รายงานวิจัยฉบับสมบูรณ์

การศึกษาฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากเตยหอมเพื่อการนำไปใช้เป็นส่วนประกอบในเครื่องสำอาง

The development of Thai pandan (*Pandanus amaryllifolius* Roxb.) extract as a natural antioxidant for cosmetic use

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The development of Thai pandan (*Pandanus amaryllifolius* Roxb.) extract as a natural antioxidant for cosmetic use

คณะผู้วิจัย

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Ampa Jimtaisong

Research Contents

Abstract

The antioxidant capacities and total phenolic content of *Pandanus amaryllifolius* Roxb. leaf and aerial root extracted in ethanol and propylene glycol solvent were investigated. Propylene glycol extract of *P. amaryllifolius* has higher DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity and total phenolic content than that of ethanol extract. DPPH radical-scavenging activity of the *P. amaryllifolius* leaf extract is higher than that of aerial root extract. The 50% inhibition concentration (IC_{50}) values of *P. amaryllifolius* leaf and aerial root crude extract are 0.810 mg/ml and 2.340 mg/ml, respectively. The antioxidant capacities of *P. amaryllifolius* crude extracts by DPPH assay and thiocyanate methods were lower than those of vitamin C and butylated hydroxytoluene (BHT). However, a neat solution extract of *P. amaryllifolius* showed higher inhibition (90.1%) of linoleic acid peroxidation than 200 ppm of vitamin C (57.1%) and butylated hydroxytoluene (BHT 71.1%). In addition, the solution extract showed good heat stability. Topical emulsion product containing the extract at 1, 3 and 5% were developed and that contains up to 3% showed acceptable properties in cosmetic point of view. The products possessed good stability after tested by centrifugation at 6000 rpm for 30 minutes as there was no sign of phase separation observed. The stability of the product was also studied at accelerated storage conditions for 1 month, i.e. at 4°C, ambient temperature, 45°C and heating-cooling cycle (4°C, 24 h; 45°C, 24 h). At initial, the product was creamy texture, medium viscosity with pandan odor. After subjected to stability test, it was found that the viscosity of the developed products was fairly stable when stored at 4°C, ambient temperature, but showed more noticeable change when stored at 45°C and heating-cooling cycle. There is no phase separation observed in all conditions, and product's color which mainly caused by the extract has a relatively small change. The product is non-irritant to the skin as performed by single patch test. The sensory test showed that most volunteers liked the overall feature of moisturizing cream expect for the odor. The product efficacy was measured every week using SELS program calculation from Visoscan® VC 98. The results illustrated that 85-90% of the volunteers possessed a decrease in wrinlkes and scaliness of the skin. Thus, the results obtained indicated that *P. amaryllifolius* extract might be useful in cosmetic industry.

Keywords: *Pandanus amaryllifolius, Extraction, Natural antioxidant, Topical emulsion, Stability.*

