DEVELOPMENT OF EMULGEL CONTAINING *ALLIUM ASCALONICUM* EXTRACT FOR COSMETIC APPLICATIONS

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A Thesis Submitted in Partial Fulfillment of the Requirements

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Pharmaceutical Technology
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วิรุจน์ จิตต์ผ่องใสกุล : การพัฒนาอิมัลเจลที่ประกอบด้วยสารสกัดหอมแดงเพื่อการนำไปใช้ทางเครื่องสำอาง (DEVELOPMENT OF EMULGEL CONTAINING *ALLIUM ASCALONICUM* EXTRACT FOR COSMETIC APPLICATIONS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ร.ต.ท. หญิง ดร. วลัยศิริ ม่วงศิริ, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.ดร.พรเพ็ญ วีระวัฒกานนท์, 82 หน้า.

งานวิจัยนี้ มีวัตถุประสงค์เพื่อศึกษาฤทธิ์การยับยั้งการก่อตัวของเม็ดสีจากสารสกัดหอมแดง เพื่อการนำไปใช้ทางเครื่องสำอาง หอมแดงถูกบดและสกัดใน เอธานอล:น้ำ (60:40 ปริมาตร/ปริมาตร) เพื่อให้ได้ ส่วนสกัดที่ 1 จากนั้นกากที่เหลือถูกสกัดด้วยตัวทำละลายเดียวกันเพื่อให้ได้ ส่วนสกัดที่ 2 น้ำคั้นที่ได้จากส่วนสกัดทั้งสองนั้นจะถูกแยกทำให้เป็นผงแห้งโดยการทำแห้งเยือกแข็ง ก่อนที่จะนำไปทำปฏิกิริยาด้วยการแยกสลายด้วยน้ำโดยใช้กรด จากนั้นทำการวิเคราะห์สารสำคัญของผงแห้งและส่วนสกัดที่ผ่านการทำปฏิกิริยาแยกสลายด้วยน้ำโดยวิธีโครมาโทกราฟีของเหลวที่สมรรถนะสูงแยกสารเคมีภายใต้ความดันของเหลว และ การแยกสารโดยโครมาโทกราฟีแบบแผ่นบาง โดยใช้ ควอเซติน เป็นสารสำคัญในการเทียบ ผลที่ได้คือ ปริมาณควอเซตินในหอมแดงในส่วนสกัดที่ 2 มีมากกว่าใน ส่วนสกัดที่ 1 เนื่องจากกากถูกสกัดด้วยตัวทำละลายที่มีปริมาณสูงกว่า และยังพบอีกว่าวิธีที่ดีที่สุดในการสกัดหอมแดงเพื่อให้ได้ควอเซตินในปริมาณมากที่สุดคือ การทำปฏิกิริยาของผงแห้งด้วยกรดไฮโดรคลอริก(0.5 โมลาร์)ใน 50% เมธานอล ที่อุณหภูมิ 60 องศาเซลเซียส เป็นเวลา 75 นาที จากนั้นทำการทดสอบฤทธิ์การยับยั้งการก่อตัวของเม็ดสีของสารสกัดหอมแดง ซึ่งผลปรากฏว่า สารสกัดหอมแดงส่วนสกัดที่ 2 มีฤทธิ์การยับยั้งเอนไซม์ไทโรซิเนสที่ดีกว่า ด้วยค่าความเข้มข้นของสารสกัดที่ยับยั้งเอนไซม์ไทโรซิเนสได้ 50% เท่ากับ 16.22 มิลลิกรัม/มิลลิลิตร ยิ่งไปกว่านั้น สารสกัดหอมแดงส่วนสกัดที่ 2 ยังมีฤทธิ์การยับยั้งการก่อตัวของเม็ดสีในเซลล์ชนิดเมลาโนมาบี16 ได้ 25% ที่ความเข้มข้นประมาณ 300 ถึง 500 ไมโครกรัม/มิลลิลิตร โดยสารสกัดหอมแดงได้ถูกนำไปศึกษาความพิษต่อเซลล์ชนิดเมลาโนมาบี16 โดยเทคนิคเพรสโตบลู ซึ่งค้นพบว่าสารสกัดหอมแดงที่ความเข้มข้น 6.25 ถึง 500 ไมโครกรัม/มิลลิลิตรไม่มีความเป็นพิษต่อเซลล์ จากนั้นจึงทำการพัฒนาอิมัลเจลที่มีส่วนผสมของสารสกัดหอมแดงปริมาณ 5 เปอร์เซ็นต์โดยน้ำหนัก และศึกษาความคงตัว อิมัลเจลมีความคงตัวเมื่อผ่านกระบวนการปั่นเหวี่ยงที่ 6000 รอบต่อนาที เป็นเวลา 30 นาที จำนวน 8 รอบ และภายใต้สภาวะร้อนสลับเย็น ที่อุณหภูมิ 40 องศาเซลเซียส 2 วัน สลับกับอุณหภูมิ 4 องศาเซลเซียส 2 วัน คิดเป็น 1 รอบ เป็นจำนวน 6 รอบ จากนั้นอิมัลเจลถูกเก็บภายใต้สภาวะเร่งที่อุณหภูมิ 30 และ 40 องศาเซลเซียส ที่ความชื้นสัมพัทธ์ 75 เปอร์เซ็นต์ เป็นเวลา 3 เดือน เพื่อทดสอบความคงตัวและปริมาณควอเซติน ผลการทดลองพบว่าอิมัลเจลมีความคงตัว และยังพบว่าปริมาณสารสำคัญ ควอเซติน ไม่เปลี่ยนแปลงภายในเวลา 3 เดือน สูตรตำรับอิมัลเจลที่มีส่วนผสมของสารสกัดหอมแดงนั้น ไม่ก่อให้เกิดการระคายเคืองบนอาสาสมัครจำนวน 20 คน ดังนั้นสูตรตำรับอิมัลเจลที่มีส่วนผสมของสารสกัดหอมแดง มีความคงตัวที่ดีภายใต้สภาวะเร่ง และไม่ก่อให้เกิดการระคายเคืองต่อผิวมนุษย์

ภาควิชา..วิทยาการเภสัชกรรมและเภสัชอุตสาหกรรม..... ลายมือชื่อนิสิต…….....

สาขาวิชา...เทคโนโลยีเภสัชกรรม..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……..

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5376855533 : MAJOR PHARMACEUTICAL TECHNOLOGY
KEYWORDS : SHALLOT / *ALLIUM ASCALONICUM* / EMULGEL / TYROSINASE / ANTI-MELANOGENESIS

WIRUT JITPHONGSAIKUL : DEVELOPMENT OF EMULGEL CONTAINING *ALLIUM ASCALONICUM* EXTRACT FOR COSMETIC APPLICATIONS. ADVISOR: ASST. PROF. WALAISIRI MUANGSIRI, Ph.D., CO-ADVISOR: ASST. PROF. PORNPEN WERAWATGANONE, Ph.D., 82 pp.

The current study majorly focused on anti-melanogenesis properties of *Allium ascalonicum* extracts (shallot) as a new active agent for cosmetic purposes. The shallot bulbs were ground and mixed with ethanol:water (60:40 v/v) to give fraction 1, the marc was further extracted with the same solvent to give fraction 2. Each fraction was separately collected and freeze-dried under a FTS system prior to hydrolysis. The lyophilized powder and its fraction were characterized using HPLC and TLC characterizations in which considered quercetin as a major compound. HPLC and TLC characterizations revealed that shallot ethanolic extract fraction 2 contained higher amount of quercetin because the solvent contained higher concentration of ethanol. The optimum condition to obtain higher concentration of quercetin is to hydrolyze shallot extracts with 0.5M HCl in 50% methanol for 75 minutes at 60 ˚C. Shallot ethanolic extracts were then evaluated for their anti-melanogenesis properties. Shallot ethanolic extract fraction 2 showed more potent in anti-tyrosinase activity with an IC_{50} value of 16 mg/mL. Moreover, shallot ethanolic extract fraction 2 also inhibited 25% of melanin production in B16 melanoma cells with a concentration range of 300-500 µg/mL. Both fractions were assayed for their cytotoxicities using presto blue method. The results indicated no toxicity of shallot extract to B16 melanoma cells at concentration of 6.25-500 µg/mL. The development of emulgel containing 5% shallot ethanolic extract fraction 2 was performed and evaluated for its stabilities. The emulgel passed 8 cycles of 30 minutes centrifugation at 6000 rpm with no separation. There was no color change, no separation, and no pH value change after subjected the emulgel to 6 cycles of heating-cooling cycle. The emulgel was stored at 30 ˚C, 75% RH and 40 ˚C, 75% RH for 3 months and determined the amount of quercetin. The result showed no significant change of quercetin from time zero to 3 months. Moreover, the emulgel showed no short term and long term irritation effect on 20 volunteers. Therefore, the emulgel containing *Allium ascalonicum* extract possesses satisfactory physical properties with good stabilities under accelerated conditions and without irritation effects.

Department: Pharmaceutics and Industrial Pharmacy Student's Signature…….

Field of Study:...Pharmaceutical Technology…….Advisor's Signature……

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LIST OF ABBREVIATIONS

%	percentage
˚C	degree Celsius
µ	micro (10^{-6})
µg	microgram
µL	microlitre
µm	micrometre
1^{st}	first
2^{nd}	second
et al.	*et alii*, and other
etc.	*et cetera* (and other similar things)
g	gram
HCl	hydrochloric acid
HLB	hydrophilic-lipophilic balance
HPLC	high performance liquid chromatography
IC	inhibition concentration
k	kilo (10^{3})
kg	kilogram
L	litre (s)
L-DOPA	L-3,4-dihydroxyphenylalanine
m	milli (10^{-3})
M	molar (s)
mg	milligram

min	minute (s)
mL	milliliter
mm	milimetre
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazoliumbromide
n	nano (10^{-9})
nm	nanometre
no.	number
o/w	oil-in-water
PBS	phosphate buffer saline
pH	potential of hydrogen
RH	relative humidity
rpm	round per minute
RT	room temperature
S.D.	standard deviation
Span®	sorbitan monooleate
TLC	thin layer chromatography
Tween®	polysorbate
UV-Vis	ultraviolet-visible
v/v	volume by volume
v/v/v	volume by volume by volume
w/o	water-in-oil
w/w	weight by weight

CHAPTER I

INTRODUCTION

1. Background and significance of the study

Nowadays, consumers of both genders spend lots of time and money on facial treatments and facial products. Cosmetic products containing ingredients with natural origins gain popularity among consumers who believe that the products possess higher safety comparing to cosmetics containing synthetic chemicals. Thailand is a tropical country, in which natural resources are plentiful. The Royal Thai Government has a policy to employ medicinal herbs and to increase its value through several strategies such as funding researches on development of cosmetic products containing herb extracts. Examples of research in this area include the formulation development of onion extract for scars treatment, the development of amla extract and studies of its cosmetic application, and the development of anti-acne property of mangosteen extracts (Kugasemrat et al., 2009, Jithavech 2005, Pothitirat et al., 2009).

Allium cepa var. *aggregatum* is the botanical variety of *Allium cepa* (onion) formerly classified as *Allium ascalonicum*; it is also known as "shallot" (Fritsch et al., 2002). Shallot is widely distributed in Asian countries including Thailand. Regular consumption of shallot can reduce risks of neurodegenerative disorders, cancer, vascular disease and heart disease. (Lu et al., 2011). Shallot is also recognized to possess an antipyretic effect, and has been used in Thai herbal medicines as a whitening agent, and a scar remover.

Major constituents in shallot are phenolic compounds and flavonoids. Phenolic compounds in shallot are reported to show several biological activities such as antioxidant, antibiotics, antifungal, and anti-angiogenesis. Flavonoids in shallot are mainly quercetin (Leelarungrayub et al., 2006, Lu et al., 2011, Amin et al., 2009, Seyfi et al., 2010, Wang et al., 2002, Wiczkowski et al., 2008). Pharmaceutical activities of quercetin have long been determined including antioxidant properties, free radical scavenging activities, anti-inflammatory effect as well as anti-melanogenesis properties (Rogerio et al., 2010, Beevi et al., 2010, Arung et al., 2011). Fresh shallot and shallot extract available in the market these days are usually used as a flavoring agent or a condiment. However, shallot extract for cosmetic purpose has never been available in the market.

Melanogenesis is a skin pigment production process which taking place in melanocytes located in epidermis. Amount of melanin produced by melanocytes defines the skin color. Ultra-violet rays stimulate the releasing of melanocyte stimulating hormone (MSH) resulting in acceleration of melanin production (Arung et al., 2011). Chemical which has an ability to inhibit or reduce melanin production can be utilized as a whitening agent. Quercetin in onion has been reported to have anti-melanogenesis properties: therefore, shallots, a plant in the same family, are expected to have quercetin and possess the same activity (Arung et al., 2011).

Emulgels is a dosage form in which a gelling agent is present in emulsions. Therefore, emulgels possess beneficial properties of both emulsions and gels. Emulgels easily incorporate both hydrophilic and hydrophobic drugs in its aqueous and oil phase of the emulsion, respectively. Emulsion attributes in emulgels have

several favorable properties for dermatological use such as its emollient, spreadability, and pleasing appearance. Gel attributes also enhances additional advantages to the formulation such as its greaselessness, and easy removability. In addition, the presence of a gelling agent in emulsion improves physical stability of emulsions by increasing viscosity resulting in minimizing chance of flocculation (Fang et al., 2011, Khullar et al., 2011, Panwar et al., 2011).

In this study, shallot crude extract and fractions were prepared, characterized for the chemical constituents and evaluated for its anti-melanogenesis properties. Emulgel preparations containing the shallot extract was formulated and characterized for its chemical and physical stabilities. Finally, the emulgel containing shallot extract was tested on human volunteers in order to determine the irritancy.

2. Objectives of the study

2.1 To prepare and evaluate anti-tyrosinase and anti-melanogenesis properties of shallot (*Allium ascalonicum*) extract, a new active agent for cosmetic purposes.

2.2 To formulate emulgel preparations containing shallot (*Allium ascalonicum*) extract and determine their stabilities and skin irritation effect.

3. Hypothesis

3.1 Shallot (*Allium ascalonicum*) extract possesses anti-tyrosinase and anti-melanogenesis properties

3.2 Preparations deliver a stable emulgel containing shallot (*Allium ascalonicum*) extract without skin irritation.

CHAPTER II

LITERATURE REVIEW

1. Shallots

1.1 Botanical aspects of shallots

Allium ascalonicum, *Allium cepa var. aggregatum*, commonly known as shallot, is a botanical variety of *Allium cepa* (onion) which was formally classified in the *Alliaceae* family. Shallots are commonly found in tropical countries such as Thailand, Indonesia and Srilanka. The variety of shallots found in Thailand is *Allium ascalonicum* (Leelarungrayub et al., 2004). The name *Allium* comes from a Celtic word "all" which means pungent, while the name *ascalonicum* comes from Ascalon, Israel, a town where originally cultivates shallots (Amin et al., 2007). The picture of *Allium ascalonicum* is shown in **Figure 1**.

Figure 1 The picture of *Allium ascalonicum* (Shallot)

Shallot bulb is distinguished from that of other *Allium cepa* varieties in terms of shape, flavor, and color. Shallot bulbs are formed in clusters with multiple cloves. Shallots show less sharpness, spiciness, tanginess, and sweetness flavor than onions (Khara., 2010). The peel color of shallots ranges from golden brown to rose red. Its flesh has reddish white color (Leighton et al., 1992). In Thailand, shallots are used as a flavoring agent or a condiment in many dishes. Therefore, most of researches have been regarding shallots as food applications.

One of the research articles in Food Industry reported the heat treatments on the quality of shallot using Chroma analysis. Microwave heat treatment of 3 minutes and 35 seconds to oil-fried shallots gave the chroma value of 5.29, 5.06, and 15.28 for *L*(lightness), *a*(redness), and *b*(yellowness), respectively in which was the closest value to the control. However, microwave heat treatment to shallots for longer than 3 minutes and 50 seconds resulted in darker and burned colored shallot, an undesirable appearance of the shallots (Chu et al., 2001).

Flavor intensity of shallots was represented by the concentration of peroxide in shallots. The observation indicated that 33% increase of peroxide value was observed from a gas heating treatment which suggested the slow evaporation of water by the gas heating treatment resulting in more-oxidized oil. The result suggested weaker flavor intensity of shallot stemmed from the increase of peroxide concentration (Chu et al., 2001).

The bulbs of *Allium ascalonicum* (shallot) have been traditionally used in Southeast Asia. Shallot plants have been known as health-promoting herbs, and also frequently used for the treatment of several health problems such as

neurodegenerative disorders, cancer, fever, vascular disease and heart disease (Lu et al., 2011). Moreover, shallot juice has long been used as a whitening agent and a scar remover in traditional cosmetics.

1.2 Phytochemical properties and biological activities of shallots

Flavonoids are major chemical compositions found in shallot (Fattorasso et al., 2002). Flavonoids are one of the most diverse and widespread group of natural compounds. Flavonoids are plant pigment, found in flowers and fruits. Flavonoids are classified based on their molecular structures as flavonol, flavone, flavanone, flavanol, isoflavone, and anthocyanidine (**Figure 2**). Each group of flavonoids is divided into subgroups; aglycones, glycosides and methylated derivatives. (**Figure 3**).

