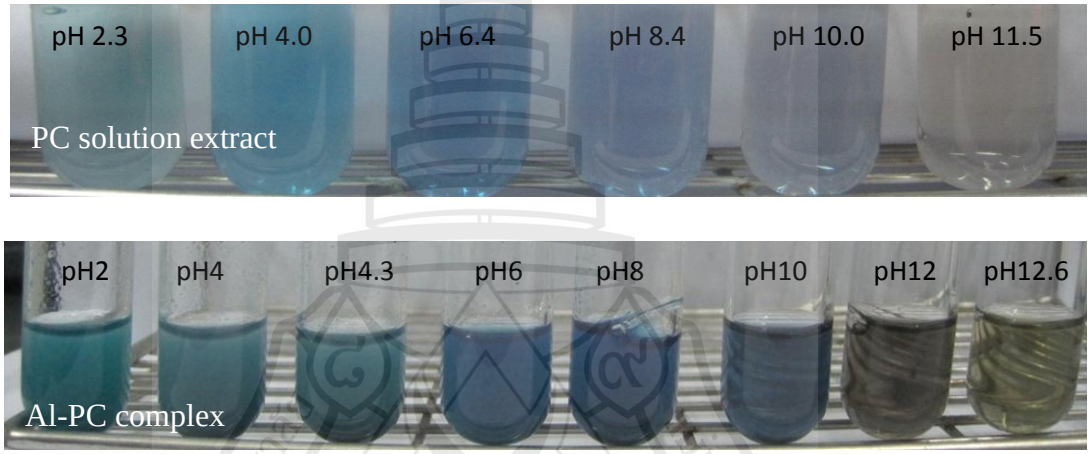


Figure 4.11 The stability of PC and its complex at different condition of pH

4.3.6 Cytotoxicity test

The cytotoxicity of PC extract and Al-PC complex was tested against human dermal fibroblast, neonatal (HDFn) C-004-5C cell using resazurin microplate assay. Both of PC extract and its complex are non-cytotoxic to HDFn C-004-5C cell (Table 4.4) which might be useful for application in the cosmetic products. Moreover, it has been reported that PC from *S. platensis* inhibited the growth of human hepatocellular carcinoma cell line SMMC-7721 (Chen & Wang, 2006) human rectal cancer cell line HR8348 (Hartwell & Kastan, 1994).

Table 4.4 The cytotoxicity of PC extract and its aluminium complex

Concentration (mg/ml)	% Survival	
	Phycocyanin	Aluminium-complex
1.000	99.12	98.69
0.500	102.61	105.92
0.250	105.35	99.62
0.125	100.74	95.62
0.0625	102.21	104.17
0.0313	103.49	100.63

4.3.7 The preparation of cosmetic product containing Al-PC complex

The colorants used in cosmetics are classified into 3 types: organic dyes and their lakes, inorganic or mineral colors and natural colors or vegetable and animal color. Dyes are synthetic, chemical compounds that exhibit their coloring power or tinctorial strength when dissolved in a solvent. Lakes, unlike dyes, are insoluble and color by dispersion. Lakes are formed by the precipitation and absorption of a dye on an insoluble base or substrate. The shade or hue of a lake varies with the pure dye content. The use of insoluble certified lakes has several advantages such as; over coloring is not a problem because the system is opaque, hence, only one shade of color will result, full color development can be achieved with a fewer number of application states and many of the problems associated with color reproducibility have been eliminated entirely (Allam & Kumar, 2011). So, the Al-PC complex was tested as lake coloring ingredient in cosmetics. Base formulas of cosmetics, i.e. powder base, emulsion base and anhydrous base were first developed. The characteristics of cosmetic products were evaluated by both visual observation and equipment.

4.3.7.1 Emulsion foundation

The product is an oil-in-water emulsion containing a small amount of oil in which the pigment is emulsified with a relatively large quantity of water as can be seen in Figure 4.12. The water phase consists of deionized water, xanthan gum (0.2%), magnesium aluminium silicate (0.3%), propylene glycol (5.0%), triethanolamine (1.0%), polysorbate 80 (0.1%) and lecithin (0.4%). The oil phase consists of glyceryl monostearate (1.0%), isopropyl palmitate (3.0%), stearic acid

(2.0%), cetyl alcohol (1.0%), hydrogenated polyisobutene (7.0%), jojoba ester (2.0%), isostearic acid (0.5%). The preservatives are methylparaben (0.2%) and propylparaben (0.2%). The pigments consist of titanium dioxide (2.5%) and Al-PC complex (10.0%). The Al-PC complex was used at 10% to give blue in the preparation with pH value is 5.5. From the result of Al-PC complex stability at different pH value, at the pH value of 2 to 4.3 the Al-PC complex showed greenish blue, at pH value of 6 to 10 it has blue shade. The Al-PC comlex showed promising potential to be used as novel, natural derived blue color for cosmetics.

