METAL COMPLEXES OF CURCUMINOIDS IN *CURCUMA PETIOLATA* EXTRACT FOR COSMETIC EMULSIONS

ANONGNUCH THAKAM

MASTER OF SCIENCE
IN
COSMETIC SCIENCE

SCHOOL OF COSMETIC SCIENCE
MAE FAH LUANG UNIVERSITY
2012

METAL COMPLEXES OF CURCUMINOIDS IN *CURCUMA PETIOLATA* EXTRACT FOR COSMETIC EMULSIONS

ANONGNUCH THAKAM

THIS THESIS IS A PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN COSMETIC SCIENCE

SCHOOL OF COSMETIC SCIENCE
MAE FAH LUANG UNIVERITY

2012

METAL COMPLEXES OF CURCUMINOIDS IN *CURCUMA PETIOLATA* EXTRACT FOR COSMETIC EMULSIONS

ANONGNUCH THAKAM

THIS THESIS HAS BEEN APPROVED
TO BE A PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF MASTER OF SCIENCE
IN
COSMETIC SCIENCE
2012

THESIS COMMITTEE

.....CHAIRPERSON
(Dr. Phanuphong Chaiwut)

.....ADVISOR
(Dr. Nisakorn Saewan)

.....CO-ADVISOR
(Dr. Krisada Kittigowittana)

.....EXAMINER
(Dr. Ampa Jimtaisong)

.....EXTERNAL EXAMINER
(Assoc. Prof. Dr. Suchada Chantrapromma)

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my supervisor Dr. Nisakorn Saewan for her patience, motivation, enthusiasm, and immense knowledge. Her guidance helped me thoughout the research and writing of this thesis. I could not have imagined having a better supervisor and mentor for my thesis study. I would like to thank my co-advisor Dr. Krisada Kittigowittana for his advice to fulfill this thesis.

Besides my advisor and co-advisor, I would like to thank my thesis committee: Assoc. Prof. Dr. Suchada Chantrapromma, Dr. Phanuphong Chaiwut, and Dr. Ampa Jimtaisong for their kindness and insightful comments.

I would like to thank all of my friends for their warm relationship, understanding and encouragement throughout this study.

Finally, I am grateful to my parents, for their love, encouragement and supporting me spiritually throughout my life. Without all these people mentioned above, this thesis would not have been completed.

Anongnuch Thakam

Thesis Title	Metal Complexes of Curcuminoids in *Curcuma petiolata* Extract for Cosmetic Emulsions
Author	Anongnuch Thakam
Degree	Master of Science
Advisor	Dr. Nisakorn Saewan
Co-advisor	Dr. Krisada Kittigowittana

ABSTRACT

Curcumin has shown a wide range of pharmacological activities including anti-inflammatory, inhibition of bacterial and fungal growth, anti-oxidant activity, inhibition of lipid peroxidation, and anti-tyrosinase activities. However, its application is usually limited due to its insoluble in water, degraded in alkaline solution and unstable to light and heat. Recent studies have demonstrated that the stability and anti-oxidant activity of curcumin would be increased when it is complexed with transition metals. The aims of this study were to determine the ability of three divalent metals, zinc, copper, and magnesium, on enhancing the stability, anti-oxidant and anti-tyrosinase activities of free curcumin. Then the most effective metal will be complex with *C. petiolata* extract and determine its anti-oxidant and anti-tyrosinase activities, stability and whitening effect in emulsion system.

The metal complexes were successfully prepared by refluxing the mixture of curcumin and metal ions in ethanol for 3 hours. The mass spectrometer demonstrated that Cu (II) form mono-ligand complex whereas Zn (II) and Mg (II) form bi-ligand complexes with curcumin. The complexes were also analyzed by UV-Vis, IR, X-Ray powder diffraction, and thermal analysis techniques. The stability of free curcumin

and its metal complexes was investigated by storage at various conditions including pH buffer solution (pH 3, 7 and 12), temperature (25, 37, 45, and 60^{o}C), light and dark condition. All buffer solutions, Cu-cur, and Zn-$(cur)_2$ showed higher stability than that of free curcumin. While Mg-$(cur)_2$ complex showed similar degradation behaviors to free curcumin. At all temperature and light exposure conditions, all metal complexes were 2 folds higher stable than that of free curcumin. The results imply that the stability of free curcumin was enhanced by chelation with metals. From the DPPH radical scavenging, ferrous reducing power, and anti-tyrosinase studies, the activities of curcumin was enhanced by complexing with Zn (II) and Mg (II). While Cu-cur showed much lower on anti-oxidant and anti-tyrosinase activities than that of free curcumin. Since Zn (II) can predominant enhance the stability and activity of curcumin. It was chosen to complex with curcumin rich extract from *C. petiolata* rhizomes (CP extract). The complex was characterized by spectroscopic techniques including UV and IR analyses. The anti-oxidant and anti-tyrosinase activities of complex were investigated. Zn-CP complex showed stronger DPPH radical scavenging, ferrous reducing power, and anti-tyrosinase activities than that of CP extract. Four whitening agent (free curcumin, *C. petiolata* extract, Zn-cur, and Zn-CP) were incorporated in o/w emulsion cream. The chemical degradation of active ingredients in various storage conditions was investigated. Metal derivatives showed more stable than free curcumin and CP extract. The products were classified as safe due to no microbial contamination and non irritation on skin. The lightening efficacy study of the product showed the whitening process of application of free form creams, F2 and F3, is complete already after 14 days. While the continued application of metal complexes creams F2 (Zn-$(cur)_2$ and F4 (Zn-CP) showed further lighten the skin..

Keywords: *Curcuma petiolata*/Metal-curcumin complexes/Skin whitening

TABLE OF CONTENT

TABLE OF CONTENT (continued)

TABLE OF CONTENT (continued)

LIST OF TABLE

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

CHAPTER 1

INTRODUCTION

1.1 Background

Curcumin is a polyphenol yellow pigment found in rhizomes of *curcuma* species. It was used as an active ingredient in many cosmetics such as anti-wrinkles, anti-acne and whitening products due to its wide range of pharmacological activities including anti-inflammatory, inhibition of bacterial and fungal growth, anti-oxidant activity, inhibition of lipid peroxidation, and anti-tyrosinase activities. However, there is limit on the use of curcumin due to its instability to alkaline, light, and heat which affect to its activities and the cosmetic product characteristics. Several studies have demonstrated that the stability of curcumin can be enhanced by complexing with transition metal ions. In basic solutions, curcumin complexes of zinc (II), copper (II), selenium (II), and magnesium (II) showed 30 folds higher stable than that of free curcumin. Moreover, complextion with copper (II) increased 6 folds of lipid peroxidation and 10 times greater superoxide dismutase (SOD) activity than those of free curcumin.

Wan-Boa-Chan (*Curcuma petiolata*) is specie in the genus *Curcuma* which is widely cultivated as an ornamental plant and has been used as a folk botanical in Asia. This plant is alternatively used as a natural source of curcumin in cosmetic industry because of the high curcumin content similarly to turmeric (7.4% w/w).

To the best of our knowledge, the use of metal curcumin complexes in cosmetics has not been previously reported. Therefore, the aims of this study were to synthesize the metal curcumin complex with allowed metal used in cosmetics (zinc,

copper, iron, and magnesium) and chosen the optimal metal that can enhance the stability of curcumin as well as showed potent anti-oxidant and anti-tyrosinase activities. After that optimal metal was used to complex with best metal with *C. petiolata* extract and determined the efficacy in whitening effect for cosmetic applications.

1.2 Objectives

1.2.1 To synthesize the curcumin complexes with various metals (zinc (II) , copper (II) , and magnesium (II))

1.2.2 To determine the stability of curcumin and its metal complexes

1.2.3 To study the anti-oxidant and anti-tyrosinase activities of curcumin and its metal complexes

1.2.4 To prepare curcumin rich extract from *C. petiolata* rhizome

1.2.5 To determine total curcumin content of curcumin rich extract

1.2.6 To synthesize the metal complex of *C. petiolata* extract with the optimal metal ion

1.2.7 To study the anti-oxidant and anti-tyrosinase activities of *C. petiolata* extract and its metal complexes

1.2.8 To prepare the whitening creams containing free curcumin, *C. petiolata* extract, metal-curcumin complex and metal-*C. petiolata* complex

1.2.9 To evaluate the stability of prepared creams

1.2.10 To evaluate the safety of prepared creams

1.2.11 To evaluate the skin whitening effect of prepared creams

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Natural products have been used throughout human history for various purposes due to their pharmacological or biological activities that played an important role in the health care of many cultures (Newman, Cragg & Snader, 2003).

Curcuma species belong to the zingiberaceae family which is a large genus of rhizomatous herbs distributed in tropical regions especially in Thailand. They have been cultivated and used since ancient times. Many species have economic value because of the widely used as spices, medicine, dyes, foods, perfumes, tonics, and as ornamentals plant (Puangpen Sirirugsa, 1996). The most known economically species which is a source of curcuminoids is *Curcuma longa* (turmeric).

Curcuma petiolata is a perennial plant in the genus *Curcuma* that grows 2–3 feet (60–90 cm) high. Its leave is lengthy and rectangular in shape with 14 inches (36 cm) long and 5 inches (13 cm) wide. Its petiole is 4–6 inches (10–15 cm) long. Its flower has a cone-shaped, golden-yellow, and pinkish white coma bract which blooms during June–August. Its root is bulbs that also produce rhizomes, which then produce stems and roots for new plants.

C. petiolata taxonomic position is as follow:

Class	Liliopsida
Subclass	Commelinids
Order	Zingiberales
Fimily	Zingiberaceae
Genus	*Curcuma*
Species	*Curcuma petiolata*

C. petiolata is widely cultivated as an ornamental plant and has long been used as a folk botanical in Asia. The rhizome of *C. petiolata* was used externally to treat bruises, sprains, skin eruptions, infections and to improve complexion. The pseudo stem of *C. petiolata* has been used as traditional medicine for wound healing properties (Prasad, Reddy, Raza & Dutt, 2008). *C. petiolata* rhizome extract contain high amount of curcumin with potent DPPH radical scavenging, ferrous reducing power and inhibition of lipid peroxidation activities (Anongnuch Thakam & Nisakorn Saewan, 2012). This plant has been suggested as an alternative source of curcumin.

(a) (b) (c)

Note. (a) Pseudo Stem and Leaves, (b) Flowers, (c) Rhizomes

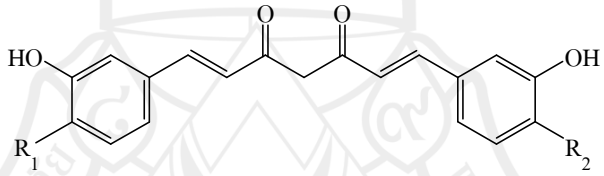
Figure 2.1 The picture of *C. petiolata*

2.2 Curcumin

The curcuminoids is the major constituent of the turmeric, which comprised of curcumin, demethoxycurcumin and bisdemethoxy-curcumin (Figure 2.2). Curcumin (diferuloylmethane) is the major constituent of curcuminoid, which was first isolated in 1815 by Pelletier (Vogel & Pelletier, 1815) and its chemical structure was determined in 1973 (Roughley & Whiting, 1973). It is a polyphenol yellowish

crystalline, odorless powder which has molecular weight of 368.37 and melting point of 183 ˚C. On UV-Vis spectrophotometric investigation, maximum light absorption of curcumin occurs around 400-430 nm depends on organic solvent (Jasim & Ali, 1989).

Curcumin is popularly used for food supplement, cosmetic and pharmaceutical purposes due to its multiple therapeutic activities including anti-cancer (Hatcher, Planalp, Cho, Torti, & Torti, 2008), anti-inflammatory, anti-bacteria and anti-fungal (Aggarwal, Sundaram & Malani, 2007) high anti-oxidant activity (Jayaprakasha, Jagan, Rao, & Sakariah, 2006), inhibition of lipid peroxidation and anti-tyrosinase activity (Patcharee Khunlad, Yongyuth Tundulawessa, Thararat Supasiri & Waradoon Chutrtong, 2008). With these features curcumin was used as active ingredients in many products such as anti-wrinkles, anti-acne and lightening cosmetics.



(1) $R_1 = R_2 = OCH3$

(2) $R_1 = OCH_3$, $R_2 = H$

(3) $R_1 = R_2 = H$

Note. (1) Curcumin, (2) Demethoxycurcumin, and (3) Bisdemethoxycurcumin

Figure 2.2 Structure of curcuminoids

The compound is poorly soluble in water, petroleum ether, and benzene; soluble in ethyl alcohols, glacial acetic acid, and in propylene glycol; very soluble in acetone and ethyl ether. In solution the principle coloring component of curcumin exhibit keto-enol tautomerism, depending on solvent, up to 95 percent is in the enol form (Figure 2.3). The coloring of curcumin solution was varies from the different pH of solution. When the pH is lower than 1, curcumin shows a red color, which indicates

the protonated form (H_4A^+). Curcumin in solution at pH 2 to 7 has yellow color. The majority of the curcumin molecules are in the neutral form (H_3A). At pH higher than 7.5, the color changed to reddish orange. The curcumin molecule was extremely unstable due to its postulation in between tree form; H_2A^-, HA^{2-}, and A^{3-} as showed in Figure 2.4.

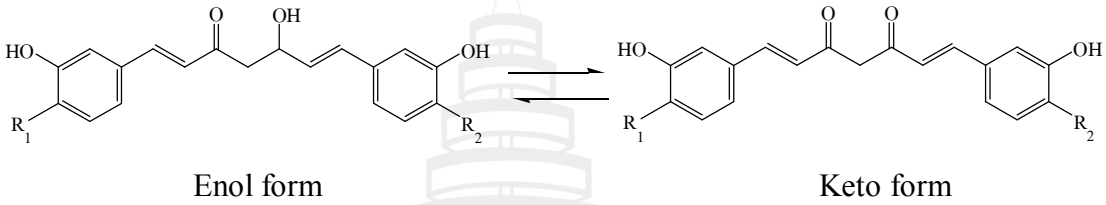


Figure 2.3 Keto-enol Tautomerism Form of Curcumin

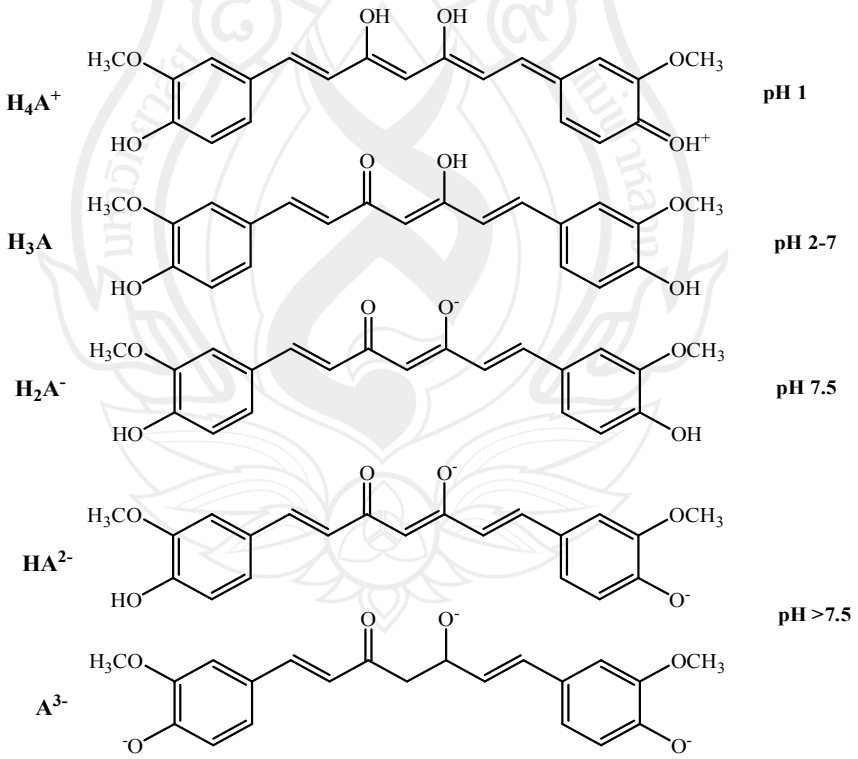


Figure 2.4 Curcumin Form in Various Buffer Solution

Tonnesen and Wang reported that the curcumin was unstable at basic pH and underwent alkaline hydrolysis in alkali (higher pH) solution. Hydrolytic decomposition study showed it is underwent photodegradation when being exposued to light in solution as well as in solid form (Tonnesen, Masson & Loftsson, 2002). Their study on basic pH solution, curcumin was unstable and was degraded within 30 minutes to ferulic acid, feruloylmethane, vanillin, and acetone (Figure 2.5). Under acidic conditions, the degradation of curcumin was much slower, with less than 20% of total curcumin decomposed at 1 hour (Wang et al., 1997)

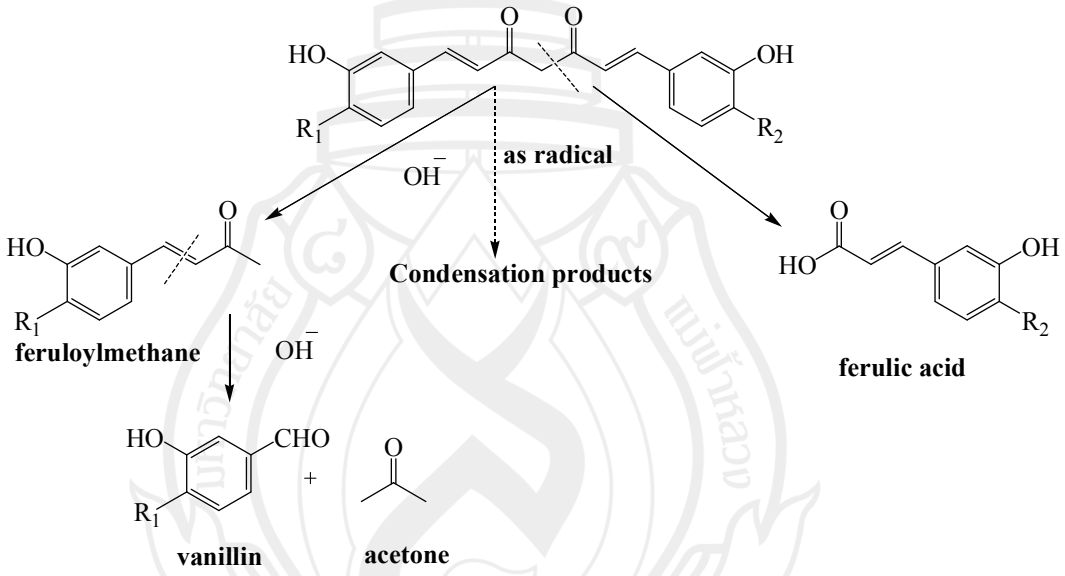


Figure 2.5 Degradation Products of Curcumin

2.3 Metal-curcumin Complexes

Several studies have demonstrated that the stability of curcumin can be enhanced by complexing with transition metal ions. Curcumin can be considered as a two feruloyl parts linked by a methylene bridge. In the middle of curcumin molecule, there is a β-diketone moiety which is resembled to acetylacetone, the most common

β-diketone ligand that forms stable metal chelate ring. Binding of acetylacetone to metal can take place in several ways; the two major forms of metal interaction which undergoes keto-enol tautomeism (Sheril, Limson, Amichand, Gareth & Santy, 2004) are showed in Figure 2.6.

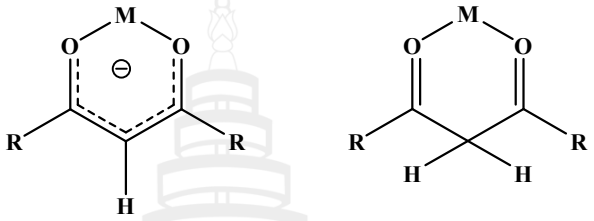


Figure 2.6 Two Major Forms of Metal Interaction at the Central *β*-Diketone

These *β*-diketone ion forms vary stable chelate complexes with a great range of metal ions. Structure activity relationships studies suggest that hydroxyl group at para position is most critical for the expression of biological activity. The strong chelating ability of diketones has been widely investigated toward a great number of metal ions. Several metallocomplexes of curcumin have been synthesized, characterized and evaluated for various biological activities. Several metal-curcumin complexes showed greater biological activity compared to free curcumin. The copper (II)-curcumin complex showed 6 folds greater inhibit on lipid peroxidation than curcumin by TBARs assay (Barik et al., 2007). Barik and Vajragupta have demonstrated the copper (II)–curcumin and manganese (II)-curcumin complexes were 10 times inhibition of superoxide dismutase (SOD) than free curcumin (Barik et al., 2007; Vajragupta et al., 2003). The study of inhibition of nitric oxide, manganese (II)-curcumin complex showed 2 folds effective more than curcumin indicating that the complex significantly attenuated KA-induced neuronal cell death than curcumin (Sumanont et al., 2004). It is well known that transition metals might exhibit both anti- and pro-oxidant effects by dismuting superoxide ion or catalyzing the decomposition of peroxide. The metal complexes mostly converted curcumin from

pro-oxidant into anti-oxidnats (Afanas'eva, Ostrakhovitch, Mikhal'chik, Ibragimova & Korkina, 2001).

Moreover, the metallocomplexes of curcumin showed greater stability compared to free curcumin. The study of the stability in basic solutions showed that zinc (II)-curcumin, copper (II)-curcumin, selenium (II)-curcumin, and magnesium (II)-curcumin complexes exhibited 30 folds greater stable than free curcumin (Zebib, Mouloungui & Noirot, 2010).

2.4 Free Radical

A radical (often, but unnecessarily called a free radical) is an atom or group of atoms that have one or more unpaired electrons. Radicals can have positive, negative or neutral charge. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can wreak havoc on a broad range of macromolecules. A prominent feature of radicals is that they have extremely high chemical reactivity, which explains not only their normal biological activities, but how they inflict damage on cells (Knight, 2000).

2.4.1 Sources of Free Radical

2.4.1.1 Internal sources:

These can be enzymatic reactions, which serve as a source of free radicals. These include those reactions involved in the respiratory chain, in phagocytosis, in prostaglandin synthesis and in the cytochrome P450 system. Some internal sources of generation of free radicals are mitochondria, xanthine oxidase, phagocytes, and reactions involving iron and other transition metals, peroxisomes, Arachidonate pathways, exercise, ischaemia / reperfusion, inflammation.

2.4.1.2 External sources:

These include non-enzymatic reactions of the oxygen with organic compounds. Free radicals also arise in reactions, which are initiated by ionizing radiations. Some external sources of free radicals are cigarette smoke, environmental

pollutant, radiations, ultraviolet light, ozone, certain drugs, pesticides, anesthetics and industrial solvents.

2.4.1.3 Physiological factors

Mental status like stress, emotion etc. and disease conditions are also responsible for the formation of free radicals

2.4.2 Types of Free Radical

There are many types of radicals, but those of most concern in biological systems are derived from oxygen, and known collectively as *reactive oxygen species*. Oxygen has two unpaired electrons in separate orbital in its outer shell. This electronic structure makes oxygen especially susceptible to radical formation. Sequential reduction of molecular oxygen (equivalent to sequential addition of electrons) leads to formation of a group of reactive oxygen species as following;

2.4.2.1 Superoxide anion ($^{\bullet}O_2^-$)

Superoxide can act either as oxidant or reductant, it can oxidize sulphur, ascorbic acid or NADPH and it can reduce Cytochrome C and metal ions. A dismutation reaction leading to the formation of hydrogen peroxide and oxygen can occur spontaneously or is catalyzed by enzyme superoxide dismutase. In its protonated form (pKa 4.8), superoxide forms and perhydroxyl radical, which is powerful oxidant but its biological relevance is probably minor because of its low concentration at physiological pH.

2.4.2.2 Hydrogen peroxide (H_2O_2)

The univalent reduction of superoxide produces hydrogen peroxide, which is not a free radical because all its electrons are paired. It readily permeates through the membranes and is therefore not compartmentalized in the cell. The main damages caused by this are breaking up of DNA, resulting in single strand breaks and formation of DNA protein crosslink. Numerous enzymes (peroxidases) use hydrogen peroxide as a substrate in oxidation reactions involving the synthesis of complex organic molecule. This is an oxidizing agent but not specially reactive and its main significance lies in it being a source of hydroxyl radical in the presence of reactive transition metal ions.

2.4.2.3 Hydroperoxyl radical ($^{\bullet}OH$)

The hydroperoxyl radical, also known as the perhydroxyl radical, is the protonated form of superoxide with the chemical formula HO^2. Hydroperoxyl is formed through the transfer of a proton to an oxygen atom. HO^2 can act as an oxidant in a number of biologically important reactions, such as the abstraction of hydrogen atoms from tocopherol and polyunstaturated fatty acids in the lipid bilayer. As such, it may be an important initiator of lipid peroxidation.

2.4.2.4 Singlet oxygen

It is not a free radical but it can be formed in some radical reactions and can trigger off others. This arises from hydrogen peroxide molecules. Singlet oxygen on decomposition generates superoxide and hydroxyl radicals.

2.4.2.5 Triplet oxygen

Triplet oxygen can react with elements and ions to form oxides, but usually not with organic compounds, which are in singlet state. However, it reacts easily with free radical molecules produced by the action of other active radicals, radiations, ultra violet light, and heat or by complex formation with oxygen and transition metal to produce active peroxide radicals and trigger auto-oxidation of unsaturated fatty acids and others.

2.5 Anti-oxidant

An anti-oxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Anti-oxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, anti-oxidants are often reducing agents such as thiols, ascorbic acid or polyphenols (Hamid, Aiyelaagbe, Usman, Ameen & Lawal1, 2010). Anti-oxidants are grouped into two classes as following;

2.5.1 Primary or Natural Anti-oxidants

They are the chain breaking anti-oxidants which react with lipid radicals and convert them into more stable products. Anti-oxidants of this group are mainly phenolic in structures and include the following:

2.5.1.1 Anti-oxidant minerals

These are co factor of anti-oxidants enzymes. Their absence will definitely affect metabolism of many macromolecules such as carbohydrates. Examples include selenium, copper, iron, zinc and manganese.