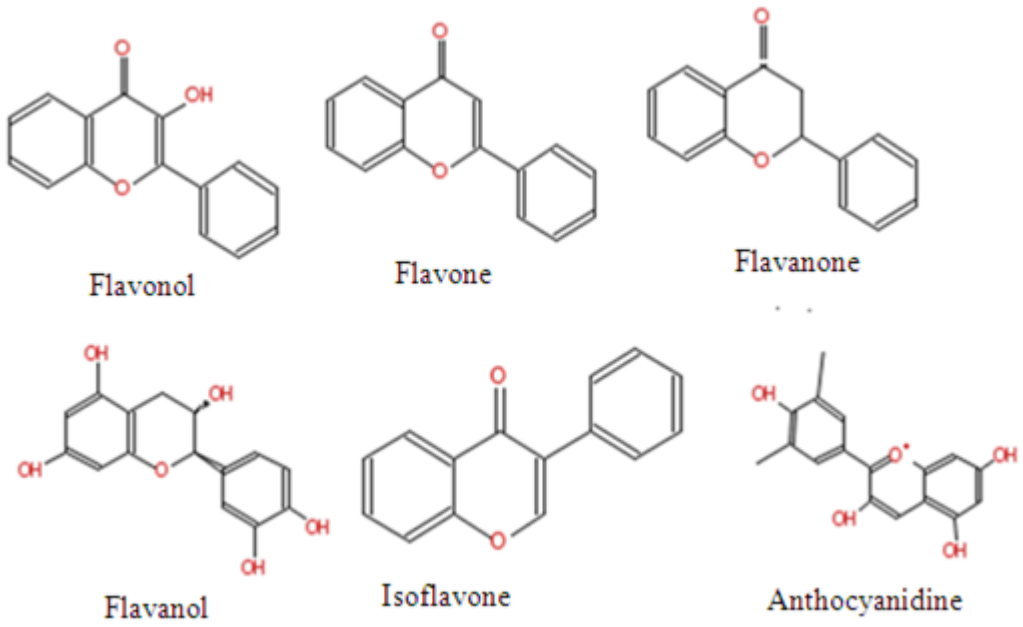

Figure 2 The molecular structure of various classes of flavonoids

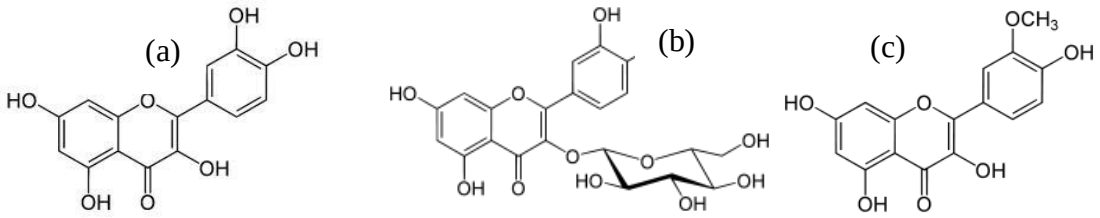

Figure 3 The chemical structure of quercetin aglycone (a), quercetin glycoside (b) and methylated quercetin metabolite (c)

Among onion varieties, shallots contain the highest amount of total flavonols, more than 800 mg/kg (Leighton et al., 1992, Fattorasso et al., 2002, Lakhanpal et al., 2007). Furthermore, quercetin derivatives in shallots were reported to be free quercetin (83.3%), quercetin 4′-O-*β*-glucoside (13.2%), quercetin 3,4′-O-bis-*β*-glucoside (3.2%), quercetin 3-O-*β*-glucoside (0.2%), and isorhamnetin 4′-O-*β*-glucoside (0.1%) (Wicskowski et al., 2008).

Total concentration of flavonoids present in shallot extract depends on extraction solvent. Ethyl acetate or hexane was used as an extracting solvent and the obtained shallot extracts were reported to yield 52.29 or 23.25 μg/mL of flavonoids, respectively. The above result illustrated the solvent effect on the content of total flavonoids (Seyfi et al., 2010).

Melanin inhibition activities of free quercetin and quercetin glycosides were examined. Free quercetin was reported to possess higher activity in anti-melanogenesis with the IC_{50} value of 26.5 μM. This result implied that the reduction of melanin inhibition ability of quercetin glucosides stemmed from the attachment of a glucoside moiety to the quercetin (Arung et al., 2011).

Moreover, flavonoids found in shallots have been reported to possess antioxidant, anti-cancer, anti-inflammatory, anti-angiogenesis, and antimicrobial properties in recent studies (Lu et al., 2011, Mohammadi-Moltlagh et al., 2010, Seyfi et al., 2010, Amin et al., 2009).

Total phenolic content in shallots and four different varieties of onions (white onion, yellow onion, red onion, and sweet onion) was determined using Folin-ciocalteu method. Shallot was shown to contain the highest total phenolic content (17.18 mg gallic acid/g) comparing to four varieties of onions. This study further evaluated the antioxidant properties of shallot and four different varieties of onions based on three assays including 2,2-diphenyl-picrylhydrazyl (DPPH) assay, trolox equivalent antioxidant capacity (TEAC) assay, and ferric reducing antioxidant power (FRAP) assay. The results also indicated that shallots showed the best antioxidant activity comparing to four different varieties of onions. The best antioxidant activity of shallots was due to the highest content of phenolic compounds found in shallots (Lu et al., 2011).

Anti-cancer activity of shallot extract was evaluated based on viability of three cancer cell lines including K562, Wehi164 and Jurkat using Trypan blue and LDH assay. The results indicated that the aqueous extract of shallot significantly diminished the viability of three cancer cell lines with IC_{50} values of 100, 100, and 400 µg/mL for K562, Jurkat, and Wehi164, respectively (Mohammadi-Moltlagh et al., 2010).

In addition, a study from the same group of researchers has determined the anti-inflammatory effect of shallot extract using acetic-acid induced vascular

permeability model. At 25, 50, 100 and 200 mg/kg of oral doses, the aqueous shallot extract showed an inhibition of 10.2%, 21.4%, 38.3% and 80.1% in vascular permeability assay, respectively. The results strongly proposed the anti-cancer and anti-inflammatory properties of the shallot extract for medicinal purposes (Mohammadi-Motlagh et al., 2010).

In medicinal field, shallot extract was shown to possess the anti-angiogenesis effect both *in vitro* and *in vivo*. Seyfi et al tested anti-angiogenesis activity of ethyl acetate, n-hexane, n-butanol, and aqueous fraction of shallot extracts (Seyfi et al., 2010). *In vitro* anti-angiogenic activity was examined on human umbilical vein endothelial cells (HUVECs). The results indicated that ethyl acetate fraction of shallot extract showed the best activity against anti-angiogenesis with the LD_{50} value of 3.2 µg/mL. Moreover, *in vivo* anti-angiogenic activity was conducted on chicken chorioallantoic membrane (CAM) model. The results also indicated that ethyl acetate fraction gave the best activity with the IC_{50} value of 1 µg/mL. This research suggested that different solvent of extraction showed different potency in an anti-angiogenesis property.

The antimicrobial activity of water extract of shallots was performed based on the determination of minimal inhibitory concentration by Epsilometer test. Seven microorganisms tested were all sensitive to water extract of shallots with the MIC value of 38, 62.5, 75, 78.1, 156.2, 156.2, and 156.2 µg/mL for *B. subtilis, A. niger, S. aureus, S. typhi, T. rubrum, E. coli,* and *C. humicolus*, respectively (Amin et al., 2009).

2. Quercetin

Among quercetin derivatives isolated from natural plants, free quercetin has been widely examined for its biological activities. Reported biological activities of quercetin include antiviral, anti-cancer, antioxidant, antimicrobial, anti-angiogenesis and anti-melanogenesis properties. (Davis et al., 2008, Zhang et al., 2012, Gao et al., 2000, Seyfi et al., 2010, Arung et al., 2010).

Quercetin is a flavonoid which classified to be a flavonol. This compound is commonly found in fruits, vegetables, flowers, berries, and tea. Quercetin composes of 3 rings and 5 hydroxyl groups (**Figure 4**) in which further forms the backbone for many other flavonoids. Quercetin itself is an aglycone which does not possess a glycoside moiety in its structure, but quercetin found in natural plants usually is in aglycone or glycoside forms. Quercetin is a hydrophobic compound with the molecular formula of $C_{15}H_{10}O_7$, molecular weight of 302.23 g/mol and the melting point of 310-317˚C. This compound is a greenish-yellow crystalline solid (Lakhanpal et al., 2007).

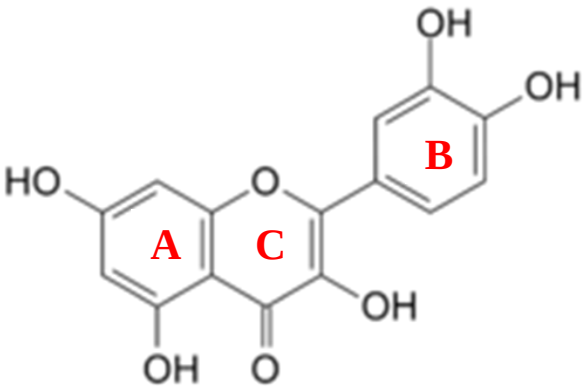

Figure 4 The molecular structure of quercetin

Quercetin is soluble in glacial acetic acid and aqueous alkaline solutions, slightly soluble in alcohol and insoluble in water. Quercetin has the highest solubility in ethanol. Solubilities of quercetin in ethanol at 25 and 37 ˚C are 3.80 and 4.00 mg/mL, respectively (Sutthiparinyanont et al., 2004). Dry powder quercetin was reported to be stable after 60 months under 30˚C, 70% humidity (Quercegen Pharma LLC).

Quercetin was reported to undergo auto-oxidation under high pH conditions (neutral and basic condition); hence, degradation of quercetin is unlikely under acidic solution (Schmalhausen et al., 2007). Another study examined the stability of quercetin at different pH values (2.7, 7, and 10) and different temperatures (room temperature, 4˚C, and -20˚C). The results demonstrated that the stability of quercetin was pH and temperature dependent, in which quercetin was more stable under acidic solution and low temperature (Moon et al., 2008). The photostability of quercetin was also evaluated by exposing quercetin to UVB irridation in which subsequently indicated that quercetin is stable under such conditions (Vicentini et al., 2007).

Quercetin possesses free radical scavenging activity and antioxidant capacity with an IC_{50} value of 10 μM (Gao et al., 2000). Free radical scavenging activity of quercetin is better than baicalein and baicalin (Gao et al., 2000). The o-di-hydroxyl structure in the B ring of quercetin is the site that can directly scavenge free radicals.

Quercetin permeation through skin was also investigated by Franz diffusion cells method using shed snake skin and human skin (Priprem et al., 2009). The permeation rate of quercetin was found to be 0.001 μg/cm^{2}min using shed snake skin; however, it was found to be 4 times lower in human skin. This study also indicated

that the permeation of quercetin through shed snake skin and human skin was occurred via passive diffusion. In addition, the permeation of quercetin through porcine ear skin depended on dosage form and formulation compositions (Fabiana et al., 2009). The results showed that quercetin in w/o microemulsions significantly increased the penetration through stratum corneum with the value of 16.94 μg/cm^2 comparing to propylene glycol, micellar system, and canola oil solutions.

3. Anti-melanogenesis activity of quercetin

Anti-melanogenesis properties of natural compounds recently caught more attention since people are exposing to the sunlight all the time and people in all ages and genders are now considering more on facial products and facial treatments. Therefore, studies on anti-melanogenesis property of natural compounds would be of commercial interest.

Melanogenesis has been described as a formation process of dark macromolecular pigments called melanin in which the amount of melanin in epidermis expresses the skin color. Whenever ultra-violet rays penetrate through human skin, melanocyte stimulating hormones will be released and stimulate melanocytes to produce more melanin resulting in darker skin. Biosynthesis pathway of melanin is illustrated in **Figure 5** (Chang., 2009). Briefly, in melanocytes, tyrosine undergoes oxidation and forms dopaquinone. This first step of melanonegenesis is catalyzed by tyrosinase enzyme. Dopaquinone undergoes auto-oxidation and forms eumelanin and pheomelanin.

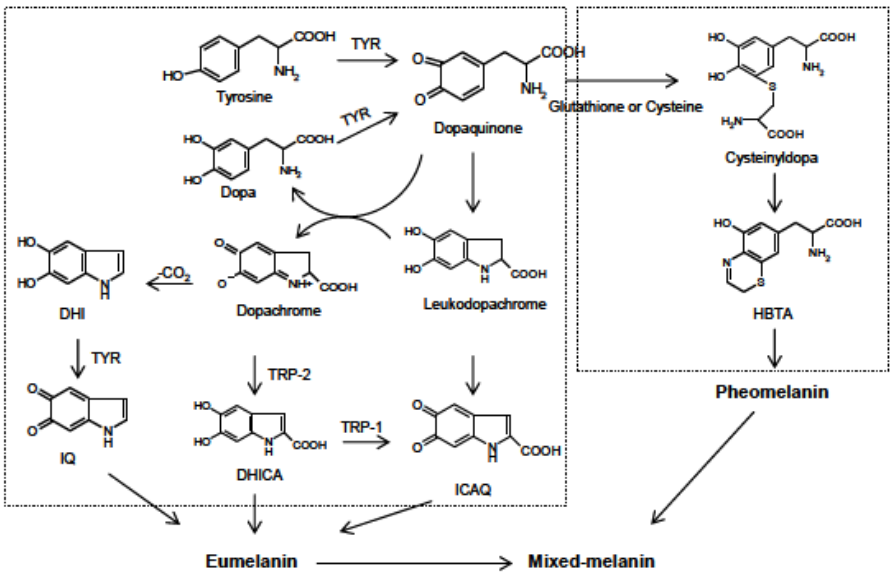

Figure 5 Biosynthesis pathway of melanin

Recent techniques in inhibition of melanogenesis include tyrosinase inhibiton, dopaquinone reduction, alpha-melanocyte stimulating hormone inhibition, and L-dopa reduction. Since tyrosinase enzyme is a catalyst in the rate limiting step of melanin formation, means to inhibit the tyrosinase activity are undergoing crucial inspection (Garcia-Molina et al., 2010, Kubo et al., 2007, Chen et al., 2002).

Tyrosinase is a copper-containing enzyme that catalyzes melanin synthesis in melanocytes. Thus getting rid of tyrosinase enzyme is an efficient way to inhibit melanogenesis. Several studies indicated the chelation positions of quercetin to copper molecules in tyrosinase enzyme to be oxygen atom from 3-hydroxy position and oxygen atom from 4-keto position (**Figure 6**) resulting in the inhibition of tyrosinase enzyme activity (Kim et al., 2006, Kubo et al., 1999).

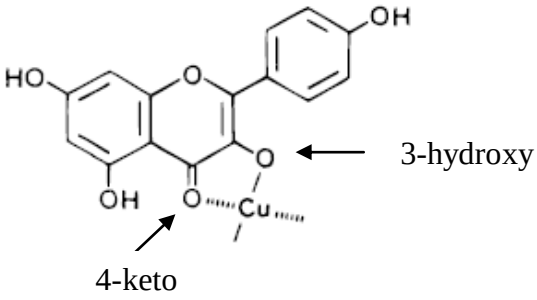

Figure 6 The chelation of quercetin to tyrosinase enzyme

B16 melanoma cell are malignant tumor cells of the melanocytes. This type of cell has long been used in many *in vitro* anti-melanogenesis experiments due to its visible melanin production and easy for culturing. Free quercetin from *Allium cepa* (onion) was reported to have an IC_{50} value of 26.5 μM against B16 melanoma cell lines along with the cell viability value of 88% (Arung et al., 2010). This study considered the 3-hydroxy position of quercetin as a crucial position to indicate the melanin inhibition in B16 melanoma cells (**Figure 6**). In addition, quercetin extracted from *Heterotheca inuloides* was reported to show the IC_{50} value of 0.07 mM against mushroom tyrosinase (Kubo et al., 2000). However, anti-melanogenesis properties of quercetin extracted from shallot has never been reported elsewhere.

Quercetin is a potent compound that possesses a lot of biological activities for medical and cosmetic uses. Moreover, Thailand is a tropical country substantial with herbs and natural plants, the extraction of quercetin from natural sources could strongly increase the market value of Thai natural plants. Furthermore, incorporating natural compounds into stable and safe formulations could further raise the usefulness of Thai plants in cosmetic industries.

4. Emulgels

Topical formulations which had been extensively used include ointments, creams, and lotions. These topical dosage forms have many disadvantages such as their stickiness, low spreadability and poor stability. Consequently, the use of transparent gels has increasingly expanded in both cosmetics and pharmaceutical preparations. However, gels have a disadvantage in term of the delivery of hydrophobic drug since gels typically compose of aqueous. A combination of emulsions and gels, emulgels, is being approached so that a successful incorporation and delivery of a hydrophobic therapeutic moiety is practical and poor physical stability of emulsions is overcome (Panwar et al., 2011).