บทคัดย่อ

โครงการวิจัยนี้ได้ศึกษาเกี่ยวกับฤทธิ์การต้านอนุมูลอิสระและ total phenolic content ในใบและรากเตยหอมซึ่งสกัดโดยใช้ตัวทำละลายคือ ethanol และ propylene glycol โดยพบว่าสารสกัดจากเตยหอมซึ่งสกัดด้วย propylene glycol มีค่า DPPH radical scavenging activity และ total phenolic content ที่สูงกว่าการสกัดด้วย ethanol อีกทั้งใบยังแสดงฤทธิ์ที่ดีกว่าราก โดยมีค่า IC_{50} ของใบและรากเป็น 0.810 mg/ml และ 2.340 mg/ml ตามลำดับ ถึงแม้ว่าค่าความสามารถในการต้านอนุมูลอิสระของสารสกัดหยาบ ยังคงน้อยกว่าสารมาตรฐาน (vitamin C และ BHT) แต่อย่างไรก็ตาม solution extract ของเตยหอมแสดงค่าการยับยั้งใน linoleic acid peroxidation ที่สูงถึง 90.1% ซึ่งสูงกว่าสารมาตรฐาน vitamin C (57.1%) และ BHT (71.1%) ที่ความเข้มข้น 200 ppm อีกทั้งยังพบว่าสารสกัดมีความคงตัวสูงภายใต้อุณหภูมิที่สูงขึ้น ในการวิจัยนี้ได้พัฒนาครีมบำรุงผิวอิมัลชัน โดยใส่สารสกัดใบเตยหอมที่ 1, 3 และ 5 % ซึ่งพบว่าครีมบำรุงผิวที่ใส่สารสกัดใบเตย 3% มีลักษณะเนื้อครีมเหมาะสม มีกลิ่นหอมของเตยอ่อนๆ แต่ถ้าใส่ถึง 5% เนื้อครีมจะมีความเหลวเกินไป จากนั้นได้ทดสอบความคงตัวของครีมโดยการปั่นเหวี่ยงที่ 6000 rpm นาน 30 นาที พบว่าไม่มีการแยกชั้น และทดสอบความคงตัวของครีมที่สภาวะเร่ง 4°C, 45°C และ heating-cooling cycle (4°C, 24 h, 45°C, 24 h) เปรียบเทียบ กับสภาวะอุณหภูมิห้อง นาน 1 เดือน พบว่า ครีมมีความคงตัวดี มีการเปลี่ยนแปลงของสีน้อย โดยสภาวะที่มีการเปลี่ยนแปลงมากที่สุดคือ 45°C และ heating-cooling นอกจากนี้ได้ทดสอบการแพ้เบื้องต้นในอาสาสมัคร โดยวิธี single patch test และไม่พบอาการแพ้ใดๆ อีกทั้งยังได้ทำการประเมินคุณภาพของครีม โดยการทำ sensory test และให้อาสาสมัครทดลองใช้ครีมโดยทาที่ผิว แล้วประเมินผลโดยการถ่ายภาพผิวด้วยเครื่อง Visoscan® VC 98 ซึ่งพบว่าอาสาสมัครมีความพอใจต่อเนื้อครีมและคุณสมบัติของครีมในระดับสูง แต่มีความต้องการให้ปรับปรุงกลิ่น และผลการถ่ายภาพผิวด้วยเครื่อง Visoscan® VC 98 พบว่าอาสาสมัครมีการลดลงของ wrinkles และ scaliness ดังนั้นผลการวิจัยนี้ได้แสดงให้เห็นว่าสารสกัดจากเตยหอมอาจเป็นตัวเลือกที่สามารถนำมาใช้ประโยชน์ในผลิตภัณฑ์เครื่องสำอางต่อไป

Keywords: เตยหอม, การสกัด, สารต้านอนุมูลอิสระธรรมชาติ, ครีมบำรุงผิวอิมัลชัน, ความคงตัว.

Executive Summary

Pandanus (Pandanaceae) is a genus of monocots with about 600 known species. *Pandanus amaryllifolius* Roxb. (Toei-hom) and *Pandanus odoratissimus* Linn. (Toei-talay) are of example species found in Thailand. *P. amaryllifolius* leaves, commonly known as pandan leaves, are often used to give a refreshing, fragrant flavor to both sweet and savory south-east Asian dishes. Besides its culinary value, pandan leaves are used in perfume industry and also medicinally important as diuretic, cardio-tonic, anti-diabetic and for skin diseasesis. Moreover, *P. amaryllifolius* leaves are used to refresh the body, reduce fever, and relieve indigestion and flatulence. Various alkaloids, such as pyrrolidine alkaloids and pandamarilactonines have been isolated from pandan leaves. The leaves also contain unglycosylated pandamin protein which exhibits antivirus activity against human viruses, herpes simplex virus type-1 (HSV-1) and influenza virus (H1N1). Moreover, pandan leaves contain quercetin, carotenoids, tocopherols, tocotrienols and essential oils. *Pandanus odoratissimus* Linn. belongs to pandanaceae family and distributes in the south part of Thailand. The ripe fruits of *P. odoratissimus* possess a pandan scent due to an ester essential oil. The leaves exhibit anti-inflammatory activity. The root part contains various chemicals, e.g., steroidal compounds, lignan, benzofuran derivatives and phenolic compounds. *P. odoratissimus* is one of the plants listed in ayurvedic anticancer treatment. Furthermore, chemical component analysis of the root part of *P. odoratissimus* led to the isolation of 3,4-bis(4-hydroxy-3-methoxybenzyl) tetrahydrofuran which shows strong antioxidative activities when compared to BHA. Traditional folk medicine has incorporated both plants as one of the component in the formula and scientists have been trying to isolate active compounds and prove for their activities. In view of cosmetic application, there has been less scientific study. This project thus has focused on the preparation of *P. amaryllifolius* extract as natural antioxidant ingredient for cosmetics. Ethanol and propylene glycol were selected as solvent in extraction because they are normally used in topical cosmetic formulation and therefore they are safe and easy to use in the preparation.

