การตั้งตำรับครีมบำรุงผิวหน้าที่มีสิ่งสกัดใบดาวอินคา *Plukenetia volubilis* และการประเมินฤทธิ์ยับยั้งไทโรซิเนสและฤทธิ์ต้านอนุมูลอิสระ

นางสาวทัศนียา ชื่นชอบ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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FORMULATION OF FACIAL CREAM CONTAINING SACHA INCHI *Plukenetia volubilis* LEAF EXTRACT AND EVALUATION OF ITS TYROSINASE INHIBITORY AND ANTIOXIDANT ACTIVITIES

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ทัศนียา ชื่นชอบ : การตั้งตำรับครีมบำรุงผิวหน้าที่มีสิ่งสกัดใบดาวอินคา *Plukenetia volubilis* และการประเมินฤทธิ์ยับยั้งไทโรซิเนสและฤทธิ์ต้านอนุมูลอิสระ (FORMULATION OF FACIAL CREAM CONTAINING SACHA INCHI *Plukenetia volubilis* LEAF EXTRACT AND EVALUATION OF ITS TYROSINASE INHIBITORY AND ANTIOXIDANT ACTIVITIES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วรินทร ชวศิริ, 61 หน้า.

งานวิจัยนี้มีจุดประสงค์เพื่อหาฤทธิ์ยับยั้งไทโรซิเนส ฤทธิ์ต้านอนุมูลอิสระ และพัฒนาผลิตภัณฑ์บำรุงผิวหน้าเพื่อผิวกระจ่างใสและผลิตภัณฑ์ต้านริ้วรอยจากสิ่งสกัดหยาบใบดาวอินคา

จากผลงานวิจัยพบว่าสิ่งสกัดหยาบเมทานอลปรากฏฤทธิ์ยับยั้งไทโรซิเนส (IC_{50} 34.55 µg/mL) ขณะที่สิ่งสกัดหยาบไดคลอโรมีเทนไม่แสดงการยับยั้งไทโรซิเนส ดังนั้นสิ่งสกัดหยาบเมทานอลจึงถูกเลือกไปทดสอบฤทธิ์ดักจับอนุมูลอิสระชนิด superoxide anion ด้วยวิธี photochemiluminescence (PCL) ผลการทดสอบพบว่าสิ่งสกัดหยาบเมทานอลมีความสามารถในการต้านอนุมูลอิสระเท่ากับ 16.1 µmoL สมมูลต่อวิตามินอี 1 g ของสิ่งสกัด และเท่ากับ 17.8 µmoL สมมูลต่อวิตามินซี 1 g ของสิ่งสกัด ความเป็นพิษต่อเซลล์ของสิ่งสกัดหยาบเมทานอลถูกทดสอบในเซลล์ไฟโบรบลาสต์ปกติของหนู (L929) และเซลล์มะเร็งตับจากมนุษย์ (HepG2) ตามลำดับ ซึ่งปรากฏค่า IC_{50} ต่อเซลล์ L929 ที่ 1,641 µg/mL และค่า IC_{50} ต่อเซลล์ HepG2 เท่ากับ 358 µg/mL ครีมจำนวน 4 ตำรับ ถูกเตรียมในรูปแบบอิมัลชันชนิดน้ำมันในน้ำที่มีสิ่งสกัดหยาบเมทานอล การประเมินทางประสาทสัมผัสในพบว่าอาสาสมัครมากกว่าร้อยละ 90 ให้การยอมรับในตำรับที่ 3 ในด้านสี ลักษณะเนื้อ ความสามารถกระจายบนผิว ความเหนอะ และ ความพึงพอใจโดยรวม ผลทดสอบฤทธิ์ทางชีวภาพของครีมตำรับที่ 3 พบว่ามีสมบัติการยับยั้งไทโรซิเนสและสมบัติการดักจับอนุมูลอิสระ นอกจากนี้ยังมีความคงตัวภายใต้ภาวะเร่งต่างๆการศึกษานี้แสดงให้เห็นประสิทธิภาพในการใช้สิ่งสกัดหยาบเมทานอลจากใบดาวอินคาเป็นสารออกฤทธิ์ในผลิตภัณฑ์เครื่องสำอาง

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The purposes of this research were to explore tyrosinase inhibitory and anti-oxidant activities of Sacha Inchi leaf, and to develop skin lightening and anti-aging product for a facial cream from the crude extract of Sacha Inchi leaf.

From the results obtained of this research, methanolic crude extract showed potent tyrosinase inbibition activity (IC_{50} 34.55 µg/mL) whereas dichloromethane crude extract did not inhibit the tyrosinase activity. Therefore, the methanolic crude extract was selected for further studies on superoxide anion radicals scavenging capacity using photochemiluminescence (PCL) assay. In the result, antioxidant capacity of the crude methanolic extract was 16.1 µmol TE/g extract and 17.8 µmoL AE/g extract, cytotoxic effect of methanolic crude extract was examined on mice fibroblast normal (L929) and Hepatocellular carcinoma (HepG2) cell lines, respectively. The IC_{50} of the methanolic crude extract exhibited on L929 cells showed at 1,641 µg/mL and HepG2 cells was 358 µg/mL. Four formulations were prepared in the form of oil-in-water emulsion containing the crude metanolic extract. The sensory evaluation, more than 90% of volunteers were accepted in formulation 3, with color, texture, spreadability, stickiness and overall of product. In the biological activities of formulated cream, the result revealed that methanolic extract showed tyrosinase inhibition and radicals scavenging properties. In addition, the formulation 3 was stable under accelerated conditions. This study suggests a potential use of the methanolic crude extract from Sacha Inchi leaf as an active ingredient in cosmetic products.

Field of Study: Biotechnology Student's Signature .....

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CONTENTS

Page

THAI ABSTRACT iv

ENGLISH ABSTRACT v

ACKNOWLEDGEMENTS vi

CONTENTS vii

LIST OF TABLES x

LIST OF FIGURES xii

LIST OF SCHEMES xiii

LIST OF ABBREVIATIONS xiv

CHAPTER I INTRODUCTION 1

1.1 Statement and significance of problem 1

1.2 Melanogenesis 2

1.3 Premature skin aging 3

1.4 Polyphenols 4

1.5 Sacha Inchi 7

1.6 Facial cream 9

1.7 Stability of cosmetic emulsion 11

1.8 Literature reviews of bioactivity effect of some cream containing plant extracts. 12

1.9 Objectives of this research 14

CHAPTER II MATERIALS AND METHODS 15

2.1 Chemicals and solvents 15

2.2 Instruments and equipments 17

2.3 Extraction of Sacha Inchi 19

Page

2.4 Biological activity tests 20
2.4.1 Determination of tyrosinase inhibitory activity 20
2.4.2 Determination of superoxide anion radicals scavenging capacity by photochemiluminescence (PCL) assay 20
2.4.3 Cytotoxicity test 21
2.4.3.1 Cell culture and preparation 21
2.4.3.2 Cell treatment 21
2.4.3.3 Cytotoxicity qualitative evaluation 21
2.4.3.5 Cytotoxicity quantitative evaluation by WST-1 assay 21
2.5 Formulation of emulsion base 23
2.6 Sensory assessment 24
2.7 Evaluations of selected facial cream containing the crude extract 24
2.7.1 Determination of tyrosinase inhibitory and antioxidant effects of selected facial cream containing the crude extract 24
2.7.2 Accelerated stability test of facial cream 25
CHAPTER III RESULTS AND DISCUSSIONS 26
3.1 The extraction of Sacha Inchi 26
3.2 Biological activity tests 27
3.2.1 Determination of tyrosinase inhibitory activity 27
3.2.2 Determination of superoxide anion radicals scavenging capacity 30
3.2.3 Cytotoxicity test 33
3.2.3.1 Cytotoxicity qualitative evaluation 33
3.2.3.2 Cytotoxicity quantitative evaluation by WST-1 assay 36
3.3 Formulation of facial creams 39

Page

3.4 Sensory assessment..... 42
3.5 Evaluations of selected facial cream containing the crude extract..... 44
3.5.1 Determination of tyrosinase inhibitory and antioxidant effects of selected facial cream containing the crude extract..... 44
3.5.2 Accelerated stability test..... 44
CHAPTER V CONCLUSION..... 48
REFERENCES..... 49
APPENDIX..... 55
VITA..... 61

LIST OF TABLES

Page

Table 2.1 List of chemicals and solvents 15

Table 2.2 Instruments and equipments 17

Table 2.3 Four formulations of O/W facial cream 23

Table 3.1 Weight and %yield of the crude extracts of SI leaf 26

Table 3.2 Tyrosinase inhibition activity of plant extracts and kojic acid 29

Table 3.3 Superoxide anion radicals scavenging capacity of plant extracts in ACL system 31

Table 3.4 Superoxide anion radicals scavenging capacity of plant extracts in ACW system 32

Table 3.5 Cytotoxicity of the CH_3OH crude extract from SI leaf against L929 cell lines by WST-1 assay 37

Table 3.6 Cytotoxicity of the CH_3OH crude extract from SI leaf against HepG2 cell lines by WST-1 assay 38

Table 3.7 Physicochemical of formulated facial creams containing CH_3OH crude extract from SI leaf 41

Table 3.8 The satisfaction point of the volunteers 42

Table 3.9 Physical characteristics of emulsion base kept at 4 and 45°C for 1 month 45

Table 3.10 Physical characteristics of formulation 3 kept at 4 and 45°C for 1 month 46

Table 3.11 Physical characteristics of emulsion base (B) and formulation (F3) creams of heating (45°C)/cooling (4°C) tests 47

Table A1 Qualitative morphological scoring of cytotoxicity of extracts 57

Table A2 Morphology score of L929 and HepG2 cells treated with SI leaf extracts and positive control 58

Table A3 Results of the volunteer satisfaction survey 59

LIST OF FIGURES

Page

Figure 1.1 Melanogenesis of melanocyte cell in skin....2

Figure 1.2 Synthesis of two melanin types and representation of the functions of major enzymes involved....3

Figure 1.3 Pathway of premature skin aging (+, induction; -, inhibition)....4

Figure 1.4 Chemical structures of some tyrosinase inhibitors....6

Figure 1.5 Sacha Inchi (*Plukenetia volubilis*)....8

Figure 1.6 Types of simple emulsion....10

Figure 1.7 The instability of emulsions....11

Figure 3.1 Characteristic of CH_2Cl_2 (A) and CH_3OH (B) crude extract from SI leaf....27

Figure 3.2 The morphology characteristics of L929 cells after treated with CH_3OH crude extract for 24 h. Untreated cell (A); 500 µg/mL concentration of the extract (B); 1,000 µg/mL concentration of the extract (C); 2,000 µg/mL concentration of the CH_3OH crude extract (D); compared with $ZnSo_4$ $7H_2O$ (positive control) at 5 mg/mL (E) and 10 mg/mL (F) concentration....34

Figure 3.3 The morphology characteristics of HepG2 cells after treated with CH_3OH crude extract for 24 h. Untreated cell (A); 500 µg/mL concentration of the extract (B); 1,000 µg/mL concentration of the extract (C); 2,000 µg/mL concentration of the CH_3OH crude extract (D); compared with $ZnSo_4$ $7H_2O$ (positive control) at 5 mg/mL (E) and 10 mg/mL (F) concentration....35

Figure 3.4 The formulated facial creams containing CH_3OH crude extract from SI leaf. F1=formulation 1(A); F2=formulation 2(B); F3=formulation 3(C) and F4=formulation 4(D)....40

Figure 3.5 Percentage of consumer acceptance of 4 product testers....43

Figure A1 Kojic acid standard curve....56

LIST OF SCHEMES

Page

Scheme 2.1 The extract procedure of Sacha Inchi leaf 19

LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percentage
µg	Microgram
µL	Microliter
µmol	Micromole
A	Absorbance
ACL	Antioxidant capacity of lipid soluble compound
ACW	Antioxidant capacity of water soluble compound
AEAC	Ascorbic Acid Equivalent Antioxidant Capacity
CH_3OH	Methanol
CH_2Cl_2	Dichloromethane
cm	Centimeter
et al.	And other
hr	Hour
HepG2	Human hepatocellular liver carcinoma cell line
g	Gram
IC50	Half maximal inhibitory concentration
L	Liter
L929	Mouse fibroblast cell line
M	Molar
min	Minute
mg	Milligram

mL	Milliliter
mm	Millimeter
mM	Millimolar
mmol	Millimole
nm	Nanometer
pH	A logarithmic measure of hydrogen ion concentration
PCL	Photochemiluminescence
S.D.	Standard deviation
TEAC	Trolox Equivalent Antioxidant Capacity
w/w	Weight by weight
w/v	Weight by volume
WST	Water soluble tetrazolium salts (2-(4-iodophenyl)-3-(4-nitro phenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt)

CHAPTER I
INTRODUCTION

1.1 Statement and significance of problem

Darkening and aging of the skin results from two main factors: intrinsic and extrinsic factors such as age, hormones, environment stress, detergents, pollution, smoke and exposure to ultraviolet (UV) light. UV exposure is a major causative factor in melanogenesis and certainly it is a critical factor in photoaging [1]. Recently, much attention has been widely studied on the use of natural substances for the development of skin care products, natural ingredients can lower skin allergy problems because they are easily absorbed by the superficial layers of the skin [2]. The sources of bioactive compounds are primarily plant polyphenols which may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks [3].