Emulgels are prepared by incorporation of hydrophobic compound into an oil phase. The oil phase is dispersed in an aqueous phase resulting in o/w emulsion. The obtained emulsion is further mixed with gel base to form emulgels. Emulgels show better physical stability since high viscosity given by the gelling agent prevents formulation from phase separation, phase inversion or phase breaking. Preparation of emulgels is simple with short steps. There is no need of special instruments for the production of emulgels resulting in high production feasibility and lower preparation cost. (Panwar et al., 2011).

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Raw material

1. Fresh shallot bulbs (Talad-Taopoon, Bangkok, Thailand)

1.2 Equipment and instruments

1. Filtering paper (Whatman no.1) (Whatman International Ltd., UK)

2. Rotary evaporator (RE 120) (Buchi, USA)

3. Lyophilizer (FTS system) (Dura-dry, USA)

4. TLC scanner (CAMAG, USA)

5. TLC spotter (LINOMAT 5, USA)

6. Aluminium silica gel plate (E. Merck, Germany)

7. Vortex mixer (Scientific industries, USA)

8. Micropipettes (50-200 µL), (100-1000 µL), (1-5 mL) (BIOHIT, Finland)

9. Micropipettes tips (BIOHIT, Finland)

10. De-ionized water (DI water) system (ELGAStat Option 3B) (ELGA, UK)

11. HPLC (Shimadzu LC-10, Japan)

12. C18 reversed phase column (4.6x150mm, 3.5 µm, AGILENT, USA)

13. Disposable syringe 1 mL without needle (NIPRO, USA)

14. Membrane filter (13 mm, 0.45 Nylon, LUBITECH, China)

15. Centrifuge (CENTRIFUGETTE 4206)

16. Microplate reader (Wallac, VICTOR 3, USA)

17. Balance (AX/MX/UMX, METTLER TOLEDO, Switzerland)

18. Vortex (VORTEX GENIE 2)

19. Incubator (MEMMERT, Germany)

20. Cooler (HETOFRIG, UK)

21. pH meter (PB20, SARTORIUS, USA)

22. High speed refrigerated micro centrifuge (MX305, TOMY, Japan)

23. Water bath (Becthai, Thailand)

24. Mobile phase filtration (KONTES, USA)

25. Microplate reader (Beckman Coulter, AD 200, USA)

26. Multimode detector (Beckman Coulter, DTX 880, USA)

27. Sonicator (Elma, Germany)

28. Vacuum pump (Waters, USA)

1.3 Chemicals

1. Methanol (HPLC grade, RCI Labscan, Thailand)

2. Acetronitrile (HPLC grade, RCI Labscan, Thailand)

3. Isopropyl alcohol (HPLC grade, RCI Labscan, Thailand)

4. Ethanol (RCI Labscan, Thailand)

5. Ultrapure water (ELGA, UK)

6. Hydrochloric acid (RCI Labscan, Thailand)

7. Toluene (RCI Labscan, Thailand)

8. Ethylacetate (RCI Labscan, Thailand)

9. Formic acid (RCI Labscan, Thailand)

10. Trifluoroacetic acid (Fluka, Germany)

11. Quercetin (Sigma-Aldrich, USA)

12. L-DOPA (Sigma-Aldrich, USA)

13. Mushroom tyrosinase (Sigma-Aldrich, USA)

14. $NaH_2PO_4 \cdot 2H_2O$ (Ajax Fine Chem, Australia)

15. Na_2HPO_4 (E. Merck, Germany)

16. Carbopol 940 (Namsian, Thailand)

17. Jojoba oil (S. Thong, Thailand)

18. Dimethicone (S. Thong, Thailand)

19. Methylparaben (S. Thong, Thailand)

20. Cetyl alcohol (Chemicals of Highest Quality, Malaysia)

21. Propylene glycol (Chemicals of Highest Quality, Malaysia)

22. Cremophor 40 (S. Thong, Thailand)

23. Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA)

24. Presto blue (Invitrogen, USA)

25. 0.25% Trypsin (Gibco, USA)

26. Tryphan Blue (Gibco, USA)

1.4 Cell line

1. B16 melanoma cell line (ATCC, USA)

2. Methods

2.1 Preparation of *Allium ascalonicum* (shallot) extract and fractions

Preparation of shallot extract and fractions was performed by grinding 3 kilograms of fresh shallot bulbs and mixing with 300 mL of ethanol : water (60:40 v/v) solution or ultrapure water (**Figure 7**). The mixture was filtered through a cotton sheet and a filtering paper (Whatman no.1). The filtrate (fraction 1) was then further collected and concentrated using a rotary evaporator (rotavapor RE120) prior to freeze-drying. The marc was further extracted with 300 mL of the same solvent and filtered through a cotton sheet and a filtering paper (Whatman no.1) under suction to obtain fraction 2. Fractions were separately collected, and concentrated using a rotary evaporator under 175 atm at 40 ˚C. Each fraction was further lyophilized using a FTS system. In order to obtain the maximum concentration of an active compound in the extracts, the lyophilized fraction 1 and 2 were then hydrolyzed by mixing 25 g of the lyophilized extract with 500 mL of 0.5 M HCl in 50% methanol and reflux at 60 ˚C for 0, 25, 50, 75, and 100 minutes prior to evaporation until dryness.

Preparation of shallot extracts was performed in triplicate. Percent yield of each extract was calculated from equation 1.

$$\% \text{ Yield} = \frac{\text{Mass of lyophilized powder (g)}}{\text{Mass of fresh shallot bulbs (g)}} \times 100 \qquad \text{Equation 1}$$

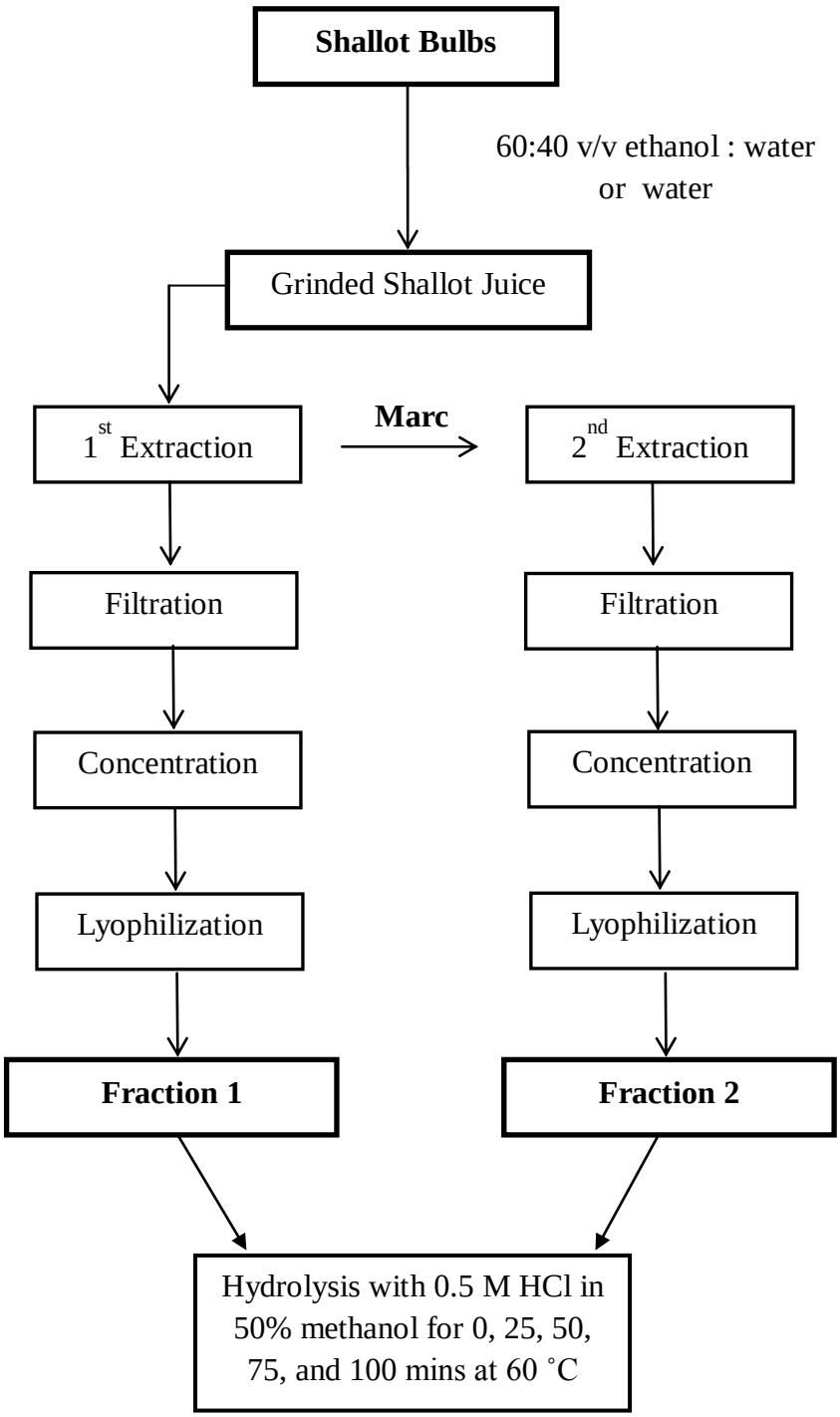

Figure 7 Preparation of shallot crude extract and its fractions

2.2 Characterization of shallot extract

Major compounds in the shallot extracts were characterized by high performance liquid chromatography (HPLC) technique and thin layer chromatography (TLC) technique.

2.2.1 HPLC Characterization

Shallot extracts or the extracts after hydrolysis was dissolved in methanol : water (80:20 v/v) to obtain a final concentration of 10 or 5 mg/mL, respectively.

A stock solution of quercetin standard was prepared at a final concentration of 40 µg/mL in methanol : water (80:20 v/v). The stock solution was further diluted with methanol : water (80:20 v/v) to acquire the concentrations of 0.1, 0.5, 1, 5, 10, 15, 20, and 25 µg/mL.

The HPLC conditions were modified from previously reported methods (Shon et al., 2004, Arung et al., 2011). The separation was achieved on a Shimadzu LC10 equipped with a C18 reversed phase column (4.6x150 mm, 3.5 µm, Agilent) using acetonitrile : water (35:65 v/v) with 0.06% trifluoroacetic acid as a mobile phase with a flow rate of 1 mL/min. An injection volume was set at 20 µL. An analytical wavelength of 371 nm was employed.

Retention times of major peaks in each extract were compared along with the retention time of quercetin standard. Peak areas of quercetin standard peak were plotted against their corresponding concentrations in order to

construct a standard curve. Concentration of quercetin in each fraction was calculated using the standard curve.

2.2.2 TLC Scanner Characterization

Shallot extracts were dissolved in methanol : water (80:20 v/v) to obtain the final concentration of 50 mg/mL. Ten microliters of each sample were separately spotted on a TLC aluminium silica gel plate using spotter (Linomat 5). The TLC plate was developed by a mixture of 5:4:0.2 v/v/v of toluene : ethlyacetate : formic acid as a mobile phase in a glass tank until the mobile phase reached the top of TLC aluminium silica gel plate (Kugasemrat et al., 2009). Quercetin was used as a standard by preparing a 250 µg/mL stock solution of quercetin in methanol : water (80:20 v/v). The stock solution was further diluted with methanol : water (80:20 v/v) to acquire concentrations of 50, 100, 125, 150, 175, and 200 µg/mL. The aluminium silica gel plate was further analyzed by a TLC scanner (CAMAG) at an analytical wavelength of 371 nm.

TLC chromatogram characteristics including peak positions (R_f value) or peak areas were compared along with those of quercetin standard. Peak areas of quercetin standard solutions were plotted against their corresponding concentrations in order to construct a standard curve. Concentration of quercetin in each fraction was calculated using the standard curve.

2.3 Anti-melanogenesis properties of shallot extract

Anti-melanogenesis property of the shallot crude extract was evaluated using the mushroom tyrosinase enzyme assay and B16 melanoma cells assay.

2.3.1 Mushroom tyrosinase enzyme assay

Mushroom tyrosinase inhibitory activity was performed in a 96-well plate. L-DOPA was used as a substrate while quercetin standard was used as a positive control. Generally, 80 µL of shallot extract solutions in methanol : water (80:20 v/v), quercetin standard solutions in methanol : water (80:20 v/v) at various concentrations, or the solvent methanol : water 80:20 v/v (control) were added into 40 µL of phosphate buffer (pH 6.8), and followed by an addition of 40 µL of mushroom tyrosinase (480 units/mL) in phosphate buffer (pH 6.8). Blank of control was prepared by an addition of 80 µL of methanol : water (80:20 v/v) into 80 µL of phosphate buffer (pH 6.8), while blank of sample was prepared by an addition of 80 µL sample solutions in methanol : water (80:20 v/v) into 80 µL of phosphate buffer (pH 6.8). Forty microliters of 0.85 mM L-DOPA substrate in phosphate buffer (pH 6.8) was then added in each well prior to incubation at 25 ˚C for 10 minutes. Test samples were varied in a concentration range of 12.5 – 87.5 mg/mL while quercetin standard solutions were varied in a range of 75 – 150 µg/mL. Formation of DOPAchrome in the wells containing samples or quercetin standard solution was observed at an analytical wavelength of 492 nm using a microplate reader (Wallac, VICTOR 3) (Jithavech 2005). The assay was performed in triplicate.

The percent inhibition of tyrosinase activity was calculated as follows:

$$\left\{\qquad\qquad\right\}$$

$$\% \text{ Inhibition} = \frac{(A - B) - (C - D)}{(A - B)} \times 100$$

A : absorbance of solution without test sample

B : absorbance of solution without test sample and enzyme

C : absorbance of solution with test sample

D : absorbance of solution with test sample, but without enzyme

The IC_{50} value (concentration at 50% tyrosinase inhibition) of each shallot extract was estimated from a plot of percent tyrosinase inhibition versus concentration. A lower IC_{50} value indicates greater anti-tyrosinase activity.

2.3.2 B16 melanoma cells assay

In this part of research, a mouse melanoma cell line (B16) was used in determination of melanin inhibition according to method reported by Arung et al., 2011. Briefly, the cells at a density of 10^4 cells/well were seeded in two 96-well plates (one plate for determining of melanin and the other for determination of cell cytotoxicity in section 2.4) and incubated at 37 ˚C in humidified atmosphere of 5% CO_2 for 24 hours prior to sample treatments. Fresh media with or without shallot ethanolic extract at various concentrations were added, quercetin was used as a positive control. Test samples were varied in a concentration range of 6.25 – 500 µg/mL while quercetin standards were varied in a concentration range of 0.625 – 50 µg/mL. The cells were then incubated for additional 72 hours. After the incubation period, 100 µL of the solutions in each well was taken to a new 96-well plate and assayed using

microplate reader (Beckman Coulter, AD 200) at 405 nm in order to determine the melanin content.

Percent melanin content was calculated by comparing absorbance values of sample treated with shallot ethanolic extract with those of control. The lower the percent melanin content depicts the better activity of the samples.

The percent inhibition was calculated as follows:

$$\% \text{ melanin} = \frac{(A - B)}{(C - B)} \times 100$$

A = absorbance of solution with test samples and cells

B = absorbance of solution without test samples and cells

C = absorbance of solution with cells and solvent, without test samples

2.4 Cell Cytotoxicity Test

Cell cytotoxicity was performed according to the method reported by alamarBlue® company. Cell viability was analyzed using presto blue technique in which the number of viable cells was detected from color change by the interaction of presto blue with active cells. B16 melanoma cells were cultured and exposed to shallot ethanolic extract at various concentrations in the same manner as mentioned in 2.3.2. After 24 and 72 hours of incubation, the medium containing corresponding substances was removed prior to an addition of PBS. PBS was furthered replaced by the addition of 100 µL of presto blue in each well. The plate was further incubated at 37 ˚C in humidified atmosphere of 5% CO_2 for 1 hour. The absorbance of each well in the plate was measured using a multimode detector (Beckman Coulter, DTX 880)

at 535 nm relative to 615 nm. Higher absorbance values at 535 nm relative to 615 nm indicate more cell viability. Cell viability was calculated by comparing absorbance values of samples treated with shallot ethanolic extract with those of control.

2.5 Development of emulgel containing shallot extract

After the preparation, characterization, and determination of anti-melanogenesis activity of the shallot ethanolic extract were achieved, emulgel formulation containing shallot ethanolic extract was performed in order to determine its stability and irritancy.