2.5.1.2 Anti-oxidant vitamins

It is needed for most body metabolic functions. They include vitamin C, vitamin E, and vitamin B.

2.5.1.3 Phytochemicals

These are phenolic compounds that are neither vitamins nor minerals.

2.5.2 Secondary or Synthetic Anti-oxidants

These are phenolic compounds that perform the function of capturing free radicals and stopping the chain reactions, the compounds include:

2.5.2.1 Butylated hydroxyl anisole (BHA)

2.5.2.2 Butylated hydroxyrotoluene (BHT)

2.5.2.3 Propyl gallate (PG) and metal chelating agent (EDTA)

2.5.2.4 Tertiary butyl hydroquinone (TBHQ)

2.5.2.5 Nordihydro guaretic acid (NDGA)

2.6 Determination of Anti-oxidant Activity

2.6.1 DPPH Radical Scavenging Assay

This assay is based on the measurement of the scavenging ability of anti-oxidants towards the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$). The free radical DPPH$^{\bullet}$ is reduced to the corresponding hydrazine when it reacts with hydrogen donors from anti-oxidant (Contreras-Guzma´n & Strong, 1982). This ability is evaluated the decolorization of DPPH$^{\bullet}$, which measure the absorbance decreased at

515–528 nm produced by the addition of the anti-oxidant to a DPPH$^{\bullet}$ solution in ethanol or methanol.

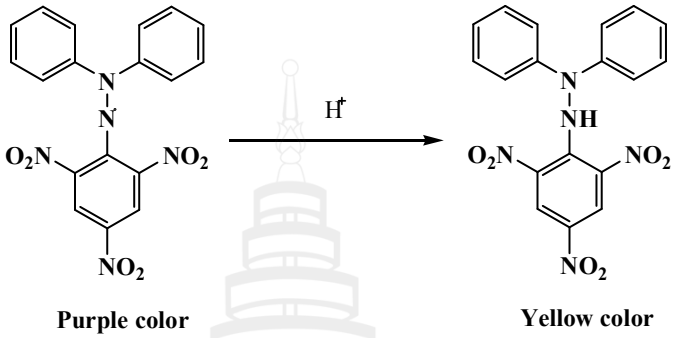


Figure 2.7 Radical and Non-Radical Forms of DPPH.

2.6.2 Ferrous Reducing Power Assay

The method is measure the reduction potential of anti-oxidant, which creates with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), then reacts with ferric chloride to form blue color of ferric ferrous complex that has an absorption maximum at 700 nm. The ferrous reducing power activity was expressed as ascorbic acid equivalents (AAE) (Aparadh, Naik & Karadge, 2012).

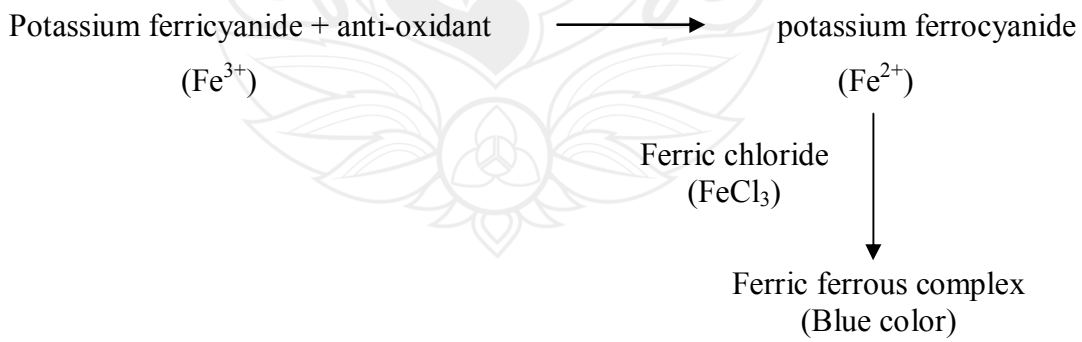


Figure 2.8 Radical and Non-Radical Forms of Ferrous Reducing Power.

2.6.3 Thiobarbituric Reactive Substance

This method measures the malondialdehyde (MDA) formed after lipid hydroperoxide decomposition (secondary products of oxidation), which forms a pink chromophore with thiobarbituric acid (TBA). This coloured complex, which absorbs at 532 nm, results in the condensation of TBA and malondialdehyde in an acidic environment. This method is not very specific, because 4-hydroxyalkenals, 2,4-alkadienals and 2-alkenals, protein and sugar degradation products, amino acids, nucleic acids and anthocyanins are also able to react with TBA, forming a chromophore (Miguel, 2010).

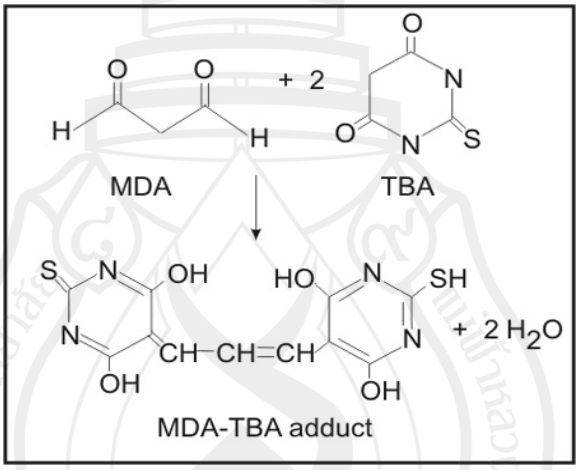


Figure 2.9 MDA-TBA Adduct Formation

2.7 Skin Structure

The skin plays an extremely important role, providing a vast physical barrier against mechanical, chemical, and microbial factors that may affect the physiological status of the body. In addition to those functions, the skin also acts as an immune network and, through its pigments, provides a unique defense system against UV radiation (UV-R). Thus, melanocytes transfer melanosomes through their dendrites to keratinocytes, where they form the melanin caps that reduce UVinduced DNA

damage in human epidermis. The skin's layers are represented by the epidermis, the dermis (whose structure will be discussed in more detail below), and the hypodermis, the latter consisting of fatty tissue that connects the dermis to underlying skeletal components (Fore, 2006) (Figure 2.10).

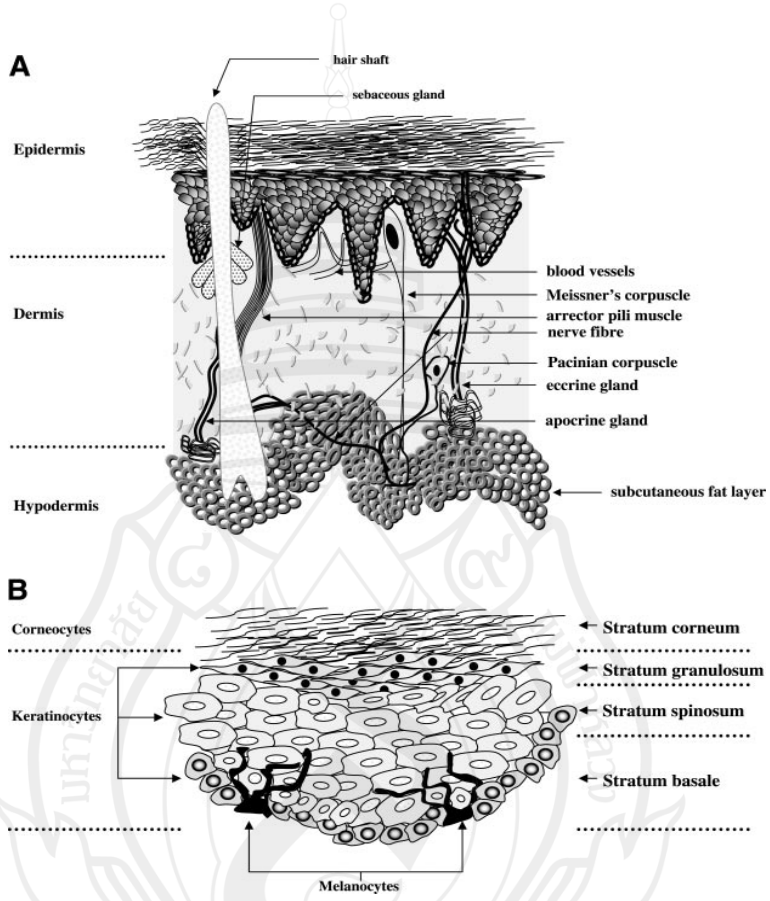


Figure 2.10 Structure of the Skin

2.8 Skin Color

Human skin color ranges in variety from the darkest brown to the lightest pinkish-white hues. Human skin shows higher variation in color than any other single mammalian species and is the result of natural selection. Skin pigmentation in humans evolved to primarily regulate the amount of ultraviolet radiation penetrating

the skin, controlling its biochemical effects (Muehlenbein, 2010). The actual skin color of different humans is affected by many substances, although the single most important substance determining human skin color is the pigment melanin.

Melanin is the major product of melanocytes and is the main determinant of differences in skin color. Melanin is synthesized in two main forms: the dark-colored brown-black, insoluble eumelanin, and the light-colored red-yellow, alkali-soluble, sulfur-containing pheomelanin. Melanogenesis is initiated with the first step of tyrosine oxidation to dopaquinone catalyzed by tyrosinase. This first step is the rate-limiting step in melanin synthesis because the remainder of the reaction sequence can proceed spontaneously at a physiological pH value (Halaban et al., 2002). The subsequent dopaquinone is converted to dopa and dopachrome through auto-oxidation. Dopa is also the substrate of tyrosinase and oxidized to dopaquinone again by the enzyme. Finally, eumelanin are formed through a series of oxidation reactions from dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic acid (DHICA), which are the reaction products from dopachrome. In the presence of cysteine or glutathione, dopaquinone is converted to cysteinyldopa or glutathionyldopa. Subsequently, pheomelanin is formed.

Although melanin has mainly a photoprotective function in human skin, the accumulation of an abnormal amount of melanin in different specific parts of the skin resulting in more pigmented patches might become an esthetic problem. In addition, enzymatic browning in fruit and fungi is undesirable in, for example, fresh fruits, beverages, vegetables, and mushrooms (Artés, Castañer & Gil, 1998). Browning after harvest is a common phenomenon in crops such as mushrooms, which decreases the commercial value of the products. Hyperpigmentation in human skin and enzymatic browning in fruits are not desirable. These phenomena have encouraged researchers to seek new potent tyrosinase inhibitors for use in anti-browning of foods and skin whitening.

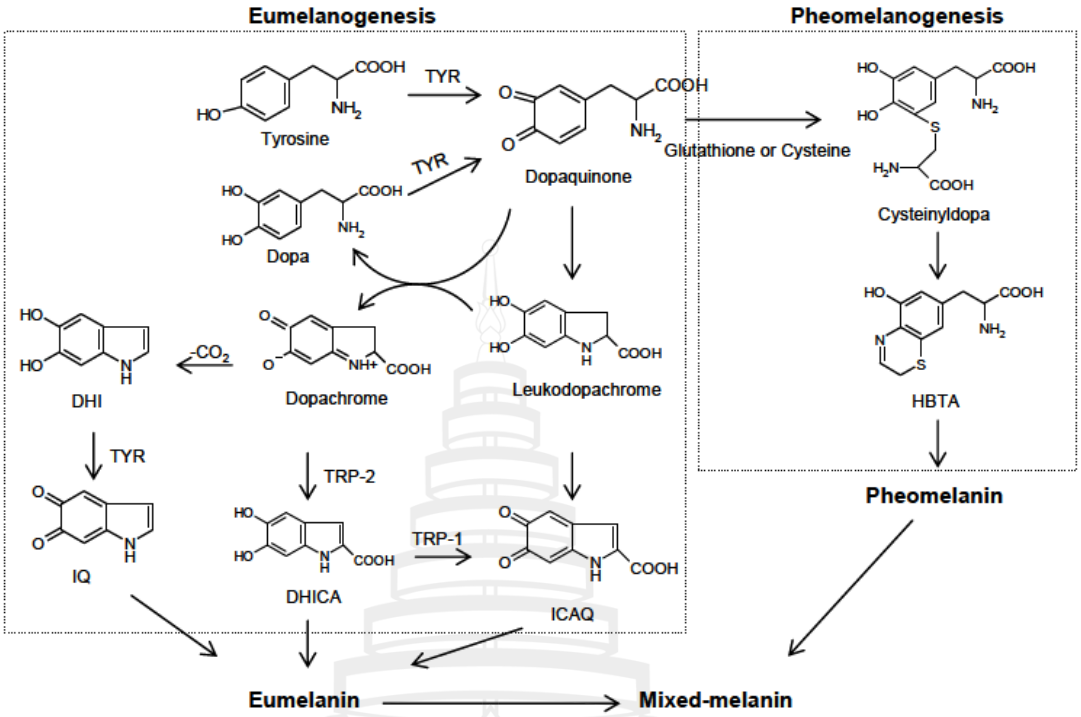


Figure 2.11 Biosynthetic Pathway of Melanin

2.9 Tyrosinase Inhibitors

Many putative inhibitors are examined in the presence of tyrosine or dopa as the enzyme substrate, and activity is assessed in terms of dopachrome formation (Chang, 2009). Thus, experimental observation of the inhibition of tyrosinase activity can be accomplished by one of following:

2.9.1 Reducing Agents

Reducing agents causing chemical reduction of dopaquinone, which used as a melanogenesis inhibitor because of its capacity to reduce back *o*-dopaquinone to dopa, thus avoiding dopachrome and melanin formations, such as ascorbic acid.

2.9.2 *o*-Dopaquinone Scavenger

o-Dopaquinone scavenger such as thio-containing compounds, which are well-known melanogenesis inhibitors and react with dopaquinone to form colorless products. The melanogenetic process is therefore slowed until all the scavenger is consumed, and then it goes at its original rate.

2.9.3 Alternative Enzyme Substrates

Alternative enzyme substrates (some phenolic compounds), whose quinoid reaction products absorb in a spectral range different from that of dopachrome. When these phenolics show a good affinity for the enzyme, dopachrome formation is prevented, and they could be mistakenly classified as inhibitors.

2.9.4 Nonspecific Enzyme Inactivators

Nonspecific enzyme inactivators, such as acids or bases, is non-specifically denature the enzyme, thus inhibiting its activity.

2.9.5 Specific Tyrosinase Inactivators

Specific tyrosinase inactivators, such as mechanism-based inhibitors, are also called suicide substrates. These inhibitors can be catalyzed by tyrosinase and form covalent bond with the enzyme, thus irreversibly inactivating the enzyme during catalytic reaction. They inhibit tyrosinase activity by inducing the enzyme catalyzing "suicide reaction."

2.9.6 Specific Tyrosinase Inhibitors

Specific tyrosinase inhibitors such as most compounds discussed in this review. The compounds reversibly bind to tyrosinase and reduce its catalytic capacity.

2.10 Tyrosinase Inhibition assay

Tyrosinase is an enzyme that is engaged in the most important rate-determining step in melanin biosynthesis. Melanogenesis is initiated by tyrosine that

is metabolized into DOPA and then dopaquinone by tyrosinase. This first reaction is the ratedetermining step in melanogenesis. Tyrosinase has various forms, including met-tyrosinase, oxy-tyrosinase and deoxy-tyrosinase. Oxy-tyrosinase is an activated form, whereas deoxy-tyrosinase is an inactivated form (Seo, Sharma & Sharma, 2003; García-Molina, Muñoz, Varón, Rodríguez-LópezGarcía-Cánovas & Tudela, 2007; Wilcox et al., 1985). The function of tyrosinase is closely related to disease. In animal studies, disorder in melanin biosynthesis has been found to lead to condition of abnormal melanin coloration such as vitiligo or hyperpigmentation. In severe cases, skin cancer may result from such disorders (Garcia et al., 2007). Therefore, studies on tyrosinase inhibitors are highly important for the development of new drugs for the treatment of abnormal melanin coloration as well as functional cosmetics intended to produce a depigmenting effect. The method used to evaluate the degree of tyrosinase inhibition is widely used in the screening of depigmenting agents. As it is easy to use and can be applied to evaluate many samples at the same time, it is considered to be the most appropriate method for early screening of candidate substances. This method, which is based on an enzyme–substrate reaction, is to induce the reaction of tyrosine and tyrosinase for a specific period and to measure the amount of products after the reaction. Mushroom tyrosinase (Wilcox et al., 1985), melanoma tyrosinase or human melanocytes tyrosinase can be used. In general, mushroom tyrosinase is widely used because it is easy to obtain. DOPA can be the end product for measurement, but it is not widely employed because of the need for the use of a radioactive isotope. HPLC, another method that can be used to measure DOPA, is not used frequently because it requires a long period of time to analyse many samples. The most appropriate method used to measure dopachrome, one of the reaction products, is to perform spectrometry at 475 nm or 490 nm.

2.11 Emulsion

Emulsions are the most common type of delivery system used in cosmetics. They enable a wide variety of ingredients to be quickly and conveniently delivered to hair and skin. The best known cosmetic products based on emulsions are creams and

lotions. Not only for the professional but also the amateur cosmetics maker there are large variety of techniques and methods to create different types of cosmetic emulsions (Mason, Wilking, Meleson, Chang & Graves, 2006).

The most typical emulsion is one in which an oil is dispersed in water. Understandably, this is called an oil-in-water (o/w) emulsion. If water droplets are dispersed in oil the resulting emulsion is called water-in-oil (w/o) emulsion. Generally, o/w-emulsions are typically chosen for applications requiring a relatively small amount of fatty material as hand, shaving or moisturizing creams. On the other hand, w/o emulsions are preferred when a large amount of oil is desired. This system has a greasier feel and leaves a longer-lasting residue. Typical products are emollient creams and sunscreens. In addition to the simple two-phase emulsion it is possible to make also multiple emulsions as w/o/w-emulsions (w/o-emulsion in water)

2.12 Cosmetic Product Evaluations

2.12.1 Stability Evaluation

Stability testing may be defined as the process of evaluating a product to ensure that key attributes stay within acceptable guidelines. In order to make this testing meaningful, it is important to accurately establish the nature of these critical product attributes, to measure how they change over time, and to define what degree of change is considered acceptable. Defining which parameters are crucial requires a combination of chemical knowledge about the formula and common sense about product usage. The factors that influence the stability of cosmetics products can be divided into two categories: intrinsic and extrinsic factor (CTFA and Colipa, 2004).

2.12.1.1 Screening stability test

The product will be submitted to a centrifugation test. It is suggest that a sample be centrifuge at 3,000 rpm for 30 minutes. The product must remain stable and any sign of instability shows the need for reformulation. If approved in this test, the product can then be submitted to the stability test.

2.12.1.2 Accelerated stability test

Accelerated conditions are internationally recognized as appropriately predicting product shelf life in many industries. Appropriate conditions, for example with regard to temperature and/or duration, should be chosen according to the product category and the in-house know-how and should be base on scientific judgment. Data acquired using various techniques at different temperature and durations can be used, possibly in conjunction with the use of mathematical models, to predict stability. Tests are often performed at 37°C, 45°C or 50°C during 1, 2, 3…months but the temperature used and the duration will depend on the product type. For instance, for certain product categories other temperature may prove to be more useful. Based on the accelerated stability results and on its scientific experience, a company may then be able to predict that actual stability for given market conditions.

2.12.1.3 Freez-thaw

Freeze-thaw testing should be considered for certain type of products. Examples of problems which can be detected by freeze-thaw testing include suspension problem (a tendency to crystallize or cloud), instability of emulsion and creams, package design issues (such as wrinkled or loss of label, cracking or distortion), corrosion of internal lacquers in aluminum tubes, ect. As products can be expected to encounter temperature and pressure extremes during transport and storage (CTFA and Colipa, 2004).

2.12.2 Safety Evaluation

2.12.2.1 Microorganism contamination test

The microorganism contamination tests typically use plate count procedures that should be evaluated for their reproducibility. Organisms from plate count are identified base on colony morphology, gram reaction, pigment production, motility, and biochemical characteristics including nutritional requirements and reactions on selective and differential media.

2.12.2.2 Test for skin irritation

Skin irritation is a less severe response than corrosion, but can span a range of responses from near corrosive at one extreme to weak cumulative or neurosensory responses at the other. The development of alternatives for skin

irritation testing has lagged behind that of skin corrosion testing, likely because of the greater urgency of developing alternatives for the more severe skin responses and because of the range of responses encompassed within the ‘‘skin irritation’’. For noncorrosive chemicals, there has been a recent effort to develop and promote the use of clinical patch testing methods for a more relevant assessment of chemical skin irritation potential than that provided by the rabbit test (Cosmetic Products Stability Guide, 2005).

2.12.3 Whitening Efficacy Evaluation

Methods for the efficacy evaluation of depigmenting agents include *in vitro*, *in vivo* and clinical trials. *In vitro* tests focus on the screening of depigmenting agents through the investigations of mechanisms for the activation or inhibition of signal transfer factors or enzymes involved in the formation of melanin. A report was recently published on a method of evaluation that employed the mechanism of inhibiting the transfer of melanosomes formed by melanocytes into keratinocytes (Hakozaki et al., 2002; Ebanks, Wickett & Boissy, 2011). For clinical studies, ultraviolet (UV) rays are irradiated to induce pigmentation. And the measurement of whitening effect is conducted for these pigmented areas. The depigmenting agent is applied to individuals with pigmented lesions, and then, measurement of the whitening effect is conducted.

Clinical studies performed to evaluate the depigmenting effect may be assessed to investigate the effect on the inhibition of melanin pigmentation or the effect on the improvement of pigmented conditions. The subject is human beings pigmented artificially by UV irradiation or had site of hyper-pigmentation, such as freckles, lentigo or blotch skin which may be investigated. Changes of skin tone can be visually measured by experts or instrumentally measured with the use of equipment such as a Chromameter. The visual evaluation method employs the scoring system to evaluate the degree of whitening. Therefore, it is important to develop well designed protocols, such as an objective scoring method and the use of qualified evaluators, to establish the reliability of results. The instrumental evaluation method has the advantage of quantitative and objective measurement. However, the results may depend on the size and condition of target sites and the experience and skill of

technicians. Therefore, it is desirable to use these two methods in parallel and to make conclusions from the results of both methods. Melanosomes move to the outside of skin with keratinocytes and are eventually sloughed off with other skin. The capacity of chemical peeling products (e.g. α-hydroxy acids) to disperse melanin pigment and/or accelerate epidermal turnover can result in skin lightening (Yoshimura et al., 2001; Ando et al., 1998). Clinical tests of the epidermal turnover rate can be used for the evaluation of whitening agents.

Chapter 3

Materials and Methods

3.1 Chemicals

The chemicals and reagent used for synthesis of curcumin complexes, and determination of anti-oxidant and anti-tyrosinase activities were analytical grade. Curcumin was purchased from sigma (83% curcumin, 12% demethoxycurcumin, and 5% bisdemethoxycurcumin). Dichloromethane, ethyl acetate, n-butanol, ethanol, methanol, DMSO, and trichloroacetic acid were purchased from Merck. Ascorbic acid was purchased from Finechem. Linoleic acid was purchased from Fluka. Zinc (II) acetate ($Zn(CH_3COO)_2$), copper (II) acetate ($Cu(CH_3COO)_2$), and magnesium (II) chloride ($MgCl_2$), and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma. Feric chloride ($FeCl_3$), potassium ferricyanide ($K_3[Fe(CN)_6]$), ammonium thiocyanate (NH_4SCN), and ferrous chloride tretahydrate ($FeCl_2 \cdot 4H_2O$) were purchased from Fisher scientific. Sodium dihydrogenphosphate ($NaH_2PO_4 \cdot 2H_2O$), and disodium hydrogen phosphate ($Na_2HPO_4 \bullet 12H_2O$) were purchased from Prolabo.

The ingredients for preparation of emulsion cream were cosmetic grade. Methyl paraben, carbopol ETD 2020, tween 60, alantoin, xanthane gum, propylene glycol, span 60, sodium behenoyl lactylate, stearic acid, glyceryl stearate and PEG-100 stearate, cetearyl alcohol, stearyl dimethicone, mineral oil, squalane, DMDM hydantoin, propyl paraben, dimethicone, and triethanolamine were purchased from Namsiang.

3.2 Plant Material

One year of mature fresh *C. petiolata* rhizomes were collected from Mae-Chan, Chiang Rai, Thailand in January 2011. The taxonomic identification of plant material was confirmed by Maxwell, J.F. of the CMU Herbarium, Department of Biology, Faculty of Science, Chiang Mai University, Thailand where a voucher specimen was deposited (CMU02-Nisakorn Saewan).

3.3 General Instruments

3.3.1. UV-visible spectrophotometer	(Libra S22, Biochrom)
3.3.2. FT-IR spectroscopy	(FTS FI-IR, Perkin Elmer)
3.3.3. X-ray diffractometer	(D8-Advance, Bruker)
3.3.4. Differential scanning calorimeter	(TGA/DSC 1, Mettler Toledo)
3.3.5. Mass spectrometer	(Thermo Finnigan MAT95XL, USA)
3.3.6. Rotary evaporator	(Eyela/ CCA-1110, Japan)
3.3.7. 2-Digital balance	(Asvanturer/ ARC 120, USA)
3.3.8. 4-Digital balance	(Sartorius/ BT 2245, Germany)
3.3.9. Microplate reader	(UVM 340, Biochrom)
3.3.10. HPLC	(Alliance/ WATER 2695, USA)
3.3.11. pH meter	(Mettler Teledo/S 20, USA)
3.3.12. Viscometer	(Brookfied/ RVDV-II+PRO, USA)
3.3.13. Colorimeter	(Hunterlab/ ULTRASCAN®, USA)
3.3.14. Centrifugation	(Spectrafuge/ 16M, UK)
3.3.15. Hot air oven	(Memmert/ UM 500, Germany)
3.3.16. Micropipette	(Labnet, USA)
3.3.17. Mexameter	(Courage&Khazaka, Germany)
3.3.18 Melting point	(MPA120 EZ-Melt, USA)

,

3.4 Preparation of Metal Curcumin Complexes

3.4.1 Preparation of Zinc-curcumin Complex (Zn-(cur)$_2$)

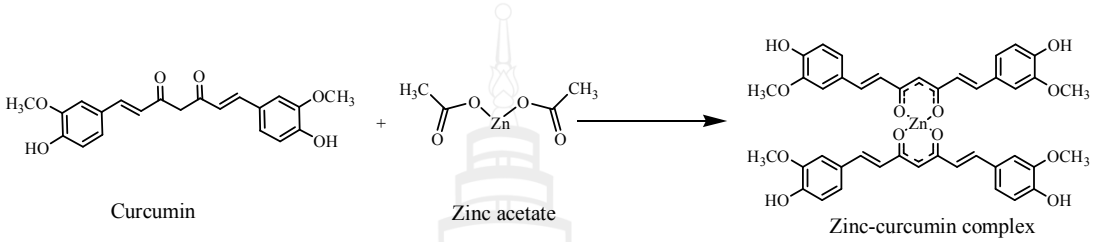


Figure 3.1 Synthesized of Zinc-curcumin Complex (Zn-(cur)$_2$)

The ethanol solution of 0.1 M zinc acetate (5 mL) was added dropwise to 5 mL of 0.1 M curcumin solution and reflux at 60 °C for 3 hours. After the reaction was complete (as assessed by the precipitate occurred) the solution was then cooled to room temperature and washed with cold ethanol-water. Zinc-curcumin Complex (Zn-(cur)$_2$) was obtained as an orange powder (64.8% yield).