Sacha Inchi, the target of this work, is a South American perennial plant in Euphorbiaceae. Previous studies have shown that seeds are an excellent source of oil and protein. Some studies have been done using leaves in order to reduce triglycerides, high density lipoprotein and cholesterol from patients with postprandial lipemia and hypercholesterolemia [4]. In addition, leaves of this plant are reported to contain flavonoids, terpenoids, saponins, and other compounds responsible for the antiproliferative and antioxidant activities [5] However, there is no scientific report about the use of leaves for cosmetic. In recent times, Thailand have started to cultivate this plant. Mature leaves are an agricultural waste of the Sacha Inchi oil industry. The purposes of this research were to examine tyrosinase inhibitory and anti-oxidant activities of Sacha Inchi mature leaves, and to develop skin lightening and anti-aging product for facial cream from the crude extract of Sacha Inchi leaf.

1.2 Melanogenesis

Melanogenesis is a process that results in melanin formation and has many functions in living systems. Melanin is secreted and produced in melanosomes by melanocytes, which are distributed in the stratum basale of the dermis [6]. (**Figure 1.1**). Tyrosinase (EC 1.14.18.1) is a multifunctional, glycosylated, and copper-containing oxidase, which involved in melanin synthesis and catalyzes the oxidation process of tyrosine to dihydroxy-phenylalanine (DOPA) and from DOPA to DOPA quinone. Finally, eumelanin is formed through a series of oxidation reactions from dihydroxyindole (DHI) and dihydroxyindole-2-carboxylic acid (DHICA) from DHI and dihydroxyindole-2-carboxylic acid (DHICA), which are the products from dopachrome. In the presence of cysteine or glutathione, dopaquinone is converted to cysteinyldopa or glutathionyldopa. Subsequently, pheomelanin is formed (**Figure 1.2**) [7]. The role of melanin is to protect the skin against UV light damage by absorbing UV [8]. The regulation of tyrosinase activity has been considered as the most common strategy to achieve skin whiteness.

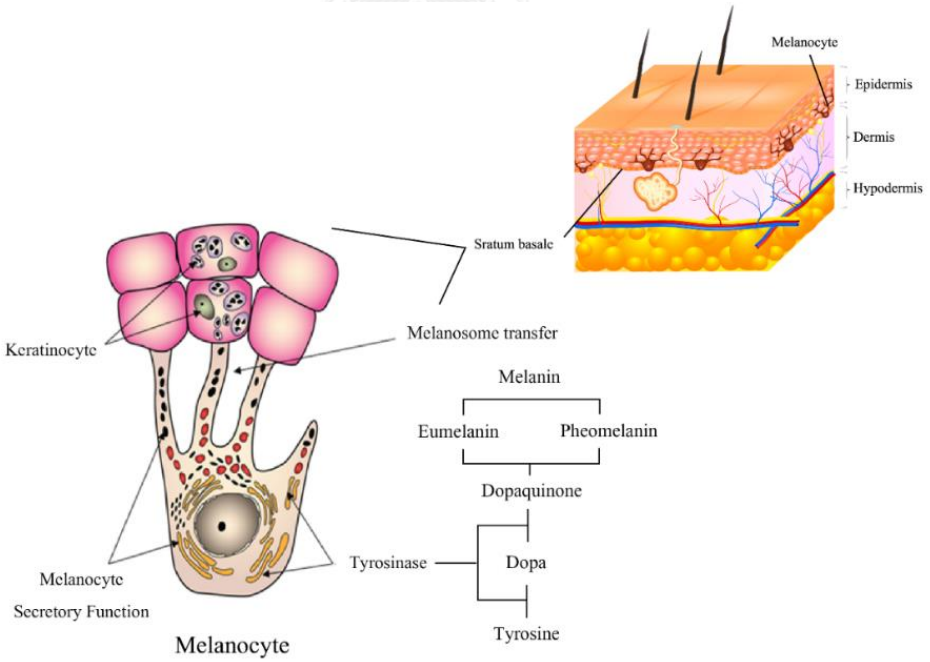


Figure 1.1 Melanogenesis of melanocyte cell in skin

(Modified from; http://www.dermamedics.com/hyperpigmentation_id60.html)

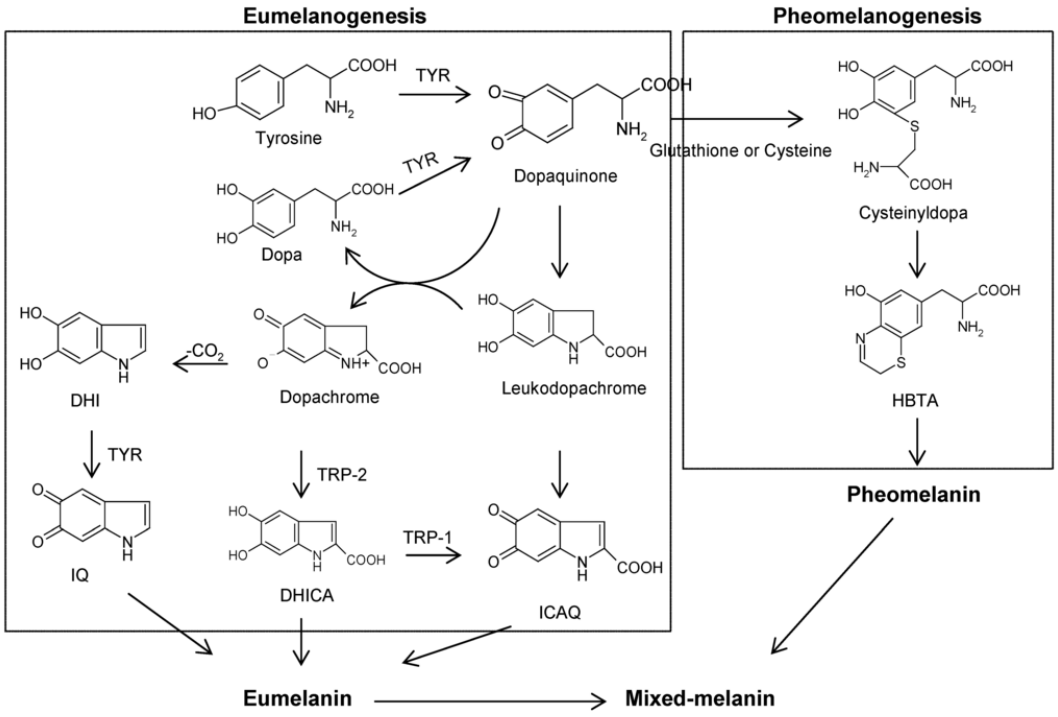


Figure 1.2 Synthesis of two melanin types and representation of the functions of major enzymes involved [5]

1.3 Premature skin aging

UV damage also underlies photoaging of the skin [1]. Photoaging is premature skin aging caused by continuous exposure of the skin to UV irradiation. UV is absorbed by skin molecules and generates reactive oxygen species (ROS) that causes "oxidative damage" to cellular components. ROS can be generated in the human body via many biochemical pathways. UV exposure induces a wound response with subsequent imperfect repair, leaving an invisible "solar scar," repetitive UV exposure eventually lead to development of visible "solar scar," manifesting as visible wrinkle over lifetime [2] **(Figure 1.3**). The progression occurs in the epidermal and dermal layers and is mainly related to extracellular matrix (ECM) degradation. The enzymes involved in ECM degradation are matrix metalloproteinases (MMPs) such as collagenase and elastase. Skin loses its tensile strength due to the effect of ECM degradation by MMPs. In this process, the wrinkling of skin occurs and roughness and dryness [3].

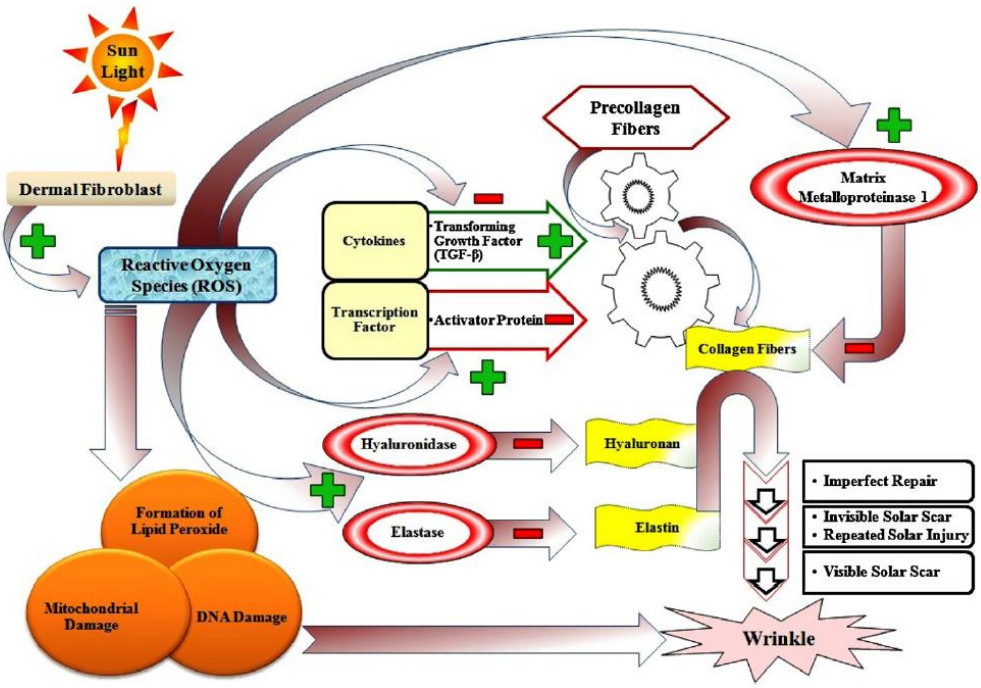


Figure 1.3 Pathway of premature skin aging (+, induction; -, inhibition) [2]

1.4 Polyphenols

Polyphenols were reported as the potent antioxidants and have been found to exhibit several beneficial effects such as antibacterial, antiviral, anti-inflammatory, antiallergic, anti-radical and anti-aging [9]. Polyphenols are a large class of chemical compounds containing multiple phenolic functionalities and are widely distributed in nature, and are antioxidant phytochemicals that tend to prevent or neutralize the damaging effects of free radicals [10]. Phytochemicals occurred naturally in plants are also the largest groups in tyrosinase inhibitors [11].

Flavonoids, the well-studied polyphenols, include flavonols, flavones, flavanones, flavanols, isoflavonoids, and chalcones. The structures of flavonoids are in principle compatible with the roles of both substrates and inhibitors of tyrosinase. In addition, flavonoids and other polyphenols, which were identified as tyrosinase inhibitors, contain stilbenes and coumarin derivatives.

Many flavonols have been isolated from plants. The inhibitory mode of flavonol inhibitors is usually competitive inhibition through the oxidation of L-dopa by tyrosinase and 3-hydroxy-4-keto moiety of flavonol structure acts as copper chelator.

Flavones, flavanones, and flavanols were identified as natural tyrosinase inhibitors including oxyresveratrol, norartocarpetin, artocarpetin, streppogenin-nobiletin, dihydromorin and taxifolin. The root and seed extracts of *Glycyrrhiza* species are effective for skin-whitening agents in East Asian countries. Isoflavonoids exhibited mainly melanogenesis inhibitory activity in the extracts. Isoflavans were also identified as potent tyrosinase inhibitors.

Chalcones consist of two aromatic rings in trans configuration, separated by three carbon atoms, of which two are connected by a double bond and the third is a carbonyl group. Some natural prenylated chalcones showed potent tyrosinase inhibitory activity. Chalcones derivatives, including licuraside, isoliquiritin, and licochalcone A were competitively inhibited the monophenolase activity of mushroom tyrosinase. In addition, the 4-resorcinol moiety in the chalcone structure is the key substituted group in exerting potent inhibitory activity, and it plays an important role in the inhibition of tyrosinase activity not only in chalcones but also in other flavonoid structures. For *N*-benzylbenzamide, an analogous to that of chalcone, the inhibitory activities of 3,5,2',4'-tetrahydroxyl, 2,4,2',4'-tetrahydroxyl, 3,5,4'-trihydroxyl and 2,4,4'-trihydroxyl substitutions were addressed (**Figure 1.4**) [7].