2.5.1 Preparation of emulgel containing shallot extract

Emulgel containing shallot ethanolic extract was prepared using beaker method. Briefly, 0.3%, 0.5%, 0.75%, 1%, 1.5%, and 2% of Carbopol 940 were employed as a gelling agent. Appropriate volume of triethanolamine was added into the carbopol solution until gel was formed. Different types of oil including mineral oil, jojoba oil, or grapeseed oil in a concentration range of 6% to 30% were employed. Emulsifiers including Cremophor40, Span80, or Brij72 were added in the oil phase while Tween80 or Brij721 were added in the aqueous phase (**Table 1, 2, 3**). Oil phase and water phase were heated to 70˚C and 75˚C, respectively, prior to a slow addition of the aqueous phase to the oil phase with continuous agitation until congeal. The carbopol gel was dispersed in water overnight prior to an addition into the obtained emulsion. Shallot ethanolic extract fraction 2 was separately dissolved in 1 mL of 50:50 v/v of ethanol : ultrapure water in the presence of propylene glycol and added to the formulation at room temperature. Methyl paraben at concentration of

1% was then dropped into the formulation and mixed well. The first part of this study, formulation 1 – 24, was the development of emulsion base; therefore, there was no addition of the shallot ethanolic extracts (**Table 1 - 3**)

In the second part of the study, 0.003% of quercetin standard was dissolved in propylene glycol and added into the formulation in order to observe stability of quercetin, the major active ingredient in the formulations (Formulation 1 – 24, **Table 1 - 3**). The formulations were selected based on their appearances, stickiness, oiliness, smoothness, spreadability, and physical stabilities. The base formulations with desirable properties were developed further by incorporating shallot ethanolic extracts instead of quercetin (**Table 3**).

In the third part of the study, 5% of the shallot ethanolic extract fraction 2 was dissolved in 1 mL of 50:50 v/v of ethanol : ultrapure water in the presence of propylene glycol prior to an addition into formulations (Formulation 25 – 27, **Table 3**). The development scheme of formulation preparation is shown in **Figure 8.**

Table 1 The % w/w of ingredients used in Formulation F1 – F10

Formulation	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Oil phase										
Stearic acid	41.67	10	6	6	6	6	6	6		
Lanolin anhydrous	6.67									
Cetyl alcohol		5	4	4	4	4	4	4	10	10
Mineral oil		20	20	20	20	20				
Jojoba oil							20		20	
Grapeseed oil								20		20
Dimethicone			2	2						
Brij72					1.2					
Span80						0.9	3.14	2.78	3.36	2.98
Cremophor RH-40										
Water phase										
Triethanolamine	2.50	3	3							
Sodium laurly sulfate				3						
Brij721					4.8					
Tween80						5.1	2.86	3.22	2.64	3.02
Propylene glycol	6.67	5	6	6	6	6	6	6	6	6
Methyl Paraben	3.33	1	1	1	1	1	1	1	1	1
Water	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Quercetin	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003

Table 2 The % w/w of ingredients used in Formulation F11 - F21

Formulation	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20	F21
Oil phase											
Stearic acid											
Lanolin anhydrous											
Cetyl alcohol	10	10	10	10	8	8	6	6	4	4	6
Mineral oil											
Jojoba oil	20		20		16		14		16		6
Grapeseed oil		20		20		16		14		16	
Dimethicone											2
Brij72											
Span80	4.8	4.6	3.36	3	3.36	3	3.6	3.2	4	3.6	2.96
Cremophor RH-40			1.32	1.5	1.32	1.5	1.2	1.4	1	1.2	1.52
Water phase											
Triethanolamine											
Sodium laurly sulfate	0.6	0.7									
Brij 721											
Tween80	0.6	0.7	1.32	1.5	1.32	1.5	1.2	1.4	1	1.2	1.52
Propylene glycol	6	6	6	6	6	6	6	6	6	6	6
Methyl Paraben	1	1	1	1	1	1	1	1	1	1	1
Water	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Quercetin	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003

Table 3 The % w/w of ingredients used in Formulation F21 - F27

Formulation	F21	F22	F23	F24	F25	F26	F27
Oil phase							
Cetyl alcohol	6	6	6	6	6	6	6
Jojoba oil	6	6	6	6	6	6	6
Dimethicone	2	2	2	2	2	2	2
Span80	2.96	2.96	2.96	2.96	2.96	2.96	2.96
Cremophor RH-40	1.52	1.52	1.52	1.52	1.52	1.52	1.52
Water phase							
Tween80	1.52	1.52	1.52	1.52	1.52	1.52	1.52
Propylene glycol	6	6	6	6	6	6	6
Paraben	1	1	1	1	1	1	1
Water	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Carbopol 940		0.3	0.5	0.75	1	1.5	2
Rose oil					0.01	0.01	0.01
Quercetin	0.003	0.003	0.003	0.003			
Shallot extract					5	5	5

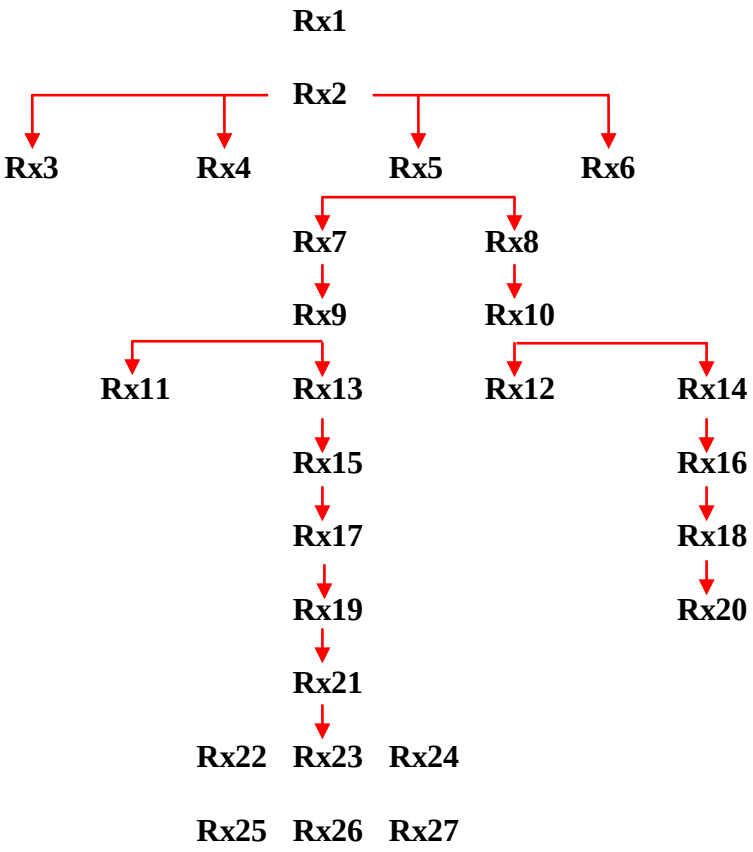

Figure 8 The scheme of formulation development

2.5.2 Physical and chemical stability test of emulgel containing shallot ethanolic extract

The selected emulgel formulations were subjected to tests for physical and chemical stability. The centrifugation and heating-cooling cycle were performed in order to evaluate the physical stabilities while the chemical stability was evaluated according to Asean Guideline on Stability Study of Drug Product (9th ACCSQ-PPWG Meeting, Philippines, 2005).

The centrifugation was conducted at 6000 rpm for 30 minutes according to Thai Industrial Standard (152-2539). Heating-cooling cycle was

performed at 40˚C for 48 hours and 4˚C for 48 hours (Marquardt et al., 1998). The selected formulations were subjected to 8 cycles of centrifugation and 6 heating-cooling cycles. At the end of each cycle, samples were taken and evaluated for their physical appearances, color change, and pH values.

The selected formulations were also stressed in stability chambers at 30˚C, 75% RH and 40˚C, 75% RH for 3 months according to Asean Guideline on Stability Study of Drug Product. Samples were taken at 0, 1, 2, 3, 4, 6, 8, 10, 12 weeks and evaluated for concentration of quercetin present in the formulation using HPLC technique as mentioned in 2.2.1.

- **Preparation of samples and standards**

Emulgel containing shallot ethanolic extract (0.2 g) was dissolved in an appropriate volume of isopropyl alcohol (IPA) prior to assay. Standard curve was constructed from peak areas of quercetin standard solution in isopropyl alcohol in a concentration range of 0.5 – 8 µg/mL. A typical standard curve showed linearity with R^2 > 0.99. Concentration of quercetin in the formulation was calculated from the corresponding standard curve.

2.6 Irritation test of emulgel containing shallot extract

Irritation test was conducted regarding to the method reported by Internatinal Contact Dermatitis Research Group (ICDRG) (Curry et al, 1991). Briefly, 20 volunteers (men or women) aged 18 to 50 years were enrolled in the test. Individuals were excluded if they had any history of skin diseases or conditions that may interfere with the evaluation of skin reaction. About 0.2 gram of test formulations (emulgel

containing shallot crude extract and emulgel base) was applied on one square inch of an individual back. An adhesive tape was placed on the applied area. The patches were left in place for 24 hours. After 24 hours, the patches were removed. The applied areas were cleaned with soaked cotton prior to immediately evaluate for any irritation by a dermatologist. The applied areas were re-evaluated at 48 and 72 hours according to Internatinal Contact Dermatitis Research Group (ICDRG) scale (**Table 4**).

Table 4 Internatinal Contact Dermatitis Research Group (ICDRG) scale

Grade	Description
0	No observable reaction occurred
+	Slight reaction occurred (small rednees of skin attached by the patch is observed)
++	Moderate reaction occurred (redness of skin attached by the patch is clearly observed)
+++	Severe reaction occurred (redness and swellness of skin attached by the patch is clearly observed)

CHAPTER IV

RESULTS AND DISCUSSION

3.1 Preparation of *Allium ascalonicum* (Shallot) extract and fractions

Shallot were extracted using ethanol : water (60:40 v/v) or ultrapure water as solvent and gave rise to lyophilized products with reddish brown crystalline powder or reddish pink crystalline powder, respectively. The appearance of lyophilized products of fraction 2 was similar to its fraction 1 (**Figure 9**). The percent yields were reported in **Table 5**.

Ethanolic extract Water extract

Figure 9 Lyophilized powder (fraction 1) extracted from *Allium ascalonicum*

Table 5 Percent yield of lyophilized powder of *Allium ascalonicum* extract

	Ethanolic extract 1st Extraction	**Ethanolic extract 2nd Extraction**	**Water extract 1st Extraction**	**Water extract 2nd Extraction**
Batch1	1.51%	1.22%	1.78%	0%
Batch2	2.17%	1.60%	2.43%	0%
Batch3	2.96%	2.24%	3.17%	0%
Mean ± SD	2.21% ± 0.73%	1.69% ± 0.52%	2.46% ± 0.70%	0%

Three batches of shallot extracts were prepared using 3 different batches of fresh shallots bought from Taopoon market, Thailand in November 2010, April 2011, and June 2011, respectively. It is well known that percent yield of herbal extract highly depends on harvest time. The results in **Table 5** confirmed that different batches gave different percent yield. Batch 3 gave the highest yield since the shallot bulbs were bought in rainy season when shallots seemed to give the highest amount of flesh and fiber after squeezing comparing to batch 1 and batch 2. Water extract tended to give higher percent yield because the water extracts were expected to contain other water soluble compounds including mineral and sugar that could pass the filtration process. Red color of the lyophilized powder from the aqueous extract was speculated to come from anthocyanin, a water soluble compound giving red color in shallots (Shigyo et al., 1997). However, the second water extraction of shallot gave negligible yield. This suggested that most of water soluble compounds were extracted by the first water extraction step. On the other hand, ethanolic solution could dissolve both polar and semi-polar components in the shallots. Therefore, less polar compounds such as flavonoids were expected to be dissolved and extracted in this solvent. This result suggested that different solvents gave different compounds due to their similarity of the polarity between solvent and compounds.

Ethanolic lyophilized powder was further hydrolyzed with 0.5 M HCl in 50% methanol for 0, 25, 50, 75, and 100 minutes in order to convert quercetin glucosides to free quercetin (Kugasemrat et al., 2009); this part will be discussed in more detail in the characterization part. The picture of the hydrolysis of shallot extract using reflux system is shown in **Figure 10**. After 25 minutes of heating by using a reflux system, the shallot solution was obviously red because anthocyanin was in an

aglycone form which giving red color under acidic conditions (**Figure 11**) (Delgado-Vargas et al., 2003). However, hydrolyzed fractions after evaporation were dark-brown viscous liquid with strong odor resulting from the use of acid solutions.

Figure 10 The picture of hydrolysis of shallot extract using reflux system

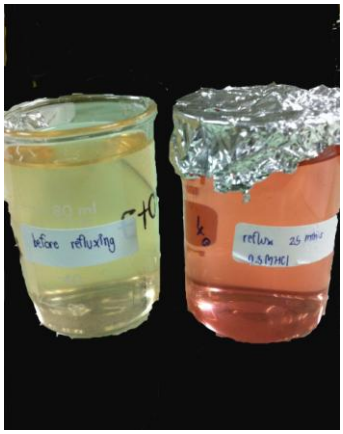

Figure 11 Appearance of shallot solution before (left) and after (right) reflux for 25 minutes

The obtained lyophilized powders including ethanolic extract fraction 1, ethanolic fraction 2, water extract fraction 1, and ethanolic fractions after hydrolysis

were further characterized for their major components using thin layer chromatography technique and high performance liquid chromatography technique.

3.2 Characterization of shallot extract

3.2.1 HPLC Characterization

The same fraction from batch 1, 2, and 3 were pooled prior to characterization. HPLC chromatograms of shallot extracts fraction 1 and fraction 2 showed one major peak with a retention time around 3 min (**Figure 12b** and **12c**) corresponding to quercetin (**Figure 12a**) while the peak at the same retention time was not found in water extract (**Figure 12d**). The results indicated that ultrapure water could not solubilize quercetin since quercetin is insoluble in water. The HPLC chromatograms of shallot fractions after hydrolysis also illustrated the presence of quercetin (**Figure 13**).

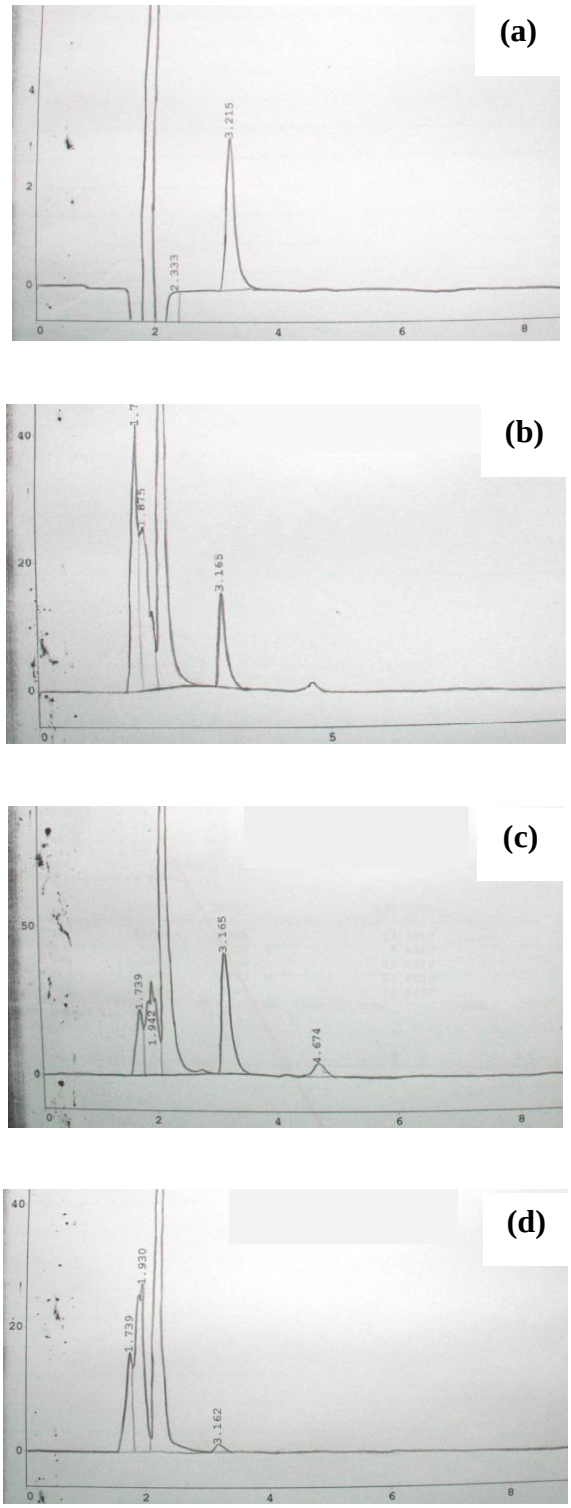

Figure 12 HPLC chromatograms of quercetin standard (a), shallot ethanolic fraction 1 (b), shallot ethanolic fraction 2 (c), and shallot water fraction 1 (d)