UV-Vis (DMSO) λ_{max} (nm)	424,446
FT-IR (KBr) ν(cm^{-1})	3376 (O-H streching), 1628, 1595 (C=O streching), 1501 (C=C aliphatic stretching), 1430 (C=C aromatic stretching), 1289 (C-O bending)
m/z (ESITOFMS)	799.3 ([M+H]$^+$)
X-ray powder diffraction spectra (2θ)	8.82, 9.27, 10.06, 10.74, 12.50, 15.63, 16.72, 18.34, 20.45, 22.07, 23.47, and 24.99

3.4.2 Preparation of Magnesium-curcumin Complex (Mg-(cur)$_2$)

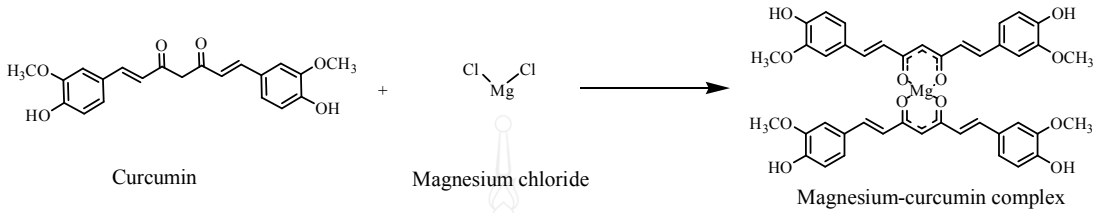


Figure 3.2 Synthesized of Magnesium-curcumin Complex (Mg-(cur)$_2$)

The mixture of 0.1 M curcumin solution (5 mL) and 0.1 M magnesium chloride (5 mL) was refluxed at 60 °C for 3 hours. The solution was then cooled to room temperature and washed with cold ethanol-water. The dried Magnesium-curcumin complex (Mg-(cur)$_2$) was obtained as a dark orange powder (67.4% yield).

UV-Vis (DMSO) λ_{max} (nm)	424
FT-IR (KBr) ν(cm^{-1})	3387 (O-H streching), 1624, 1590 (C=O streching), 1512 (C=C aliphatic stretching), 1430 (C=C aromatic stretching), 1289 (C-O bending)
m/z (ESITOFMS)	759.3 ([M+H]$^+$)
X-ray powder diffraction spectra (2θ)	at 7.00, 8.99, 12.31, 14.65, 15.24, 15.97, 16.42, 17.36, 18.24, 18.96, 19.56, 21.30, 23.44, 23.86, 24.73, 25.76, 26.24, 26.84, 27.53, 28.29, and 29.83

3.4.3 Preparation of Copper-curcumin Complex (Cu-cur)

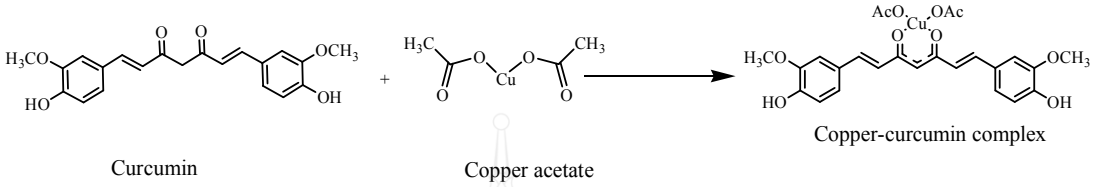


Figure 3.3 Synthesized of Copper-curcumin Complex (Mg-$(cur)_2$)

Copper-curcumin complex (Cu-cur) was prepared by slowly added dropwise to 0.1 M copper acetate (5 mL) to 0.1 M curcumin solution (5 mL) and reflux at 60 ^{o}C for 3 hours. The solution was then cooled to room temperature and washed with cold ethanol-water. Manganese-curcumin Complex (Mg-$(cur)_2$) was obtained as dark gold powder (79.2% yield).

UV-Vis (DMSO) λ_{max} (nm)	422, 446
FT-IR (KBr) $\nu(cm^{-1})$	3510 (O-H streching), 1622, 1593 (C=O streching), 1509 (C=C aliphatic stretching), 1405 (C=C aromatic stretching), 1283 (C-O bending)
m/z (ESITOFMS)	566.2 ($[M+H_2O]^+$)
X-ray powder diffraction spectra (2θ)	9.07, 12.90, and 24.83

3.5 Stability Study of Free Curcumin and Its Metal Complexes

To study the stability of free curcumin and its metal complexes, the compounds were storage in various conditions including pH buffer solution (pH 3, 7 and 12), temperature (25, 37, 45, and 60^{o}C), light and dark condition. The absorbance of the substances was recorded in range 300-600 nm using microplate reader. The results was expressed as the percent residual of tested compounds which calculated by

comparing the absorbance of the compounds with their calibration curve. The calibration curve were prepared by plotting the absorbance value of curcumin and its metal complexes (Y-axis) against their concentrations (X-axis) to obtain linear regression equation (Y = aX + b). The stability of tested compounds in various conditions was calculated from their calibration curve and expressed as percentage residual.

To study the effect of pH on stability of free curcumin and its metal complexes, the solution of 1 mg/ mL of samples in various pH buffer solutions (pH 3, 7 and 12) were prepared. The absorption spectrum of tested compounds was measured every 1 hour for the first 10 hours and every 10 hours between 10-60 hours.

To study the effect of temperature and light on stability of free curcumin and its metal complexes, ten mg of samples were dissolved in 10 mL of ethanol and keep at various temperature (25, 37, 45, and 60^{o}C), light and dark conditions for 7 weeks. The absorption spectra of curcumin and its metal complexes were measured every week.

3.6 Determination of Anti-oxidant and Anti-tyrosinase Activities of Curcumin and Its Metal Complexes

3.6.1 DPPH Radical Scavenging Activity

This assay is a decolorization assay that measures the capacity of anti-oxidants to directly react with (scavenge) DPPH radicals. The 5 µl of different concentration of each sample was reacted with 195 µl of 100 µM DPPH in a 96-well microplate and incubated at 37 ºC for 30 minutes in dark. The absorbance of reaction was measured at 515 nm. The result was expressed in IC_{50} value which calculated using the formula given below.

$$\% \text{ DPPH radical scavenging} = \frac{A_{sample} - A_{blank\ of\ sample}}{A_{control}} \times 100$$

3.6.2 Ferrous Reducing Power Activity

This assay was determine the reduction of $[Fe^{3+}(CN^{-})_6]$ to $[Fe^{2+}(CN^{-})_6]$ by anti-oxidants, which is form the green complex from excess Fe^{3+} ions. The 25 µl of each sample, 25 µl of 0.1 M phosphate buffer (pH 7.2) and 50 µl of 1% potassium ferricyanide were added to 96-well microplate. After incubation at 37ºC for 60 minutes, 25 µl of 10% trichloroacetic acid and 100 µl of DI water were added and the absorbance was measured at 700 nm (A_1). Then, 25 µl of 0.1% ferric chloride was added to the mixture and the absorbance was measured again (A_2). The reducing power (OD) of samples was determined using the formula given below and expressed as ascorbic acid equivalents (AAE). The reducing power of samples was.

$$\text{The reducing power (OD)} = (A_{sample2} - A_{sample1}) - (A_{control2} - A_{control1})$$

3.6.3 Inhibition of Lipid Peroxidation Activity

The inhibition of lipid peroxidation activity of samples was determined by measured the amount of free radical MDA (Malondialdehyde) formed after lipid hydroperoxide decomposition. Thiobarbituric acid (TBA) was used to determine the amount of MDA which reacts with TBA to produce the pink chromophore adduct and can be measure by spectrophotometry. Each sample (100 µl) was mixed with linoleic acid 900 µl. Then the mixture was incubated at 100 °C for 20 minutes. The pH 3.5 buffer solution (1 mL) and the mixture of 20 mM thiobabituric acid in 10% trichloroacetic acid were added, and then incubated at 100 °C for 30 minutes. The absorbance of the pink solution was measured at 532 nm. The percentage inhibition of lipid peroxidation was calculated using the following formula and expressed in IC_{50} value.

$$\%\ \text{Inhibition of lipid peroxidation} = \frac{A_{sample} - A_{blank\ of\ sample}}{A_{control}} \times 100$$

3.6.4 Anti-tyrosinase Activity

The tyrosinase activity of curcumin and its metal complexes was determined using the L-tyrosine oxidation assay. The absorbance at 490 nm decreases as a result of the reaction of melanin synthesis was interrupted. The 40 µl of 1.7 mM L-tyrosine solution was mix with 40 µl of 0.1M phosphase buffer (pH 6.8). Then 40 µl of samples was added and incubation at room temperature for 10 minutes. 40 µl of mushroom tyrosinase (245 U/mL in phosphase buffer (pH 6.5)) was added to the solution. The absorbance was recorded after 20 minutes of incubation at room temperature at 490 nm using microplate reader. The percentage inhibition of tyrosinase was calculated by following equation and expressed in IC_{50} value.

$$\% \text{ Inhibition of tyrosinase} = \frac{A_{sample} - A_{blank\ of\ sample}}{A_{control}} \times 100$$

3.7 Extraction of Curcumin from *Curcuma petiolata* Rhizomes

Dried powder of *C. petiolata* rhizome (100 g) was extracted with ethanol at room temperature for 24 hours. Then the ethanolic extract was washed with hexane to eliminate the oily substances. The solvent was removed by using rotary evaporator and kept at 4°C until further analysis.

3.8 Determination Curcuminoid Content of *C. petiolata* Extract

The curcumins content of *C. petiolata* extract was determined by UV-Vis spectrophotometer and high performance liquid chromatography (HPLC) technique.

3.8.1 UV-Vis Spectrophotometer

The absorption spectum of *C. petiolata* extract was measured in range 300-600 nm. The curcumin content of *C. petiolata* extract was calculated from a standard curve of curcumin. The standard curve of curcumin was prepared by plotting the

absorbance values (Y-axis) at 434 nm of curcumin standard solutions against their concentrations (X-axis) to obtain a linear regression equation (Y = ax + b), which is used to calculate the total curcumins content of *C. petiolata* extract (mg curcumins/mg sample).

3.8.2 High Performance Liquid Chromatography (HPLC)

Analytical HPLC analysis for determine the amount of curcumin derivatives, curcumin, bismethoxycurcumin, and demethoxycurcumin, was carried out using a model 1090 M series II DR5 ternary pumping system with integral variable volume autosampler, column oven and model 1040 series II photodiode array detector with HP workstation (Hewlett–Packard, Stockport, UK). The column used was a 250 × 4.6 mm HRPB 5 μm (octyl-/octadecylsilane, fully end-capped 14% carbon loading; HiChrom, Theale, UK) and the mobile phase consisted of solvent A (0.4% v/v acetic acid in acetonitrile) and solvent B (0.4% v/v aqueous acetic acid) delivered at 1 mL/min. The mobile phase was fixed at a ratio of 65%A:35%B. Photodiode detection was carried out at 425 nm for curcumin. The samples were filtered through a 0.2 μm membrane syringe filter prior to analysis. The injection volume was 20 μl. The *C. petiolata* extract (1 mg) was dissolved in 1 mL of ethanol. Curcumin, bismethoxycurcumin, and demethoxycurcumin were accounted as the standard. A calibration curve was prepared from standard concentration 10-1000 ppm. The sample was analyzed in triplicate, content of curcumin, bismethoxycurcumin, and demethoxycurcumin in the sample was calculated and expressed as mg/g plant.

3.9 Preparation of Zinc-*C. petiolata* extract complexes

CP extract + Zinc acetate ⟶ Zinc-CP complex

Figure 3.4 Synthesized of Zinc-*Curcuma Petiolata* Extract Complex (Zn-CP)

50 mg of *C. petiolata* extract in ethanol solution (5 mL) was mixed with 0.1 M of zinc acetate and reflux at 60 ^{o}C for 3 hours. After the precipitate occurred, the solution was then cooled to room temperature and washed with cold ethanol-water. The dried compound of Zn-CP complex obtained as a yellow powder (36.15 mg).

UV-Vis (DMSO) λ_{max} (nm)	424, 441
FT-IR (KBr) ν(cm^{-1})	3407 (O-H streching), 1595 (C=O streching), 1509 (C=C aliphatic stretching), 1418 (C=C aromatic stretching), 1285 (C-O bending).

3.10 Formulation of Whitening Cream

The ingredients of cream base were shown in Table 3.1. The O/W base cream was prepared as follows. Carbopol ETD 2020 solution was added by Tween 60, allatoin, and methyl paraben. Dispersion of xanthane gum in propylene glycol was added in the mixture. Oil part (span 60, sodium behenoyl lactyclate, stearic acid, cetearyl alcohol, stearyl dimethicone, glyceryl stearate and PEG-100 stearate, mineral oil, squalane, dimethicone, and propyl paraben) was added to water phase at 75 ^{o}C. Triethanolamine and DMDM hydantoin were added to the mixture with homogenize (at 6,000 rpm for 10 min). Then the emulsion was cooled down (45 ^{o}C). The 0.5 % w/w of free curcumin, *C. petiolata* extract, Zn-cur, and Zn-CP were added to base cream to obtained whitening creams, F1, F2, F3, and F4**,** as shown in Table 3.1.

Table 3.1 The Ingredient of Whitening Cream

Part	Ingredient	Function	% w/w			
			F1	**F2**	**F3**	**F4**
A	DI water	Solubilizer	79	79	79	79
	Methyl paraben	Preservative	0.1	0.1	0.1	0.1
	Carbopol ETD 2020	Thickening agent	0.4	0.4	0.4	0.4
	Tween 60	Emulsifier	4	4	4	4
	Allantoin	Smoothening	0.3	0.3	0.3	0.3
B	Xanthane gum	Thickening agent	0.2	0.2	0.2	0.2
	Propylene glycol	Solubilizer, Humactant	3	3	3	3
C	Span 60	Emulsifier	1	1	1	1
	Sodium behenoyl lactylate	Emulsifier	1	1	1	1
	Stearic acid	Thickening agent	1	1	1	1
	Cetearyl alcohol	Thickening agent	2	2	2	2
	Stearyl dimethicone	Skin conditioning agent	0.5	0.5	0.5	0.5
	Glyceryl stearate	Thickening agent	1	1	1	1
	Mineral oil	Emollient	1	1	1	1
	Squalane	Smoothening	2	2	2	2
	Dimethicone	Occlusive agent	2	2	2	2
	Propyl paraben	Preservative	0.15	0.15	0.15	0.15
D	DMDM hydantoin	Preservative	0.5	0.5	0.5	0.5
E	Triethanolamine	Chelating agent	0.3	0.3	0.3	0.3
F	Free curcumin	Active	0.5	-	-	-
	C. petiolata extract	Active	-	0.5	-	-
	Zn-cur	Active	-	-	0.5	-
	Zn-CP	Active	-	-	-	0.5

3.11 Stability Study

The cream containing free curcumin, *C. petiolata* extract, and metal-curcumin complexes (15.0 g) was centrifuged at 5,000 rpm for 30 minutes. Then, the product (85.0 g) was weighted into a transparent bottom and screwed on the top. Each bottom was stored in ambient temperature, heating-cooling (4 °C for 24 hrs and 45 °C for 24 hrs, as one cycle), heating (50 °C), cooling (4 °C), and light exposure, for one month.

The physical properties of products were evaluated for every week including;

Appearance of the product

pH value

The product was triplicate measured with pH meter (Eutech, pH 510).

Color value

The product (25.0 g) was placed on the lid and triplicate measured the color using chroma meter. The color value of products is expressed as the *L*, a** and *b** value

Viscosity

The viscosity of product was measured using Brookfield Rotational Digital Viscometer (DV II RVTDV-II). The spindle was rotated at 10 rpm. Creams were allowed to settle over 30 minutes at the assay temperature (25 ± 1°C) before the measurements were taken.

3.12 Degradation of Active Ingredient in Cream at Different Storage Conditions

The stability of formula F1-F4 was determined by storage at various condition including; ambient temperature, light exposure, heating-cooling (4 °C for 24 hrs and 45 °C for 24 hrs, as one cycle), heating (50 °C), and cooling (4 °C) over 1 month period. One gram of cream was dissolved in ethanol 5 mL, and centrifuged at 5,000 rpm for 10 minutes. The absorbance and spectrum of the obtained solution was measured by using UV-visible spectrophotometer. The degradation of the active

ingredient was followed between 0-4 weeks. The percent residual of an actives were calculated by comparing the absorbance with their standard curves.

3.13 Safety evaluation

3.13.1 Microorganism Contamination Test

Microorganism contamination test of the whitening products was performed by using mikrocount® combi. The products (0.2 g) were dissolved in DI water before smeared on both side of the slide then the slide was placed back in the tube and screwed on the top. The slide was incubated at 37 °C for 3 days. Then, observed the colonies of microorganism on both side of the slide.

3.13.2 Single Close Patch Test

Skin irritation was evaluated using placed patch test on 10 volunteers. The 0.1 g of product was placed in Finn chamber® and placed on volunteer forearm for 48 hours. Scoring of patch test reactions was shown in Table 3.2. The score were completed by the calculation of the mean irritation index (M.I.I) according to the equation. The interpretation of the calculated M.I.I scale is shown in Table 3.3.

M.I.I = [Σ of the grade (erythema + oedema)/ number of subject]

Table 3.2 Scoring of Patch Test Reaction

Score	Interpretation
-	Negative
?+	Doubtful reaction
+	Weak (non-vesicular) reaction
++	Strong (oedematous or vascular) reaction
+++	Extreme (bullous or ulcerative)
NT	Not tested
IR	Irritation reaction of different types

Table 3.3 The Mean Irritation Index

M.I.I	Class
M.I.I<0.20	Non irritating (NI)
0.20≤M.I.I<0.50	Slightly irritating (SI)
0.50≤M.I.I<1	Moderately irritating (MI)
M.I.I≥1	Irritating (I)

3.14 Efficacy evaluation

The trial was performed in accordance with the Declaration of Helsinki and its subsequent amendments. The patients gave their informed consent after the whole procedure of the study had been fully explained. The emulsion creams (0.5 g), F1-F4, were topically applied twice times daily on forearm of 10 volunteers, aged between 25 and 50 years old. During the study the volunteers were asked to avoid direct sun light exposure. The melanin value of skin was measured by the Mexameter® MX 18 and monitored between 0-4 weeks. The decreasing of melanin value after 4 weeks was calculated and expressed as percentage increasing of skin whitening.

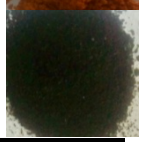
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Preparation and Characterization of Metal-curcumin Complexes

Several studies have been reported that curcumin complexes of zinc (II), copper (II), and magnesium (II) were higher activities such as lipid peroxidation, superoxide dismutase, and xanthine oxidase than that of free curcumin. In this study, three divalent metals, zinc (II), copper (II), and magnesium (II) were chosen to complex with curcumin. The selection of metal salts were based on their color, toxicity, and economic. In cosmetics, zinc acetate was usually used as an astringent, anti-biotic for the topical treatment of acne, and topical anti-itch. Since, zinc acetate was colorless compound and less toxicity. Magnesium chloride was usually used as a flavoring agent and thickening agent. Copper acetate was usually used in preparation of pigments from natural source. Zinc acetate, magnesium chloride and copper acetate were non toxic. Therefore, they were chosen to complex with curcumin. The complexes were successfully prepared by refluxing the mixture of curcumin and metal salts (ratio 1:1 mol) in ethanol for 3 hours. The complexes were obtained as various colored powder (Table 4.1).

Table 4.1 The Physical Appearance and Percentage Yield of Curcumin and Its Metal Complexes

Compound	% Yield	Physical appearance	
Free curcumin	-	Yellow powder	
Zinc-curcumin ($Zn\text{-}(cur)_2$)	68.4	Orange powder	
Magnesium-curcumin ($Mg\text{-}(cur)_2$)	67.4	Dark yellow powder	
Copper-curcumin (Cu-cur)	79.2	Dark gold powder	

4.1.1 Mass spectrometer

The ESITOFMS were obtained using a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate mass. The molecular formula of complex of Zn and curcumin ($Zn\text{-}(cur)_2$ complex) was deduced as $C_{42}H_{38}O_{12}Zn$ (observed *m/z* 799.3 $[M+H]^+$, calcd for $C_{42}H_{39}O_{12}Zn$, 799.3). $Mg\text{-}(cur)_2$ complex molecular formula was deduced as $C_{42}H_{38}O_{12}Mg$ (observed *m/z* 759.3 $[M+H]^+$, calcd for $C_{42}H_{39}O_{12}Mg$, 759.3). Cu-cur complex molecular formula was deduced as $C_{25}H_{25}O_{10}Cu$ (observed *m/z* 566.2 $[M+H_2O]^+$, calcd for $C_{25}H_{27}O_{11}Cu$, 566.1). The results demonstrated that Cu ion formed complexes with one molecule of curcumin whereas Zn and Mg formed di-ligand complexes with two molecules of curcumin (Figure 4.1). The difference of coordination structure is probably due to the atomic radius of metal ions. The smaller atomic radius (copper; 128 pm), is not preferable to form bi-ligand due to more steric hindrance between two ligands, while the bigger atomic radius, (Zn; 134 pm and Mg; 160 pm) can accommodate with two curcumin ligands with less steric effect.

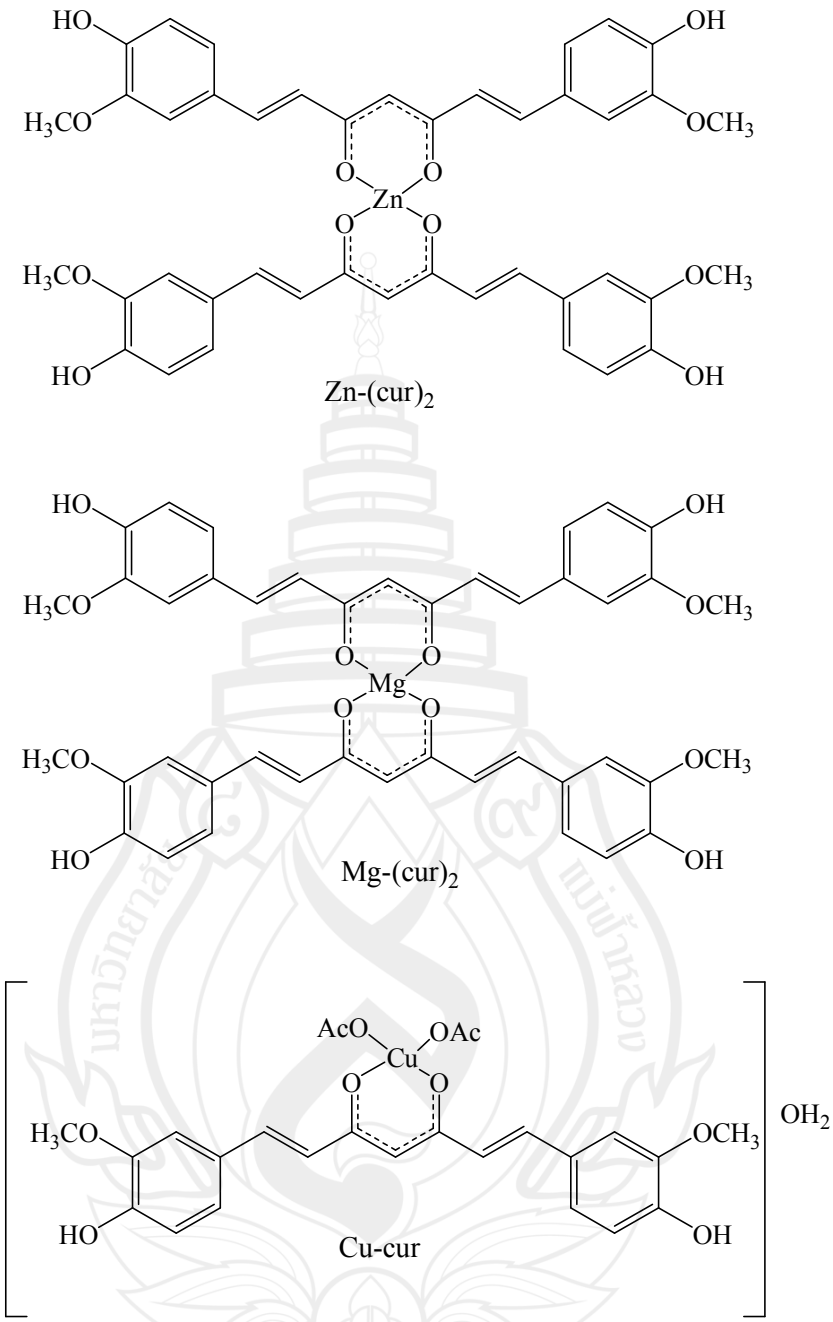


Figure 4.1 Structure of Metal Complexes

4.1.2 UV-visible Absorption Spectra

The UV-vis absorption spectra of curcumin and its metal complexes was recorded between 300-600 nm. All complexes were darker color than that of free curcumin and their spectra showed broader visible absorption than that of free curcumin and the lighter color powder, Zn-$(cur)_2$ and Mg-$(cur)_2$ complexes, absorb narrow visible region than the dark color of Cu-cur complex. Curcumin shows an intent band of n→π* transition at 435 nm. The maximum absorption of its complexes showed a large blue shift and decreased in intensity. The maximum absorption wavelength of Zn-$(cur)_2$, Mg-$(cur)_2$, and Cu-cur were 424, 424, and 422 nm, respectively (Figure 4.1).