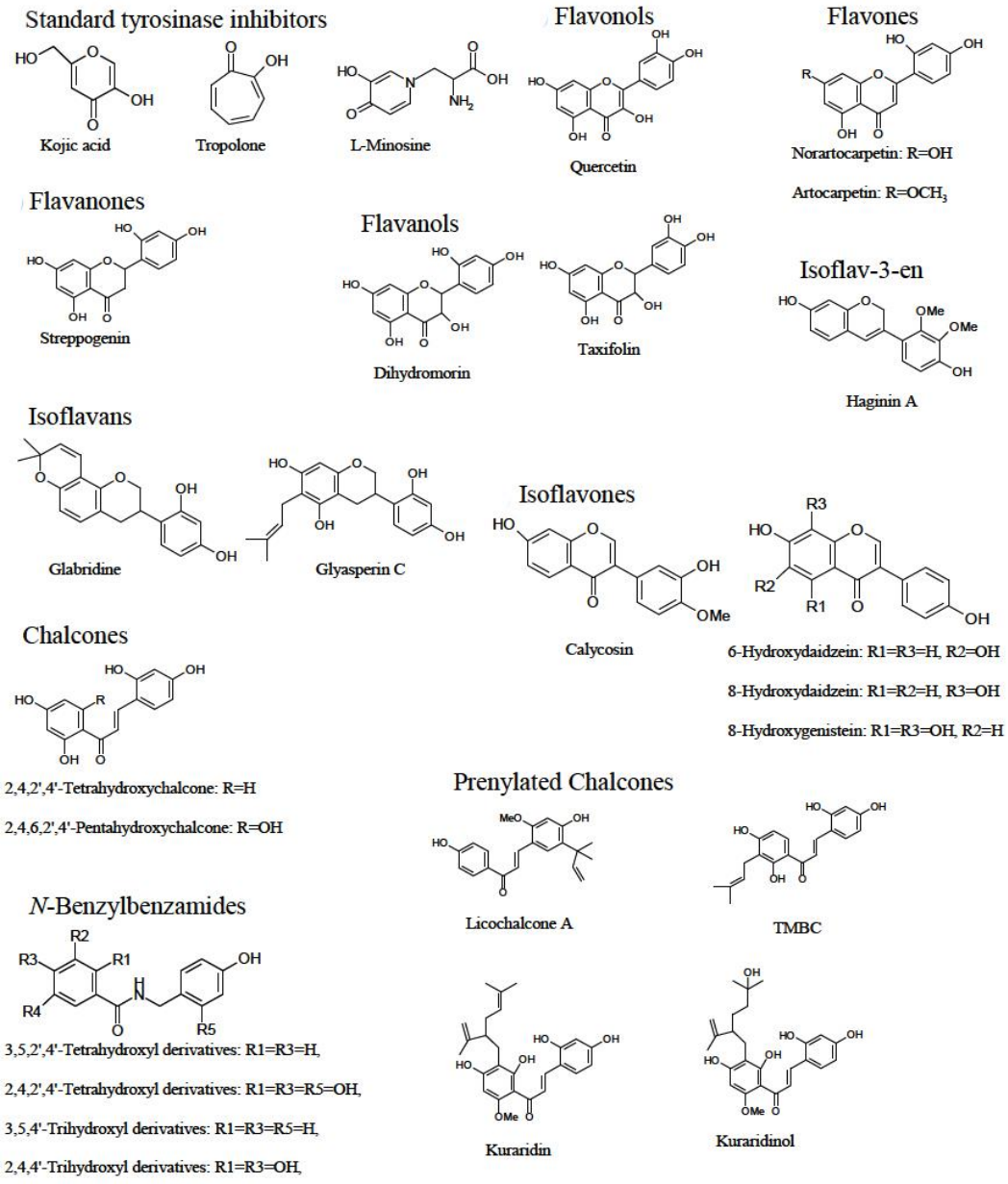


Figure 1.4 Chemical structures of some tyrosinase inhibitors [7]

The antioxidant properties of polyphenols have been widely studied, the chemical structure of polyphenolic compounds act as antioxidants and free radical scavengers. Tea (*Camellia sinensis* L.) is one of the best sources of phenolic antioxidants especially green tea and white tea and possess potent antioxidant and anti-tyrosinase properties [12]. The major polyphenols in tea are flavan-3 - ols, particularly epigallocatechin-3 - gallate (EGCG), epigallocatechin, epicatechin and epicatechin-3-gallate [13]. Beside tea, Grape (*Vitis vinifera* L.) is an excellent source of polyphenols [14]. The antioxidant activity of grapes depended on the content of anthocyanins and flavonoids increases with increasing color intensity of grapes [15]. Thus, antioxidant activity of plant extracts often correlates with the total phenolic content [16].

1.5 Sacha Inchi

Family: Euphorbiaceae

Subfamily: Acalyphoideae

Tribe: Plukenetieae

Subtribe: Plukenetiinae

Genus: Plukenetia

Species: P. volubilis

Sacha Inchi (*Plukenetia volubilis*) known as “Inca peanut” [17] (**Figure 1.5)**, is a climbing shrub plant in Euphorbiaceae that founds mostly in the Amazon region [18]. Nowadays, it is cultivated commercially in South East Asia, most notably in Thailand, known as Dao-inka (Inka star). Euphorbiaceae is formed by more than 6,000 species with extreme diversity of secondary compounds produced [5]. The plant reaches a height of 2 m, with heart shaped, 10 to 12 cm long and 8 to 10 cm wide, that have petioles 2–6 cm long. The male flowers are small, white, and arranged in clusters. Two female flowers are located at the base of the inflorescence. In tropical locations it is often a vine requiring support and producing seeds nearly year-round. The fruits are

capsules of 3 to 5 cm in diameter with 4 to 7 points, are green and ripen blackish brown. On ripening, the fruits contain a soft black wet pulp that is messy and inedible, so are normally left to dry on the plant before harvest. By two years of age, often up to a hundred dried fruits can be harvested at a time, giving 400 to 500 seeds a few times a year. Fruit capsules usually consist of four to five lobes. Inside are the seeds, oval, dark-brown, 1.5 to 2 cm in diameter and 45 to 100 grams of weight [19]. Sacha Inchi seeds have high protein (27–33%) and oil (35–60%), it contains a high concentration of polyunsaturated fatty acids. The oil is excellent source for Omega 3 (α-linolenic acid) and Omega 6 (linoleic acid). It contains antioxidants, vitamin A, vitamin E, essential and non-essential amino acids that are important for good health.

Recent study, total antioxidant capacity of Sacha Inchi oil was 133.42 mM gallic acid.kg^{-1} [18]. In 2003, Chirinos *et al*. reported that total antioxidant capacity of Sacha Inchi seed extract of the 16 cultivars were within the ranges of 6.5-9.8 μmoL TE/g of seed [20]. Sacha Inchi leaves are reported to contain terpenoids, saponins, phenolic compounds and other components [5]. In 2013, Nascimento *et al*. studied antioxidant activity from Sacha Inchi leaf. The total antioxidant capacity was observed that the extracts showed values ranging from 59.31 to 97.76 EAA/g. Furthermore, the DPPH assay values ranged from 62.8% to 88.3% [19].

Figure 1.5 Sacha Inchi (*Plukenetia volubilis*)

1.6 Facial cream

Creams are emulsions of oil and water. The formulas are made by heating up the oil and water phases separately, mixing them together (along with emulsifying agents) [21]. The face is usually the first part of the body that people see upon meeting each other and the source of most people's first visual impression. In order to preserve the youthful and fresh appearance of the facial skin, cosmetologists and dermatologists recommend that individuals use a facial cream daily to keep the skin soft and healthy. It is important for preventing the signs of premature aging, Facial skin also secretes more oil sebum from its pores than other body parts, especially when exposed to UV. This makes the face is a high risk area for skin damage, and it must be protected early on [22].

Emulsions are a class of disperse systems consisting of two unblendable substances. One substance is dispersed in the other [23]. The common types can be distinguished: water-in-oil (W/O) and oil-in-water (O/W) [24] (**Figure 1.6**). O/W emulsions are comfortable and cosmetically acceptable as they are less greasy and more easily washed off using water. W/O emulsions are more difficult to handle but many drugs which are incorporated into emulsions are hydrophobic and will be released more readily from W/O emulsions than O/W emulsions. W/O emulsions consist of water droplets dispersed in oil. The type of emulsifier used is a decisive factor in the type of emulsion formed, O/W or W/O [25]. Hydrophilic surfactants induce formation of O/W emulsions, lipophilic surfactants favor W/O emulsions. Use of water-soluble macromolecular surfactants also results in formation of O/W emulsions [26]. To disperse two immiscible substances, one needs the third component, namely, emulsifier. The choice of emulsifier is crucial in the formation of emulsion and its long-term stability [23]. The preparation of emulsions that are kinetically stable over a time period that is of practical use to the cosmetic industry requires the incorporation of substances known as stabilizers. Stabilizers can be distinguished according to their mode of operation as either “emulsifiers” or “texture modifiers” [27].

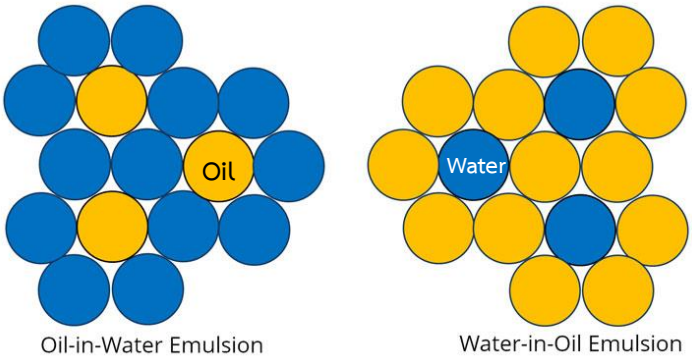


Figure 1.6 Types of simple emulsion
(Source: http://www.molecularrecipes.com/emulsions/emulsion-types)

An emulsifier is a surface-active substance that when present at low concentration in a system, has the properties of absorbing on to the surfaces of interfaces of the system, and altering to marked degree the surface or interfacial free energies of the surfaces (or interfaces) [28]. The simplest type is ions such as OH^- that can be specifically adsorbed on the emulsion droplet thus producing a charge. An electrical double layer can be produced, which provides electrostatic repulsion. This has been demonstrated with very dilute O/W emulsions by removing any acidity. The most effective emulsifiers are nonionic surfactants that can be used to emulsify O/W or W/O. In addition, they can stabilize the emulsion against flocculation and coalescence. Ionic surfactants such as sodium dodecyl sulfate (SDS) can be used as emulsifiers, but the system is sensitive to the presence of electrolytes. Surfactant mixtures can be more effective in emulsification and stabilization of the emulsion. Nonionic polymers are more effective in stabilization of the emulsion, but they may suffer from the difficulty of emulsification unless high energy is applied for the process [23].

A texture modifier is a substance that thickens or gels the continuous phase [29]. Its purpose is to improve emulsion stability by retarding or preventing droplet movement is a substance that either increases the viscosity of the continuous phase (thickening agent) or forms a gel network within the continuous phase (gelling agent).

The texture modifiers help slowing down the movement of droplets due to gravity. And most of the texture modifiers form hydrocolloids that form multimolecular layers around emulsion droplets. Hydrocolloid stabilizer have little or no effect on interfacial tension, but exert have a protective colloidal effect, reducing the potential for coalescence by providing a protective sheath around droplets, imparting a charge to the dispersed droplets, and swelling to increase the viscosity of the system. Hydro colloidal emulsifiers may be classified as vegetable derivatives, animal derivatives, semi-synthetic agents and synthetic agents [27], [30].

1.7 Stability of cosmetic emulsion

An important parameter for emulsion based products stability pertaining to their emulsion stability. An emulsion is considered to be physically unstable in the case of the internal phases tend to form aggregates of globules, large or aggregates of globules rise to the top or fall to the bottom of the emulsion to form a concentrated layer of the internal phase, If all or a part of the liquid of the internal phase becomes unemulsified on the top or bottom of the emulsion. The instabi lity of emulsions can be classified into four types: Flocculation, creaming, coalescence and breaking [30] (**Figure 1.7**).