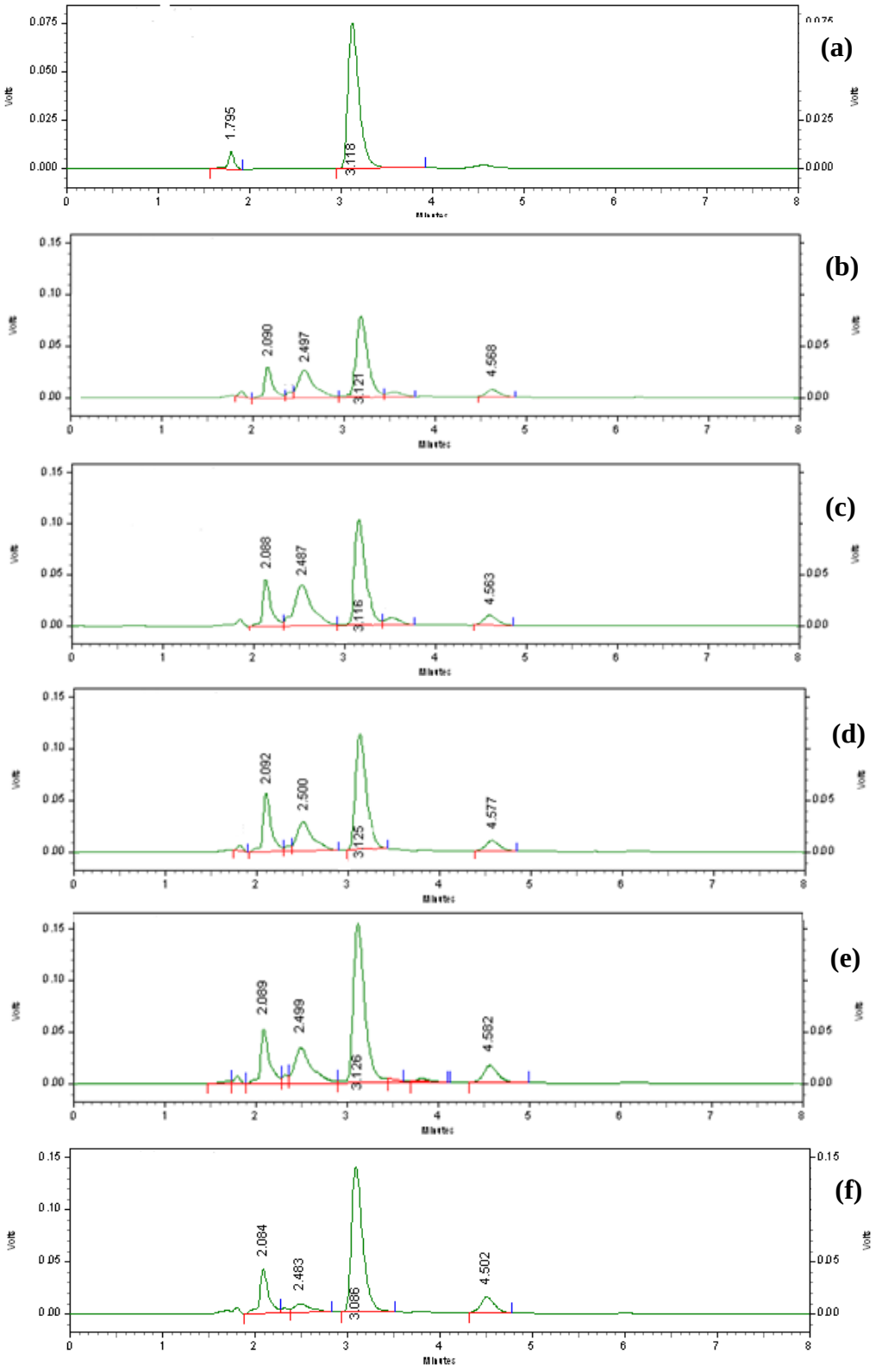

Figure 13 The HPLC chromatograms of quercetin standard (a), shallot fraction 2 after hydrolysis with heat for 0 (b), 25 (c), 50 (d), 75 (e), and 100 minutes (f)

The quercetin standard curve was constructed in order to determine quercetin concentration found in each fraction (**Figure 14**). **Table 6** showed that ethanolic extract fraction 2 gave higher amount of quercetin comparing to ethanolic extract fraction 1. Ethanol in the second extraction was not much further diluted by water present in the marc; thus, higher amount of quercetin could be dissolved. In other words, during the first ethanolic extraction, ethanolic solvent was diluted by water present in shallot flesh. The mixture of ethanolic solvent and shallot flesh was then squeezed. The marc contained lower amount of water than that of fresh shallot. Therefore, ethanol in the solvent of the second extraction step was not much further diluted by water in shallot flesh and could solubilized quercetin at higher amount.

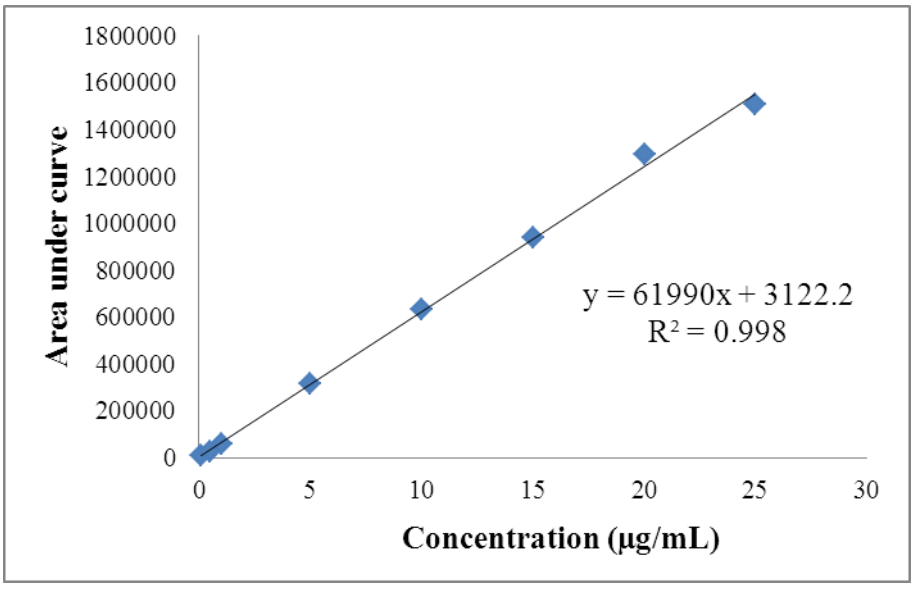

Figure 14 Quercetin standard curve (HPLC Characterization)

The results also showed that quercetin concentration was increased as hydrolysis time was increased (**Table 6**). The concentration of quercetin reached the highest level at the hydrolysis time of 75 minutes and then the concentration of quercetin was slowly decreased. In nature, quercetin is presented as free quercetin and

quercetin glycosides. In addition, quercetin glycoside was reported to show lower biological activity due to its glucoside moiety (Arung et al., 2011). Hydrolysis of shallot extract under heat was an attempt to convert quercetin glucoside to free quercetin (Kugasemrat et al., 2009). Nevertheless, too long reflux time may cause quercetin decomposition (Costa et al., 2002).

Table 6 Concentration of quercetin in shallot fractions using HPLC characterization

	Fraction 1	**Fraction 2**	**Hydrolyzed time (min)**				
			0	**25**	**50**	**75**	**100**
Quercetin µg/mg extract	0.60 ± 0.03	2.58 ± 0.05	2.14 ± 0.05	2.65 ± 0.05	2.99 ± 0.06	3.81 ± 0.04	3.76 ± 0.06

(mean ± SD, n = 3)

3.2.2 Thin layer chromatography characterization

TLC chromatograms of shallot ethanolic extract fraction 1 and 2 (**Figure 15b** and **15c**) showed one major peak with a R_f value of 0.4 corresponding to quercetin (**Figure 15a**). Percentage of quercetin presented in each fraction of each batch were estimated based on a standard curve of quercetin (**Figure 16**) and reported in **Table 7**. Chromatograms of shallot fractions before hydrolysis, shallot fractions after hydrolysis for 0, 25, 50, 75, and 100 minutes with heat are shown in **Figure 17**.

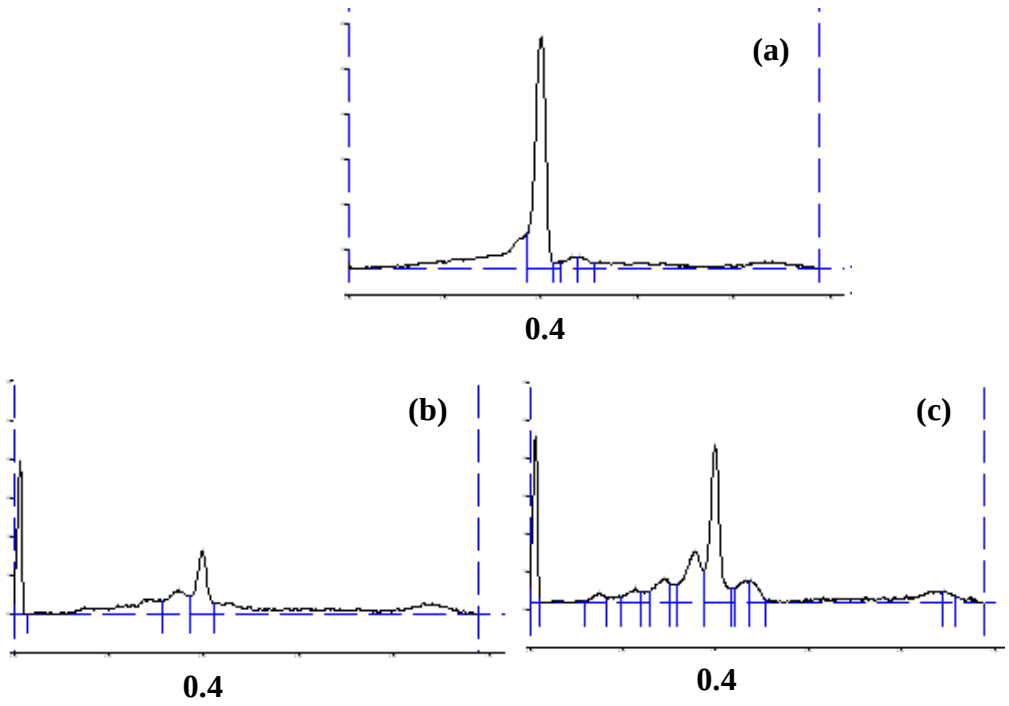

Figure 15 TLC chromatograms or quercetin standard (a), shallot ethanolic extract fraction 1 (b), and shallot ethanolic extract fraction 2 (c)

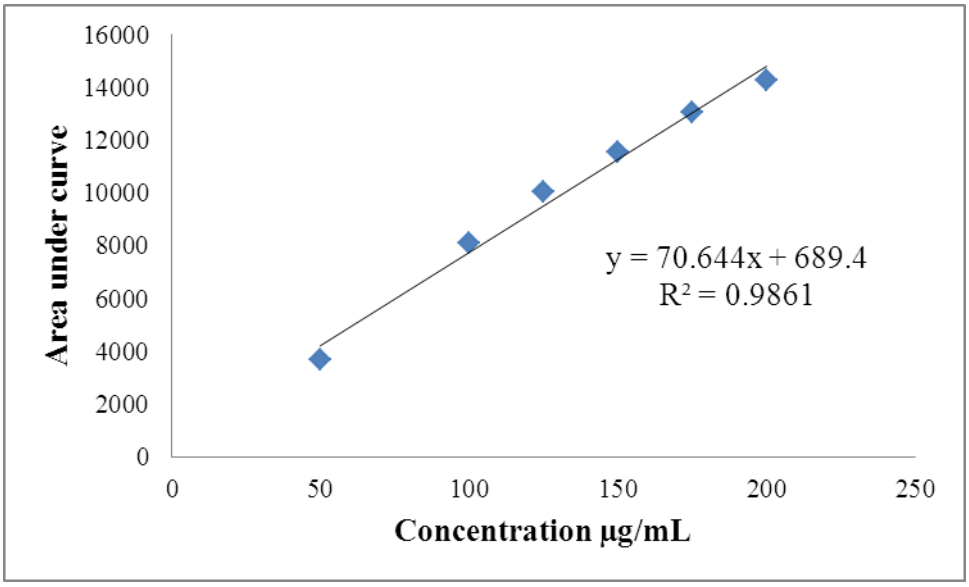

Figure 16 Quercetin standard curve (TLC characterization)

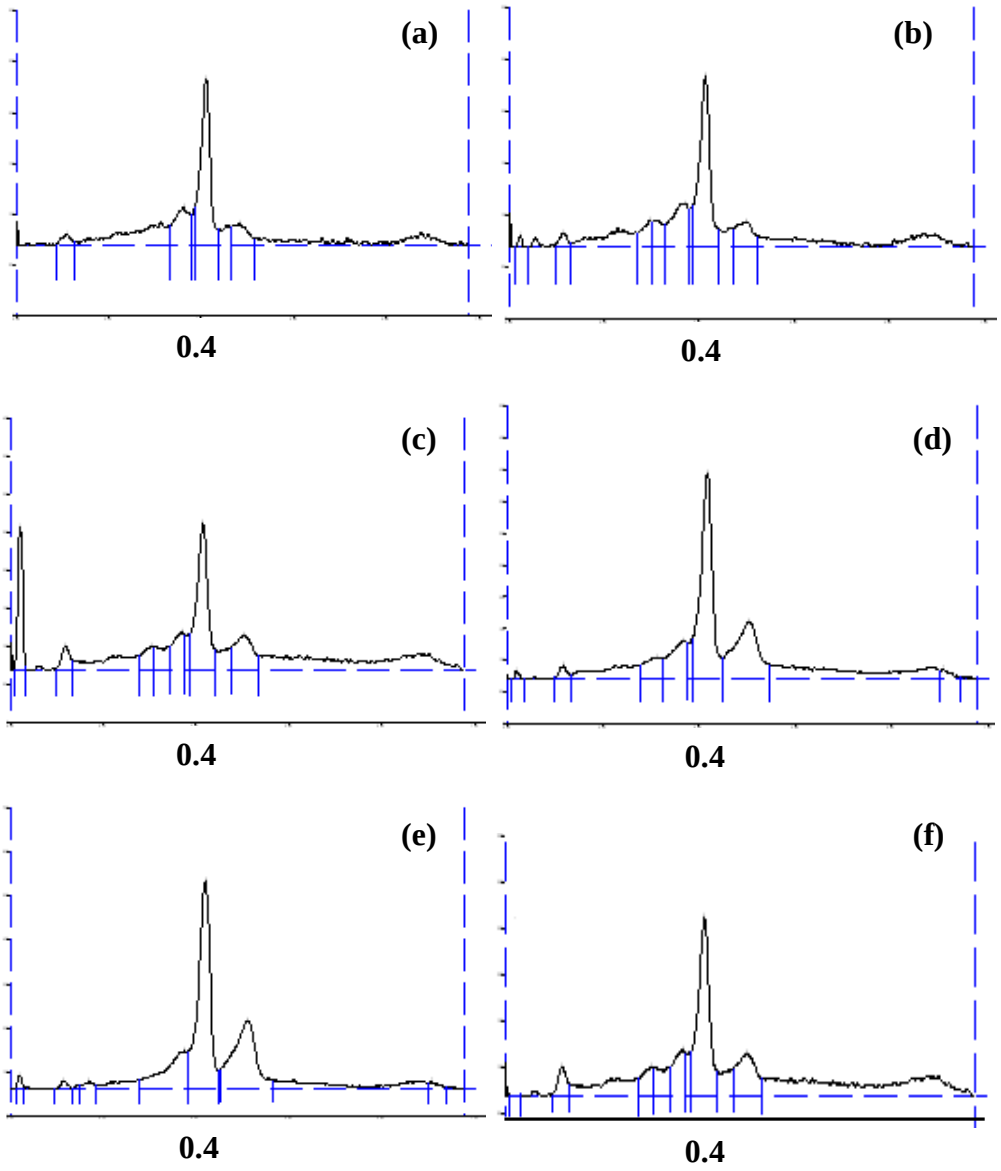

Figure 17 TLC chromatograms of shallot fraction before hydrolysis (a), shallot fraction after hydrolysis for 0 (b), 25 (c), 50 (d), 75 (e), and 100 minutes (f)

Table 7 Concentration of quercetin in shallot fractions using TLC characterization

Batch number	Fraction 1	Fraction 2	before hydrolysis	Hydrolyzed time (min)				
				0	25	50	75	100
1	0.33	1.04	1.00	1.09	1.31	1.64	3.22	2.48
2	0.98	3.04	1.68	1.88	2.26	2.84	3.40	3.02
3	0.28	1.26	0.80	0.89	1.17	2.14	3.44	2.95
Mean ± SD	0.53±0.39	1.78±1.10	1.16±0.46	1.29±0.52	1.58±0.59	2.21±0.60	3.36±0.12	2.81±0.29

Note: The unit of quercetin concentration was reported in µg/ mg extract

In all batches, concentration of quercetin in the fraction 2 was higher than that of in the fraction 1 which was consistent with the result previously reported in section 3.2.1. The ethanolic extract fraction 1 and 2 before hydrolysis was combined. The extract was refluxed with 0.5 M HCl in 50% methanol up to 100 minutes. The results showed that concentration of quercetin determined by TLC scanner was increased as hydrolysis time increased (**Table 7**). These results were consistent with the results previously reported in section 3.2.1. Therefore, the optimum condition to hydrolyze quercetin glucoside in the shallot extract was to hydrolyze at 60 ˚C for 75 minutes.