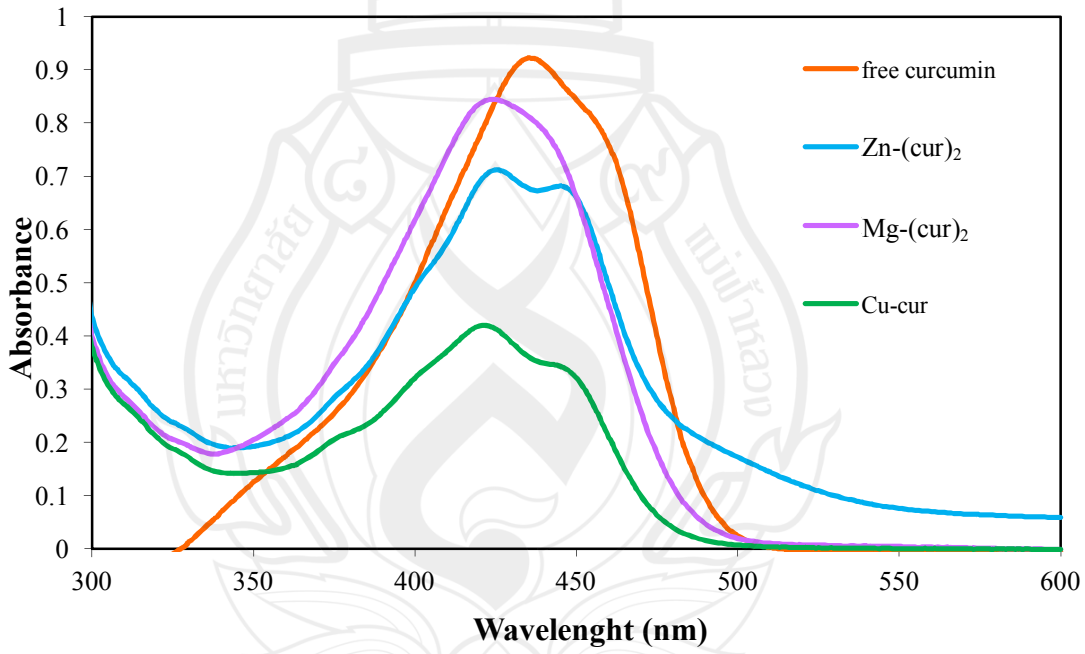


Figure 4.2 UV-Vis Absorption Spectra of Free Curcumin and Its Metal Complexes

4.1.3 Infrared Spectra

Infrared spectroscopy is a technique used for studying the functional group of compounds. The central β-diketone and the phenolic group (at both ends) are two possible functional groups of curcumin molecule that can be coordinated to the metal ions (Figure 4.2). Two major forms of metal interaction with keto-enol tautomerism are shown in Figure 4.3. Type A is associated with chelation in the ionic enolic form. Type B is associated with chelation in the neutral ketonic form (Daniel, Limson, Dairam, Watkins & Daya, 2004).

Infrared spectra of curcumin and its metal complexes were collected by using KBr pellets in the range 4,000-400 cm^{-1}. Curcumin shows a strong sharp O-H stretch at 3,512 cm^{-1}, ascribed to the hydrogen-bonded enol form of the β-diketone moiety, and medium broad O-H stretching indicating strong hydrogen bonding in range 3,340 cm^{-1}, assigned to the phenoxyl group. The metal derivatives show broad band with the shift to lower wavenumber but the sharp O-H band disappeared. Curcumin posses two strong carbonyl bond (C=O) at 1,627 and 1,604 cm^{-1}. The decrease of intensity of carbonyl bond (C=O) and shifted towards lower wavenumber ($\Delta\nu$ =2-14 cm^{-1}) indicating that some interaction has occurred at these sites and its involvement in complexation by new created link between the metal and curcumin. In addition, several other bands in the infrared spectra are indicator for complexation. There is a collapse of a group of three weak bands at 574, 562, and 543 cm^{-1} into a single band (551 cm^{-1} for Zn-$(cur)_2$, 547 cm^{-1} for Mg-$(cur)_2$, and 559 cm^{-1} for Cu-cur).

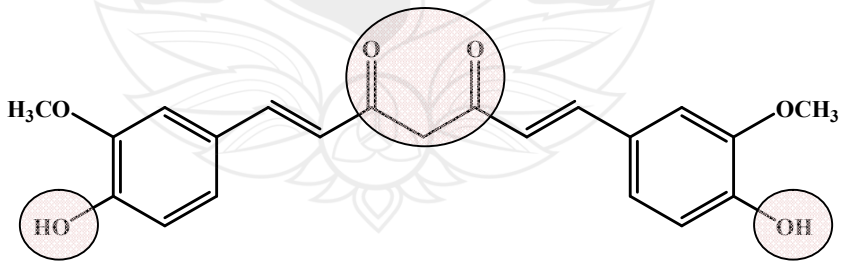
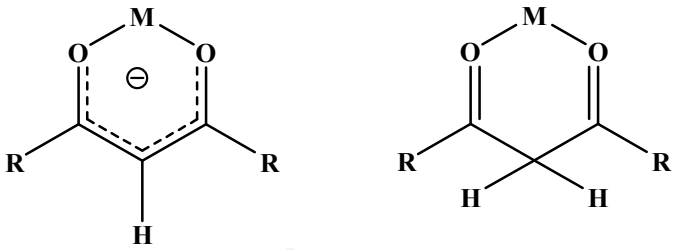


Figure 4.3 The Possible Functional Groups of Curcumin that can be Coordinated to the Metal Ion



Type A **Type B**

Figure 4.4 The Major Forms of Metal Interaction at the Central β-diketone

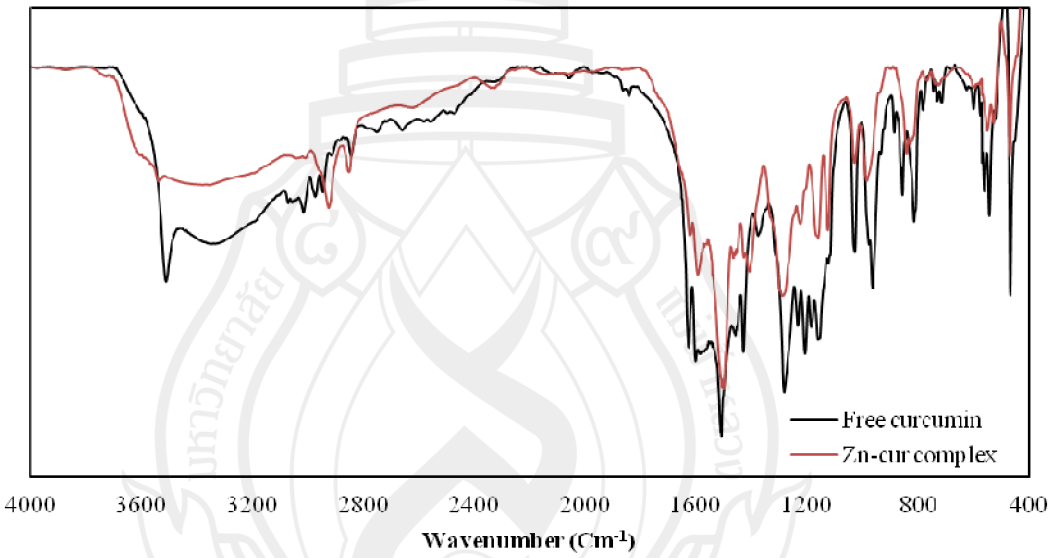


Figure 4.5 FT-IR Spectra of Curcumin and Zn-$(cur)_2$

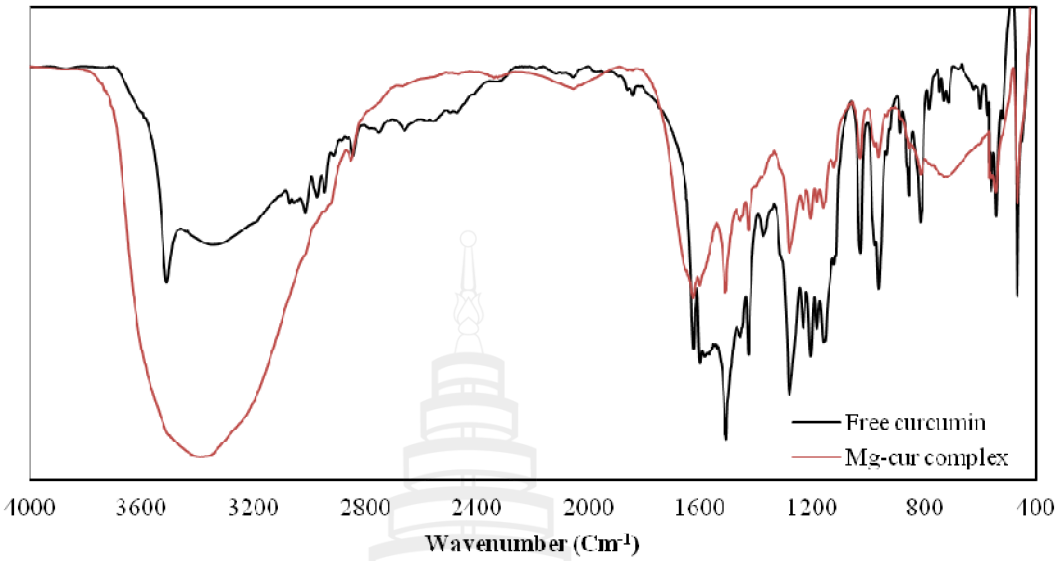


Figure 4.6 FT-IR Spectra of Curcumin and Mg-$(cur)_2$

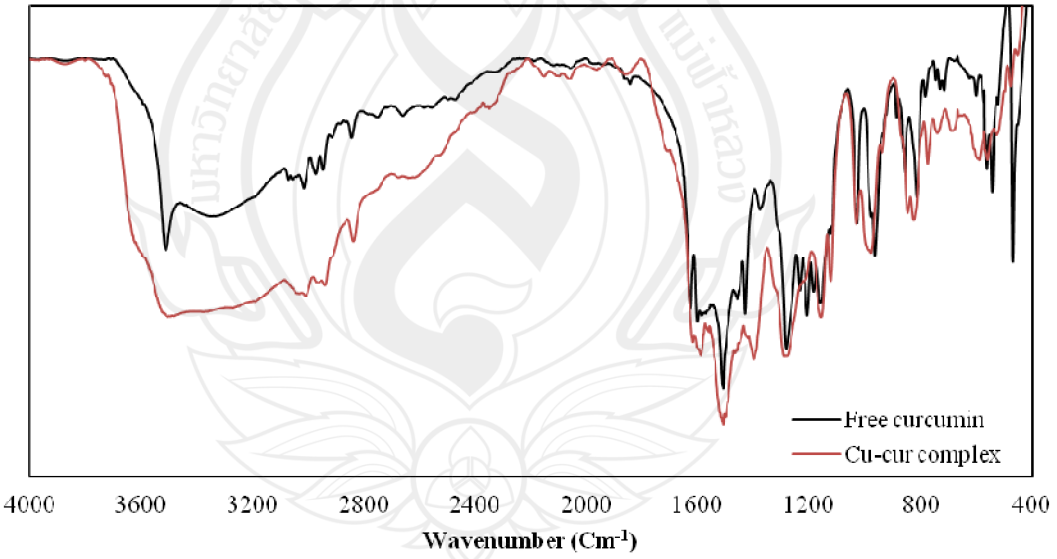


Figure 4.7 FT-IR Spectra of Curcumin and Cu-cur

Table 4.2 Wavelength Changes from Infrared (KBr pellets) Spectral Data of Curcumin and Its Metal Complexes.

Compound	IR (cm^{-1})						
	$\nu_{(OH)\ enol}$	$\nu_{(OH)\ phenol}$		$\nu_{(C=O)}$	$\nu_{(C=C)\ aliphatic}$	$\nu_{(C=C)\ aromatic}$	$\delta_{(C-O)}$
Free curcumin	3,512 (s)	3,340 (br)	1,627	1,604	1,509	1,430	1,283
Zn-$(cur)_2$	-	3,376 (br) ($\Delta\nu$ = 136)	1,628	1,595 ($\Delta\nu$ = 9)	1,501($\Delta\nu$ = 8)	1,410 ($\Delta\nu$ = 20)	1,289 ($\Delta\delta$ = 6)
Mg-$(cur)_2$	-	3,387 (br) ($\Delta\nu$ = 125)	1,624 ($\Delta\nu$ = 3)	1,509 ($\Delta\nu$ = 5)	1,512 ($\Delta\nu$ = 3)	1,430	1,283
Cu-cur	-	3,360 (br) ($\Delta\nu$ = 152)	1,622 ($\Delta\nu$ = 5)	1,593 ($\Delta\nu$ = 11)	1,509	1,405 ($\Delta\nu$ = 25)	1,283

4.1.4 X-ray Powder Diffraction Spectra

X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material and provides information on unit cell dimensions. The crystalline atoms cause a beam of X-rays to diffraction into many specific directions, which showed different diffraction peak.

The X-ray powder diffraction patterns of curcumin, and its metal complexes are shown in Figure 4.8. The X-ray powder diffraction in the range of $0^{o}<2\theta<35^{o}$ of curcumin showed 20 patterns. The characteristic peaks of curcumin appeared at a diffraction angle of 2θ at 7.96, 8.95, 12.30, 14.56, 15.92, 17.37, 17.90, 18.24, 18.96, 19.53, 21.32, 23.40, 23.83, 24.69, 25.66, 26.19, 26.79, 27.46, 28.25, and 29.05 revealing that curcumin is present in a highly crystalline state.

The diffraction spectrum of Mg-$(cur)_2$ complex exhibits the similar characteristic diffraction peaks with free curcumin demonstrated that the crystalline structure of Mg-$(cur)_2$ complex was similarly to free curcumin. The diffraction patterns of Zn-$(cur)_2$ showed twelve reflections with diffraction angle of 2θ at 8.82, 9.27, 10.06, 10.74, 12.50, 15.63, 16.72, 18.34, 20.45, 22.07, 23.47 and 24.99, and Cu-cur showed three reflections with diffraction angle of 2θ at 9.07, 12.90, and 24.83 indicating that the crystallinity of complexes was difference from free curcumin.

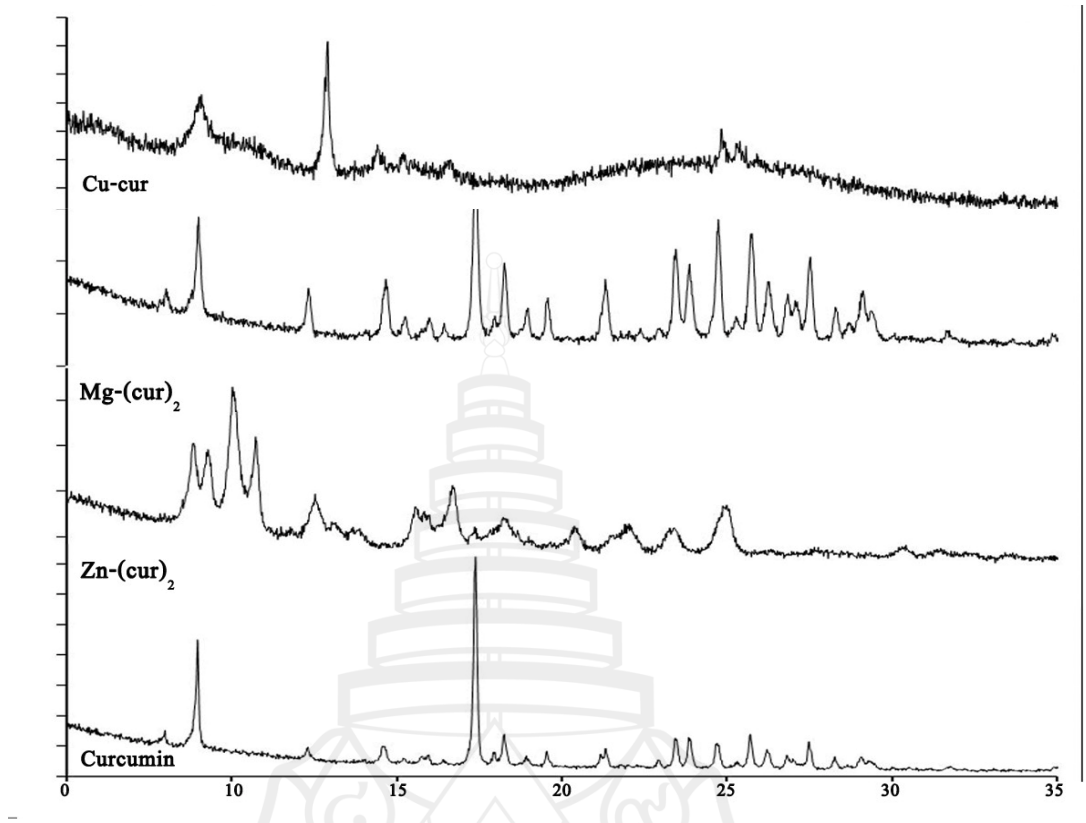


Figure 4.8 X-ray Powder Diffraction Spectra of Curcumin and Its Complexes

4.1.5 Thermogravimetric Analysis

The thermal stability of curcumin and its metal complexes were followed in the temperature range between 20-600 °C as shown in Table 4.3 and Figure 4.9.

Curcumin has melting point at 183 °C. At 275 °C the degradation of curcumin was observed corresponding to the dehydroxylation of OH groups by eliminated two water molecules (Weight loss: 8.64%). Above 573 °C curcumin was totally decomposed.

The TGA curve of Zn-$(cur)_2$ complex showed the dehydroxylation of OH groups of two curcumin ligands connected to one zinc atom by the elimination of four water molecules (deduced by obtained weight loss) in range of 100-315 °C (Weight

loss: 8.02%). Then, the complex exhibited single step decomposition from 328 to 369 °C (Weight loss: 73.97%).

The TGA curve of Mg-$(cur)_2$ complex indicates that the loss of weight start around 95 °C (Weight loss: 8.44%) related to the dehydroxylation of OH groups of two curcumin ligands connected to one magnesium atom by the elimination of four water molecules. Then, the complex exhibited single step decomposition from 100 to 520 °C (Weight loss: 81.35%) due to the decomposition of curcumin ligand.

The thermogram of Cu-cur complex was observed the removal of uncoordinated water molecules in the temperature range 50-95 °C (Weight loss: 3.18%). In range 100-260 °C, the elimination two molecules of OH and OCH_3 group was observed (Weight loss: 16.26%). The curcumin ligand was totally decomposed in range 270 to 420 °C to obtain the constant weight region from formation of copper oxide (CuO) contamination (Weight loss: 61.31%).

Table 4.3 The Weight Loss of Curcumin and Its Metal Complexes

Compound	**Weight loss**		
	I (%)	**II (%)**	**III (%)**
Free curcumin	8.64	91.36	-
Zn-$(cur)_2$ complex	8.02	73.97	-
Mg-$(cur)_2$ complex	8.44	91.56	-
Cu-cur complex	3.18	16.26	61.31

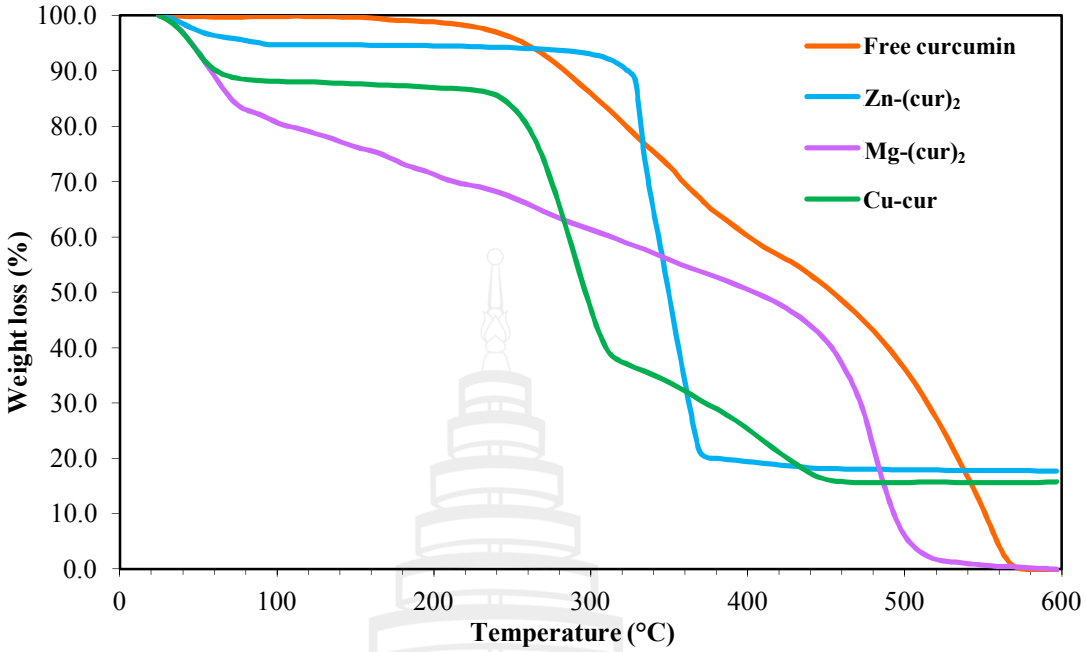


Figure 4.9 Thermogram of Curcumin and Its Metal Complexes

4.2 Stability Study of Curcumin and Its Metal Complexes

4.2.1 Effect of pH Solution

The curcumin molecule was extremely unstable. The kinetics of hydrolytic degradation reactions of curcumin has been proposed by Tonnesen and Karlsen (Tonnesen & Karlsen, 1985). It is postulation in between tree form; H_2A^-, HA^{2-}, and A^{3-} as shown in Figure 4.10. Curcumin exist in the protonated form (H_4A^+) and appear as red color when the pH is lower than 1. The neutral form (H_3A) of curcumin has yellow color in pH 2 to 7 solution. At pH 7.5 and above, the anionic form (H_2A-, HA^{2-}, and A^{3-}) of curcumin are the major which have reddish orange color.

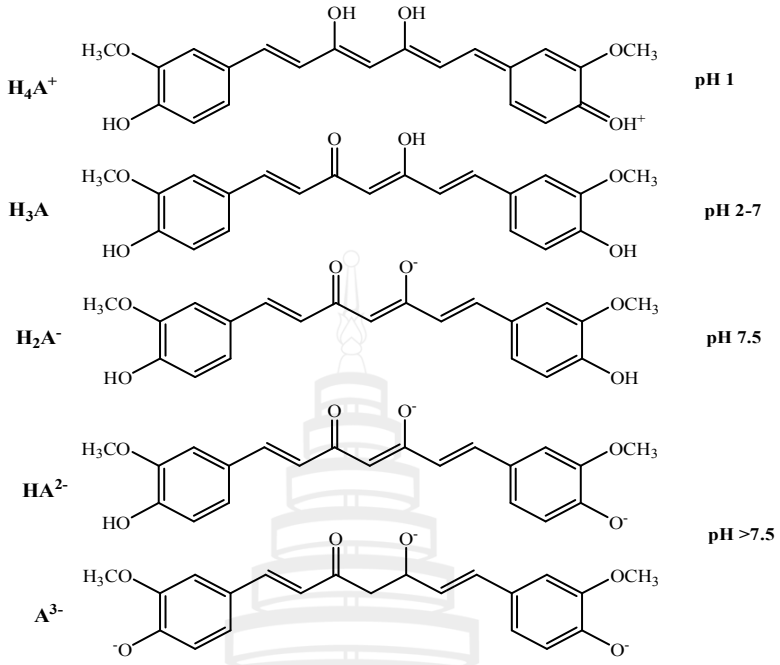


Figure 4.10 Curcumin Form in Various Buffer Solution

To study the stability of curcumin and its complexes in various pH solutions (pH 3, 7, and 12), the chemical degradation was investigated in "*in vitro*" over a 60 hours period.

When curcumin was added to phosphate buffer pH=3, it was majority degradation (40%) within two hours. After 2 hours, curcumin was continuously degraded and remain 25% residual at 60 hours. At neutral and basic conditions, curcumin was totally decomposed after 2 hours. The results showed that curcumin was more stable in acidic condition than neutral and basic condition due to the conjugated diene structure, whereas it can readily donate H-atom of phenolic group at neutral and basic conditions, leading to the decomposition. The study have demonstrated that metal complexes was stable than that of free curcumin. Cu-cur complex was the most stable in all conditions with more than 70% residual at the end of study. Zn-$(cur)_2$ complex showed majority degradation (15% in acid and neutral, 45% in basic conditions) at first two hours. In range 2 to 60 hours, it showed equilibrium stable with more than 50% residual. While Mg-$(cur)_2$ complex showed similar degradation behaviors to free curcumin. The more stable of metal derivatives

might be due to bonding between metal and β-diketone group which cause the difficult decomposition.