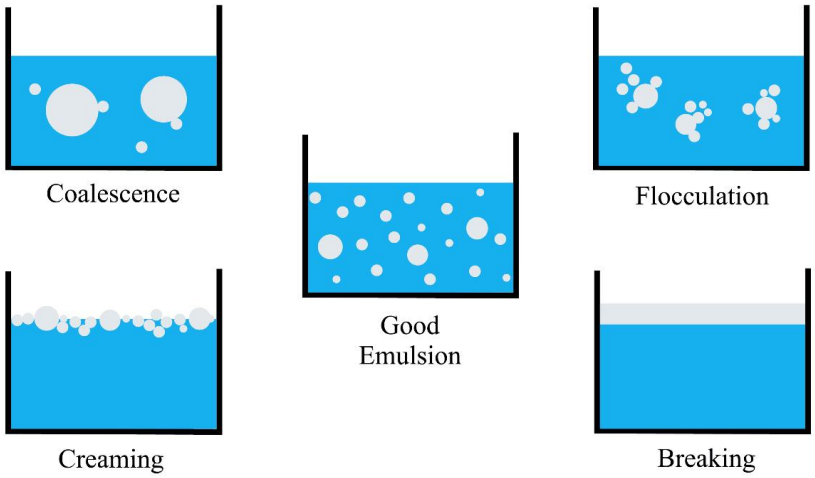


Figure 1.7 The instability of emulsions

The objective of stability testing cosmetic products is to ensure that a product meets the intended physical and chemical quality standards as well as functionality and aesthetics when stored under appropriate conditions.

Accelerated tests, developed because of the relatively short development cycle for cosmetic products, enable the prediction of stability. A commonly accepted practice is to support the forecasts obtained from accelerated stability testing by carrying out periodic post-launch monitoring of retained samples stored at ambient temperatures. Accelerated test conditions are internationally recognized as appropriately predicting product shelf life in cosmetic companies. Data acquired using many techniques at different temperatures and durations can be used, possibly in conjunction with the use of mathematical models, to predict stability. Common test procedures such as temperature variations, temperature cycling and centrifuge testing. High temperature testing is now commonly used as a predictor of long-term stability. Temperature cycling or "cooling-heating" tests can reveal some types of inadequacies more quickly than can storage at a constant temperature. Cooling-heating tests should be considered for certain types of products. Instability of emulsions can be detected by cooling-heating tests. As products can be expected to encounter temperature and pressure extremes during transport and storage [31]. In addition, centrifugation is a good test method to predict creaming [32].

1.8 Literature reviews of bioactivity effect of some cream containing plant extracts.

The cosmetic industry is in a constant search for plant extracts with relevant bioactive properties, which became valuable ingredients to design cosmeceutical formulations. Nowadays, plant extracts have been widely used to study bioactivity effect for the development of cosmetic products.

In 2011, Bernatoniene et al. reported that the topical cream with Calendula officinalis L. was proved to contain the significant antioxidant activity and suitable chemical and microbial stability. Therefore, this cream may be sufficient for the regular

topical application as the effective long term protection of the skin against ROS caused damage [33].

In 2013, Elya et al. presented the antioxidant activity of Solanum lycopersycum L. (tomato) cream by DPPH method. This research showed that the tomato cream with concentration of 0.5%, 1%, 2% and 3% had a physical stability with storage at cool temperature 4oC, room temperature and high temperature 40±2oC. The tomato cream with concentration of 1%, 2% and 3% reached a minimum value of DPPH retention (EC50) but the tomato cream 0.5% did not reach a minimum value of DPPH retention (EC50). The tomato cream 1 % had the best physical stability and the tomato cream extract 3% had the best antioxidant activity [34].

In 2014, Jayanthi and Lalitha studied tyrosinase inhibition of cream containing ethyl acetate extract of Eichhornia crassipes (Mart.) Solms. The results of the study revealed 8 to 11% tyrosinase inhibition even at lower concentrations and the plant extract can be formulated into creams with other active ingredients and can be used in cosmeceutical industry [35].

In 2014, Mishra et al. studied antioxidant effect of herbal face cream containing ethanol extract of Nardostachys jatamansi (Valerianceae). This study revealed that an ethanol fraction analyzed from a sample of N. jatamansi showed a significant antioxidant activity with an IC50 of 58.39 µg/mL while for ascorbic acid the IC50 was 46.68 µg/mL. Among the six formulations (F1-F6) F5 and F6 showed good spread ability, good consistency, homogeneity, appearance, pH. Also the formulations F5 and F6 showed no redness or edema or erythema and irritation [36].

In 2015, Kusumawati et al. determined tyrosinase inhibitor activity of edamame extract and formulated the extract into skin whitening cream, the result indicated that edamame extract had good tyrosinase inhibitor activity (IC50 92.8 µg/mL), and can be formulated into skin whitening cream with good cosmetological. Genistein content was retained in cream, indicating that the formulation process did not affect the genistein content in extract [37].

In 2015, Sekar et al. formulated and evaluated an antiaging cream containing Rambutan fruits extract. The SMEF (Successive methanol extract (Flesh)), CMEF (Crude methanol extract (Flesh)), SMEP (Successive methanol extract (Peels)) and CMEP (Crude methanol extract (Peels)) produced significant antioxidant activities and tyrosinase inhibition with low IC50. The results showed that the formulated antiaging creams and its ingredients were consistent in quality and can be easily used. In addition, the formulation containing SMEF and CMEF was safe and usable for the skin [38].

In 2016, Muthukumarasamy et al. formulated and evaluated the antioxidant cream comprising the methanolic leaf extract of Piper betel. The in-vitro free radical scavenging activity was studied by using DPPH assay. The IC50 result revealed that leaves of P. betel shows good antioxidant properties with IC50 value of 56.88 µg/mL. The evaluation of the formulated cream containing 2% P. betel extract showed good results and can be good potential for cosmetic product development [39].

In the same year, Taofiq et al. performed A. bisporus-based cosmeceutical formulations. Results displayed the highest anti-tyrosinase activity among the studied mushroom species (EC500.16 mg/mL). In the antioxidant activity, the formulations with A. bisporus and P. ostreatus showed the highest radical scavenging activity (EC507.04 and 7.69 mg/mL, respectively) and reducing power (EC50 2.34 and 2.36 mg/mL, respectively). Therefore, mushrooms can further be exploited as natural cosmeceutical ingredients [40].

1.9 Objectives of this research

The purposes of this research are to explore tyrosinase inhibitory and anti-oxidant activities of Sacha Inchi leaf, and to develop skin lightening and anti-aging product for a facial cream from the crude extract of Sacha Inchi leaf.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals and solvents

The chemicals and solvents are listed in **Table 2.1**

Table 2.1 List of chemicals and solvents

Chemical and solvent	Supplier
Antibiotic/Antimycotic	GIBCO, USA
Antioxidant capacity of lipid soluble compound kits	Analytik Jena, Germany
Antioxidant capacity of water soluble compound kits	Analytik Jena, Germany
C12-15 alkyl benzoate	Namsiang, Thailand
Cetyl alcohol	Thai Sanguanwat, Thailand
Cyclomethicone	Chemico, Thailand
Dichloromethane	Merck, Germany
Disodium hydrogen phosphate	Sigma-aldrich, Germany
Dulbecco's Modified Eagle Medium (DMEM)	Biowest, France
Ethanol	Merck, Germany
Fetal bovine serum (FBS)	GIBCO, USA
Glycerin	Namsiang, Thailand
Glyceryl monostearate	Thai Sanguanwat, Thailand

Table 2.1 (continued)

Chemical and solvent	**Supplier**
Green tea leaf extract	Cream building, Thailand
Isohexadecane	Chemico, Thailand
Jojoba oil	Namsiang, Thailand
Methanol	Merck, Germany
Minimum Essential Media (MEM)	Biowest, France
Mineral oil	Namsiang, Thailand
Modified Dulbecco's PBS (DPBS)	Biowest, France
Mushroom tyrosinase	Sigma-aldrich, Germany
Phenoxyethanol (and) Ethylhexylglycerin	DKSH, Thailand
Phynyltrimethicone	Chemico, Thailand
Propylene glycol	Thai Sanguanwat, Thailand
Stearic acid	Brenntag, Germany
Stearyl alcohol	Thai Sanguanwat, Thailand
Sulfuric acid (Analytical Grade)	Sigma-aldrich, Germany
Sodium PCA	Namsiang, Thailand
Sodium dihydrogen phosphate	Sigma-aldrich, Germany
Trypan blue stain	GIBCO, USA
Trypsin-versene / ETDA	GIBCO, USA
WST-1 Reagent	Roche Applied Science, Germany

2.2 Instruments and equipments

The instruments and equipments are listed in **Table 2.2**

Table 2.2 Instruments and equipments

Instrument and equipment	Supplier
Biological Safety Cabinet, Class II	SANYO, Japan
Centrifuge	Kubuta, Japan
CO_2 incubator	SANYO, Japan
4-Digits balance	Sartorius, Germany
Haemocytometer	BOECO, Germany
Homoginizer	Charn intertech, Thailand
Hot air oven	Memmert, Germany
Hot plate stirrer	Biobase, China
Rotary evaporator	BUCHI, Germany
Laboratory fume hood	Asian chemicals & engineering, Thailand
Inverted microscope	Nikon, Japan
Microcentrifuge tube	Axygen, USA
Micropipettes	GILSON, USA
Multichannel pipette	GILSON, USA
Pipette tips	Corning, USA
pH meter	SI analytics, Germany

Table 2.2 (continued)

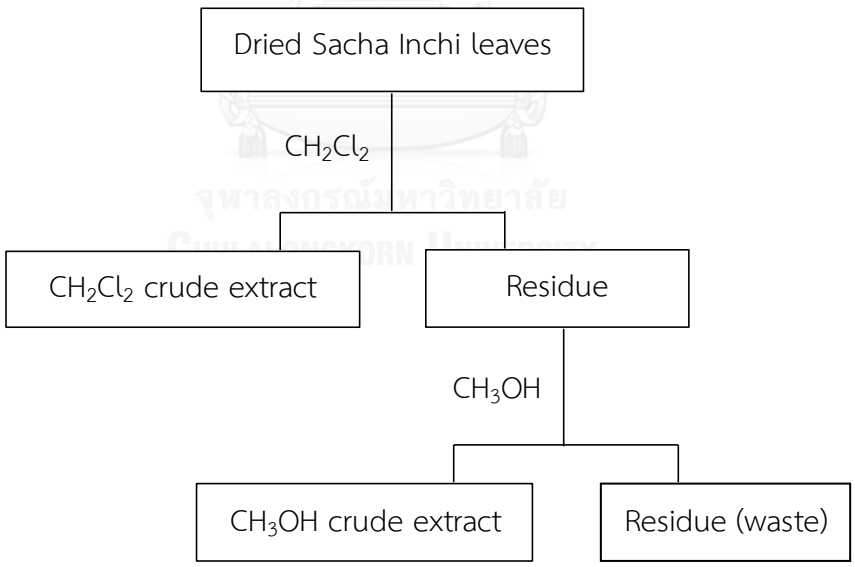
Instrument and equipment	**Supplier**
Photochem	Analytik Jena, Germany
Refrigerator	SANYO, Japan
Refrigerated Centrifuge	Kubuta, Japan
Sterlile Syringe	Corning, USA
Vortex	Scientific Industries, USA
Vacuum pump	Millivac, USA
Water bath	Memmert, Germany
96-well plate	Corning, USA

2.3 Extraction of Sacha Inchi

Sacha Inchi (SI) leaf was collected from Sukhothai province, Thailand in September 2016. The leaves (5 kg) were cut into small pieces and extracted with dichloromethane for five days and the residue was extracted with methanol, respectively for three times. The extracts were obtained by filtration the mixture through a filter paper (Whatman No.1) using vacuum filtration, the filtrates were evaporated to dryness under vacuum. The extraction procedure for the plants is shown in **Scheme 2.1**. The percentage yield of the crude extract was determined by following equation.

% yield = (the dry weight of crude extract/soaked samples material) * 100

The crude extracts were used for further studies including tyrosinase inhibitory activity, antioxidant activity, cytotoxicity test and cosmetic formulation.



Scheme 2.1 The extract procedure of Sacha Inchi leaf

2.4 Biological activity tests

2.4.1 Determination of tyrosinase inhibitory activity

Tyrosinase inhibitory activity was employed with some modifications from that of Saewan *et al*. CH_2Cl_2 and CH_3OH crude extracts were dissolved in DMSO and diluted to the concentrations of 100, 80, 60, 40 and 20 µg/mL, respectively. 50 µl of 1.7 mM L-tyrosine solution was dissolved in 0.1 M sodium phosphate buffer (pH 6.8) and then added to 50 µl of the samples and incubated for 10 minutes at room temperature, mushroom tyrosinase solution (250 U/mL in pH 6.8 PBS) was added. 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

$$\% \text{ Inhibition of tyrosinase} = [(A_{control} - A_{sample})/A_{control}] * 100$$

Where A is the absorbance

The IC_{50}'s were determined from plots of percent inhibition 50 *vs* tyrosinase inhibitor concentration and were calculated by linear regression analysis from the mean inhibitory values. Kojic acid and green tea leaf extract were used as standard tyrosinase inhibitors. All tests were performed in triplicate. The sample exhibiting tyrosinase inhibitor was used for antioxidant activity test [41].