Although hydrolysis of shallot extract would give a higher amount of free quercetin, the active anti-melanogenesis compound, the dark-brown color with strong odor of the extract after hydrolysis was unpleasant for cosmetic use. Moreover, reflux process not only needs a special set of equipment but also requires additional manufacturing time and cost. Therefore, shallot ethanolic fraction 2 which contained high concentration of free quercetin was selected for further studies. The attempt to increase free quercetin concentration in the extract by hydrolysis process was then dropped out.

3.3 Anti-melanogenesis properties of shallot extract

3.3.1 Mushroom tyrosinase enzyme assay

The anti-tyrosinase activity of *Allium ascalonicum* was evaluated based on DOPAchrome enzymatic method using L-DOPA as a substrate (Jithavech, 2005). Anti-tyrosinase activities of shallot ethanolic fraction 1 and shallot ethanolic fraction 2 were determined in triplicates. The final concentration of standards and samples in each well at the beginning of the reaction were re-calculated and the percent inhibitions of tyrosinase were reported in **Table 8** and **9**.

Table 8. The percent inhibition of tyrosinase enzyme of shallot ethanolic extract fraction 1 and shallot ethanolic extract fraction 2 (mean ± SD, n = 3)

Concentration of extract (mg/mL)	**% Inhibition**	
	Shallot ethanolic fraction 1	**Shallot ethanolic fraction 2**
	Mean ± SD	**Mean ± SD**
5	-19.78 ± 0.61	31.84 ± 2.55
10	-7.57 ± 0.69	40.27 ± 2.01
15	-2.26 ± 0.83	47.48 ± 2.62
20	7.97 ± 1.74	56.33 ± 1.78
25	11.82 ± 1.51	63.95 ± 1.03
30	15.94 ± 1.38	72.11 ± 0.47
35	19.79 ± 1.22	80.27 ± 1.31

Table 9. The percent inhibition of tyrosinase enzyme of quercetin standard (mean ±SD, n = 3)

Concentration of quercetin (µg/mL)	**% Inhibition**
	Mean ± SD
30	22.72 ± 0.85
40	30.07 ± 1.03
50	37.28 ± 2.72
60	45.31 ± 2.04
70	53.33 ± 0.63

Table 8 and **9** showed that anti-tyrosinase activity of ethanolic extract fraction 1, ethanolic extract fraction 2 and quercetin standard increased as concentration of the extract and quercetin increased. Shallot ethanolic extract fraction 2 possessed higher inhibition activity than shallot ethanolic extract fraction 1 because fraction 2 contained higher concentration of quercetin as previously determined in section 3.2.1 and 3.2.2. Quercetin found in shallot ethanolic extract fraction 1 previously estimated in section 3.2.1 was certainly low, thus shallot ethanolic extract fraction 1 tended not to inhibit tyrosinase enzyme. Moreover, amount of ethanol in the first extraction was lower than that of in the second extraction, this ratio of ethanol probably solubilized compounds that enhanced Dopachrome formation instead of Dopachrome inhibition. In contrast, the ethanol ratio in the second extraction mainly solubilizes quercetin which shows anti-tyrosinase property.

The IC_{50} values of shallot ethanolic extract fraction 2 and quercetin were estimated from **Figures 18** and **19**, respectively. Since the percent inhibitions of shallot ethanolic fraction 1 were relatively low, the IC_{50} value of shallot fraction 1 was not calculated. The mean value of IC_{50} of shallot ethanolic extract

fraction 2 and quercetin standard were 16.22 ± 1.12 mg/mL and 66.11 ± 2.16 µg/mL, respectively. From HPLC characterization, shallot ethanolic extract fraction 2 contained about 0.25 % of quercetin (**Table 6**). Thus, 16.22 mg/mL of shallot ethanolic extract fraction 2 would contain 41.86 µg/mL of quercetin. By comparing the IC_{50} values between quercetin found in shallot ethanolic extract fraction 2 and quercetin standard (41.86 and 66.11 µg/mL), it can be concluded that shallot ethanolic extract fraction 2 probably contained other compounds that enhanced anti-tyrosinase activity.

This result consistent with the report from Kubo et al., 1999 that quercetin was able to chelate copper atoms of tyrosinase enzyme and further inhibited tyrosinase activity. Moreover, kaempferol, another flavonoid found in shallot was reported to show the anti-tyrosinase activity; accordingly, this compound would be another compound that enhanced anti-tyrosinase activity in shallot ethanolic fraction 2 (Kubo et al., 1999, Horbowicz et al., 2000).

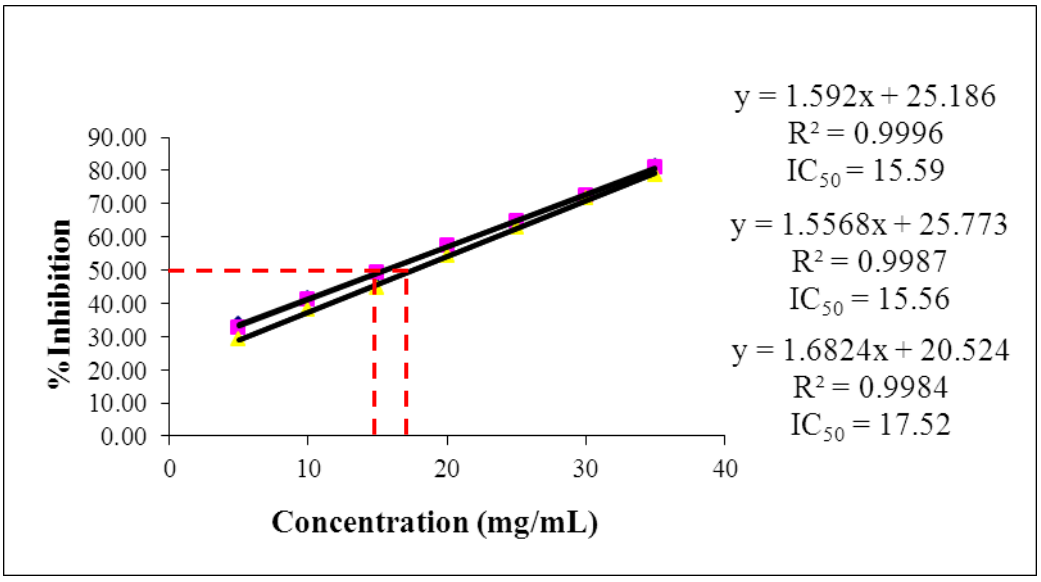

Figure 18 The percent tyrosinase inhibition of shallot ethanolic extract fraction 2

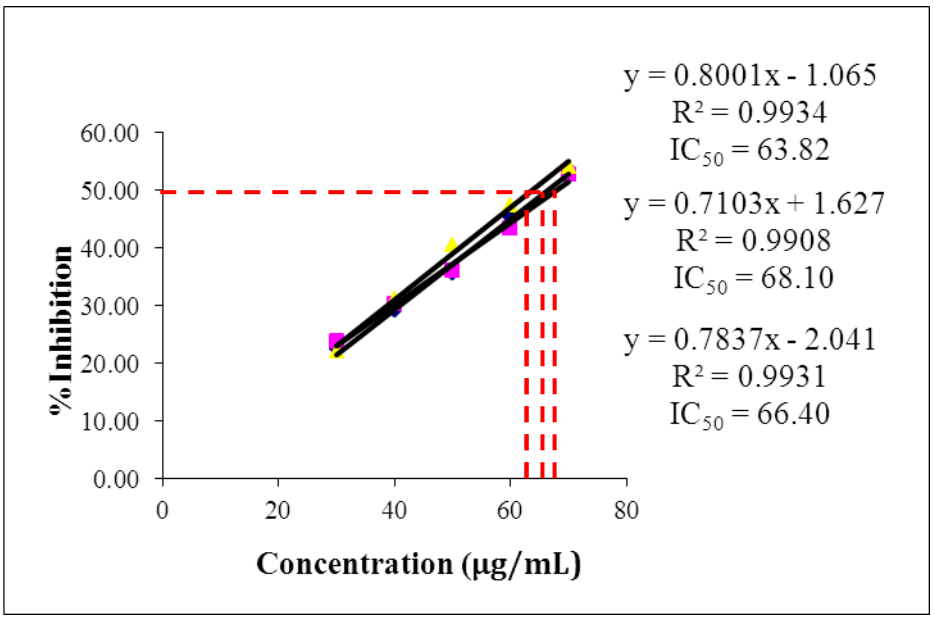

Figure 19 The percent tyrosinase inhibition of quercetin standard

3.3.2 B16 melanoma cells assay

The anti-melanogenesis activity on B16 melanoma cells was evaluated based on the detection of melanin inhibition as previously reported (Arung et al., 2011). Anti-melanin activity of shallot ethanolic extract fraction 1 and shallot ethanolic extract fraction 2 were determined in five replicates. The percentage of melanin content was reported as mean value and shown in **Figures 20 - 22**.

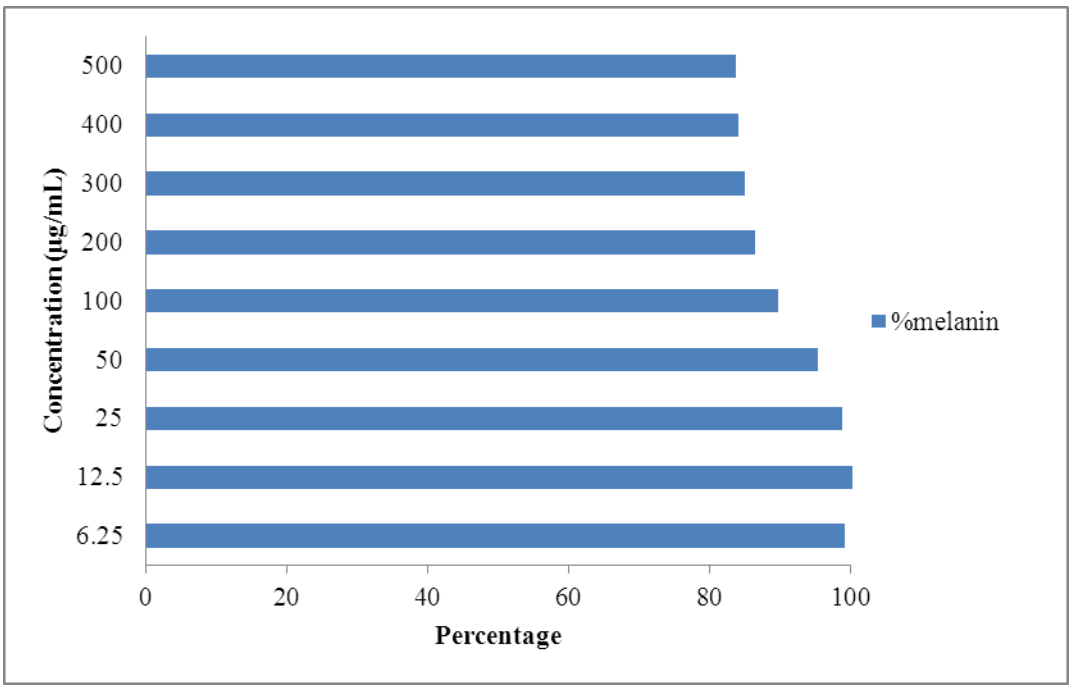

Figure 20 Percent melanin production (after 72 hours of incubation) at various concentration of shallot ethanolic extract fraction 1

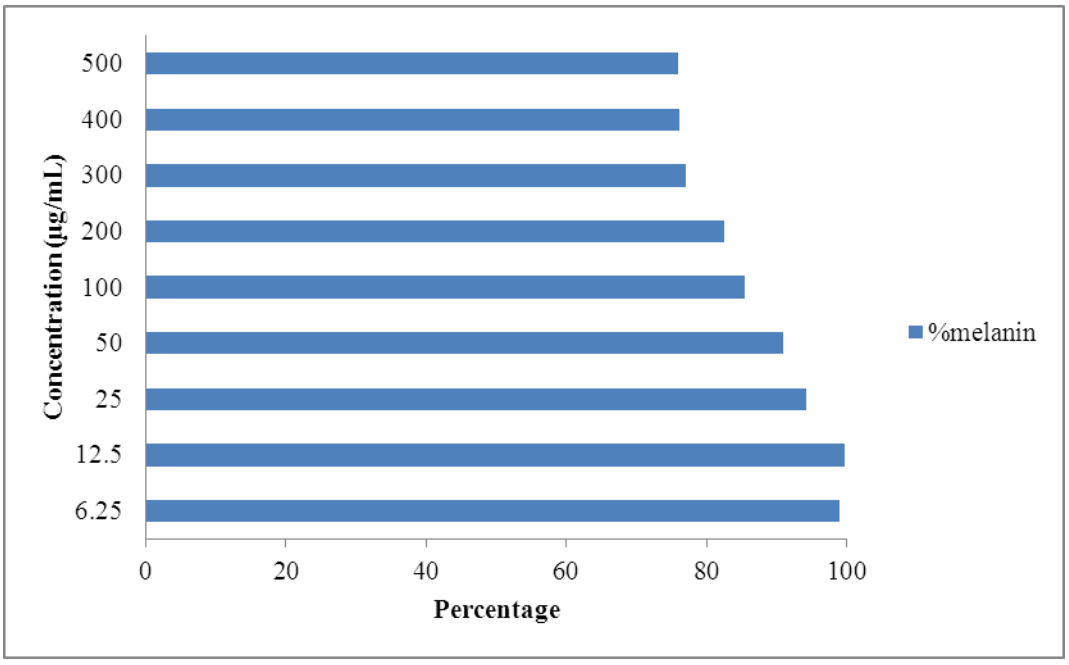

Figure 21 Percent melanin production (after 72 hours of incubation) at various concentration of shallot ethanolic extract fraction 2

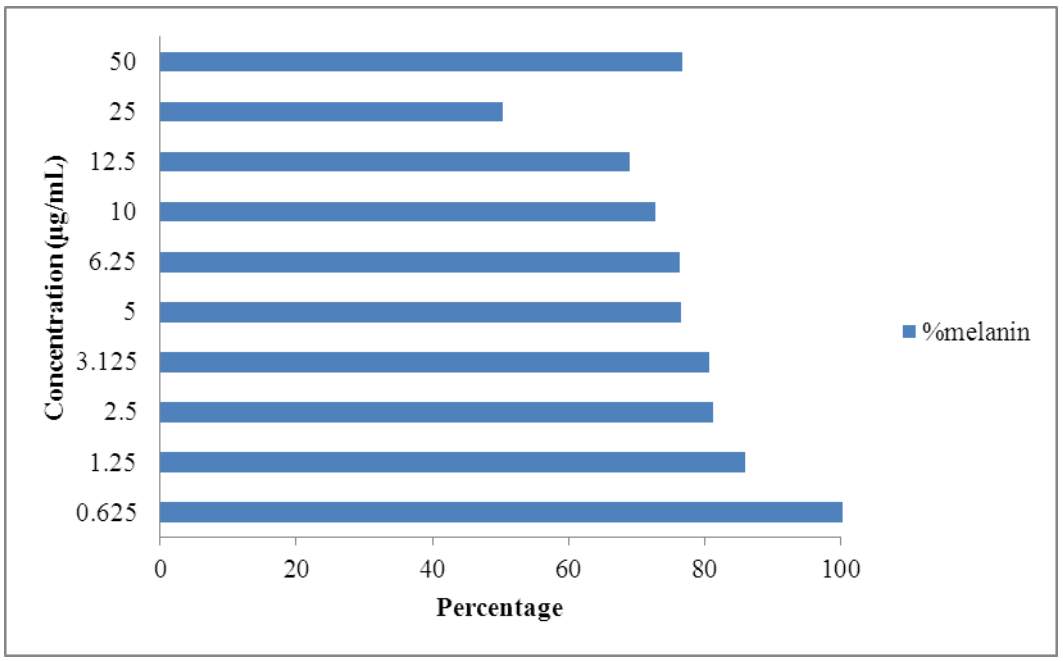

Figure 22 Percent melanin production (after 72 hours of incubation) at various concentration of quercetin standard

Figures 20, 21, 22 showed that anti-melanin activity of shallot ethanolic extract fraction 1, shallot ethanolic extract fraction 2 and quercetin standard increased as concentrations increased. At the concentration of 300 µg/mL, shallot ethanolic extract fraction 2 possessed higher inhibition activity than shallot ethanolic extract fraction 1 because shallot ethanolic extract fraction 2 contained higher concentration of quercetin, a known compound with anti-melanogenesis properties, as previously determined in section 3.2.1 and 3.2.2.

Shallot ethanolic extract fraction 1 inhibited melanin formation in B16 melanoma cells only 10-15% at even concentrations of 200 to 500 µg/mL. However, the inhibition of 20-25 % melanin formation in B16 melanoma cells of shallot ethanolic extract fraction 2 was noticeable at concentrations of 200 to 500 µg/mL.