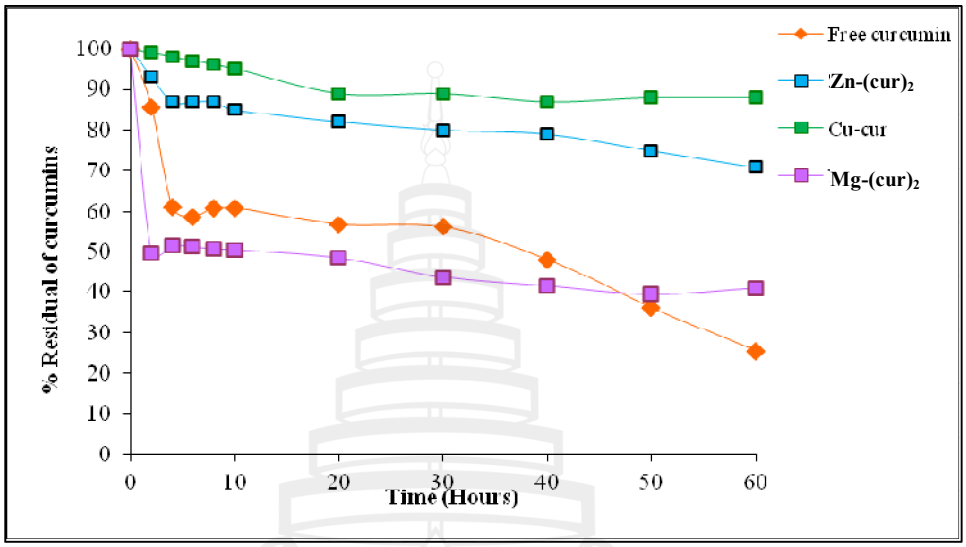


Figure 4.11 Degradation of Curcumin and Its Metal Complexes in Acid Solution

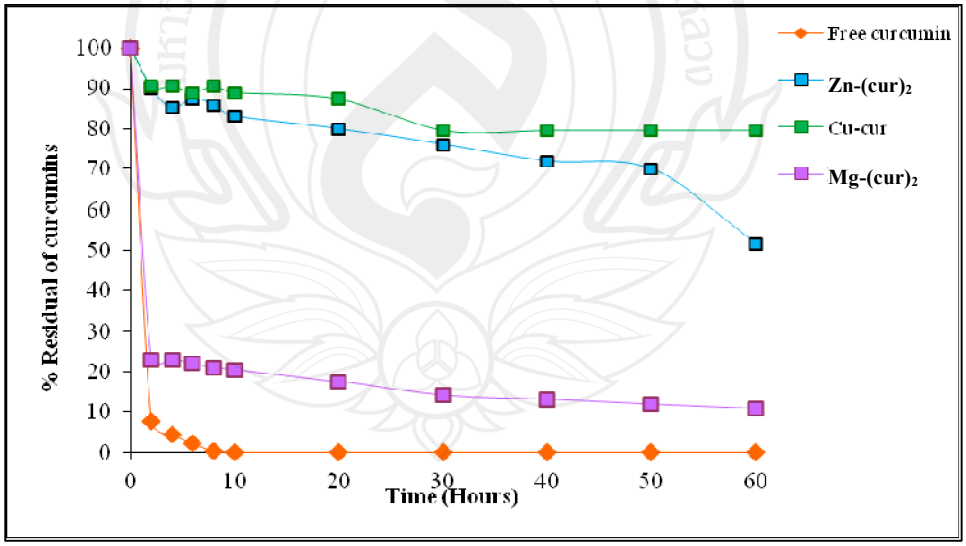


Figure 4.12 Degradation of Curcumin and Its Metal Complexes in Neutral Solution

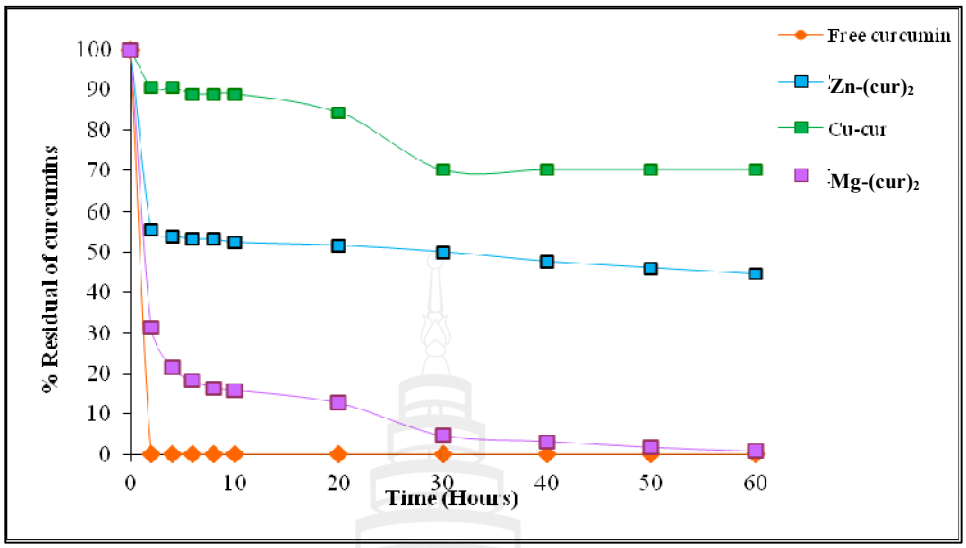


Figure 4.13 Degradation of Curcumin and Its Metal Complexes in Alkaline Solution

4.2.2 Effect of Temperature

Under high temperature and light exposure, the dione group of curcumin was break to obtain feruloylmethan, ferulic acid, vanillin, and acetone as the degradation products (Wang et al., 1997) (Figure 4.14).

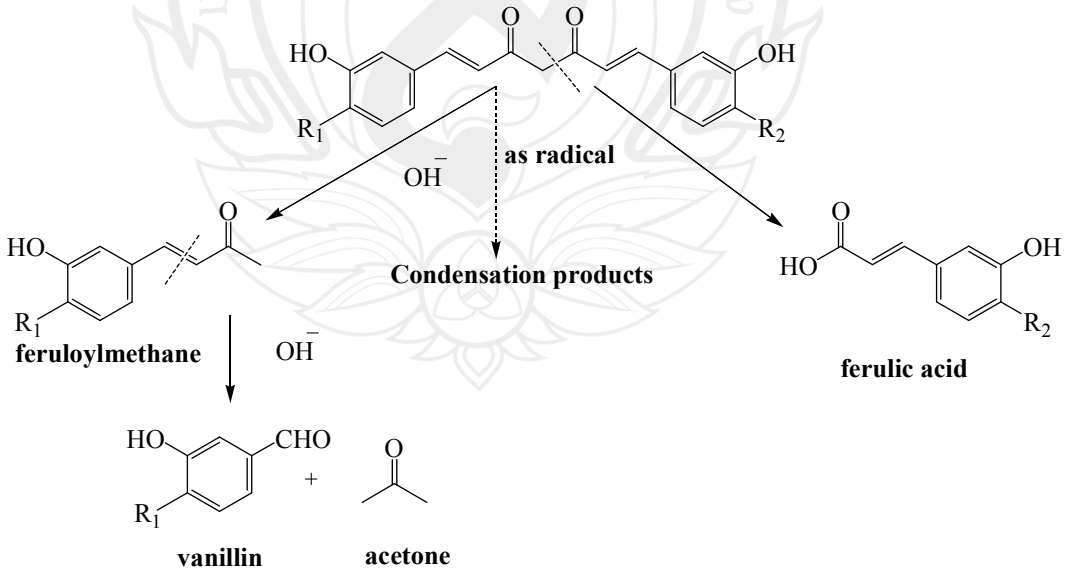


Figure 4.14 Degradation Products of Curcumin

To study the stability of curcumin and its complexes in various temperature conditions, the solutions of samples were storage at room temperature, 37, 45, and 60 °C and investigated the chemical degradation over a 7-week period.

At the end of experiment, curcumin was 50% degradation at room temperature. The increasing temperature induced higher rate of curcumin degradation, more than 90% of curcumin was decomposed at higher temperature as a result of the breaking of dione group of curcumin was activated. All metal derivatives were more stable than that of free curcumin. At room temperature and 37 °C, Zn-$(cur)_2$ and Cu-cur complexes were higher stable. While Mg-$(cur)_2$ complex showed identically degradation with curcumin. At high temperature (45 and 60 °C), all metal complexes was 2 folds higher stable than that of free curcumin. These results are consistent with those of other studies and suggest that the difficult breaking of dione group due to is inconsequence of the electron delocalization through six membered ring of the metal and dione group.

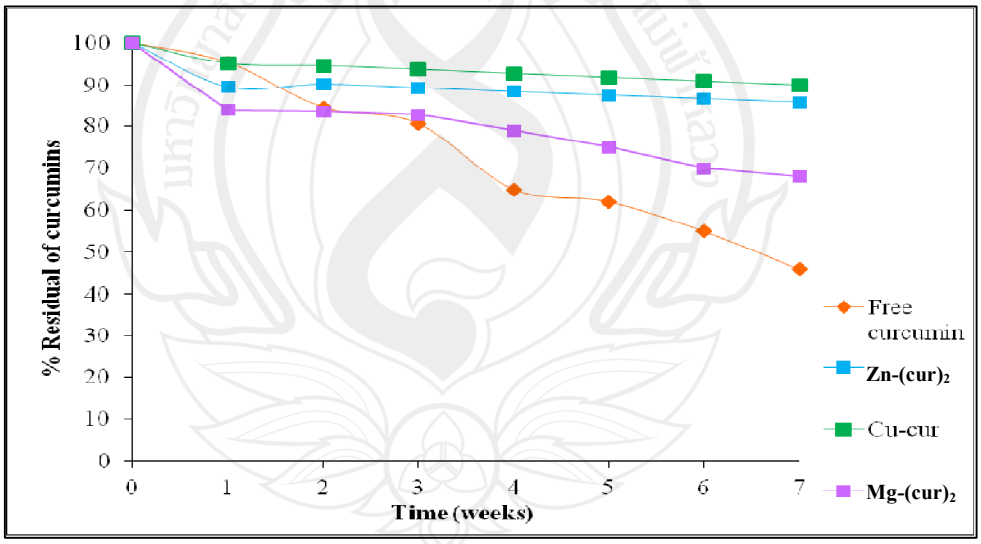


Figure 4.15 Degradation of Curcumin and Its Metal Complexes at Room Temperature

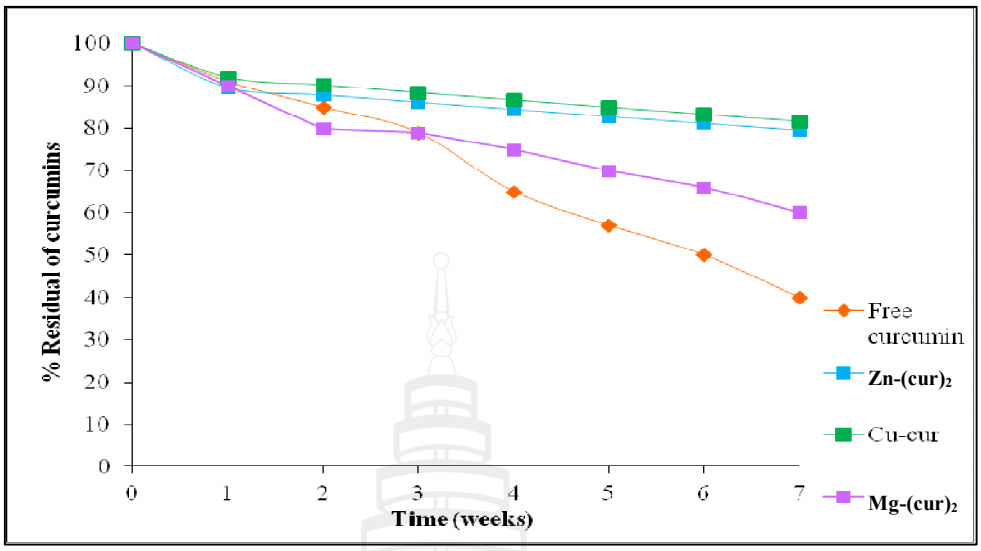


Figure 4.16 Degradation of Curcumin and Its Metal Complexes at 37 °C

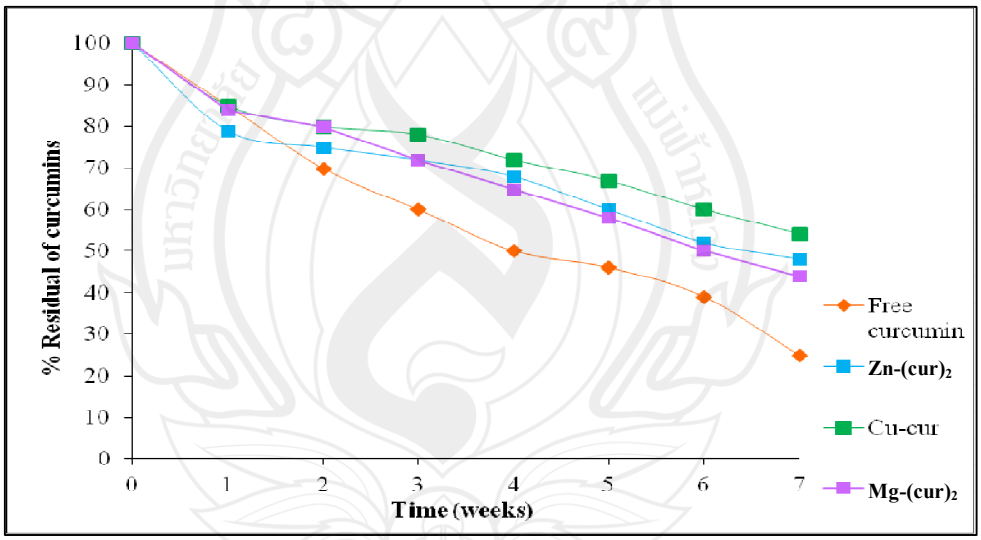


Figure 4.17 Degradation of Curcumin and Its Metal Complexes at 45 °C

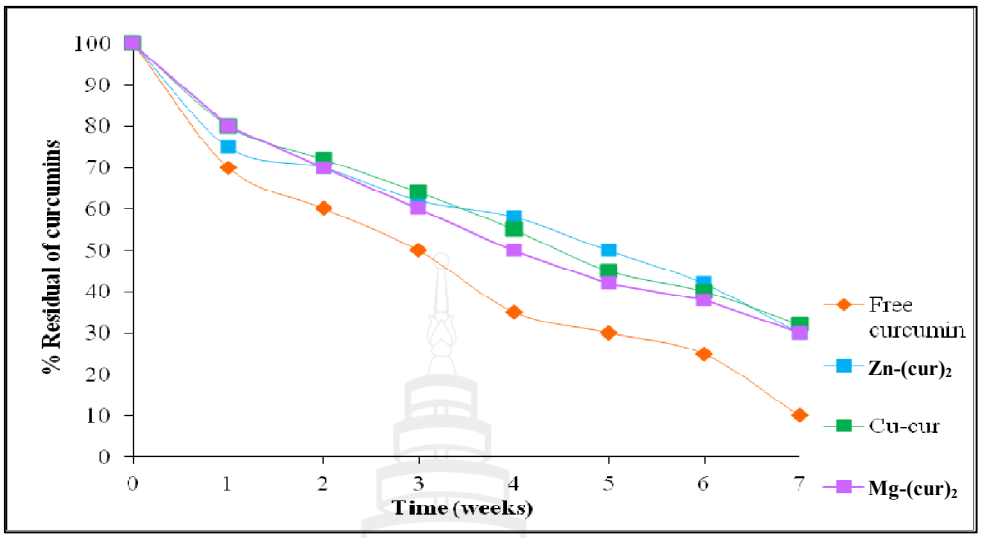


Figure 4.18 Degradation of Curcumin and Its Metal Complexes at 60 °C

4.2.3 Effect of Light

In order to study the effect of light on the stability of curcumin and its complexes, the chemical degradation of samples storaged under light exposure and dark condition over a 7-week period was investigated.

In the dark condition, curcumin and its complexes remained more than 80% relatively stable during 7 weeks. The stability of complexes was in order of: Cu-cur ≅ Zn-$(cur)_2$ > Mg-$(cur)_2$ > curcumin (Figure 4.19). Under light exposure, curcumin and its complexes were consistently decomposed. Curcumin was degraded to 75% after 7 weeks. The stability of all complexes was remained 2 folds higher than that of free curcumin (Figure 4.20). The higher stability of metal complexes caused by the easily break group of curcumin was form the stable six-membered ring with metal. Therefore, they were slower degradation than that of free curcumin.

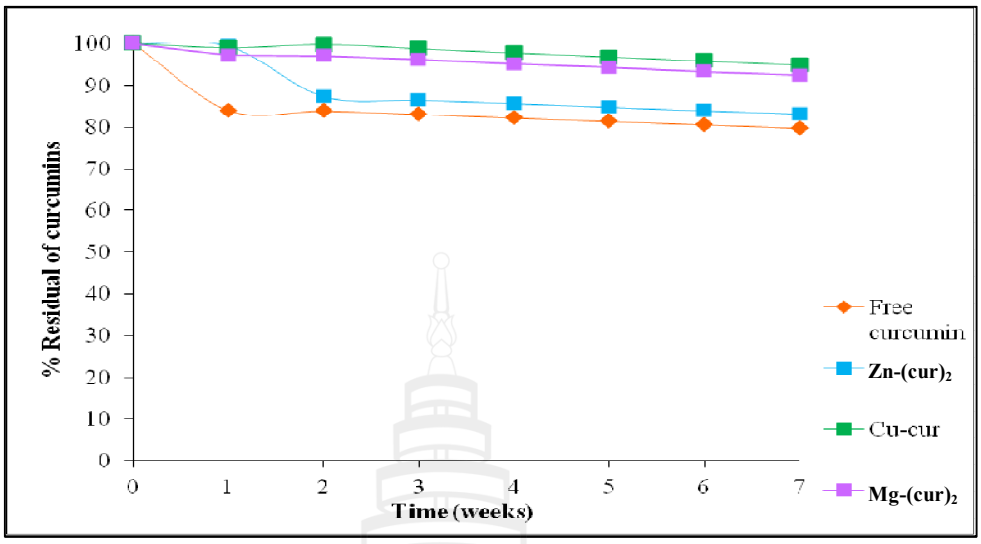


Figure 4.19 Degradation of Curcumin and Its Metal Complexes in Dark Condition

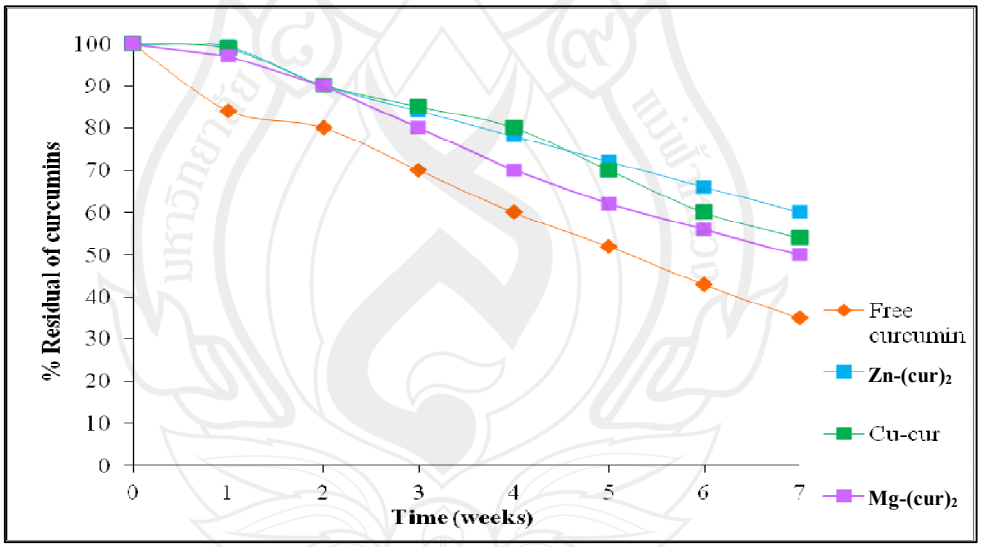


Figure 4.20 Degradation of Curcumin and Its Metal Complexes under Light Exposure Condition

4.3 Anti-oxidant and Anti-tyrosinase Activities of Curcumin and Its Metal Complexes

4.3.1 Anti-oxidant Activity

The anti-oxidant activities of curcumin and its complexes were tested by three assays including DPPH radical scavenging, reducing power, and thiobarbituric acid reactive substance as shown in Table 4.4.

For DPPH radical scavenging, the absorbance at 515 nm decreased as a result of DPPH color changed from purple to yellow due to the radical scavenged by anti-oxidants which donated the hydrogen atom to form the stable DPPH-H. The result was expressed in IC_{50} value. Lower IC_{50} value indicated higher DPPH radicals scavenging activity. Curcumin showed DPPH radical scavenging activity with IC_{50} 0.034 mM. The anti-oxidant activity of the compounds depends on their molecular structures. The metal ions may affect the chemical properties of ligand molecules hence the resulting complexes may be of higher or lower activity. Zn-$(cur)_2$, and Mg-$(cur)_2$ complexes showed the higher DPPH radical scavenging activity as free curcumin with IC_{50} 0.013 and 0.015 mM, respectively. The higher activity of both complexes might be due to they have more phynoxy group, more electron and H atom donating. While, Cu-cur showed much lower DPPH radical scavenging activity (IC_{50} 0.108 mM) than that of free curcumin. It was probably due to solubility and electronic configuration.

The reduction of $[Fe(CN)_6]^{3-}$ to blue complex of $[Fe(CN_6)]^{4-}$ by anti-oxidants was measured. The ferrous reducing power activity was expressed as ascorbic acid equivalents (AAE). The more value of ascorbic acid equivalents, the higher reducing power activity. Curcumin showed potent reducing power (0.464 g AAE/mM). The reducing power of curcumin increased by complexing with Zn(II) and Mg(II) (1.699 and 1.700 g AAE/mM, respectively). Curcumin usually reductions of Fe^{3+} to Fe^{2+} by electron transfer through phenoxy and enol group. Then it was converted to a derived curcumin radical. The bonding with metal ions at β-diketone group gives structure stabilizes through resonance, the ArO-H bond strength weaker, which make electron donating easily. Therefore, they are stronger ability of electron

transfer when compared with free curcumin. While Cu-cur showed much lower reducing power than that of free curcumin with 0.004 g AAE/mM. There are many factors that effect to the reducing power activity. For example, electro-negativity, polarity, and solubility. More studies would need to be performed to further investigate the decrease of ferrous reducing power of Cu-cur complex.

The inhibition of lipid peroxidation activity of curcumin and its metal complexes was determined by measured the amount of free radical malondialdehyde (MDA) formed after lipid hydroperoxide decomposition. Thiobarbituric acid (TBA) was used to determine the amount of MDA by measuring the pink chromophore adduct. The result was expressed in IC_{50} value. Curcumin showed lower inhibition of lipid peroxidation than BHT and ascorbic acid. The inhibition of lipid peroxidation of metal derivatives was lower than that of free curcumin. This study produced results which corroborated the finding of Halliwell & Gutteridge (Halliwell & Gutteridge, 1984). In the presence of transition metal ions, ROOH can give rise to the generation of radicals capable of re-initiating lipid peroxidation by redox-cycling of these metal ions

4.3.2 Anti-tyrosinase Activity

The effect of metal complexes on tyrosinase activity was determined using the L-tyrosine oxidation assay. This amino acid was oxidized by tyrosinase enzyme to give melanin which absorbs visible light at 490 nm. The decrease of the absorbance at 490 nm is a result of inhibition of melanin synthesis. The result was expressed in IC_{50} value. Enzyme activity was directly related to inhibitor concentration.

Curcumin showed inhibition of melanin synthesis with IC_{50} 0.086 mM. The anti-tyrosinase activity of metal derivatives was in order of; Zn-$(cur)_2$ > Mg-$(cur)_2$ > Cu-cur. Among the metal derivatives, Zn-$(cur)_2$ and Mg-$(cur)_2$ showed the higher tyrosinase inhibition than that of free curcumin with IC_{50} 0.032 and 0.044 mM, respectively. The high activity of both complexes might be due to they have more phenoxyl group to chelate with active site of tyrosinase enzyme. In contrast, Cu-cur showed 2 folds lower tyrosinase inhibition than that of free curcumin (Table 4.4). This result may be due to it has less binding position with tyrosinase enzyme.

Table 4.4 The Anti-oxidant and Anti-tyrosinase Activities of Curcumin and Its Metal Complexes

Sample	Anti-oxidant activities			Anti-tyrosinase activity (IC_{50} mM)
	DPPH radical scavenging (IC_{50} mM)	Reducing power (g of AAE/mM)	Inhibition of lipid peroxidation activity (IC_{50} ug/ml)	
Free curcumin	0.034 ± 0.001^{b}	0.464 ± 0.013^{b}	59.864 ± 0.208^{a}	0.086 ± 0.000^{b}
Zn-$(cur)_2$	0.013 ± 0.000^{a}	1.699 ± 0.005^{a}	65.209 ± 0.122^{b}	0.032 ± 0.000^{a}
Mg-$(cur)_2$	0.015 ± 0.000^{a}	1.700 ± 0.001^{a}	72.132 ± 0.030^{c}	0.044 ± 0.000^{b}
Cu-cur	0.108 ± 0.001^{d}	0.004 ± 0.000^{d}	97.200 ± 0.320^{d}	0.175 ± 0.001^{c}

Note. Different letters in the same column indicate significant differences among means of treatments ($P < 0.05$)

4.4 Preparation of Curcumin Rich Extract from *Curcuma petiolata* Rhizomes

The *C. petiolata* (CP) rhizome was dried and grinded to powder. The powder was extracted with ethanol at room temperature for 24 hours. The ethanolic extract was washed with hexane to eliminate the oily substances. The solvent was removed by using rotary evaporator to obtain dark orange-brownish crude extracts as shown in Figure 4.21.

(a) (b)

Note. (a) Rhizome Powder, (b) Ethanolic Extract

Figure 4.21 The Physical Appearance *C. petiolata* (CP) Extract

4.5 Determination of Curcuminoid Content in CP Extract

The curcuminoid content of the extract was determined by UV-Visible spectrophotometer and high performance liquid chromatography.

From the UV-Vis spectrophotometer method, the curcumins content of the extract was calculated from the linear regression equation ($y = 0.8388x + 0.0888$, $R^2 = 0.9993$) of standard curcumin and expressed as mg curcumin/g plant. The results

showed that the extract contained high amounts of total curcumins with 133.36 mg/g plant.