2.4.2 Determination of superoxide anion radicals scavenging capacity by photochemiluminescence (PCL) assay

The protocol was modified from that of Klungsupya *et al*. The crude extract was dissolved in methanol and diluted to concentration of 100 mg/mL. The solution was sonicated for 10 min to facilitate complete solubility and stored at -20 °C. For the measurement, test samples were prepared to 5, 10, 20, 40 and 50 µl in each test tube for ACL and ACW kits. The antioxidant capacity was determined using Photochem, the reaction was initiated by adding standard antioxidant compound (Trolox and ascorbic acid) or test samples to the mixture of R1, R2, and R3 (see in **appendix**). All samples were conducted and measured in triplicate. The results were presented in equivalent

units (μmoL) of ascorbic acid (vitamin C) for the antioxidative capacity of the water-soluble substances (ACW) system of Trolox (synthetic vitamin E) units for the lipid-soluble substances (ACL) system [42].

2.4.3 Cytotoxicity test

2.4.3.1 Cell culture and preparation

The L929 mouse fibroblast (ATCC®CCL-1™) was cultured in MEM medium supplemented with 10% fetal bovine serum (FBS) whereas HepG2 (ATCC® HB-8065™) was cultured in DMEM with 10% FBS and added 1% (v/v) penicillin-streptomycin to both L929 and HepG2 in tissue culture flask, each cell line at a density of $2x10^5$ cells/mL was seeded onto 96-well plate and incubated at 37°C of 5% CO_2 for 24 hours.

2.4.3.2 Cell treatment

The crude extract was prepared to 100 mg/mL (dissolved in 95% ethanol) and diluted to concentration of 62.5, 125, 250, 500, 1,000 and 2,000 μg/mL. 75 μl of test samples were added into each well of the 96-well culture plates and incubated at 37°C in 5% CO_2 incubator for 24 hr. $ZnSO_4$ $7H_2O$ was used as a positive compound that high toxicity at concentration of 5 μg/mL and 10 μg/mL.

2.4.3.3 Cytotoxicity qualitative evaluation

Treated and untreated cells were stained with 0.4% Trypan blue and examined using inverted microscope. General morphology change of cells was evaluated by morphology score (see in **Appendix**), and was recorded in the test report descriptively [43].

2.4.3.5 Cytotoxicity quantitative evaluation by WST-1 assay

The cytotoxic property of the crude extract on L929 and HepG2 cells was determined by mitochondrial dehydrogenase activity assay or WST (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay. For the measurement, 10 μl of WST-1 solution per 100 μl of DMEM was added to each well. The plates were kept in darkness for 30 min before measuring the absorbance at

450 nm by the microplate reader system. Values of three independent experiments obtained from WST assay were calculated the percentage viability of the cells using the equation below. A graph of absorbance plotted against sample concentration was constructed. The cytotoxicity of methanolic crude extract from SI leaf was presented as IC_{50}. The IC_{50} value of the extract on each cell was determined the highest concentration tested did not achieve 50% on the cytotoxicity index and was used as active ingredient in formulation of facial cream.

$$\% \text{ Cell viability} = (A_{\text{treated cells}} / A_{\text{untreated cells}}) * 100$$

Where A is the absorbance

2.5 Formulation of emulsion base

Table 2.3 Four formulations of O/W facial cream

Part	Ingredient	Function	Amount (%)				
			Master formula	F1	F2	F3	F4
A	Stearyl alcohol	Stiffening agent	4.00	4.00	2.00	3.00	2.00
	Cetyl alcohol	Stiffening agent	3.50	3.00	2.00	2.00	1.50
	Stearic acid	Emulsifier	1.50	1.00	0.20	0.20	0.20
	Glyceryl-monostearate	Emulsifier	4.00	4.00	2.50	3.00	2.50
	Cyclomethicone	Emollient	2.50	2.00	2.00	1.50	1.50
	Phynyltrimethicone	Emollient	0.20	0.20	0.20	0.20	0.20
	Sodium PCA	Humectant	0.50	0.50	0.50	0.50	0.50
	Isohexadecane	Emollient	1.00	1.00	1.00	1.00	1.00
	C12-15 alkyl-benzoate	Emollient	2.00	2.00	2.00	2.00	2.00
	Mineral oil	Emollient	2.00	2.00	1.00	1.00	1.00
	Jojoba oil	Emollient	1.00	1.00	0.50	0.50	0.50
B	Glycerin	Emollient	2.50	2.50	3.00	2.00	1.00
	Propylene glycol	Humectant	2.00	2.00	2.50	1.00	0.50
	Distilled water	Vehicle	72.30	73.80	79.60	81.10	84.60
C	Phenoxyethanol	Preservative	1.00	1.00	1.00	1.00	1.00

F1= formulation 1; F2= formulation 2; F3= formulation 3; F4= formulation

Four formulations were prepared via emulsification process. Part A was melted at 80°C to form oil phase, and water phase in part B heated up to 80°C. Oil phase was slowly poured into the water phase and mixed with homogenizer until a smooth and uniform mixture of cream was obtained, and then added part C and mixed. The cream was cooling down at room temperature, then 0.03% extract was incorporated directly into the cream and mixed until homogeneous. The ingredients in all the formulation creams are listed in **Table 2.3.**

2.6 Sensory assessment

Sensory assessment was conducted in 20 healthy volunteers, the product was evaluated in color, odor, texture, spreadability, stickiness and overall properties. The test uses 5 point hedonic scales of satisfaction (scales 1 = slightly, 2 = few, 3 = medium, 4 = good and 5 = excellent). The volunteer who satisfied the product in each parameter at scale of 4 and 5 will be assessed to satisfy or accept with this parameter (1 customer = 5%).

2.7 Evaluations of selected facial cream containing the crude extract

2.7.1 Determination of tyrosinase inhibitory and antioxidant effects of selected facial cream containing the crude extract

The protocol was slightly modified from that of O¨zer et al (2007). Facial cream containing the crude extract and base cream was extracted three times from 5 g of the sample. 10 mL of methanol was added and sonicated for 30 min. The remaining suspension was centrifuged at 4000 rpm for 30 min. The supernatants were further diluted 1:10 with methanol [44], evaluated percentage of tyrosinase inhibition by mushroom tyrosinase assay in Section 2.4.1 and antioxidant capacity as equivalent of standard antioxidants by PCL assay in Section 2.4.2, respectively.

2.7.2 Accelerated stability test of facial cream

The emulsion base and facial cream containing the extract were divided into three samples separately in a 100 ml glass bottle. These samples were kept at different storage conditions; at 45°C in hot air oven and at 4°C in refrigerator, for 1 month. The physical changes of samples were observed after storage for every week at room temperature. A heating/cooling cycle test was performed and the samples were stored in a refrigerator/hot air oven and temperature was changed, respectively, between 45 °C and 4 °C every 48 hr for 6 cycles. The physical changes were recorded at cycle 0 and cycle 6.

The centrifugation test, 5 mL of all samples were tested for gravitational stability under centrifugation at 3000 rpm for the duration of 30 min. The changes of samples were investigated for every week.

CHAPTER III
RESULTS AND DISCUSSIONS

Plukenetia volubilis (Euphorbiaceae) known as Sacha Inchi (Dao-inka) is currently cultivated commercially in Thailand. The seed is excellent source for Omega 3 and Omega 6. It is high in protein, essential and non-essential amino acids that are important for good health. However, there is no scientific data about the use of leaves for cosmetic. The main objectives of this research were to studied tyrosinase inhibitory and antioxidant activities of Sacha Inchi leaf, and to develop skin lightening and anti-aging product for a facial cream from the crude extract of Sacha Inchi leaf.

3.1 The extraction of Sacha Inchi

The dried leaves of Sacha Inchi (SI) were cut into small pieces and extracted by soaking in dichloromethane for five days and residue was repeatedly extracted by methanol, respectively for three times. The extract was filtered and evaporated with a rotatory evaporator to obtain CH_2Cl_2 (192.5, 3.85%yield) and CH_3OH (115.5, 2.31 %yield), respectively. The summary of the extraction is depicted in **Table 3.1** and **Figure 3.1.**

Table 3.1 Weight and %yield of the crude extracts of SI leaf

Sample	Solvent	Weight (g)	Yield (%w/w)	Characteristic
SI leaves	CH_2Cl_2	192.5	3.85	Dark green liquid (**Fig.3.1A**)
	CH_3OH	115.5	2.31	Dark green solid (**Fig.3.1B**)

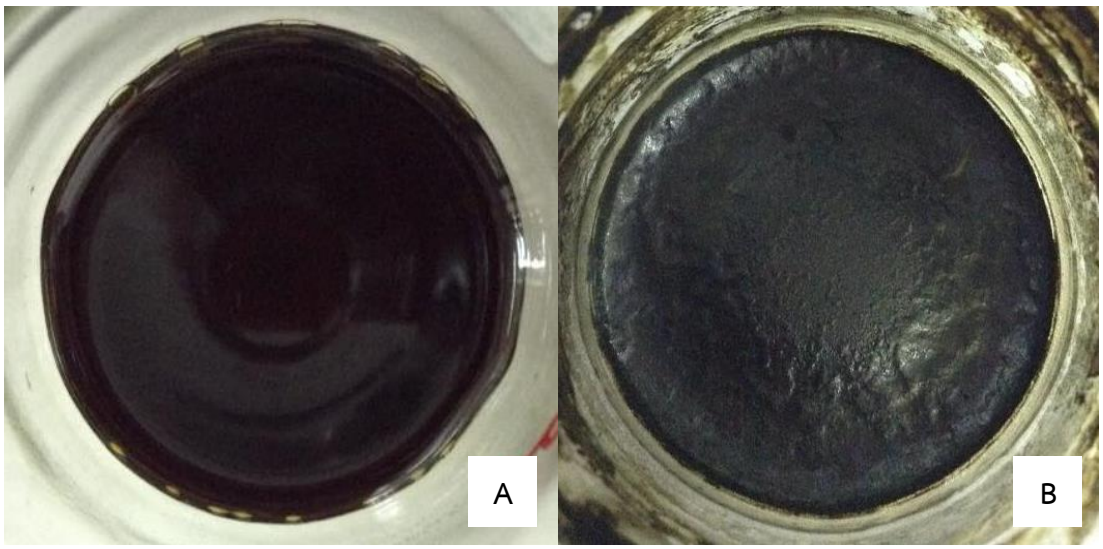


Figure 3.1 Characteristic of CH_2Cl_2 (A) and CH_3OH (B) crude extract from SI leaf

3.2 Biological activity tests

3.2.1 Determination of tyrosinase inhibitory activity

Tyrosinase is a major enzyme of the melanin synthetic pathway in melanocytes. Therefore, inhibition of tyrosinase could be an important strategy for blocking melanogenesis [44]. In the experiment, tyrosinase inhibition activity of SI leaf extract was determined using mushroom tyrosinase assay. The absorbance at 490 nm decreases as a result of the reaction of melanin synthesis being interrupted.

The inhibition was expressed as IC_{50} value, CH_3OH crude extract showed IC_{50} value of 34.56 µg/mL (**Table 3.2**). On the other hand, CH_2Cl_2 crude extract did not inhibit the tyrosinase activity (**data not shown**) whereas green tea leaf extract and kojic acid (positive control) showed IC_{50} at 53.52 µg/mL and 7.25 µg/mL, respectively (**Table 3.2**). Recent studies reported the use of arbutin, kojic acid and hydroquinone as tyrosinase inhibitors in cosmetics. However, some of these inhibitors involve several side effects [44].

Nowadays, available tyrosinase inhibitors suffer from toxicity and there is a constant quest for better inhibitors from natural sources as they are expected to be free of harmful side effects [45]. The majority of natural tyrosinase inhibitors consist of

a phenolic structure or are a metal chelater [46]. Phenolics contained in our samples may play a role in inhibition of tyrosinase activity in CH_3OH crude extract. In addition, previous studies revealed that Sacha Inchi composed of alpha-tocopherol [18]. Alpha-tocopherol is usually known to inhibit melanin formation by suppressing oxidative polymerization of phenylalanine [47].

CH_3OH crude extract exhibited the strongest tyrosinase inhibitory activities IC_{50} value of 34.55 µg/mL. Therefore, the CH_3OH crude extract was further used as a sample in antioxidant capacity analysis.