By comparing melanin inhibition activity of shallot ethanolic extract fraction 2 and quercetin standard, about 300 µg/mL of shallot ethanolic extract fraction 2 and 6.25 µg/mL of quercetin standard inhibited 25% of melanin formation in B16 melanoma cells (**Figures 21 and 22**). From HPLC characterization, the shallot ethanolic extract fraction 2 contained 0.25% quercetin. In other words, the shallot ethanolic extract fraction 2 containing 0.75 µg/mL of quercetin showed the same inhibition activity as 6.25 µg/mL of quercetin standard. It implied that shallot ethanolic extract fraction 2 probably contained other compounds that increased anti-melanogenesis activity of quercetin on B16 melanoma cells. Quercetin glycosides would be another compound that helps increased the anti-melanogenesis activity since shallot contained quercetin-4'-O-β glucoside, a compound inhibited anti-melanogenesis activity in B16 melanoma cells (Arung et al., 2011).

3.4 Cell cytotoxicity Test

The percentage of B16 melanoma viable cells was evaluated after 24 and 72 hours of incubation of cells with and without sample solutions. The percentage of viable cells after exposure to shallot extract or quercetin standard were reported in **Table 10** and **Table 11**, respectively.

Percentage of B16 melanoma viable cells after 24 hours exposure to 500 µg/mL of shallot ethanolic extract fraction 1 and 2 were about 101.75 ± 1.08 % and 102.71 ± 0.92 %, respectively, while percentage of B16 melanoma viable cells after 24 hours exposure to 50 µg/mL of quecetin standard was decreased to about 73.30 ±

1.04 % (**Tables 10 and 11**). In addition, after 72 hours of exposure to 500 µg/mL of shallot ethanolic extract fraction 1, shallot ethanolic extract fraction 2, or 50 µg/mL of quercetin standard, percentage of B16 melanoma viable cells was decreased to about 94.52 ± 2.06, 94.61 ± 0.78, and 50.52 ± 0.54 %, respectively.

From **Table 10** and **Table 11**, the results indicated that both shallot ethanolic extract fraction 1 and fraction 2 caused no cytotoxicity to B16 melanoma cells after 24 and 72 hours of incubation at concentration lower than 500 µg/mL. Quercetin standard at a concentration range of 0.625 – 25 µg/mL also caused no cytotoxicity to B16 melanoma cells. On the other hand, quercetin standard at a concentration of 50 µg/mL caused about 50 % of cell death resulting in higher detection of melanin leaking out from cells. This suggested that shallot extracts would be more proper to use in cosmetic applications since it could show the desirable anti-tyrosinase activity with lower cell cytotoxicity when comparing with the quercetin standard.

Table 10 The percent cell viability of B16 melanoma cells of shallot ethanolic extract fraction 1 and shallot ethanolic extract fraction 2 (mean ±SD, n = 5)

Concentration of extract (µg/mL)	**%Cell viability**			
	Shallot fraction 1		**Shallot fraction 2**	
	24 hrs	**72 hrs**	**24 hrs**	**72 hrs**
6.25	99.74±0.67	102.32±2.17	101.38±1.98	99.70±1.23
12.50	98.66±1.14	101.89±1.76	100.58±1.36	99.18±1.16
25.00	101.23±1.34	99.97±0.89	98.98±0.69	98.48±0.83
50.00	101.75±0.58	98.85±0.97	100.27±0.52	98.97±0.60
100.00	100.04±0.79	95.09±0.68	100.08±0.79	95.16±1.18
200.00	102.60±0.94	94.94±0.75	99.36±0.84	94.46±1.01
300.00	101.54±1.02	95.07±1.01	100.09±0.78	94.86±0.66
400.00	100.25±0.66	94.62±0.92	100.48±0.64	94.38±0.59
500.00	101.75±1.08	94.52±2.06	102.71±0.92	94.61±0.78

Table 11 The percent cell viability of B16 melanoma cells of quercetin standard (mean ± SD, n = 5)

Concentration of standard (µg/mL)	%Cell viability Quercetin standard	
	24 hrs	72 hrs
0.625	102.81±2.24	97.18±2.17
1.250	102.32±1.96	95.75±2.02
2.500	101.91±2.13	95.32±1.74
3.125	100.01±1.54	99.75±0.99
5.000	99.63±0.87	95.57±0.65
6.250	100.80±0.86	98.69±0.68
10.000	99.61±0.61	97.06±0.77
12.500	99.45±0.70	99.31±0.81
25.000	98.45±0.95	100.96±1.16
50.000	73.30±1.04	50.52±0.54

3.5 Development of emulgel containing shallot extract

3.5.1 Preparation of emulgel containing shallot extract

Shallot ethanolic extract fraction 2 in propylene glycol was a turbid pink solution; therefore, incorporating this solution into gel was likely caused unpleasant gel appearance. The emulsion was then prepared in order to hinder the turbidity of shallot ethanolic extract in propylene glycol and acquire the occlusive film when applied to the skin. Moreover, emulsions provide skin hydration and enhance skin penetration of an active compound. However, emulsions often encounter with its poor stability; therefore, an addition of gel is employed to improve emulsion stability. This had led to the preparation of emulgel, a combination of emulsion and gel.

Various formulations were prepared prior to the addition of shallot ethanolic extract fraction 2. The amounts of surfactants used were calculated based on HLB value of the formulation. The example of HLB calculations is represented in **Figure 23**.

During the formulation development process, in order to roughly evaluate compatibility and physical stability of shallot ethanolic extract fraction 2 in the formulations, quercetin (a known active compound in the extract) was added at a predetermined concentration in the formulation instead of an addition of the shallot ethanolic extract fraction 2. After formulations containing quercetin with compatibility and physical stability were selected, quercetin was replaced by 5% shallot ethanolic extract fraction 2.

According to the content of formulation listed in **Tables 1, 2 and 3**, all formulations were prepared using beaker method in which an oil phase and a water phase were heated prior to an addition of the water phase to the oil phase. Formulation F1 and F2 were primary formulations for vanishing cream and o/w cream, respectively. Based on formulation F1, stearic acid and lanolin anhydrous were chosen as stiffening agents, triethanolamine was used as a soap foaming agent resulting in a formation of triethanolamine stearate, an emulsifier. The vanishing cream formulation gave a very sticky solid cream. Therefore, the formulation needed further development for pleasant feeling after skin application.

The example of HLB calculations of formulation F21 (7 g of oil phase, 3 g of surfactant is needed)

The required HLB = (3 x 15)/7 + (3 x 6)/7 + (1 x 5)/7 = 6.43 + 2.57 + 0.71 = 9.71

HLB of Cetyl alcohol Jojoba oil Dimethicone

	HLB value	**Required HLB**		**Amount needed**
Span80	4.3	5.29	(10.58x3)/21.4	1.48
		5.29		
		9.71		
Tween80	15	5.41	(5.41x3)/21.4	0.76
Cremophor RH-40	15	5.41	(5.41x3)/21.4	0.76
		Total 21.4		(3 g)

Figure 23 The example of HLB calculations of F21

Formulation F2 contained stearic acid, cetyl alcohol and mineral oil in its oil phase. Triethanolamine stearate was also the emulsifier in this formulation. Although formulation F2 gave rise to viscous cream, it gave a better feeling and less sticky than the formulation F1 after application. Formulation F3 contained dimethicone with less amount of stearic acid was prepared in order to reduce viscosity of the formulation. Formulation F2 and F3 containing quercetin were off-yellow cream with pH values of 7.72 and 7.79, respectively. Both formulations showed discoloration after storage at room temperature and changed its color to off-red due to instability of quercetin under alkaline conditions (Schmalhausen et al., 2007 and Moon et al., 2008).

In formulation F3 - F6, emulsifying systems were varied from triethanolamine stearate, sodium lauryl sulfate, Brij72 – Brij 721, and Span80 – Tween80, respectively. Employing sodium lauryl sulfate, formulation F4 resulted in foaming cream. The formulation containing Brij72 and Brij 721 (F5) yielded physically unstable cream as creaming and cracking were observed. The formulation containing Span80 and Tween80 (F6) gave physically stable cream. The formulation F6 gave good appearance and texture but still gave oily feeling after application. Moreover, discoloration of the formulation F6 containing quercetin was not observed because this formulation did not contain a base.

Composition of the oil phase was varied by replacement of mineral oil with jojoba oil or grapeseed oil in formulation F6 - F8 while concentrations of emulsifiers, Span80 and Tween80, were calculated based on HLB value of the oil phase. Formulation containing jojoba oil or grapeseed oil, (F7 or F8, respectively)

gave less oily feeling than formulation containing mineral oil (F6), but they still gave sticky feeling after application. In addition, formulation F7 or F8 showed good physical stability without creaming or cracking effects. Formulation F7 and F8 containing quercetin also showed good physical stability without discoloration because the triethanolamine was not employed in these formulations. Therefore, jojoba oil and grapeseed oil were chosen instead of mineral oil for further formulation development.

Formulation F9 and F10 was an attempt to reduce sticky feeling after application by a replacement of stearic acid with cetyl alcohol. These two base formulations gave better cream appearance as well as smoother texture and lighter feeling after an application on skin. Therefore, stearic acid was dropped out from the formulations.

Effect of co-emulsifiers, sodium lauryl sulfate or Cremophor RH-40, on formulation characteristics were accessed in formulations F11 – F15 in the presence of jojoba oil or grapeseed oil in order to maximize physical stability of the formulations. Formulations containing sodium lauryl sulfate resulted in yellowish and smooth texture cream, nevertheless, formulation F11 and F12 tended to undergo creaming after left the formulations for a week at room temperature. Cremophor RH-40 contains fatty acid ester of glycerol and polyethylene glycol; therefore, Cremophor RH-40 as a co-emulsifier would give more stable cream. However, formulations F11 – F15 still gave oily feeling after application because the oil phase in the formulations F11 – F15 was as high as 30%.

Concentration of the oil phase was varied from 30 to 20% in formulation F14 - F20 in order to minimize oil content in the formulation. When the content of cetyl alcohol was less than 4% (F19 and F20), the formulations were too thin. Comparing jojoba oil to grapeseed oil, jojoba oil was finally chosen as oil in the formulation since it gave less oily feeling. Furthermore, in the presence of 2% dimethicone, an amount of jojoba oil could decrease as low as 6% (F21). Formulation F21 gave good appearances, textures and non-heavy feeling, and then F21 was further subjected to 6 heating-cooling cycles and 8 centrifugation cycles as previously mentioned in section 2.5.2. The result indicated that F21 passed both tests without any separation; however, this formulation showed creaming effect after left it at room temperature for a month. Thus, a viscosity inducing agent giving no greasy feeling such as a polymer should be added in the formulations.

The addition of a gelling agent was employed in formulation F22 - F27. Carbopol 940, a polyacrylate polymer forms gel upon neutralization, providing high viscosity suitable to be used as a rheology modifier for gels or cream. Morevoer, Carbopol polymers have been reported to show extremely low irritancy and non-sensitizing effects after repeated usage (Hosmani, 2006). The concentration of Carbopol 940 was varied from 0.3 – 2.0% in F22 – F27. In this study, the carbopol 940 solution was neutralized by triethanolamine and adjust the pH value to about 5. At pH around 5, Carbopol 940 could undergo cross-linking and form gel and pH value of the formulation was close to skin's pH value. Emulgel formulation (F26), was the best formulation since it gave very good appearance and good feeling with physical stability and no discoloration after one month storage at room temperature (**Figure 24**).

Figure 24 The picture of formulation F26 (no shallot extract)

Five percent of the shallot ethanolic fraction 2 was added by dissolving the shallot powder in small amount of 50:50 v/v ethanol:water and 6% propylene glycol prior to well-mix with the emulgel base. The resulting pink emulgel was obtained (**Figure 25**). Formulation F26 was further accessed for its chemical and physical stabilities.

Figure 25 The picture of formulation F26 (with shallot extract)

3.5.2 Stability test of emulgel containing shallot extract

Both chemical and physical stabilities of formulation F26 were evaluated. The centrifugation of the emulgel was subjected to 8 cycles at 6000 rpm at room temperature. The results indicated neither phase separation nor creaming. The pictures of the formulation in after centrifuged for 1 cycle and 8 cycles are shown in **Figure 26**.

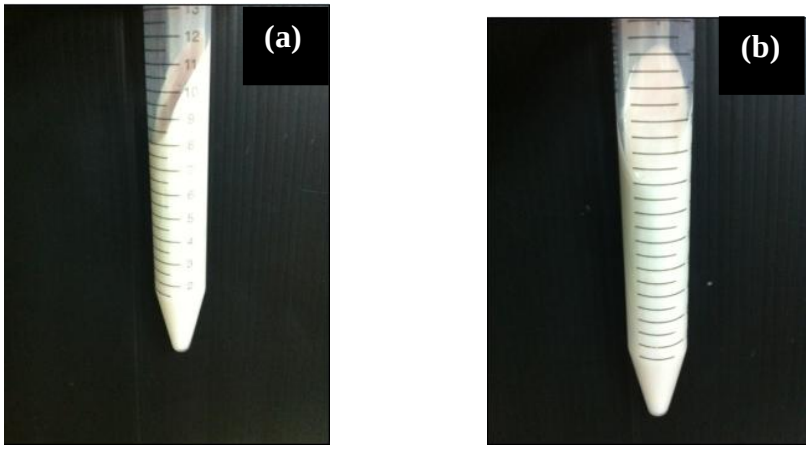

Figure 26 The pictures of the formulation after centrifuged for 1 (a) and 8 cycles (b)

The formulation was then subjected to heating-cooling cycle for 6 cycles. The results illustrated that there was no phase separation, color change and creaming observed during and after the test as shown in **Figure 27**. Moreover, the pH value of the formulation was quite stable as presented in **Figure 28**. This suggested that formulation F26 containing shallot ethanolic fraction 2 was physically stable, then F26 was further evaluated for its chemical stability.

Figure 27 The picture of formulation during heating-cooling cycle

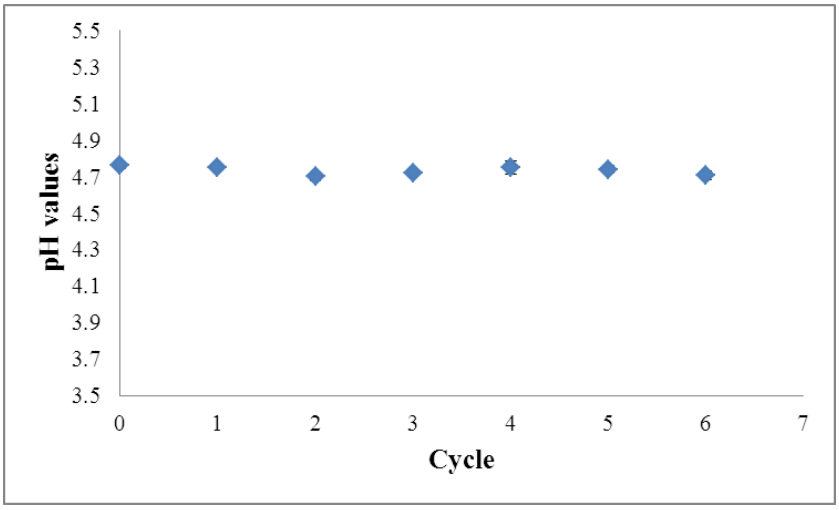

Figure 28 The pH values of the formulations after 6 cycles of heating-cooling cycle

The formulation F26 was further kept in stability chambers at 30 ˚C, 75% RH and 40 ˚C, 75% RH for 3 months. At the end of week 1, 2, 3, 4, 6, 8, 10 and 12, the concentration of quercetin remaining in the formulation and pH value were analyzed using the HPLC method and pH meter. The chromatograms of quercetin standard, formulation containing shallot extract, blank formulation, and formulation containing quercetin were compared and they are presented in **Figure 29**. The quercetin was eluted at the retention time of 3 minutes (**Figure 29a**, **29b** and **29c**),

while there was no quercetin peak found in the chromatogram of blank emulgel (**Figure 29d**).