From HPLC analysis, the extract contained 22.26% of bisdemethoxycurcumin (BDMC), 12.09% of demethoxycurcumin (DMC), and 65.65% of curcumin (Cur) (Figure 4.21). The total curcumin content was 137.81 mg/g plant (Table 4.5). The extract was considered as a high amount of curcumin source which was equivalent to that of turmeric extract (10-13 %w/w) (Li et al., 2011).

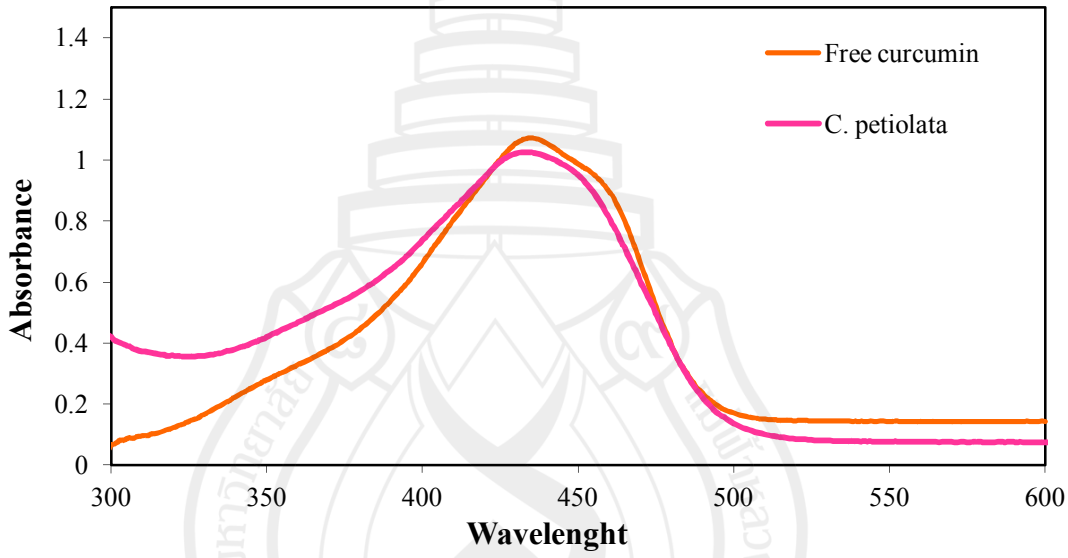


Figure 4.22 Spectra of CP Extracts and Free Curcumin

Table 4.5 The Curcuminoid Content of CP Extracts

Compound	Content (mg/g plant)	Content (%)
Bisdemethoxycurcumin content	30.67	22.26
Demethoxycurcumin content	16.66	12.09
Curcumin content	90.48	65.65
Total	137.81	100.00

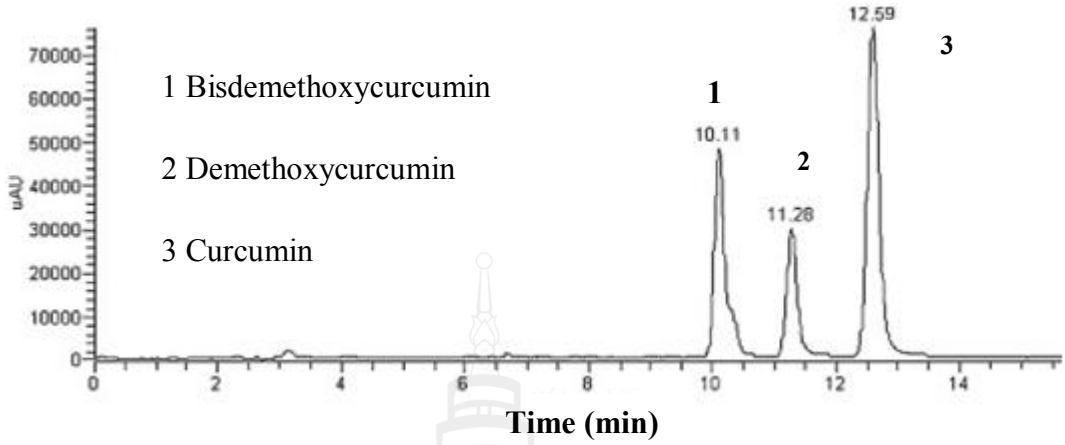
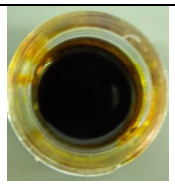


Figure 4.23 HPLC Chromatogram of CP Extracts

4.6 Preparation and Characterization of Metal-CP Extract Complexes

The Zn-CP complex was successfully prepared by reflux the mixture of curcumin rich extract from *C. petiolata* extract and zinc acetate in ethanol for 3 hours. The product was obtained as yellow powder (Table 4.6).

Table 4.6 The Physical Appearance of CP and Its Complexes

Compound	Physical appearance
CP extract	
Zinc- CP complex (Zn-CP)	

4.6.1 UV-visible Absorption Spectra

CP extract displays an intent band at 422 nm, and Zn-CP complex showed the decrease in intensity as well as the shift of maximum absorption wavenumber in the presence of metal ions as shown in Figure 4.23.

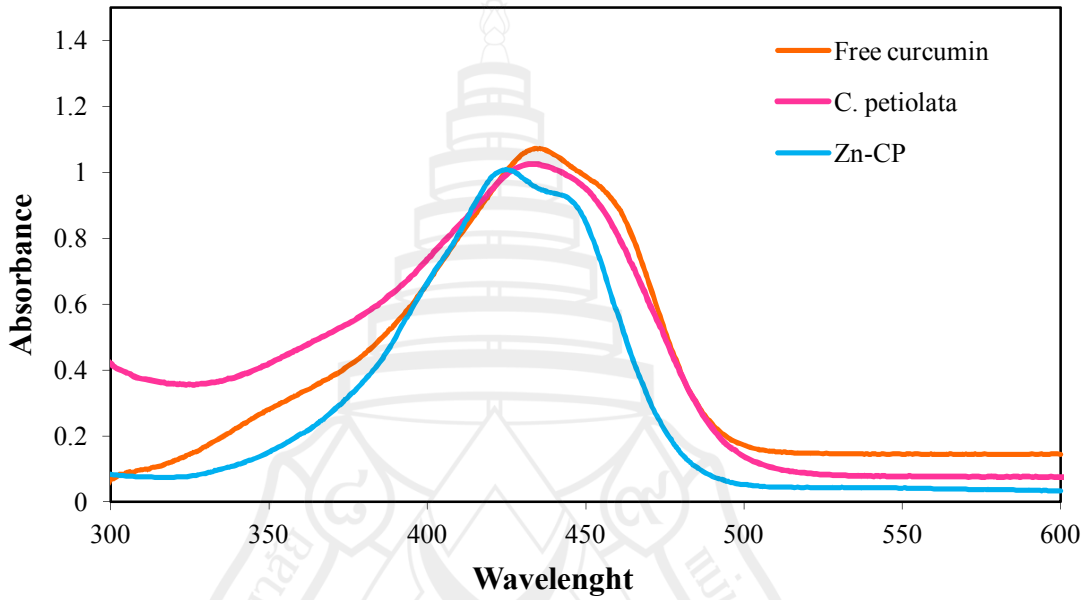


Figure 4.24 UV-Vis Spectra of CP Extract and Its Complexes

4.6.2 Infrared Spectra

IR spectrum of CP extract was resemble with curcumin which showed strong sharp O-H stretching (hydrogen-bonded enol form) at 3,491 and 3,435 cm^{-1}, and medium broad O-H stretching (the phenoxyl group) in range 3,331 cm^{-1}. The strong sharp O-H stretching was disappeared and the intensity of O-H stretching was decreased in presence of metal. CP extract showed strong carbonyl bond (C=O) at 1,630, while Zn-CP showed shifted to lower wave number of carbonyl (C=O) bands indicated that the reaction between metal ion and β-diketone group of CP extract was occurred (Figure 4.25).

Table 4.7 Wavelength Changes from Infrared (KBr pellets) Spectral Data of *C. petiolata* Extract and Its Metal Complexes.

IR (cm^{-1})	Compound	
	CP extract	**Zn-CP extract complex**
$\nu_{(OH)enol}$	3,491, 3435 (s)	-
$\nu_{(OH)phenol}$	3,331 (br)	3,407 (br) ($\Delta\nu$ = 76)
$\nu_{(C=O)}$	1,631	-
	1,604	1,595 ($\Delta\nu$ = 9)
$\nu_{(C=C)\ aliphatic}$	1,513	1,509 ($\Delta\nu$ = 4)
$\nu_{(C=C)\ aromatic}$	1,431	1,418 ($\Delta\nu$ = 13)
$\delta_{(C-O)}$	1,287	1,285 ($\Delta\nu$ = 2)

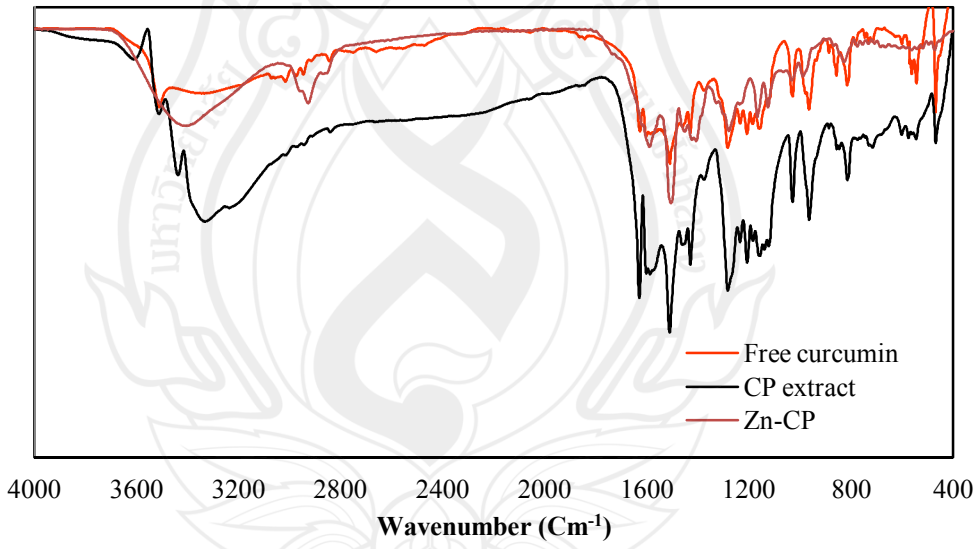


Figure 4.25 FT-IR Spectra of CP Extract and Zn-CP Extract Complex

4.7 Anti-oxidant and Anti-tyrosinase Activities of CP Extract and Its Metal Complex

4.7.1 Anti-oxidant Activities of CP Extract and Its Metal Complex

The anti-oxidant activities of CP and its Zn complex were determined by DPPH radical scavenging, ferrous reducing power and thiobarbituric acid reactive substance assays. Pure curcumin was used as standard (Table 4.9.).

The radical scavenging activity of CP extract was similar to that of pure curcumin (IC_{50} 12.463 and 13.237 μg/ml, respectively). The DPPH radical scavenging of CP extract was enhanced by complexing with Zn(II) (IC_{50} 9.334 μg/ml).

CP extract showed lower ferrous reducing power (0.762 mg AAE/mg sample) and lipid peroxidation (82.768 μg/ml) activities than that of pure curcumin (1.253 mg AAE/mg sample and 59.864 μg/ml, respectively). Zn-CP complex showed higher reducing power activity than that of CP extract with 1.124 mg AAE/mg sample. The inhibition of lipid peroxidation activity of Zn-CP was similar with CP extract with IC_{50} 85.444 μg/ml.

The higher DPPH radical scavenging and ferrous reducing power activities of Zn-CP complex might be due to the bonding with metal ions at *β*-diketone group gives ArO-H bond strength weaker, which make electron donating easily.

4.7.2 Anti-tyrosinase Activity of CP Extract and Its Metal Complex

The anti-tyrosinase activity of samples was determined by monophenolase assay. CP extract showed lower tyrosinase inhibition than free curcumin with IC_{50} 39.331 ug/ml as shown in Table 4.9. Zn-CP showed stronger inhibition of monophenolase than that of CP extract, and free curcumin (IC_{50} 11.218 μg/ml).

Table 4.8 The Anti-oxidant and Anti-tyrosinase Activities of CP Extract and Its Metal Complex

Sample	Anti-oxidant activities			Anti-tyrosinase activity (IC_{50} ug/ml)
	DPPH radical scavenging (IC_{50} ug/ml)	Reducing power (mg of AAE/mg sample)	Inhibition of lipid peroxidation activity (IC_{50} ug/ml)	
Free curcumin	12.463 ± 0.320^{b}	1.253 ± 0.013^{a}	59.864 ± 0.208^{a}	35.224 ± 0.112^{b}
CP extract	13.237 ± 0.128^{b}	0.762 ± 0.001^{b}	82.768 ± 0.121^{b}	39.331 ± 0.012^{c}
Zn-CP	9.334 ± 0.108^{a}	1.124 ± 0.005^{a}	85.444 ± 0.432^{b}	11.218 ± 0.044^{a}

Note. Differ letters in the same column indicate significant differences among means of treatments ($P < 0.05$)

4.8 Formulation of Whitening Cream

The preferable O/W base cream was prepared and performed the screening study. The base cream had appreciable texture, spreadability, thickness, and smoothness which showed no phase separation when centrifuged at 5,000 rpm for 30 minutes (Figure 4.27). The pH value of base cream was 5.85 which is suitable for skin. The 0.5 % w/w of free curcumin, CP extract, Zn-$(cur)_2$, and Zn-CP were added to base cream (Figure 4.28).

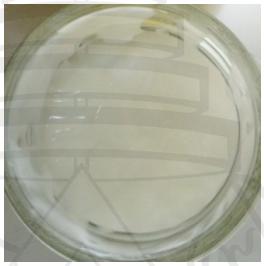


Figure 4.26 Physical Appearance of Emulsion Base

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

Note. (a) Free Curcumin, (b) CP Extract, (c) Zn-$(cur)_2$, d) Zn-CP

Figure 4.27 Physical Appearance of Whitening Cream

The pH value of formulas 1-3 was showed in Table 4.10. After free curcumin, CP extract, and metal derivatives were separately added to base cream, the pH value of products was slightly changed. Under centrifugation (3,000 rpm, 30 minutes), all of prepared products showed no phase separation indicate the stability of cream base.

Table 4.9 pH Value of Whitening Products

Formula	pH value
Base cream	5.85
F1 (free curcumin)	5.80
F2 (CP extract)	5.82
F3 (Zn-$(cur)_2$)	5.83
F4 (Zn-CP)	5.81

Then all cosmetic products were subjected to the stability test at five different conditions including; ambient temperature, high temperature (50 ^{o}C), low temperature (4 ^{o}C), heating-cooling cycle (50 ^{o}C for 24 hrs and 50 ^{o}C for 24 hrs, as one cycle), and light exposure for 1 month.

The physical properties of the cosmetic products including; appearance, viscosity, pH value, and color value were observed every week.

4.8.1 Physicochemical of Cosmetic Products

The texture and odor of all cosmetic products was unchanged in all storage condition as shown in Table 4.11.

Table 4.11 Physical Appearance of Whitening Cream

Formula/Conditions	Week 0	Week 4				
		Ambient temperature	High (50 °C)	Low (4 °C)	Heating-cooling	Light exposure
Base cream						
F1 (free curcumin)						
F2 (CP extract)						
F3 ($Zn\text{-}(cur)_2$)						
F4 (Zn-CP)						

4.8.2 pH Value

The pH values of the tested products over one month period are given in Table 4.12 and Figure 4.29-4.33. The pH value of base cream showed paltry alteration at all storage condition. The whitening cream containing free curcumin showed slightly change in pH at ambient temperature (Figure 4.29) and cooling condition (Figure 4.33). While, the pH value of products storage under light exposure, heating, heating-cooling conditions decreased might due to the degradation products of curcumin to ferulic acid. The pH value of CP extract cream over a one month period was similarly to free curcumin cream. The whitening cream containing metal derivatives showed slightly change in pH value at all storage conditions, which remained stable for the entire period. The percent change of pH value of whitening cream containing metal derivatives were lower than free curcumin and CP extract creams indicating the metal-curcumin complexes creams was more stable than free curcumin.

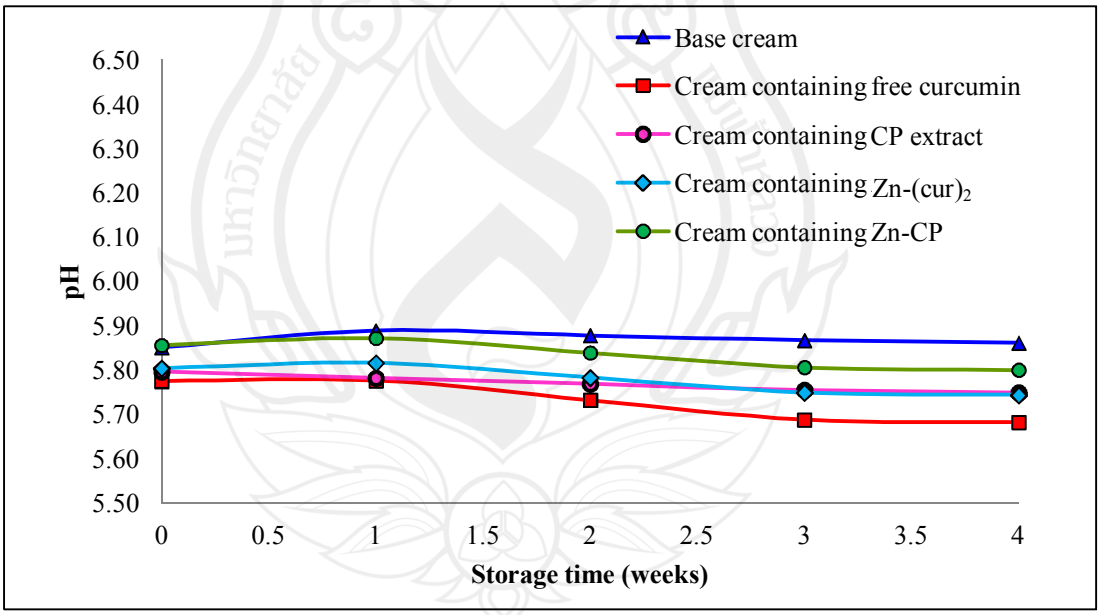


Figure 4.28 The pH Value of Product at Ambient Temperature Condition

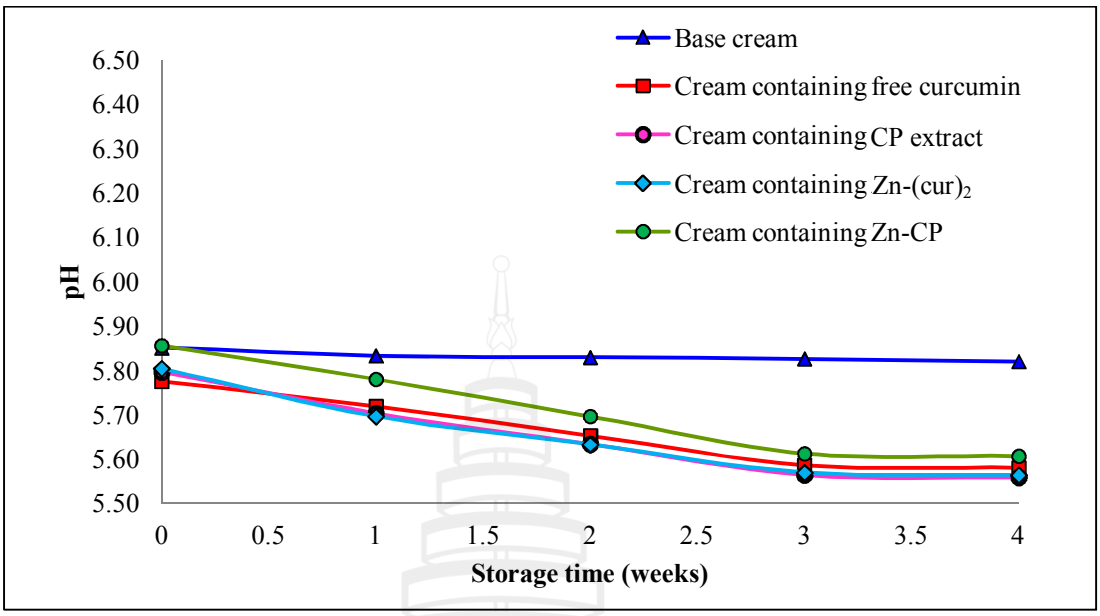


Figure 4.29 The pH Value of Product at Heating-cooling Condition

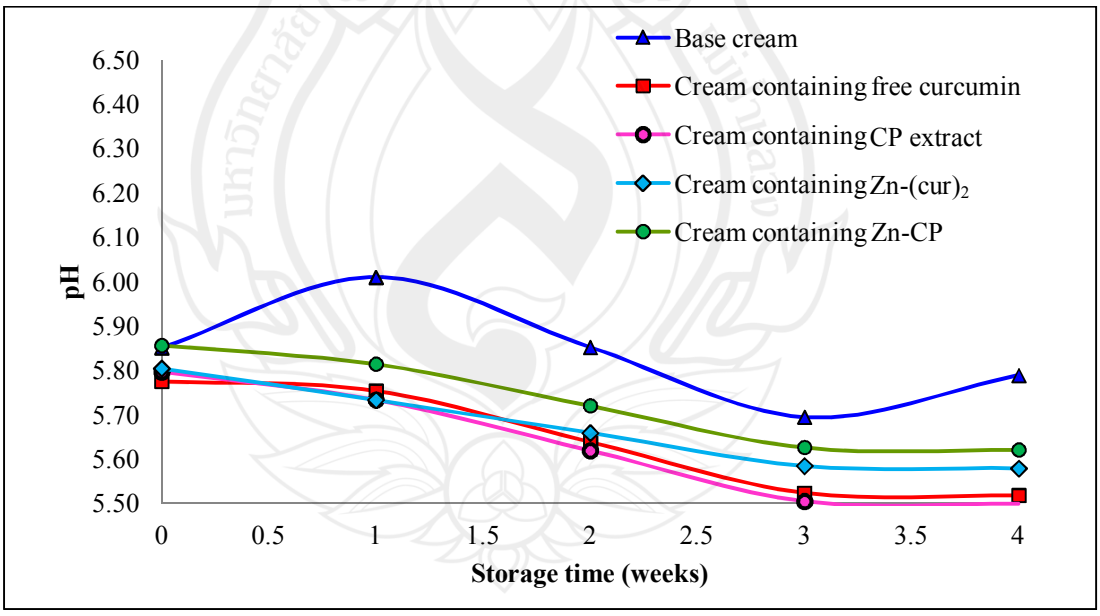


Figure 4.30 The pH Value of Product at High Temperature

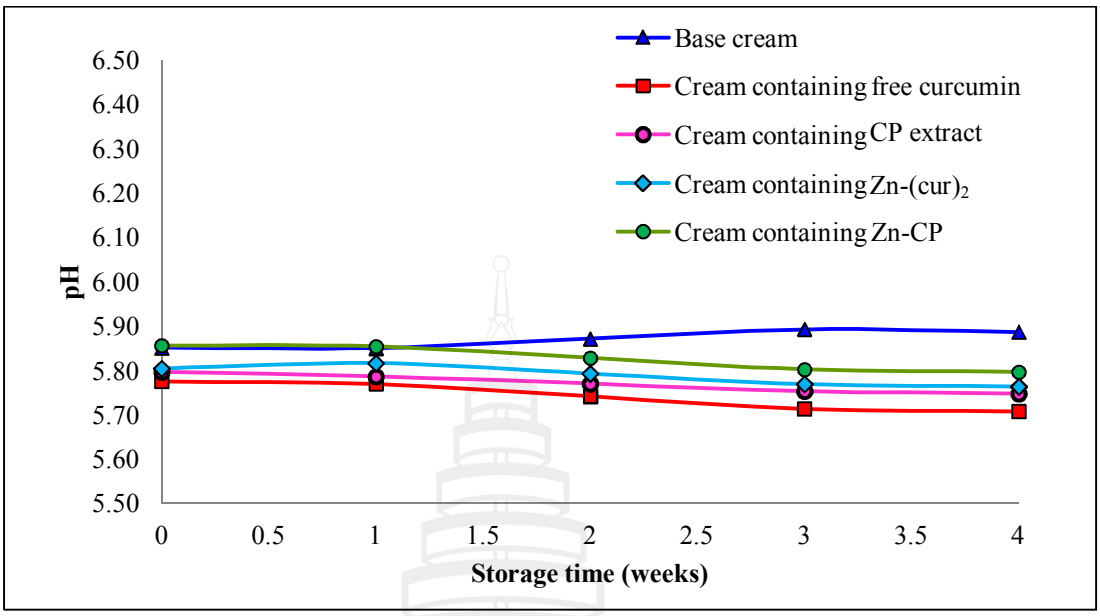


Figure 4.31 The pH Value of Product at Cool Temperature

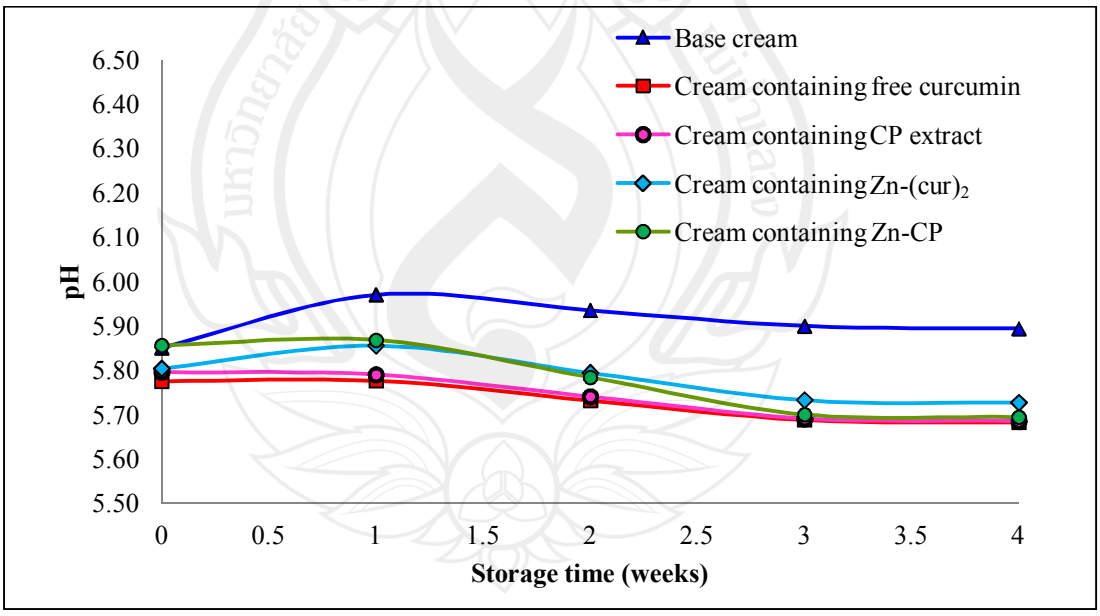


Figure 4.32 The pH Value of Product at Light Exposure Condition

Table 4.11 pH Value of Whitening Cream

Formula/Conditions	Week 0	Week 4				
		Ambient temperature	Heating (50 ^{o}C)	Cooling (4 ^{o}C)	Heating-cooling	Light exposure
Base cream	5.85	5.86	5.79	5.85	5.82	5.79
F1 (free curcumin)	5.80	5.78	5.52	5.79	5.58	5.68
F2 (CP extract)	5.82	5.81	5.78	5.81	5.76	5.80
F3 (Zn-$(cur)_2$)	5.87	5.75	5.62	5.76	5.67	5.79
F4 (Zn-CP)	5.81	5.80	5.72	5.80	5.75	5.78

4.8.3 Viscosity

The viscosity of cosmetic products was measured once a week for one month period using a viscometer. The viscosity results of the cream over the period are shown in Figure 4.34-4.38 and Table 4.13. Within 2 weeks of storage, the viscosity of all products increase and was relatively constant after 3 weeks. Cream containing CP extract, Zn-$(cur)_2$, and Zn-CP showed the lower percent change of viscosity indicating the extract and metal-curcumin complexes creams was more stable than free curcumin.