Table 3.2 Tyrosinase inhibition activity of plant extracts and kojic acid

Sample	Concentration (µg/mL)	%Inhibition	IC_{50} (µg/mL)
SI leaf extract	20	37.75±0.87	34.55±0.92
(crude CH_3OH)	40	57.90±0.86	
	60	66.41±0.72	
	80	80.36±1.41	
	100	89.73±1.65	
Green tea leaf	20	22.73±1.22	53.52±1.17
extract*	40	43.95±1.51	
	60	56.44±0.85	
	80	68.36±0.54	
	100	82.00±1.19	
Kojic acid**	2	13.20±1.12	7.25±0.80
	4	29.54±0.78	
	6	42.10±0.72	
	8	57.88±0.68	
	10	65.35±0.82	

* Natural active ingredient in cosmetic; ** Positive control

3.2.2 Determination of superoxide anion radicals scavenging capacity

The PCL assay was performed in the presence of a superoxide anion radical ($O_2^{\bullet-}$), which is one of the most reactive oxygen species present in the human body [39]. The antioxidant capacity was determined using Photochem, the principle is based on measurement of PCL. Superoxide anion radicals were generated in the system by optical excitation of luminol (photosensitizer) [42]. The measure of radical quantity in the system is the intensity of the emitted light, and the radical scavenging compounds attenuate the photochemiluminescence intensity in proportion to the amount and activity of the tested antioxidant compared with the standard antioxidant (constructed a calibration curve) [47]. PCL measures the potential antioxidant property of the CH_3OH crude extract from SI leaf by ACL and ACW protocols, which measure the antioxidant capacity of the lipid-and water- soluble components, respectively.

In this study, the result exhibited $O_2^{\bullet-}$ scavenging activity of both ACL (16.1 μmol TE/g extract) and ACW (17.8 μmol AE/g extract) systems, respectively which had potential of both hydrophilic and lipophilic. The antioxidant capacity of CH_3OH crude extract from SI leaf and green tea leaf extract were no different.

However, cytotoxicity test is important for developing new skin care products, we studied on determining the dose of the formulation by cytotoxicity test.

Table 3.3 Superoxide anion radicals scavenging capacity of plant extracts in ACL system

Sample	Volume (µl)	Sample content (µg)	Trolox equivalent (mmol)	µmol TEAC/g extract
SI leaf extract (crude CH_3OH)	5	50	0.686±0.035	16.1±0.002 (4.030 mg Trolox)
	10	100	1.843±0.137	
	20	200	3.113±0.309	
	30	300	5.215±0.600	
	40	400	6.092±0.476	
Green tea leaf extract*	5	50	0.637±0.005	14.8±0.001 (3.704 mg Trolox)
	10	100	1.192±0.011	
	20	200	2.701±0.080	
	30	300	4.027±0.287	
	40	400	6.450±0.195	

* Natural active ingredient in commercial skincare products

Table 3.4 Superoxide anion radicals scavenging capacity of plant extracts in ACW system

Sample	Volume (µl)	Sample content (µg)	Ascorbic acid equivalent (mmol)	µmol AEAC/g extract
SI leaf extract (crude CH_3OH)	5	50	0.760±0.045	17.8±0.001 (3.135 mg ascorbic acid)
	10	100	1.893±0.063	
	20	200	3.528±0.119	
	30	300	5.747±0.193	
	40	400	6.776±0.239	
Green tea leaf extract*	5	50	0.853±0.044	18.2±0.001 (3.205 mg ascorbic acid)
	10	100	1.950±0.091	
	20	200	3.592±0.137	
	30	300	6.150±0.225	
	40	400	6.737±0.685	

*Natural active ingredient in commercial skincare products

3.2.3 Cytotoxicity test

3.2.3.1 Cytotoxicity qualitative evaluation

Cell morphology was observed under microscope. The effect of CH_3OH crude extract from SI leaf on L929 cells was presented in **Figure 3.2**. The CH_3OH crude extract showed neither cytotoxicity nor morphological change to L929 cells even the concentration of 500 µg/mL (**Figure 3.2B)**. At 1,000 µg/mL concentration, the result showed slightly change in cell morphology **Figure 3.2C**) meanwhile, the highest concentration at 2,000 µg/mL found morphology change in all cells (**Figure 3.2D**). The positive control treated with $ZnSo_4$ $7H_2O$ showed severe change in cells at 5 and 10 mg/mL (**Figure 3.2E** and **3.2F**, respectively)

The **Figure 3.3** expressed effect of the extract on HepG2 cells, the result found that HepG2 cells showed neither cytotoxicity nor morphological change to L929 cells even the concentration of 250 µg/mL (**Figure not shown**). As the **Figures 3.3B** and **3.3C** showed slightly change in morphology cells at 500 and 1,000 µg/mL concentration, respectively. At 2,000 µg/mL concentration of the extract exhibited mild change (some cells round or spindle shaped). The positive control treated with $ZnSo_4$ $7H_2O$ showed severe change in cells at 5 and 10 µg/mL (**Figures 3.3E** and **3.3F**, respectively) whereas all cells show morphological changes after treated with $ZnSo_4$ $7H_2O$ at 5 and 10 mg/mL (**Figure 3.3E** and **3.3F**, respectively).

Our data demonstrated that CH_3OH crude extract from SI leaf induced morphological changes in L929 and HepG2 cells at the concentration more than 1,000 µg/mL and 500 µg/mL, respectively. However, qualitative evaluation means are appropriate for screening purposes. Thus, quantitative evaluation of cytotoxicity is preferable.

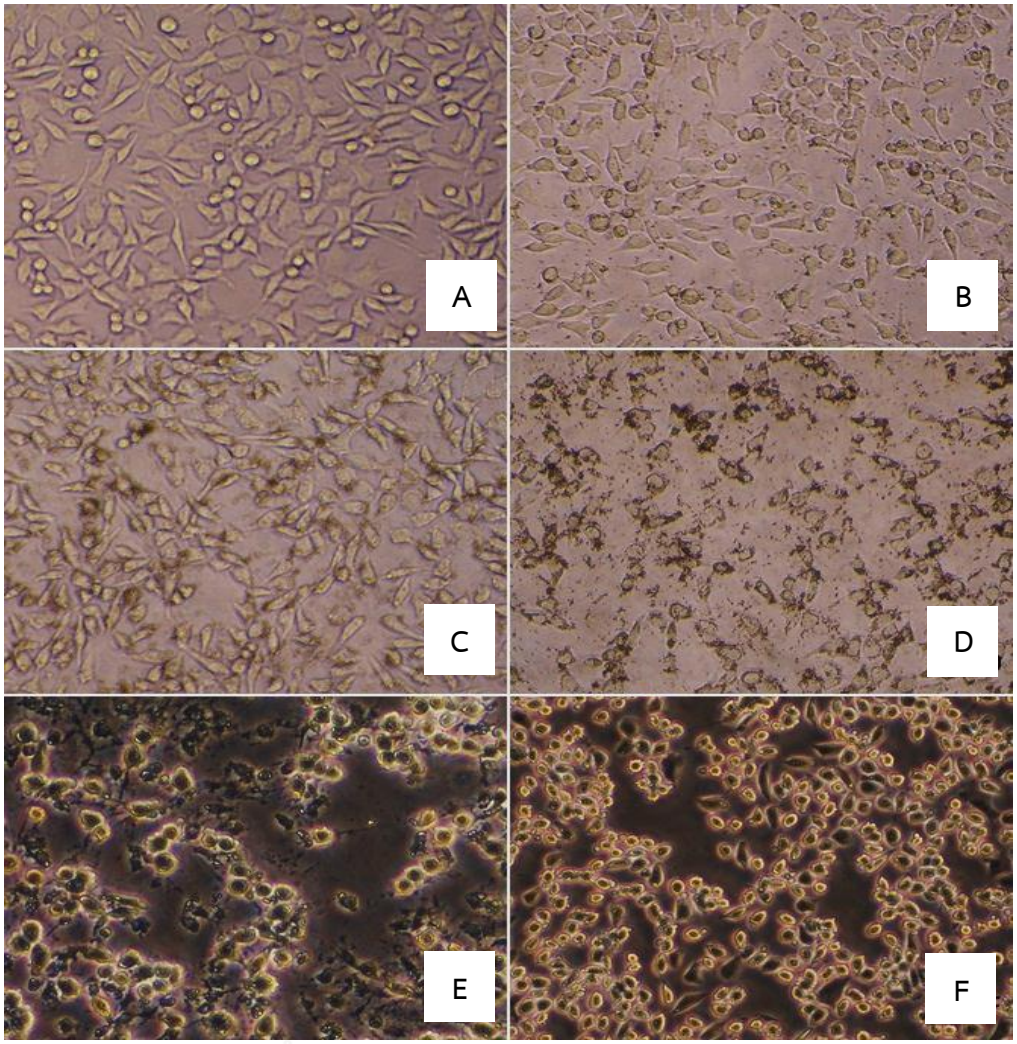


Figure 3.2 The morphology characteristics of L929 cells after treated with CH_3OH crude extract for 24 h. Untreated cell (A); 500 µg/mL concentration of the extract (B); 1,000 µg/mL concentration of the extract (C); 2,000 µg/mL concentration of the CH_3OH crude extract (D); compared with $ZnSo_4\ 7H_2O$ (positive control) at 5 mg/mL (E) and 10 mg/mL (F) concentration

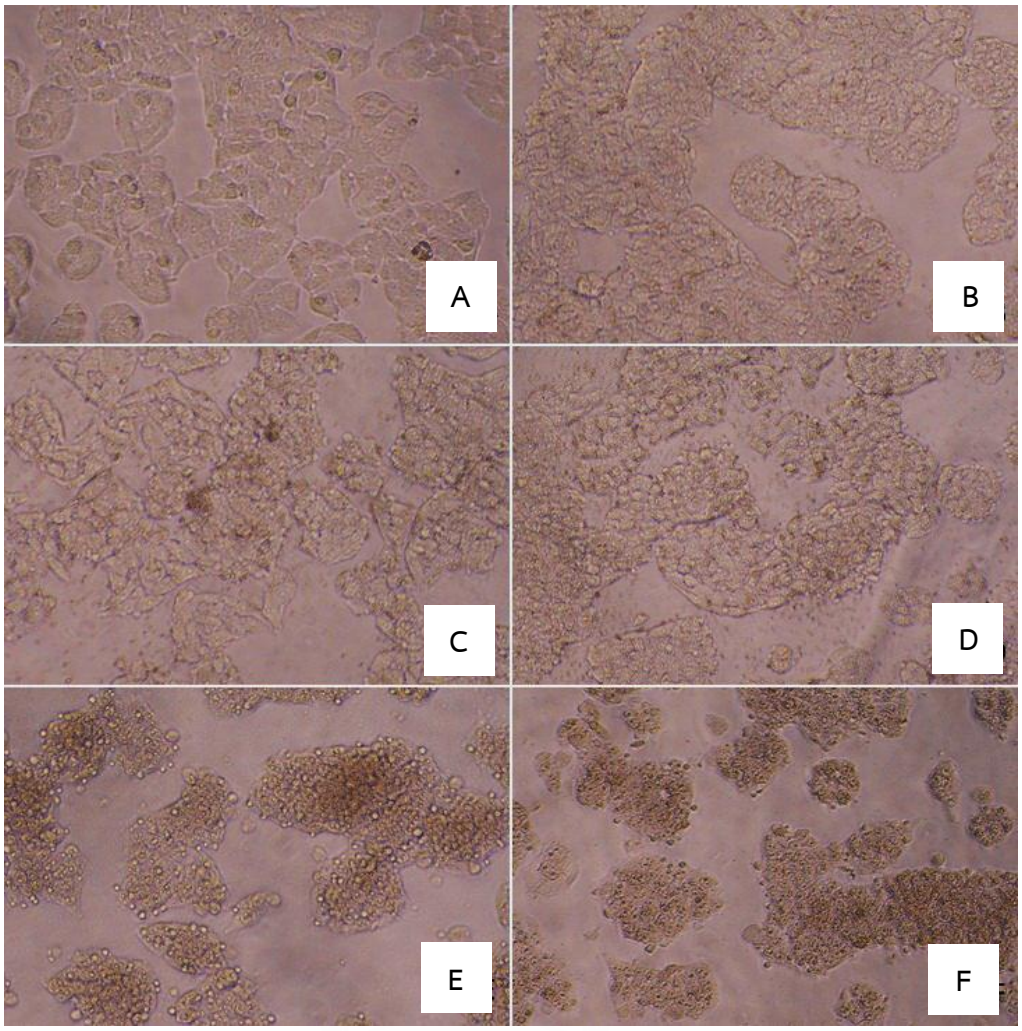


Figure 3.3 The morphology characteristics of HepG2 cells after treated with CH_3OH crude extract for 24 h. Untreated cell (A); 500 µg/mL concentration of the extract (B); 1,000 µg/mL concentration of the extract (C); 2,000 µg/mL concentration of the CH_3OH crude extract (D); compared with $ZnSo_4$ $7H_2O$ (positive control) at 5 mg/mL (E) and 10 mg/mL (F) concentration

3.2.3.2 Cytotoxicity quantitative evaluation by WST-1 assay

WST works similarly to MTT by reacting with the mitochondrial succinate-tetrazolium reductase forming the formazan dye. The WST-1 reagent produces a water-soluble formazan rather than the water-insoluble product of the MTT assay. The mitochondrial dehydrogenase activity was determined with the WST-1 colorimetric assay [48]. The L929 cell line is an established substrate and has been commonly used for cytotoxicity evaluation of biomaterials. In a previous study, toxic substances showed similar results on L929 fibroblasts and human gingival fibroblasts, indicating that L929 fibroblasts assays may represent sufficient screening models for *in vitro* evaluation of cytotoxicity [49]. HepG2 cells are derived from human liver. It is a perpetual cell line consisting of carcinoma cells [50]. These cells are highly differentiated and display many of the genotypic features of normal liver cells. Consequently, HepG2 cells can be used to screen the cytotoxicity potential of compounds [51].The cytotoxicity of CH_3OH crude extract was assessed by WST-1 assay in L929 and HepG2 cells were treated with different concentrations (62.5-2,000 µg/mL) of the extracts for 24 hours and the viability of cells was determined according to its principle as described above. The result of cytotoxicity test of the CH_3OH crude extract in L929 cells found that the cells remained more than 80% viable at 62.5-500 µg/mL concentration. The cell viability decreased up to 69.1 and 39.28% for 1,000 and 2,000 µg/mL concentration, respectively, compared with the $ZnSo_4 7H_2O$ (positive control), the cell viable remained at 35.80 and 33.26% of concentration 5 and 10 mg/mL, respectively (**Table 3.5**).