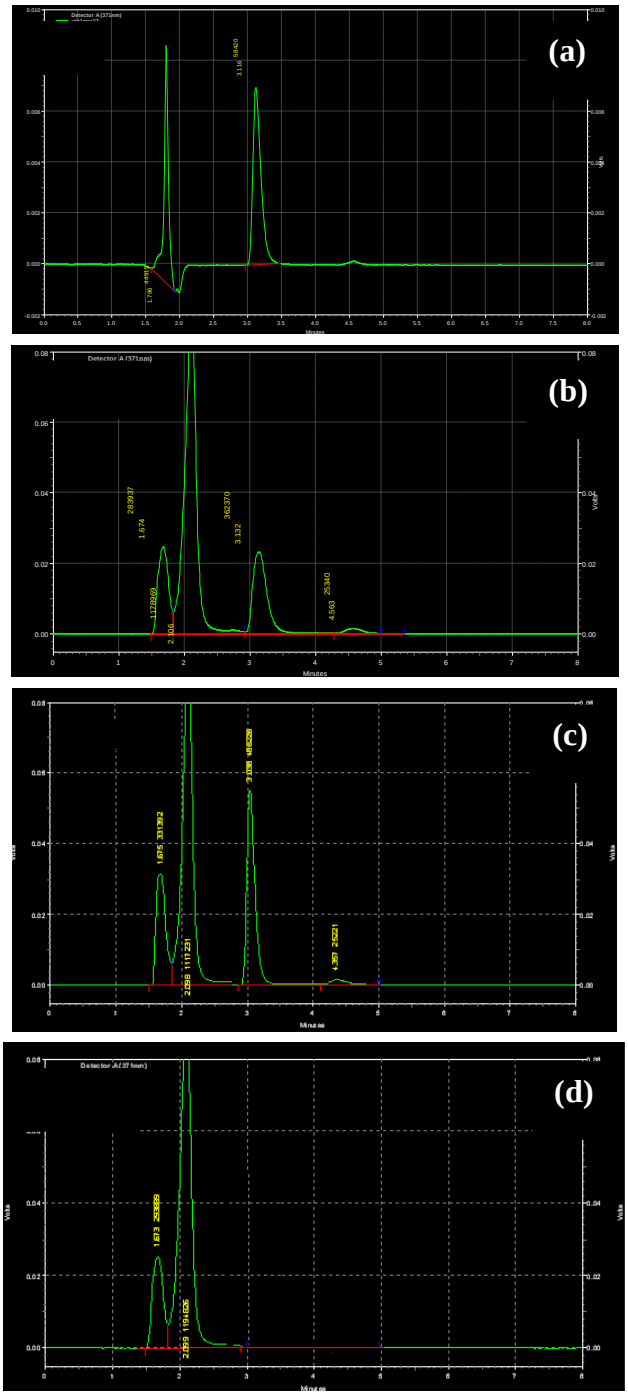

Figure 29 The HPLC chromatograms of quercetin standard (a), formulation containing shallot extract (b), formulation containing quercetin (c) and blank formulation (d)

The pH values of the formulation under the storage at 30 ˚C and 40 ˚C were measured and showed in **Figure 30**. The results revealed constant pH values of the formulations over 12 weeks at about 4.5-4.6.

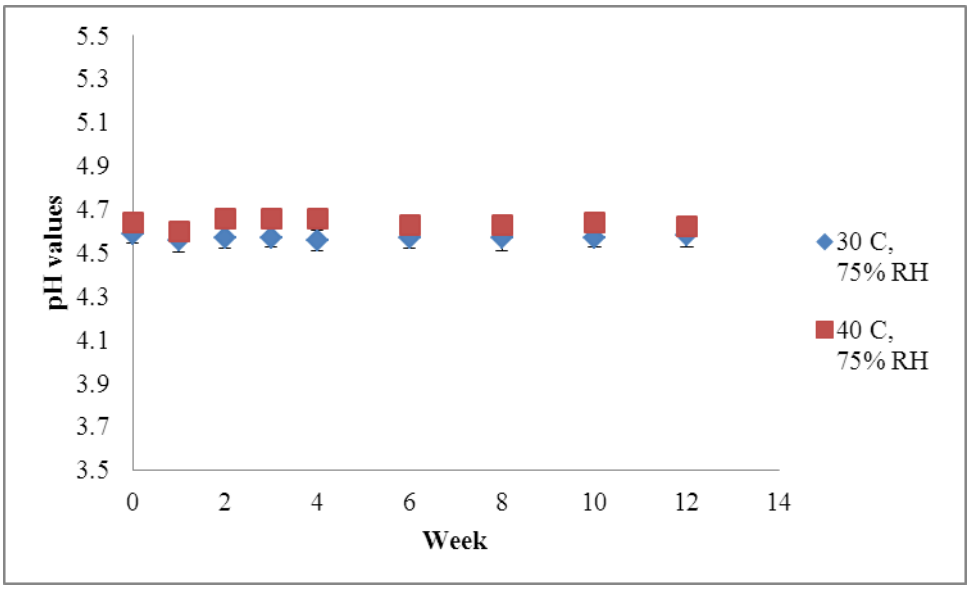

Figure 30 The pH values of emulgel containing shallot ethanolic extract fractions at various time points

The concentrations of quercetin in the formulation F26 after stored at 30˚C and 40˚C were calculated as percent quercetin remaining to evaluate the chemical stability of quercetin in the formulation (**Figure 31**). From the results, there was no significant change in the concentration of quercetin in the emulgel over 12 weeks (99% and 99% remaining at 30˚C and 40˚C, respectively). This can be suggested that the F26 containing shallot extract was physically and chemically stable at 30˚C and 40˚C storage for at least 3 months. Then formulation F26 was subjected to irritation testing.

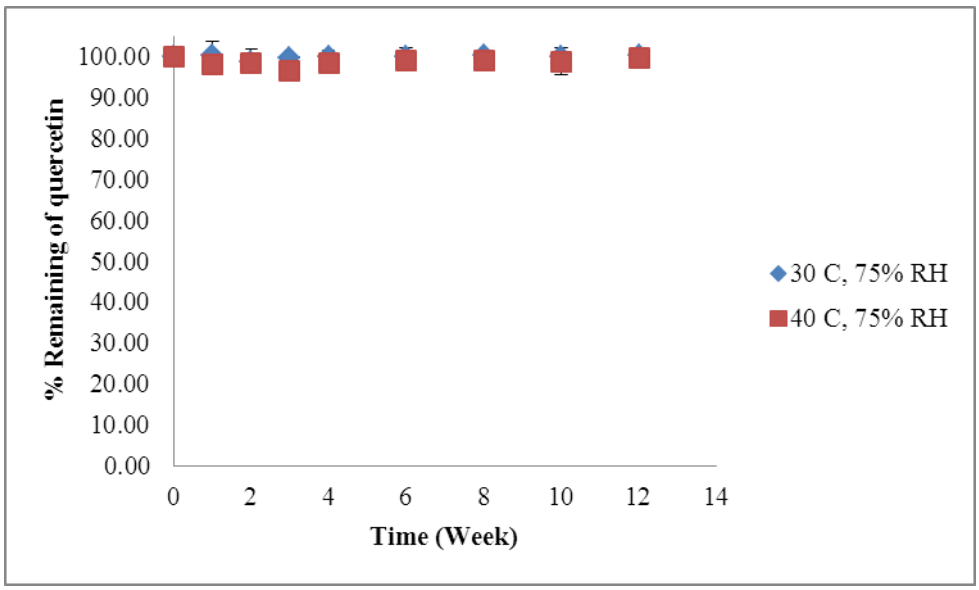

Figure 31 Graph plotting between the percent remaining of quercetin (mean) versus time

3.6 Irritation testing of emulgel containing shallot extract

Emulgel containing shallot ethanolic extract fraction 2 and emulgel base were subjected to irritation testing. Nineteen females in the age range of 26-48 and one male at the age of 36 were enrolled to the test. The emulgels with and without shallot ethanolic extract were applied to the right side and left side of volunteer's back, respectively for 24 hours (**Figure 32**). The irritancy was observed after 24, 48, and 72 hours according to Internatinal Contact Dermatitis Research Group (ICDRG) scale (**Figure 33**).

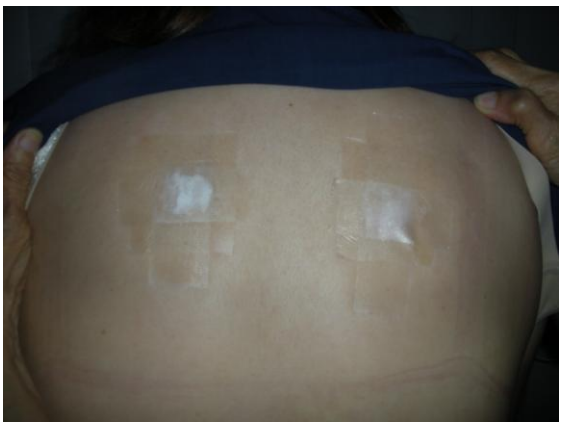

Figure 32 Patch test of emulgel with and without shallot crude extract

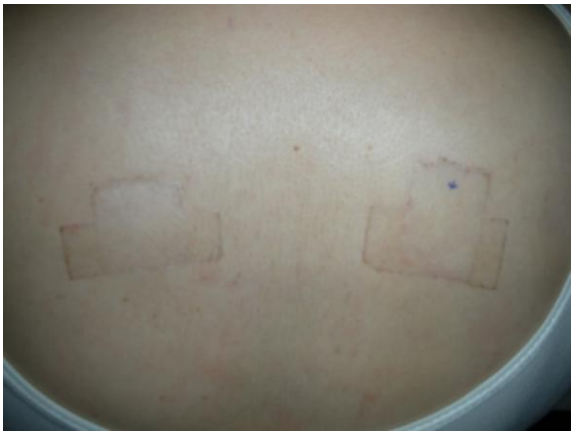

Figure 33 Volunteer's back after left the patch for 24 hours

The results indicated that there was no short-term irritation effect in 20 volunteers after left the patches for 24 hours for both emulgel containing shallot extract and emulgel base as shown in **Figure 33**. There was no redness or swelling of the skin. Moreover, after 48 and 72 hours, the results did not show any long-term irritation on the back of 20 volunteers. Therefore, this emulgel formulation is safe to use as an actual product in cosmetic industries.

From overall study, several further studies should be accomplished including shallot odor elimination and other active compounds determination. Shallot ethanolic extract was given an unpleasant odor resulting from Allinase enzyme in shallot bulbs. To overcome this obstacle, a protein precipitation method may be performed or further extractions would be achieved. Furthermore, the identification of other compounds in the shallot ethanolic extract fraction 2 that possess anti-tyrosinase and anti-melanogenesis properties should be performed. The compounds could be kaempferol and flavonoids found in shallot, possess anti-tyrosinase activity. Therefore, the evaluation of stability and skin penetration of other active compounds should be well-estimated. Nevertheless, efficacy of quercetin that retains in viable epidermis should be evaluated whether quercetin still possesses anti-melanogenesis when passed through skin. Finally, *in vivo* efficacy study should be conducted.

CHAPTER V

CONCLUSION

The extraction of *Allium ascalonicum* bulbs was performed. The extracts and fractions were further characterized for their major constituent "quercetin" using HPLC and TLC. The biological activities in anti-tyrosinase and anti-melanogenesis were evaluated based on mushroom tyrosinase enzyme assay and B16 melanoma cell assay, respectively. The extracts were determined for their cytotoxicities on B16 melanoma cells. The selected extract was developed into emulgel preparation and further investigated for its physical and chemical stabilities. Finally, the emulgel containing *Allium ascalonicum* extract was subjected to irritation test on human volunteers. The research indicated to following conclusions:

The percent yield of shallot ethanolic extract fraction 1 was higher than shallot ethanolic extract fraction 2. By using HPLC and TLC characterization, shallot ethanolic extract fraction 2 contained higher content of quercetin.

Shallot ethanolic extract fraction 2 gave higher potent in anti-tyrosinse activity with an IC_{50} value of 16.22 mg/mL and inhibited 25% of melanin formation in B16 cells at a concentration range of 300-500 µg/mL with no cytotoxicity to cells.

The emulgel containing *Allium ascalonicum* extract (shallot ethanolic extract fraction 2) was physically and chemically stable for at least 3 months without any short-term and long-term irritation effects to human skin.

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APPENDICES

Appendix A: Data

Table A1 The percent tyrosinase inhibition of shallot ethanolic extract fraction 1 and shallot ethanolic extract fraction 2

Concentration of extract (mg/mL)	% Inhibition					
	Shallot ethanolic fraction 1			Shallot ethanolic fraction 2		
	run1	run2	run3	run1	run2	run3
5	-20.32	-19.92	-19.12	33.88	32.65	28.98
10	-7.97	-6.77	-7.97	41.63	41.22	37.96
15	-1.99	-1.59	-3.19	48.57	49.39	44.49
20	9.96	6.77	7.17	57.14	57.55	54.29
25	13.55	11.16	10.76	64.08	64.90	62.86
30	17.53	15.14	15.14	71.84	72.65	71.84
35	21.12	18.73	19.52	81.22	80.82	78.78

Table A2 The percent tyrosinase inhibition of quercetin standard

Concentration of quercetin (µg/mL)	% Inhibition		
	run1	run2	run3
30	21.51	23.11	22.71
40	30.28	30.28	29.88
50	37.05	38.65	37.85
60	46.61	45.42	45.42
70	52.59	54.58	53.78

Table A3 The IC_{50} values of shallot ethanolic extract fraction 2 and quercetin standard in mushroom tyrosianse inhibition

		IC_{50}	Mean	SD
Shallot fraction 2 (mg/mL)	**run1**	15.59	16.22	1.12
	run2	15.56		
	run3	17.52		
Quercetin standard (µg/mL)	**run1**	63.82	66.11	2.16
	run2	68.1		
	run3	66.4		

Table A4 The percent of melanin produced by B16 melanoma cells of shallot ethanolic extract fraction 1, and shallot ethanolic extract fraction 2 (mean value) and percent cell viability (mean value)

Concentration of	**Shallot fraction 1**		**Shallot fraction 2**	
extract (µg/mL)	**%Melanin**	**%Cell viability**	**%Melanin**	**%Cell viability**
6.25	99.11	102.32	99.02	99.70
12.5	100.35	101.89	99.65	99.18
25	98.67	99.97	94.14	98.48
50	95.21	98.85	90.95	98.97
100	89.60	95.09	85.49	95.16
200	86.37	94.94	82.49	94.46
300	84.91	95.07	77.00	94.86
400	84.10	94.62	76.19	94.38
500	83.59	94.52	75.97	94.61

Table A5 The percent of melanin produced by B16 melanoma cells of quercetin standard (mean value) and percent cell viability (mean value)

Concentration of	**Quercetin standard**	
standard (μg/mL)	**%Melanin**	**%Cell viability**
0.625	100.66	97.18
1.25	85.93	95.75
2.5	81.17	95.32
3.125	80.66	99.75
5	76.56	95.57
6.25	76.40	98.69
10	72.82	97.06
12.5	68.94	99.31
25	50.31	100.96
50	76.75	50.52

Table A6 The pH values after 6 cycles of heating-cooling cycle of formulations

	Cycle0	**Cycle1**	**Cycle2**	**Cycle3**	**Cycle4**	**Cycle5**	**Cycle6**
pH	4.77	4.74	4.7	4.71	4.73	4.73	4.69
Values	4.74	4.76	4.72	4.74	4.79	4.75	4.74
	4.76	4.74	4.68	4.7	4.73	4.71	4.71
Mean	4.76	4.75	4.70	4.72	4.75	4.73	4.71
SD	0.015	0.012	0.020	0.021	0.035	0.020	0.025

Table A7 The pH values of formulations under the storage at 30˚C and 40˚C over 12 weeks

		N1	N2	N3	Mean	SD
30 ˚C	**Week0**	4.54	4.61	4.63	4.59	0.047
	Week1	4.5	4.58	4.6	4.56	0.053
	Week2	4.52	4.57	4.62	4.57	0.050
	Week3	4.52	4.58	4.6	4.57	0.042
	Week4	4.51	4.58	4.6	4.56	0.047
	Week6	4.53	4.56	4.62	4.57	0.046
	Week8	4.51	4.58	4.63	4.57	0.060
	Week10	4.53	4.57	4.61	4.57	0.040
	Week12	4.55	4.55	4.64	4.58	0.052
40 ˚C	**Week0**	4.62	4.65	4.65	4.64	0.017
	Week1	4.58	4.63	4.6	4.60	0.025
	Week2	4.64	4.7	4.65	4.66	0.032
	Week3	4.64	4.68	4.66	4.66	0.020
	Week4	4.63	4.69	4.66	4.66	0.030
	Week6	4.59	4.66	4.63	4.63	0.035
	Week8	4.61	4.64	4.64	4.63	0.017
	Week10	4.61	4.66	4.64	4.64	0.025
	Week12	4.6	4.65	4.62	4.62	0.025

Table A8 The percent w/w of quercetin found in formulations from time zero to 12 weeks

		N1	N2	N3	Mean	SD
30 ˚C	**Week0**	0.0114	0.0118	0.0115	0.0116	0.00019
	Week1	0.0116	0.0112	0.0113	0.0114	0.00019
	Week2	0.0114	0.0113	0.0116	0.0114	0.00018
	Week3	0.0111	0.0114	0.0112	0.0112	0.00014
	Week4	0.0114	0.0115	0.0115	0.0114	0.00004
	Week6	0.0110	0.0119	0.0116	0.0115	0.00043
	Week8	0.0112	0.0118	0.0115	0.0115	0.00031
	Week10	0.0113	0.0116	0.0115	0.0115	0.00017
	Week12	0.0114	0.0118	0.0115	0.0116	0.00019
40 ˚C	**Week0**	0.0103	0.0098	0.0105	0.0102	0.00037
	Week1	0.0103	0.0098	0.0106	0.0102	0.00037
	Week2	0.0101	0.0097	0.0104	0.0101	0.00036
	Week3	0.0102	0.0098	0.0105	0.0102	0.00036
	Week4	0.0103	0.0099	0.0106	0.0102	0.00035
	Week6	0.0103	0.0098	0.0105	0.0102	0.00034
	Week8	0.0103	0.0100	0.0104	0.0103	0.00022
	Week10	0.0101	0.0102	0.0103	0.0102	0.00012
	Week12	0.0102	0.0100	0.0105	0.0102	0.00027

VITA

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