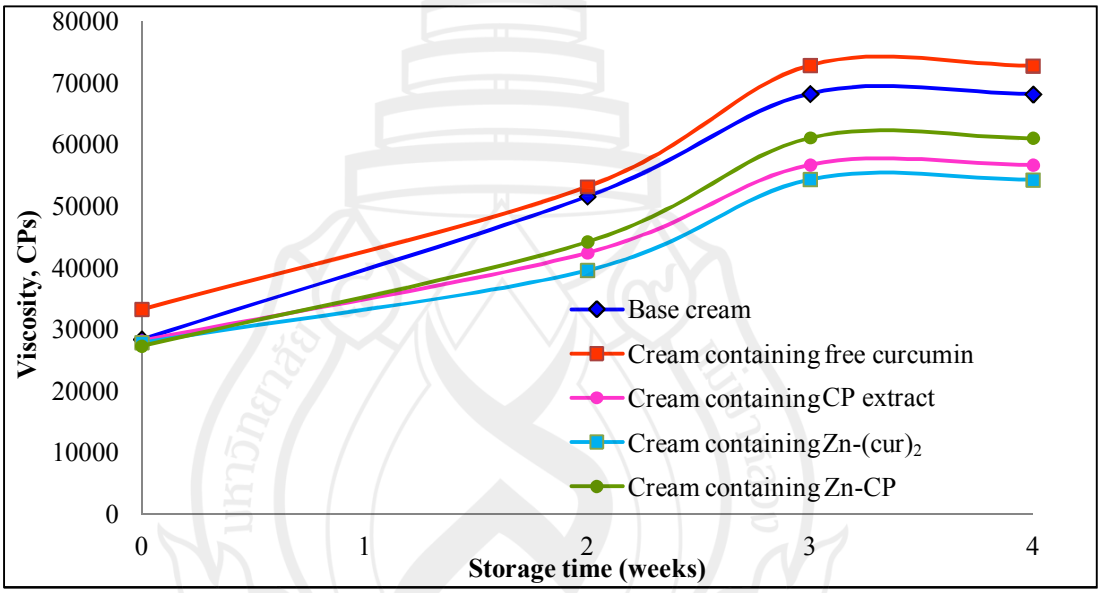


Figure 4.33 The Product's Viscosity at Ambient Temperature Condition

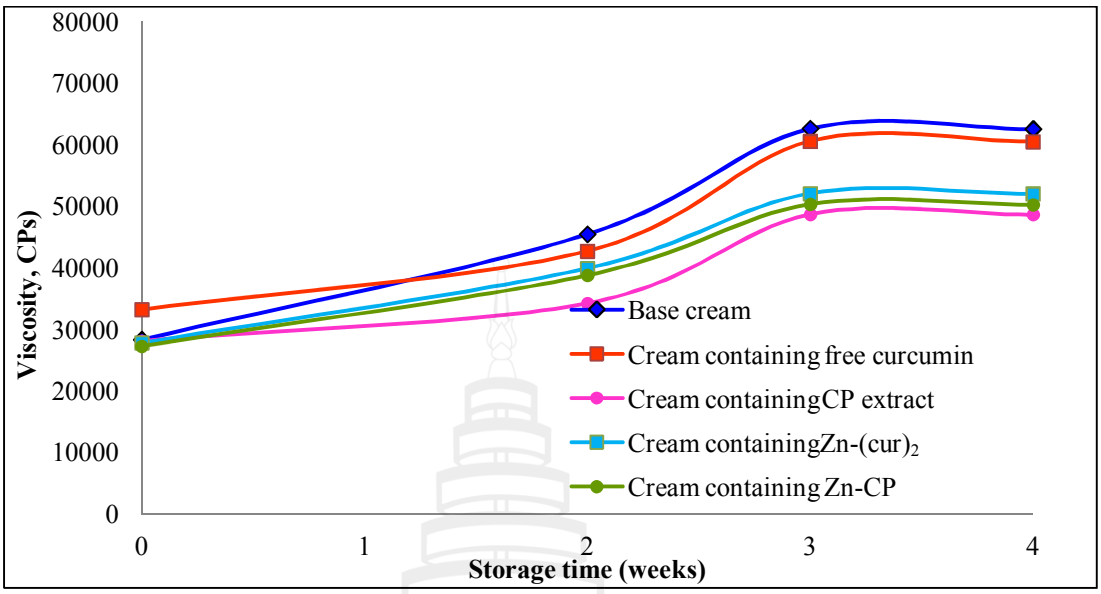


Figure 4.34 The Product's Viscosity at Heating-Cooling Condition

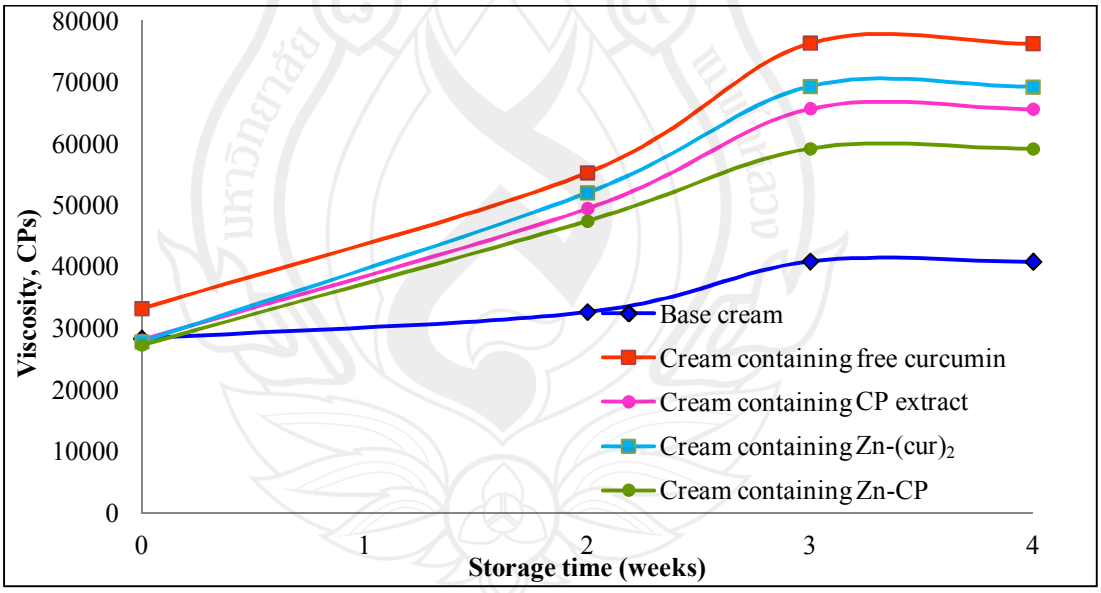


Figure 4.35 The Product's Viscosity at High Temperature

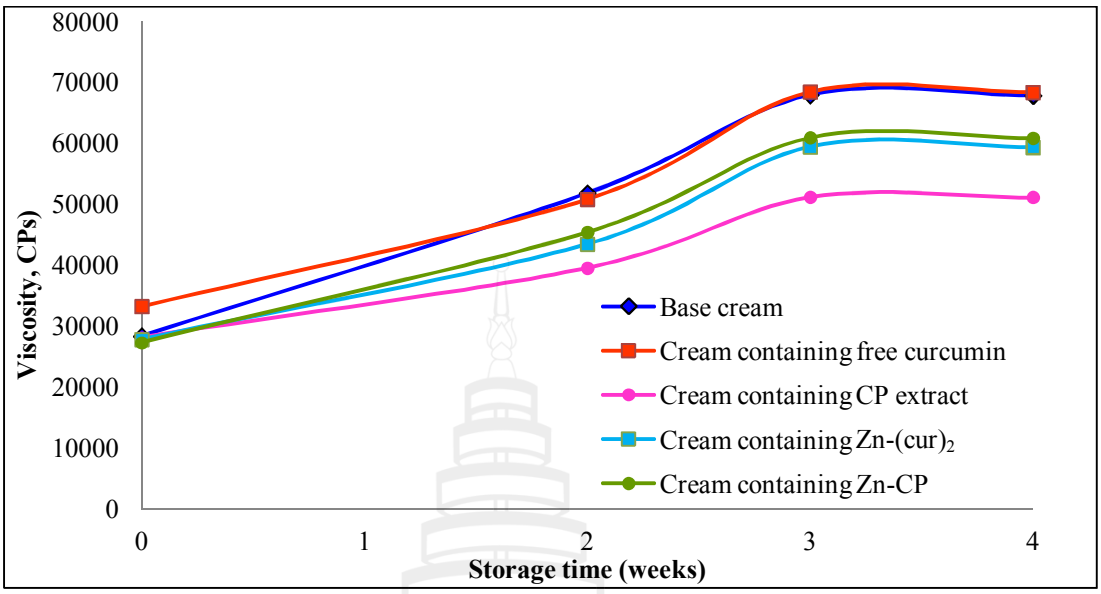


Figure 4.36 The Product's Viscosity at Cool Temperature

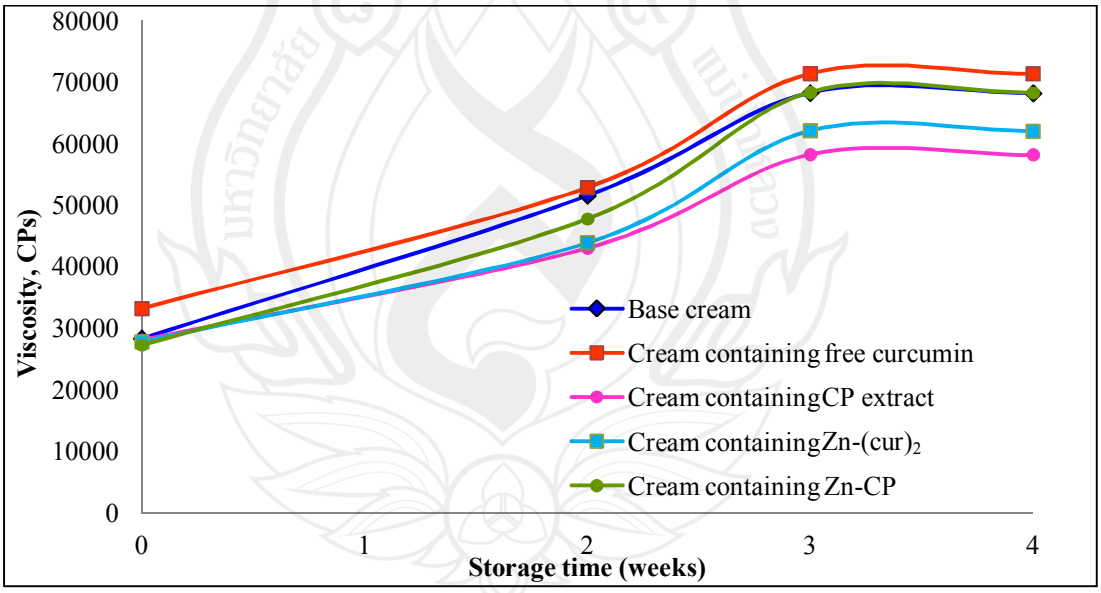


Figure 4.37 The Product's Viscosity at Light Exposure Condition

Table 4.12 Viscosity Value (cPs) of Whitening Cream

Formula/Conditions	Week 0	Week 4				
		Ambient temperature	Heating (50 ^{o}C)	Cooling (4 ^{o}C)	Heating-cooling	Light exposure
Base cream	28,360	68,240	69,920	60,880	62,640	68,240
F1 (free curcumin)	33,240	72,807	76,244	68,412	60,500	71,329
F2 (CP extract)	27,840	54,266	69,231	59,381	52,028	62,018
F3 (Zn-$(cur)_2$)	28,000	56,720	65,680	51,200	48,720	58,240
F4 (Zn-CP)	27,280	61,059	68,252	60,899	50,270	59,141

4.8.4 Color Value (L*,a*, b*)

The color value of cosmetic products was visually observed and also measured by a chromameter (Minolta CR-400) once a week for one month period. The results were recorded in L*a*b* values as shown in Figure 4.39-4.43.

The L* value describes the lightness of the product. All storage products showed decreasing in L* value over time. The results indicating that the products appeared darker.

The a* value is the color correlation between green and red where the negative values indicate green color while positive values indicate red color. The results showed all products at all storage conditions were paltry alteration changed of a* value. All of products are not changed by visually observed.

The b* value refers to blue and yellow color where negative values indicate blue and positive values indicate yellow. The b* values of the products at all storage condition showed decreasing in b* values indicating the increasing of yellow color.

From the color stability, cream containing free form of curcumin and metal complexes was change to darker and increasing of yellow color over time. The suitable condition of product storage was kept in dark bottle at room temperature and cool condition.

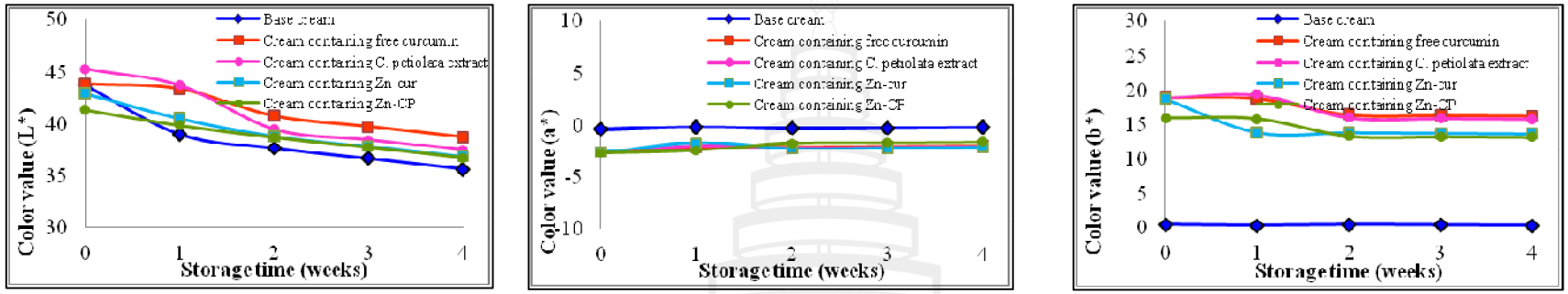


Figure 4.38 The color value of products storage at ambient temperature condition

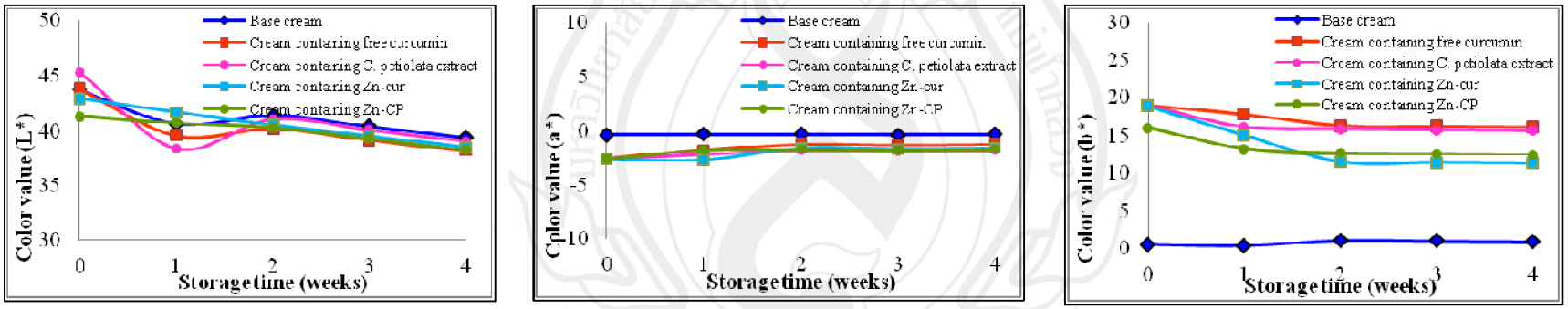


Figure 4.39 The color value of products at heating-cooling condition

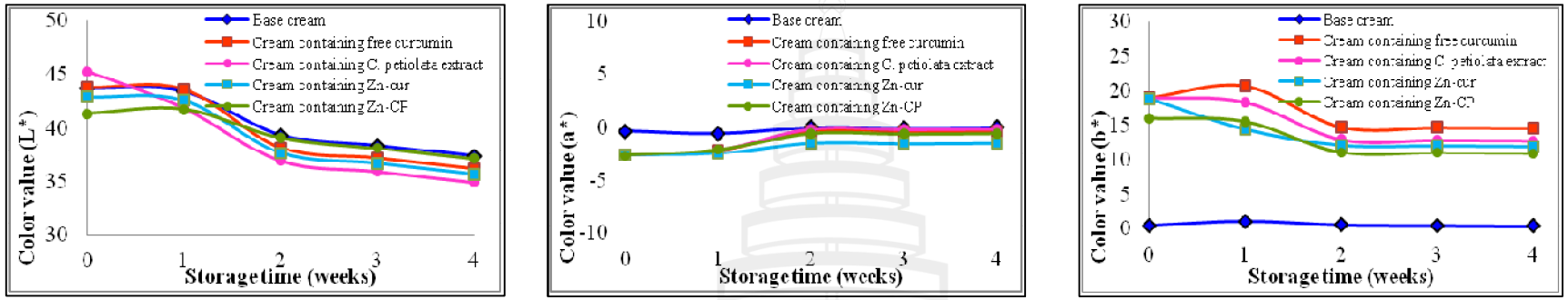


Figure 4.40 The color value of products storage at high temperature

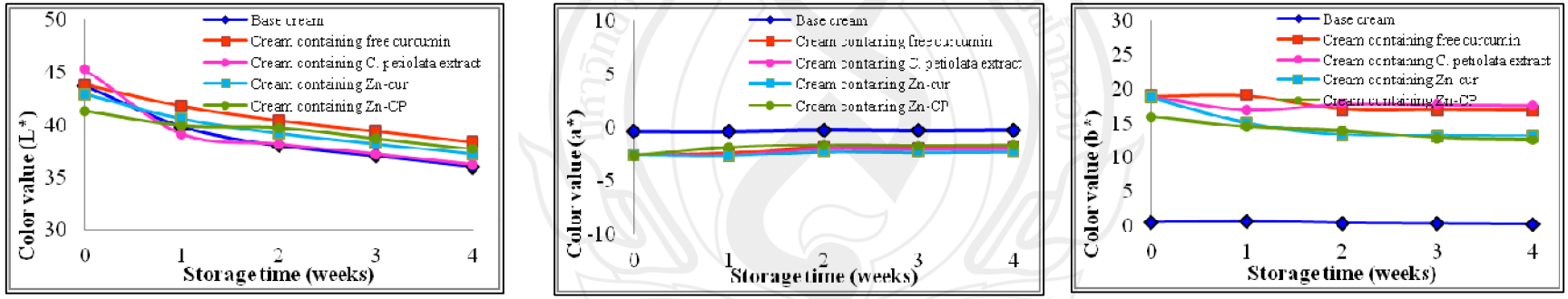


Figure 4.41 The color value of products storage at cool temperature

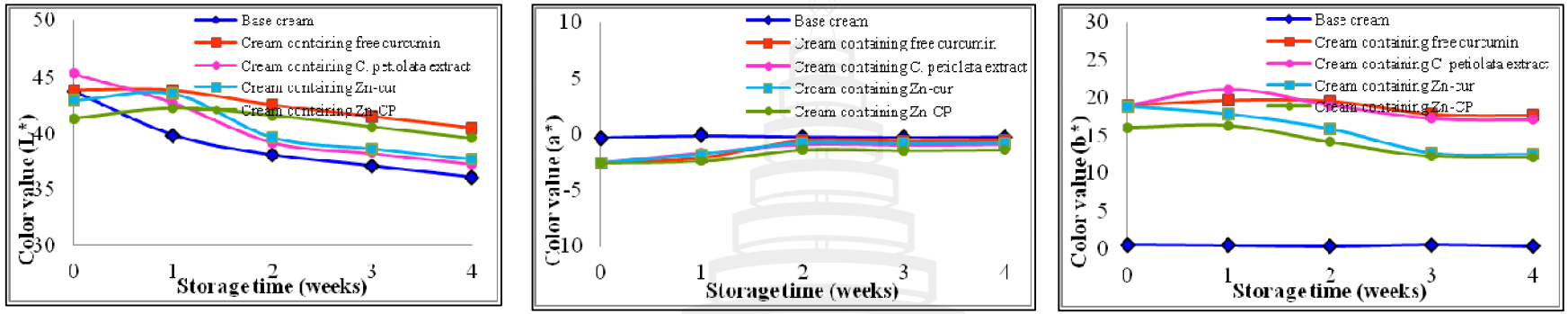


Figure 4.42 The color value of products storage at light exposure condition

4.8.5 Degradation of Active Ingredient

The stability of cream containing curcumin, CP extract, Zn-$(cur)_2$, and Zn-CP was investigated the chemical degradation in "*in vitro*" over 1 month period.

The active ingredients remains stable at all storage and metal derivatives showed more stable than their free form as shown in Figure 4.44-4.48.