The viability of HepG2 cells were more than 80% at low concentration range (62.5-250 µg/mL), and were decreased up to 31.36, 28.75 and 23.84% at concentration of range 500-2,000 µg/mL, respectively. The $ZnSo_4 7H_2O$ showed cell viable at 20.47 and 19.21% for 5 and 10 mg/mL concentration, respectively (**Table 3.6**).

Table 3.5 Cytotoxicity of the CH_3OH crude extract from SI leaf against L929 cell lines by WST-1 assay

Samples	Concentration	% Cell Viability of L929	IC_{50} (µg/mL)
SI leaf extract (crude CH_3OH)	62.5 µg/mL	97.66±2.44	1,641.50±10.61
	125 µg/mL	90.42±4.07	
	250 µg/mL	86.99±3.54	
	500 µg/mL	83.94±3.38	
	1,000 µg/mL	69.1±0.55	
	2,000 µg/mL	39.28±0.94	
$ZnSo_4$ $7H_2O$*	5 mg/mL	35.80±0.40	-
	10 mg/mL	33.26±1.03	

***** Positive control

Table 3.6 Cytotoxicity of the CH_3OH crude extract from SI leaf against HepG2 cell lines by WST-1 assay

Sample	Concentration	% Cell Viability of HepG2	IC_{50} (µg/mL)
SI leaf extract (crude CH_3OH)	62.5 µg/mL	96.31±1.36	358.00±7.07
	125 µg/mL	90.47±3.39	
	250 µg/mL	82.28±9.13	
	500 µg/mL	31.36±3.11	
	1,000 µg/mL	28.75±8.65	
	2,000 µg/mL	23.84±7.26	
$ZnSo_4$ $7H_2O$*	5 mg/mL	20.47±2.07	-
	10 mg/mL	19.21±2.87	

***** Positive control

The report expressed that IC_{50} of the CH_3OH crude extract was 1,642 µg/mL of L929 cells and 358 µg/mL of HepG2 cells. Therefore, concentration below IC_{50} value of the CH_3OH crude extract on HepG2 cells may be suitable for used as active ingredient in facial cream.

3.3 Formulation of facial creams

The characteristic of cream base was its emulsion property, which can be obtained by combining C12-15 alkyl benzoate, glyceryl monostearate and stearic acid (fatty acid) with distilled water. The excipients which were used for the cream should not interfere the bioactivity of active ingredient [37]. Hence, we carefully chose the excipients to ensure that the extract as the active compound retained its bioactivity. Glycerin, cyclomethicone, sodium PCA and phenyltrimethicone were used as emollients, which soften the skin by slowing evaporation of water. Jojoba oil, propylene glycol and Sodium PCA were chosen as humectant, since it can give humidity for skin by water absorption from its surrounding environment, and help the active ingredient crossing the layer to achieve target cells. Cetyl alcohol and stearyl alcohol were used as stiffening agent, cetyl alcohol gave smooth texture on cream, and stearyl alcohol gave good consistency. SI leaf extract was used as active ingredient (skin whitening and anti-aging agents) at 0.03% (w/v). In addition, phenoxyethanol mixture was used as preservative at concentration of 1%. It had antibacterial properties and was effective against strains of *Pseudomonas aeruginosa* [52]. The formulations intended for application to the skin should have pH which close to the pH of skin (4.5 - 6.5) to prevent skin irritation [37].

All formulations had yellowish green shade, and different in physical characteristics (**Figure 3.4** and **Table 3.7**). Hence, the formulated creams had good physicochemical properties.

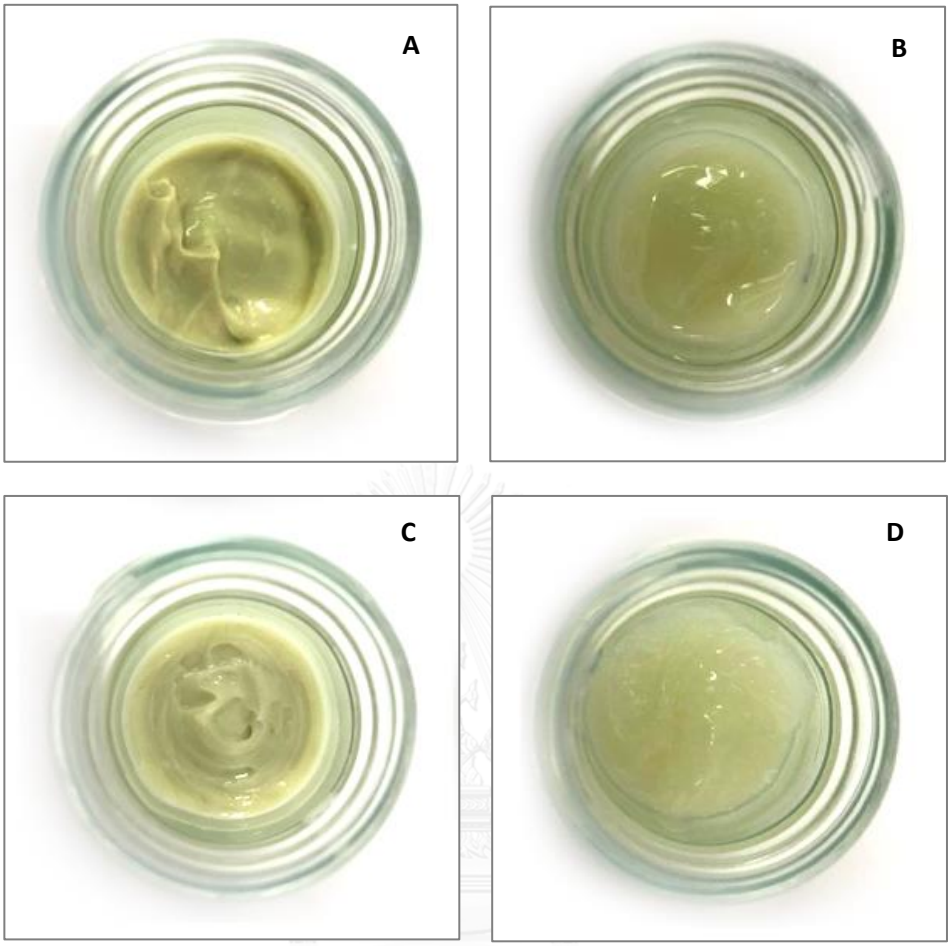
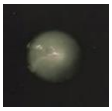


Figure 3.4 The formulated facial creams containing CH_3OH crude extract from SI leaf. F1=formulation 1(A); F2=formulation 2(B); F3=formulation 3(C) and F4=formulation 4(D)

Table 3.7 Physicochemical of formulated facial creams containing CH_3OH crude extract from SI leaf

Formula	Charac-teristic	Color	Texture	Homo-genous	Ease of removal	pH	Remark
F1	Opaque	Yellowish green	Heavy, Greasy	Good	Easy	5.58	
F2	Translucent	Light yellowish green	Heavy, smooth, sticky	Good	Easy	6.00	
F3	Opaque	Yellowish green	Light, smooth	Good	Easy	5.65	
F4	Translucent	Light yellowish green	Light, smooth, sticky	Good	Easy	6.05	

F1 = formulation 1; F2 = formulation 2; F3 = formulation 3; F4 = formulation 4

3.4 Sensory assessment

The sensory test was performed on 20 healthy female volunteers, 18–50 years old. The satisfaction point of volunteers expressed in **Table 3.8** and **Appendix**. In the result, F3 showed the highest satisfaction point at scales 4 (good) in color, texture, spreadability, stickiness and overall of product (except in odor). Sensory assessment is the method of analysis for the examination of the product by evaluation of the properties which are detected by the five sense organs such as color, odor, taste, touch, texture and noise. They are used in many fields such as foods, cosmetics, pharmaceuticals, textiles and household products. In the cosmetic industry, sensory evaluation is used to evaluate the consumer acceptance of the cosmetic; especially for products of general topical use, sensory evaluation data has been used as a part of marketing decision. It has been used to identify and quantitatively model the key drivers for a product's acceptance [53].

Table 3.8 The satisfaction point of the volunteers

Formula	Satisfaction point (Mean ± SD)					
	Color	Odor	Texture	Spread-ability	Stickiness	Overall properties
F1	2.7±0.67	2.5±0.83	2.8±0.85	3.4±0.60	3.0±0.46	2.9±0.55
F2	3.0±0.46	2.7±0.88	3.9±0.55	3.8±0.79	3.5±0.51	3.8±0.79
F3	4.2±0.75	3.1±0.72	4.1±0.31	4.3±0.55	4.2±0.37	4.3±0.64
F4	3.8±0.97	3.3±0.64	4.0±0.32	4.0±0.56	3.9±0.85	3.8±0.44

F1 = formulation 1; F2 = formulation 2; F3 = formulation 3; F4 = formulation 4

In the percentage of consumer acceptance, most of the consumers were satisfied with F3, 90% of consumers were satisfied with the color and overall of product, 30% of consumers were satisfied with the odor, and 100% of consumers were satisfied with the texture, spreadability, and stickiness. However, for the odor, most of the volunteers gave mark less than 4 which implies that the consumers were not satisfied with the odor of the formulated cream (**Figure 3.3**). Also, considering the consumer acceptance, F3 was acceptance from all volunteers.

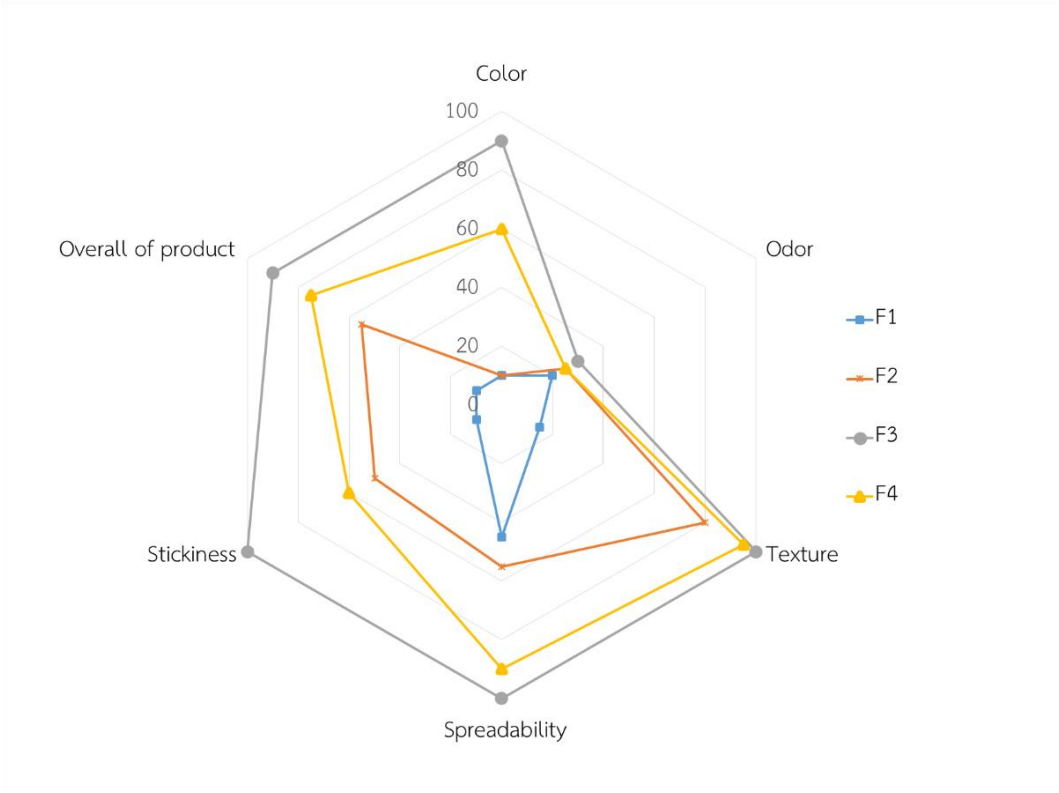


Figure 3.5 Percentage of consumer acceptance of 4 product testers

3.5 Evaluations of selected facial cream containing the crude extract

3.5.1 Determination of tyrosinase inhibitory and antioxidant effects of selected facial cream containing the crude extract.