Figure 4.12 Emulsion foundation

4.3.7.2 Anhydrous foundation

Anhydrous foundation was prepared by suspension of pigments in a mixture of waxes and oils. It has a very high degree of waterproofing and smudges resistance. The raw materials consisted of carnauba wax (3.0%), beeswax (5.0%), mineral oil (30.0%), isopropyl myristate (10.0%), petrolatum (6.0%), Phytosqualene (5.0%), Cyclomethicone (5.0%), Titanium dioxide (2.0%) and Talcum. The Al-PC complex 10% was added to give blue shade in the preparation. All ingredients are heated to 75 to 80 °C and mix all melted it together well. This product was evaluated the stability of Al-PC complex when used under heating condition for formulation process. The finish product containing of Al-PC complex had the blue color shade and good texture as can be seen in Figure 4.13.

Figure 4.13 Anhydrous foundation

4.3.7.3 Eyeshadow

Eyeshadow is a cosmetic which is applied on the eyelids, under the eyebrows and corners of the eyes to create shadow. The ingredients consists of talcum (54.0%), zinc stearate (2.5%), kaolin (4.0%), PVP K-30 (0.5%), titanium dioxide coated mica (5.0%), isopropyl myristate (2.0%), jojoba oil (2.0%). The finished product containing 25.0% of Al-PC complex is easy to apply and good adhesion to skin (Figure 4.14).

Figure 4.14 Eyeshadow product

CHAPTER 5

CONCLUSION

Silver nanoparticles were successfully prepared by an eco-friendly synthesis method using *A. concinna* pods and *P. emblica* extracts as reducing agent. Water was used as a green solvent in the preparation. The Emblica has reduction capacity relatively close to BHT and strong reducing agent when compared with Soap Pod solution. The Emblica solution (0.4 ml) was mixed with 50 ml of 0.5 mM silver nitrate and stirred at 80 °C for 4 h is the best condition for synthesis silver-Emblica nanoparticle. For silver-Soap pod nanoparticle, 3 ml of Soap pod solution was mixed with 50 ml of 0.5 mM silver nitrate and stirred at ambient temperature for 3 h. The obtained silver-Soap pod nanoparticles have average particle size of 20.9 nm and silver-Emblica at 41.2 nm. The morphology of the silver-Soap Pod nanoparticles revealed the silver particles were dispersed on the plant extract and the size is normally rounded and the silver-Emblica particles exhibited small size and agglomerated on the plant extract. They showed the antimicrobial activities against gram positive (*S. aureus*) and gram negative (*E. coli*) bacterial. Additionally, the AgNPs were not cytotoxic to human healthy cells (HDFn cell lines) which might be useful for cosmetic topical application. The cosmetic emulsion product was developed using silver nanoparticles as antimicrobials. The product was prepared as oil in water (O/W) cream gel emulsion with 1% AgNPs solution. The viscosity of product was change about 58-60% in viscosity values from the initial. The color of the product recorded in L*a*b* values showed slightly change in all storage conditions. From above study, the silver nanoparticles were applied as antimicrobial substances in cosmetic preparation. For further studies, the controlled synthesis size of silver colloid particles may prepare using a two-step reduction process. The strong reducing agent

was first applied to produce small silver particles, which were then enlarged in a secondary step using a weaker reducing agent.

The aluminium complex of phycocyanin from *S. platensis* was also successfully prepared. The PC is extracted from *S. platensis* powder (10 g) in 100 ml deionized water (4 °C for 8 h). The purity of PC extracts is evaluated based on the ratio between absorbencies from phycocyanobilin at 620 nm and aromatic amino acids at 280 nm. The clear blue phycocyanin was obtained after protein precipitation using ammonium sulfate precipitation method with increased the purity from 1.18 to 1.52. The Al-PC complex was successfully prepared. The PC extracts 25 ml was mixed with Alum 5 ml (10%w/v solution) stirred at ambient temperature for 30 min. The complex is dark greenish-blue. The FT-IR spectrums of carboxyl and amide groups of Al-PC complex were shift from PC extract which may indicate that the carboxyl and amide groups of the PC extract are coordinated with the metal salt. The XRD pattern of PC extract exhibited the characteristic crystalline peak which indicated that the structure of PC was changed after reacted with aluminium salts and new crystalline was formed. Moreover, the morphology of PC extract investigated by scanning electron microscopy showed the exhibited small shape and agglomerated which is different from its complex. The extract and its complex showed non-cytotoxic effect to HDFn C-004-5C cell. The stability of the complex was investigated at ambient temperature, 4°C, 45°C, 50°C, UV light and fluorescent light condition for 4 weeks. The change of ΔL* (lightness), Δa* (green and red) and Δb* (blue and yellow) of PC extract at week 4 is higher than that of the complex in all conditions which indicated that Al-PC complex can helps maintaining and improving stability of PC.Finally, the utilization of Al-PC complex as colorant in cosmetic products was studied and it showed promising potential to be used as novel, natural derived blue color for cosmetics.

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