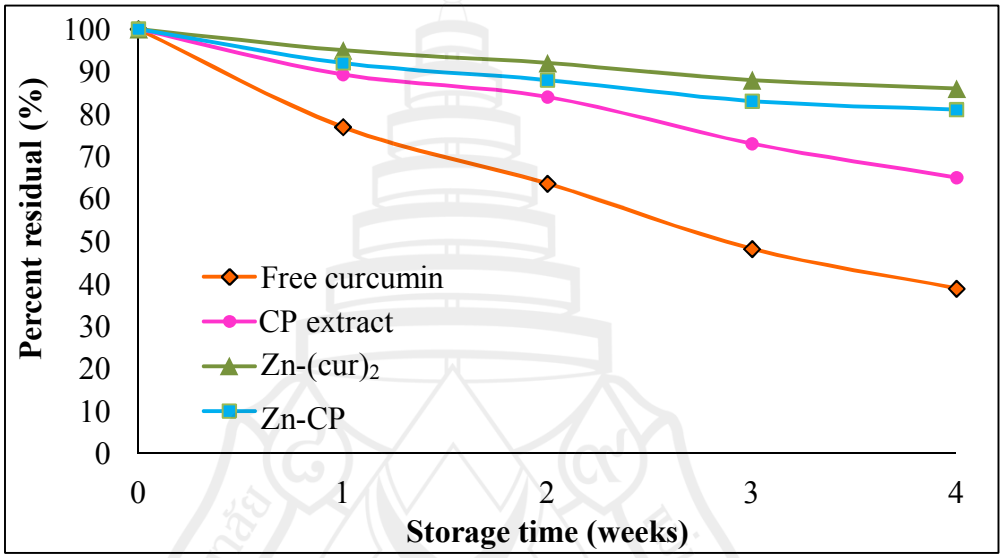


Figure 4.43 Degradation of Curcumin, CP Extract, Zn-$(cur)_2$, and Zn-CP in Cream at Ambient Temperature

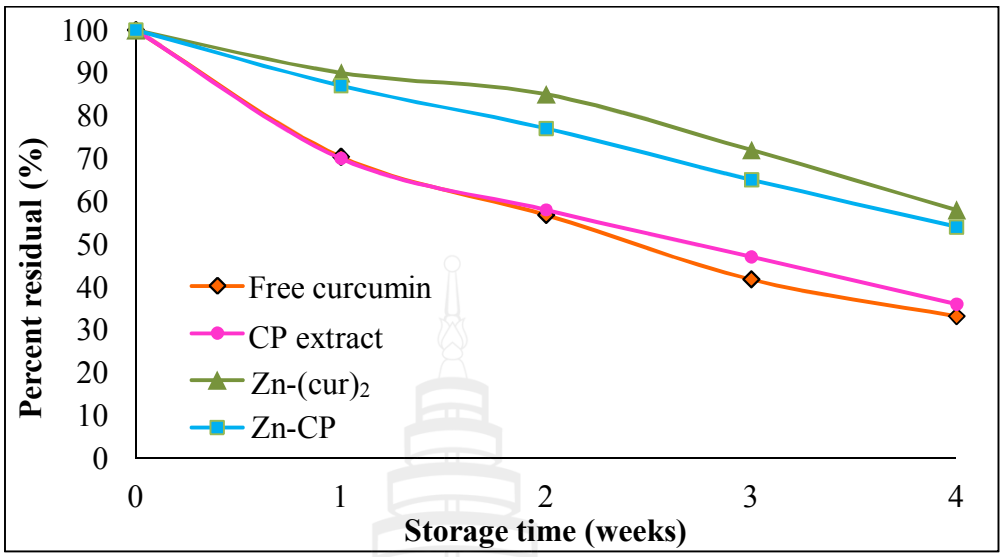


Figure 4.44 Degradation of Curcumin, CP Extract, Zn-$(cur)_2$, and Zn-CP in Cream at Heating-Cooling Condition

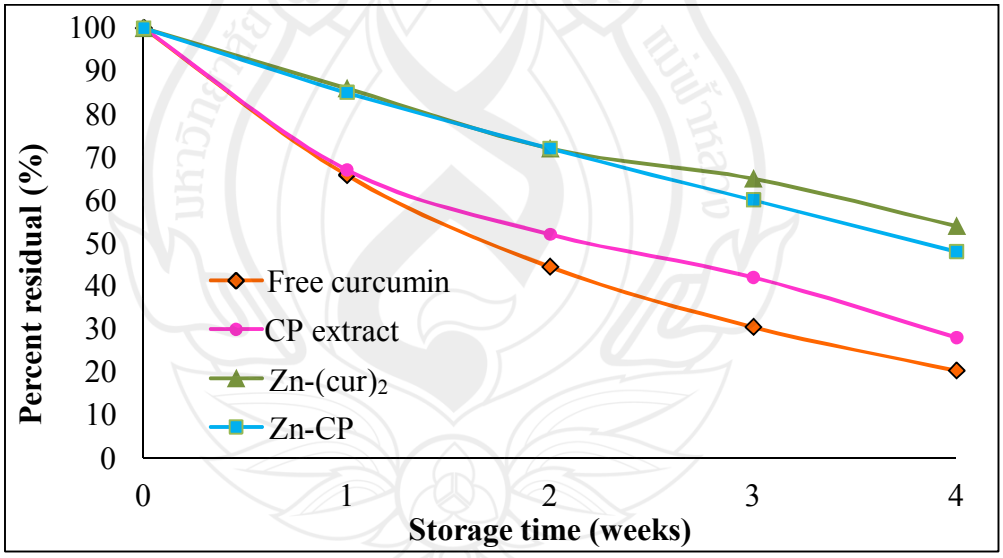


Figure 4.45 Degradation of Curcumin, CP Extract, Zn-$(cur)_2$, and Zn-CP in Cream at High Temperature

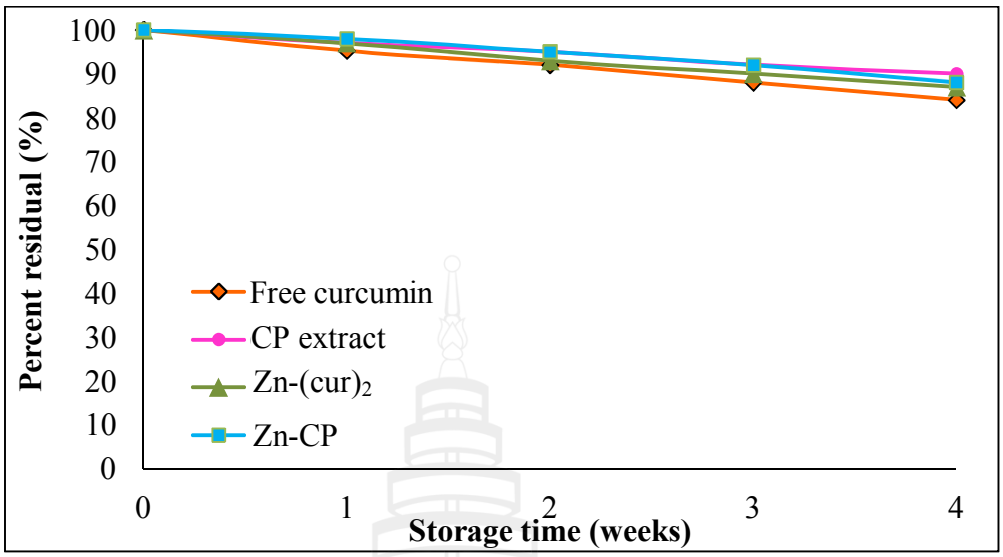


Figure 4.46 Degradation of Curcumin, CP Extract, Zn-$(cur)_2$, and Zn-CP in Cream at Cool Temperature

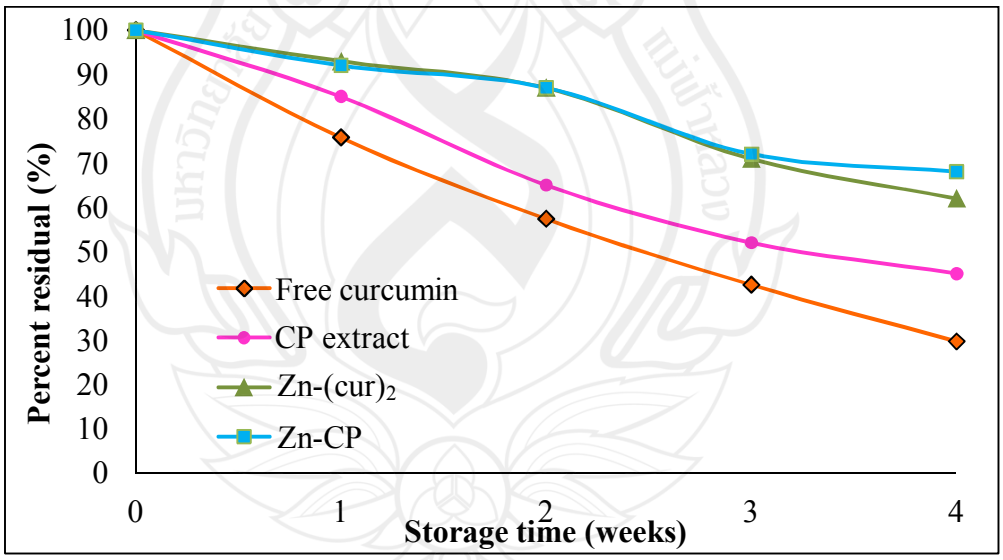


Figure 4.47 Degradation of Curcumin, CP Extract, Zn-$(cur)_2$, and Zn-CP in Cream at Light Exposure Condition

4.9 Safety Evaluation

4.9.1 Microbial Contamination Test

Mikrocount® combi test kit was used to determine the microbial contamination of the product. The result showed that the product had no colonies on both sides suggesting that the products have no microbial contamination.

4.9.2 Safety Evaluation

The safety of whitening cosmetics was evaluated using single closed patch test at 24 and 48 hrs. The M.I.I values of all volunteers were 0. Therefore, the product can be classified as non-irritation on the skin. The M.I.I value was mean irritation index which calculated from the patch test reaction scoring.

4.10 Whitening Efficacy Evaluation

The whitening efficacy of prepared creams (F1-F4) was evaluation on 10 volunteers. The whitening cream were applied twice daily on volunteer's forearm and the skin color was evaluated during treatment on day 7, 14, 21, and 28. The L-values reflected the skin color was measured with a Minolta Chromameter CR 300 in compliance with the Commission International de l'eclairage (CIE) system. The results were expressed as percent increase skin whitening. Already after 14 days of application a tremendous effect could be observed (Figure 4.48). Skin lightening was increased to 11.30, 10.11, 8.39, and 7.25 % for formulas F2 (Zn-$(cur)_2$), F4 (Zn-CP), F1 (curcumin) and F3 (CP extract), respectively, while after 28 days to 25.46, 22.57, 13.93, and 12.75%. These results show that the whitening process of application of free form creams, F2 and F3, is complete already after 14 days. While the continued application of metal complexes creams F2 (Zn-$(cur)_2$ and F4 (Zn-CP) showed further lighten the skin.

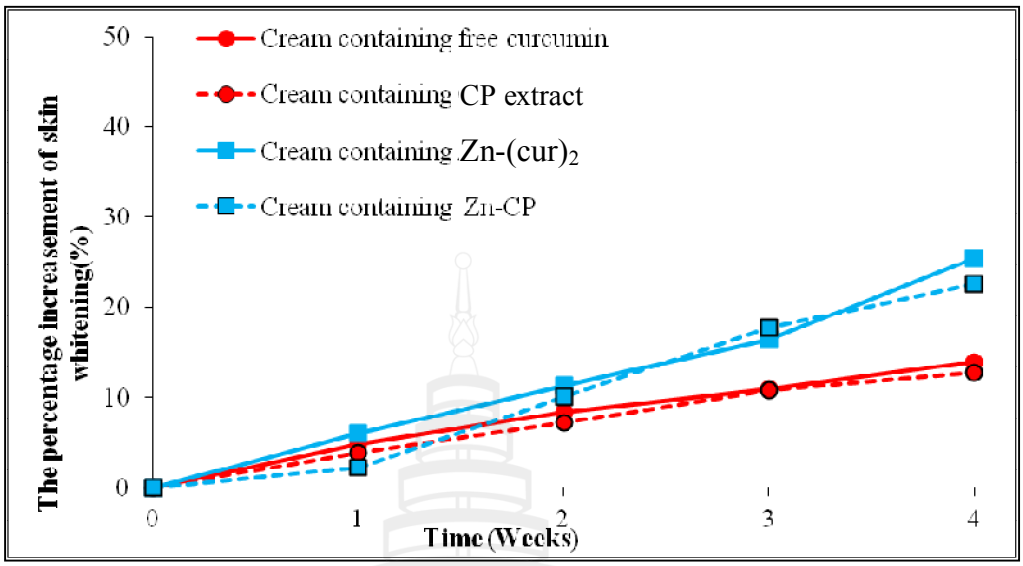


Figure 4.48 The Percentage Increasement of Skin Whitening

CHAPTER 5

CONCLUSION

Zn-$(cur)_2$, Mg-$(cur)_2$, and Cu-cur complexes were successfully prepared by reflux method and obtained as orange, dark yellow and dark gold powders. The mass spectrometer demonstrated that Cu (II) ion form mono-ligand complex whereas Zn (II) and Mg (II) form bi-ligand complexes. From the UV-Vis, IR, X-Ray powder diffraction, and thermal studies, the results indicating some interaction occurred between the metal and diketone group of curcumin. The effect of pH buffer solution (pH 3, 7 and 12), temperature (25, 37, 45, and 60^{o}C), and light exposure on the stability of free curcumin and its metal complexes was established. The results suggested that the stability of free curcumin was enhanced by bonding with metal. Cu (II) and Zn (II) enlarged the stability of curcumin at various buffer solutions. All metal raised the stability of curcumin at high temperature (45 and 60^{o}C) and light exposure. The DPPH radical scavenging, ferrous reducing power, and anti-tyrosinase activities of curcumin were enhanced by complexing with Zn (II) and Mg (II).

Zn (II) was chosen to complex with curcumin in CP extract. The Zn-CP was obtained as yellow powder. The results of UV, IR, and thermal analysis of CP extract showed curcumin characteristic. Zn-CP complex showed stronger DPPH radical scavenging, ferrous reducing power, and anti-tyrosinase activities than that of CP extract.

Then, free curcumin, CP extract, Zn-$(cur)_2$, and Zn-CP were incorporated in o/w base cream and evaluated for their stability, safety, and efficacy. In all storage condition including ambient temperature, low temperature, high temperature, heating-cooling cycle, dark and light exposure conditions showed that the texture and odor of all cosmetic products was not changed, while the pH and color value showed slightly change at all storages. The stability of all active ingredients in various storages remain stable which metal derivatives showed more stable than that of free curcumin and CP

extract. From microbial contamination and single closed patch test evaluation, the products were classified as safe due to no microbial contamination and non skin irritation. The evaluation of whitening efficacy of prepared creams showed that the application of metal complex creams F2 (Zn-$(cur)_2$ and F4 (Zn-CP) showed higher increment of skin whitening (25.46 and 22.57 %, respectively) than that of the free metal creams (curcumin, 13.93 %) and (CP extract, 12.75 %).

REFERENCES

REFERENCE

Afanas'eva, I. B., Ostrakhovitch, E. A., Mikhal'chik, E. V., Ibragimova, G. A., & Korkina, L. G. (2001). Enhancement of antioxidant and anti-inflammatory activities of bioflavonoid rutin by complexation with transition metals. **Biochemical Pharmacology, 61**(6), 677-684.

Aggarwal, B. B., Sundaram, C., & Malani, N. (2007). Curcumin: The Indian solid gold. **Advance in Botanical Reasearch, 595**, 1-75.

Ando, H., Funasaka, Y., Oka, M., Ohashi, A., Furumura, M., Matsunaga, J., Hearing, V. J., & Ichihashi, M. (1999). Possible involvement of proteolytic degradation of tyrosinase in the regulatory effect of fatty acids on melanogenesis. **Journal of Lipid Research, 40**, 1312–6.

Aparadh, V. T., Naik, V. V., & Karadge, B. A. (2012). Antioxidative propertile (TPC, DPPH, metal chelating ability, reducing power and TAC) with some Cleome species. **Annali Di Botanica, 2**, 49-56.

Artés, F., Castañer, M., & Gil, M.I. (1998). Review: enzymatic browning in minimally processed fruit and vegetables. **Journal of Agriculture & Food Chemistry, 4**, 377-389.

Barik, A., Mishra B., Kunwar, A., Kadam, R. M., Shen, L., Dutta, S., Padhye, S., Satpati, A. K., Zhang, H. Y., & Priyadarsini, K. I. (2007). Comparative study of copper(II)-curcumin complexes as superoxide dismutase mimics and free radical scavenger. **European Journal Medicinal Chemistry, 42**(4), 431-439.

Chang, T. S. (2009). An Updated Review of Tyrosinase Inhibitors. International **Journal of Molecular Science, 10**, 2440-2475

Contreras-Guzman, E., Strong, F. C., & da Silva, W. J. (1982). Fattyacid and vitamin E content of nutrimaiz, a sugary / opaque-2 corncultivar. Journal of **Agricutural Food and Chemistry, 30**, 1113–1117.

Daniel, S., Limson, J.L., Dairam, A., Watkins, JM., & Daya, S. (2004). Through metal binding, curcumin protects against lead-and cadmium-induced lipid peroxidation in rat brain homogenates and against lead-induced tissue damage in rat brain. **Journal of Inorganic Biochemistry, 98**(2), 266-275.

Ebanks, J. P., Wickett, R. R., & Boissy, R. E. (2009). Mechanisms regulating skin pigmentation: the rise and fall of complexion coloration**. Internation Journal of Molecule Science, 10**, 4066-4087.

Fore, J. (2006). A review of skin and the effects of aging on skin structure and function. **Ostomy Wound Manage, 52**(9), 24-35.

García-Molina, F., Muñoz, J.L., Varón, R., Rodríguez-López, J.N., García-Cánovas, F., & Tudela, J. (2007). A review on spectrophotometric methods for measuring the monophenolase and diphenolase activities of tyrosinase. **Journal of Agricultue & Food Chemistry, 55**, 9739-9749.

Hakozaki, T., Minwalla, L., Zhuang, J., Chloa, M., Matsubara, A., Miayamoto, K., Greatens, A., Hillebrand, G.G., Bissett, D.L., & Boissy, R.E. (2002). The effect of niaciamide on reducing cutaneous pigmentation and suppression of melanosome transfer. **Journal of dermatological, 147**, 20–31.

Halaban, R., Patton, R. S., Cheng E., Svedine, S., Trombetta, E.S., Wahl, M.L., Ariyan, S., & Hebert, D.N. (2002). Abnormal acidification of melanoma cells induces tyrosinase retention in the early secretory pathway. **Journal of Biological Chemistry, 277**, 14821-14828.

Halliwell, B. & Gutteridge, J.M.C. (1984). Oxygen toxicity, oxygen radicals, transition metals and disease. **Biochemical Journal, 218**, 1-14.

Hamid, A., Aiyelaagbe, O. O., Usman, L. A., Ameen, O. M., & Lawal1, A., (2010). Antioxidants: Its medicinal and pharmacological applications. **African Journal of Pure and Applied Chemistry, 4**(8), 142-151.

Hatcher, H., Planalp, R., Cho, J., Torti, F. M., & Torti, SV. (2008). Curcumin: from ancient medicine to current clinical trials. **Cellular Molecular Life Science 65**(11), 1631-1652.

Jara, J.R., Solano, F., Garcia-Borron, J.C., Aroca, P., & Lozano, J.A. (1990) Regulation of mammalian melanogenesis. II: The role of metal cations. **Biochim Biophys Acta, 1035**, 276–285.

Jasim, F., & Ali, F. (1989). Measurement of some spectrophotometric parameters of curcumin in 12 polar and nonpolar organic solvent. **Microchemical Journal, 39**, 156-159.

Jayaprakasha, G. K., Jagan, L., Rao, M., & Sakariah, K. K. (2006). Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxycurcumin. **Food Chemistry, 98**(4), 720-724.

Knight, J. A. (2000). Review: Free radicals, antioxidants, and the immune system. **Annals of Clinical & Laboratory Science, 30**(2), 145-158.

Li, S., Yuan, W., Deng, G., Wang, P., Yang, P., & Aggarwal, B.B. (2001). Chemical composition and product quality control of turmeric (*Curcuma longa* L.). **Pharmaceutical crops, 2**, 28-54

Mason, T. G., Wilking, J. N., Meleson, K., Chang, C. B., Graves, S. M., (2006). Nanoemulsions: formation, structure, and physical properties, **Journal of Physics: Condensed Matter, 18**(41), 635-666.

Miguel, M.G. (2010). Antioxidant activity of medicinal and aromatic plants. **Molecules, 25**, 291-312.

Muehlenbein, Micheal (2010). **Human Evolutionary Biology**. Cambridge University Press.

Newman, D. J., Cragg, G. M., & Snader, K. M. (2003). Natural Products as Sources of New Drugs over the Period 1981-2002**. Journal of Natural Product, 66**, 1022-1037.

Patcharee Khunlad, Yongyuth Tundulawessa, Thararat Supasiri & Waradoon Chutrtong (2008). Tyrosinase Inhibitory Activity of Curcuminoids from Powder of Turmeric (Curcuma longa Linn.) **Journal of SWU Science, 1**, 24.

Prasad, P. R. C., Reddy, C. S., Raza, S.H., & Dutt, C. B. S. (2008). Folklore medicinal plants of North Andaman Islands, **India. Fitoterapia, 79**(6), 458-464.

Puangpen Sirirugsa. (1966). **The genus *Curcuma* (Zingiberaceae) in Thailand**. Department of Biology, Faculty of Science Prince of Songkla University.

Roughley, P.J., & Whiting, D.A. (1973). Experiments in the biosynthesis of curcumin. **Journal of Chemical speciation, 20**, 2379-2388.

Seo, S.Y., Sharma, V.K., & Sharma, N. (2003). Mushroom tyrosinase: recent prospects. **Journal of Agricultue & Food Chemistry, 51**, 2837-2853.

Sheril, D., Limson, J.L., Amichand, D., Gareth, M.W., & Santy, D. (2004). Through metal binding, curcumin protects against lead- and cadmium-induced lipid peroxidation in rat brain homogenates and against lead-induced tissue damage in rat brain. **Journal of Inorganic Biochemistry, 98**, 266-275.

Sumanont Y, Murakami Y, Tohda M, Vajragupta O, Matsumoto K, & Watanabe H. (2004). Evaluation of the nitric oxide radical scavenging activity of manganese complexes of curcumin and its derivative. **Biological & Pharmaceutical Bulletin, 27**, 170–3.

Tonnesen, H.H., Masson, M., Loftsson, T. 2002. Studies of Curcumin and Curcuminoids XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability, **International Journal of Pharmacological 244**, 127–135.

Vajragupta, O., Boonchoong, P., Watanabe, H., Tohda, M., Kummasud, N., & Sumanont,Y. (2003). Manganese complexes of curcumin and its derivatives: Evaluation for the radical scavenging ability and neuroprotective activity. **Free Radical Biology & Medicine, 35**(12), 1632–1644.

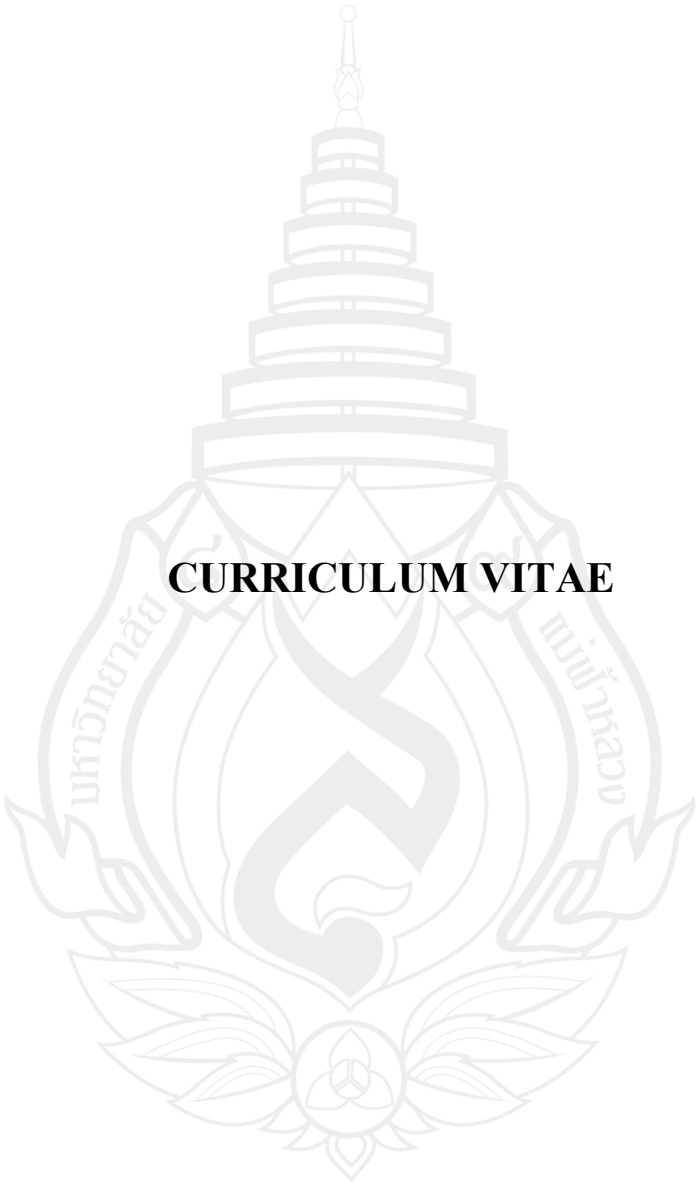
Vogel, H. A., Pelletier, J. (1815). Curcumin-biological and medicinal properties. **Journal of Pharmacol, 2**, 50–50.

Wang, Y.J., Pan, M.H., Cheng, A.L., Lin, L.I., Ho, Y.S., Hsieh, C.Y., & Lin, J.K. 1997. Stability of curcumin in buffer solutions and characterization of its degradation products. **Journal of Pharmacological Biomedical Analysis, 15**(12), 1867-1876.

Wilcox, D.E., Porras, A.G., Hwang, Y.T., Lerch, K., Winkler, M.E., & Solomon, E.I. (1985). Substrate analogue binding to the coupled binuclear copper active site in tyrosinase. **Journal of the American Chemical Society, 107**, 4015-4027.

Yoshimura, K., Tsukamoto, K., Okazaki, M., Virador, V. M., Lei, T. C., Suzuki, Y., Uchida, G., Kitano, Y., & Harii, K. (2001). Effects of alltrans retinoic acid on melanogenesis in pigmented skin equivalents and monolayer culture of melanocytes. **Journal of Dermatol ogical Science, 27**, 68–75.

Zebib, B., Mouloungui, Z., & Noirot, V. (2010). Stabilization of Curcumin by Complexation with Divalent Cations in Glycerol/Water System. **Bioinorganic Chemistry and Applications,** 1-8.



CURRICULUM VITAE

CURRICULUM VITAE

NAME Miss Anongnuch Thakam

DATE OF BIRTH 4 April 1987

ADDRESS 126/1 M.4 Maechan, Chiang Rai, Thailand 57240

EDUCATION BACKGROUD

2009 Bachelor Degree in Science (Cosmetic science)
Mae Fah Luang University, Thailand

PUBLICATIONS

Anongnuch Thakam & Nisakorn Saewan. (2012). Chemical Composition of Essential Oil and Antioxidant Activities of *Curcuma Petiolata* Roxb. Rhizomes. Advance **Material Research, 506**, 393-396.

INTERNATIONAL CONFERENCES

Anongnuch, Thakam., Nisakorn, Saewan., Krisada, Kittigowittana., & Ampa, Jimtaisong. (2012). Lightening effect of curcumin- Zn^{2+}complex cream. **Natural Products for Health and Beauty International Conference.**

Anongnuch, Thakam., Nisakorn, Saewan., Krisada, Kittigowittana., & Ampa, Jimtaisong. (2012). Antioxidant and antityrosinase activities of metal complexes of *Curcuma Petiolata* for cosmetic applications. **Mae Fah Luang University International Conference.**

Anongnuch, Thakam., & Nisakorn, Saewan. (2011). Antioxidant Activities of Curcumin-metal Complexes. **Thai Journal of Agricultural Science, 44 (5),** 188-193.

Anongnuch, Thakam., Nisakorn, Saewan., & Ampa, Jimtaisong. (2012). Anti-oxidant and anti-tyrosinase activities of curcumin-metal complexes**. International Federation of Societies of Cosmetic Chemists Conference.**

Nisakorn, Saewan., Anongnuch, Thakam., & Sorwaporn, Koysomboon. (2012). Inhibition effect of *Blumea Balsamifera* DC flavonoids on monophenolase and diphenolase activities of tyrosinase. **Pure and Applied Chemistry International Conference.**