Evaluation of the formulation effects on tyrosinase inhibition demonstrated that F3 containing 0.03% CH_3OH crude extract has the tyrosinase inhibitory activity at 19.6±1.32%, and the emulsion base showed no tyrosinase inhibitory effect. In conclusion, the plant extracts in the formulation may be effective in treating uneven skin pigmentation. The antioxidant capacity was then examined and compared with the emulsion base as the reference sample. As expected, emulsion base displayed no potency as compared to F3 containing 0.03% SI leaf extract. The formulation showed antioxidant capacity at 0.92±0.07 µmol TE/g and 1.31±0.09 µmol AE/g. Such potency is compatible with a potential efficacy on skin.

3.5.2 Accelerated stability test

Stability test is the crucial areas in the cosmetic testing program, because the instability of the product involved in the safety, efficacy, and quality of cosmetic formulations [54].

In this study, F3 and emulsion base were observed with change in liquefaction, color, odor, phase separation, gravitational stability (centrifugation) and pH. Result indicated that no change in the color of base and formulation of observation periods may be attributed to different factors contributing to the emulsion stability, such as the components of oil phase, i.e. jojoba oil, mineral oil which is a colorless and transparent, Silicone, propylene glycol and glycerin which is a clear and colorless, the extract had yellowish green color maybe containing chlorophyll pigments and polyphenols. Phenoxyethanol mixture may protect the formulation components from microbial growth of those organisms which might produce such substances, which are able to change the color of the formulation during the storage time. Liquefaction, no liquefaction during kept the samples under 4°C and 45°C (**Table 3.9** and **Table 3.10**), and heating/cooling cycle (**Table 3.11**).

After centrifugation, no phase separation on centrifugation was seen in any of the samples kept under different storage conditions for 1 month, it may be said that proper homogenization speed during emulsion formulation prevented the base and the formulation breakage during stress testing [55]. While odor slightly change under 45°C at week 4. The result revealed that the F3 were stable at all conditions.

Table 3.9 Physical characteristics of emulsion base kept at 4 and 45°C for 1 month

Parameters		Physical changes				
	Weeks	**0**	**1**	**2**	**3**	**4**
Color (visual observation)	45°C	W	W	W	W	W
	4°C	W	W	W	W	W
Liquefaction	45°C	-	-	-	-	-
	4°C	-	-	-	-	-
Odor	45°C	-	-	-	-	+
	4°C	-	-	-	-	-
Phase separation	45°C	-	-	-	-	-
	4°C	-	-	-	-	-
Centrifugation	45°C	-	-	-	-	-
	4°C	-	-	-	-	-
pH (mean±SD)	45°C	5.70±0.00	5.70±0.01	5.70±0.01	5.71±0.01	5.70±0.01
	4°C	5.70±0.00	5.70±0.01	5.72±0.01	5.70±0.01	5.71±0.01

- =No change; +=slight change; W=white

Table 3.10 Physical characteristics of formulation 3 kept at 4 and 45°C for 1 month

Parameter		Physical change				
	Week	0	1	2	3	4
Color (visual observation)	45°C	YG	YG	YG	YG	YG
	4°C	YG	YG	YG	YG	YG
Liquefaction	45°C	-	-	-	-	-
	4°C	-	-	-	-	-
Odor	45°C	-	-	-	-	+
	4°C	-	-	-	-	-
Phase separation	45°C	-	-	-	-	-
	4°C	-	-	-	-	-
Centrifugation	45°C	-	-	-	-	-
	4°C	-	-	-	-	-
pH (mean±SD)	45°C	5.68±0.00	5.69±0.00	5.69±0.01	5.68±0.01	5.70±0.02
	4°C	5.68±0.01	5.70±0.00	5.68±0.02	5.65±0.02	5.67±0.02

- =No change; +=slight change; W=white; YG=yellowish green

Table 3.11 Physical characteristics of emulsion base (B) and formulation (F3) creams of heating (45°C)/cooling (4°C) tests

Parameter	Physical change				
	Cycle	0		6	
	°C	B	F3	B	F3
Liquefaction	45/4	-	-	-	-
Color (visual observation)	45/4	W	YG	W	YG
Odor	45/4	-	-	-	-
Phase separation	45/4	-	-	-	-
Centrifugation	45/4	-	-	-	-
pH (mean±SD)	45/4	5.69±0.00	5.68±0.00	5.60±0.01	5.60±0.02

- =No change; +=slight change; W=white; YG=yellowish green

CHAPTER V
CONCLUSION

Sacha Inchi leaf extract was used to evaluate for their potency as tyrosinase inhibitory and antioxidant activities. Results indicated that the methanolic crude extract from SI leaf exhibited the effectiveness and can be used as an active ingredient in skin lightening and anti-aging products because it showed effective in mushroom tyrosinase inhibition and PCL assays. Results obtained from WST-1 assay, using L929 and HepG2 cell lines suggested that the methanolic crude extract may be safe for applied in facial cream. In the formulation of facial creams, formulated creams had good cosmetological properties, and the satisfaction of formulation 3 was acceptance from all volunteers and had good stability at all accelerated storage conditions. However, further studies has to be conducted to enhance the pleasant odor of the formulated cream, and are needed to properly assess in clinical and in *vivo* studies.

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APPENDIX

Preparation of buffer and reagents

- **M Sodium phosphate buffer pH 6.8**

Dissolved 138 g of NaH_2PO_4 in deionized water up to 1 L (stock solution A) and dissolved 142 g of Na_2HPO_4 in deionized water up to 1 L (stock solution B), and mixed 510 mL of stock solution A and 490 mL of stock solution B. The PBS buffer pH 6.8 was checked by using pH meter

- **Mushroom tyrosinase solution**

Dissolved tyrosinase in 0.1 M Sodium phosphate buffer pH 6.8 at a concentration of 250 U/mL

- **1.7 mM L-tyrosine solution**

Dissolved 0.015 g of L-tyrosine in 50 mL of deionized water and vortex.

- **Antioxidant capacity of lipid soluble compound kit**

Reagent R1: Methanol

Reagent R2: Reaction buffer

Reagent R3: Luminol (photosensitizer and detection reagent)

Reagent R4: Trolox®

- **Antioxidant capacity of water soluble compound kit**

Reagent R1: ACW-Diluent (sample solvent)

Reagent R2: Reaction buffer

Reagent R3: Luminol (photosensitizer and detection reagent)

Reagent R4: Ascorbic acid

IC_{50} Determination

IC_{50} is a median concentration of measurement of the effective inhibitor on *in vitro* which is reduced by 50% inhibition of tyrosinase inhibitory activity. IC_{50} was drawn a graph between various concentrations)X-axis(and percentage inhibition)Y-axis. And IC_{50} value was calculated from slope of this graph.

For example, Kojic acid exhibited high tyrosinase inhibitory activity with IC_{50} value of 7.25 µg/mL.

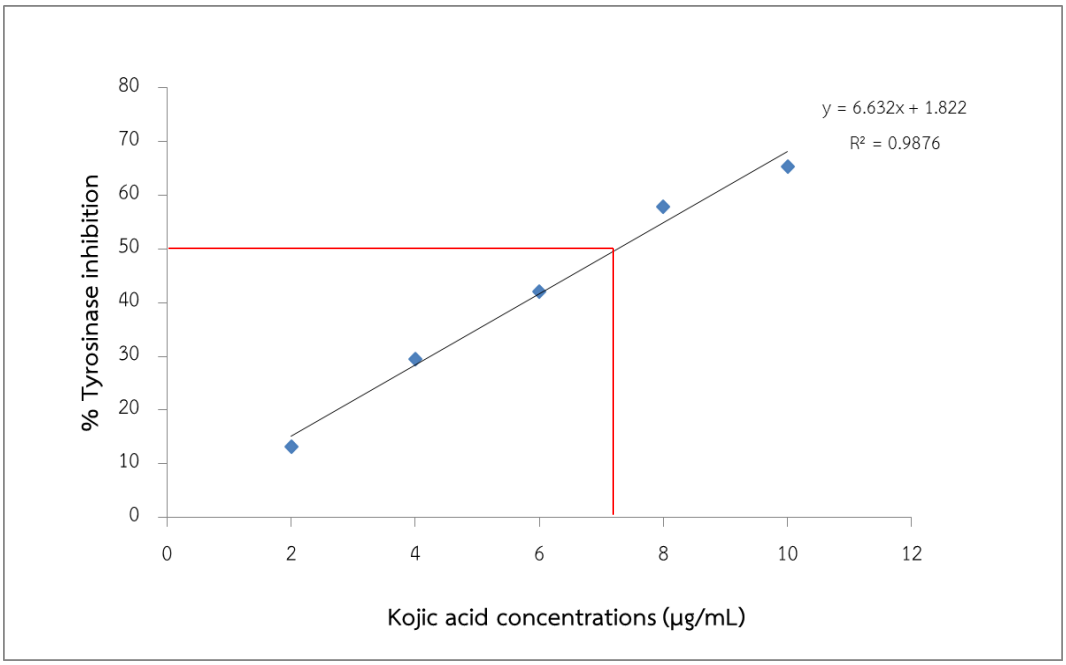

Figure A1 Kojic acid standard curve

Table A1 Qualitative morphological scoring of cytotoxicity of extracts

Score	Reactivity	Change of cellular morphology
0	None	No changes
1	Slightly toxic	Slightly changes, few cells affected
2	Mildly toxic	Mild changes, some cells round/spindle shaped
3	Moderately toxic	Moderate changes, many cells round/spindle shaped
4	Severely toxic	Severe changes, about all cells show morphological change

Table A2 Morphology score of L929 and HepG2 cells treated with SI leaf extracts and positive control

Sample	Concentration	Scoring	
		L929	HepG2
Untreated cells	-	0	0
SI leaf extract (CH_3OH)	62.5 µg/ml	0	0
	125 µg/ml	0	0
	250 µg/ml	0	0
	500 µg/ml	0	1
	1,000 µg/ml	1	1
	2,000 µg/ml	4	2
Positive control ($ZnSo_4$ $7H_2O$)	5 mg/ml	4	4
	10 mg/ml	4	4

Table A3 Results of the volunteer satisfaction survey

Parameter	Volunteer satisfaction (persons)					
	Formula	Scale 1 (slightly)	Scale 2 (few)	Scale 3 (medium)	Scale 4 (good)	Scale 5 (excellent)
Color	1	-	9	9	2	-
	2	-	2	16	2	-
	3	-	1	1	13	5
	4	-	2	6	7	5
Odor	1	-	14	2	4	-
	2	-	10	3	7	-
	3	-	4	10	6	-
	4	-	1	14	4	1
Texture	1	-	9	8	2	1
	2	-	12	4	2	2
	3	-	-	-	18	2
	4	-	-	1	18	1
Spread-ability	1	-	1	10	9	-
	2	-	-	9	7	4
	3	-	-	-	14	6
	4	-	-	3	14	3

Table A3 (Continued)

Parameter	Volunteer satisfaction (persons)					
	Formula	Scale 1 (slightly)	Scale 2 (few)	Scale 3 (medium)	Scale 4 (good)	Scale 5 (excellent)
Stickiness	1	-	2	16	2	-
	2	-	-	10	10	-
	3	-	-	-	17	3
	4	-	-	8	6	6
Overall properties	1	-	4	14	2	-
	2	-	-	9	7	4
	3	-	-	2	11	7
	4	-	-	5	15	-

VITA

Miss Thadsaneeya Chuenchob was born on September 23, 1989 in Srisatchanalai district, Sukhothai province, Thailand. She received her Bachelor's degree of Science (Cosmetic science) from the School of Cosmetic science, Mae Fah Luang University in 2012. She graduated in master's degree of Science in Biotechnology in 2016 from Chulalongkorn University.

Miss Thadsaneeya Chuenchob has attended the following conference for poster presentation.

The 5th Academic Science and Technology Conference 2017 (ASTC2017) scheduled on May 25, 2017 at Bangkok (Thailand).

Proceeding:

Chuenchob, T. and Chavasiri, W. Formulation of facial cream containing Sacha Inchi (Plukenetia volubilis) leaves extract for tyrosinase inhibition and antioxidant activities. The 5th Academic Science and Technology Conference 2017, pp. 75-81. Bangkok, Thailand, 2017.