Propylene glycol extract of *P. amaryllifolius* has higher DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity and total phenolic content than that of ethanol extract. DPPH radical-scavenging activity of the *P. amaryllifolius* leaf extract is higher than that of aerial root extract. The 50% inhibition concentration (IC_{50}) values of *P. amaryllifolius* leaf and aerial root crude extract are 0.810 mg/ml and 2.340 mg/ml, respectively. The antioxidant capacities of *P. amaryllifolius* crude extracts by DPPH assay and thiocyanate methods were lower than those of vitamin C and butylated hydroxytoluene (BHT). However, a neat solution extract of *P. amaryllifolius* showed higher inhibition (90.1%) of linoleic acid peroxidation than 200 ppm of

vitamin C (57.1%) and butylated hydroxytoluene (BHT 71.1%). Topical emulsion systems generally consist of multiple phases in which lipid and water coexists with some emulsifiers. Thus an antioxidant study using a linoleic acid emulsion can be simulated with heterogeneous emulsion or with the biological lipid system. The studied results show that *P. amaryllifolius* extracts are able to slow down the peroxidation of linoleic acid in the heterogeneous system. The solution extracts was also showed good stability after subjected to the elevated temperature over time.

The oil in water (O/W) emulsion cream was developed as general moisturizing cream containing antioxidant. The oil phase consisted of cyclopentasiloxane (4%), jojoba oil (5%), dimethicone (2%), shea butter (2.5%), jojoba ester (3.5%), Span-80 (1.7%), Tween-80 (1.3%), Sepigel-305 (2.5%) and propylparaben (0.15%) while the aqueous phase was comprised of propylene glycol (3%), methylparaben (0.15%) and deionized water. *P. amaryllifolius* extract was added at 40-45 $^{\circ}$C after an emulsion is formed. The concentration of the extract in the preparation was varied as 1, 3 and 5%. It was found that the products containing up to 3% extract had superior texture emulsion cream with light pandan odor.

Stability test was performed to ensure that the products meet the intended physical, chemical and performance characteristics when they stored under various conditions. The developed creams containing 3% *P. amaryllifolius* extract were subjected to centrifugation at 6000 rpm for 30 minutes and there was no sign of phase separation observed. Then, the product was further studied for accelerated stability test at different storage conditions for 1 month, i.e. at 4°C in refrigerator, ambient temperature, 45°C in hot air oven and heating-cooling cycle (4°C, 24 hours; 45°C, 24 hours). These samples were monitored every week with respect to changes in appearance, color, odor, pH and viscosity.

Appearance, texture, odor and color of the developed products were visually observed. At initial, the product was creamy texture, medium viscosity with pandan odor. After subjected to stability test under various conditions, it was found that the viscosity of the products in hot air oven and heating-cooling cycle conditions were decreased over time, but there is no phase separation observed in all conditions. The color of the product changed to slightly lighter-green compared with the initial. The odor of the product was somewhat oily and the pandan odor was less significant. Additionally, pH was practically unchanged except for at 45 °C.

The viscosity of the developed products was fairly stable when stored at 4°C, ambient temperature, but showed more noticeable change when stored at 45°C and heating-cooling cycle. Thus, the results are very useful in selecting optimal storage condition for the product over time.

The total color differences (ΔE*) between initial (W0) and each week (W1, W2, W3 and W4) were calculated using the equation: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. The results indicated that the highest

change is when the product was kept under heating-cooling condition followed by elevated 45 °C, ambient temperature and 4 °C.

Single patch test was performed on 23 volunteers using occlusive patch test to determine the potential irritation of the product. The acute irritation index or mean irritation index (M.I.I) was calculated according to an equation below and the results are shown in Table xx The M.I.I. values of all volunteers is 0, 0, 0 and 0 after removed, at 24 h, 48 h, and 72 h., respectively. Therefore, the product may be classified as non-irritation on the skin.

Thirties volunteers aged between 18-30 years old were selected for sensory evaluation. Volunteers were asked to use the product and fill out their opinion regarding the properties of the product in sensory evaluation form. It was found that most volunteers liked the overall feature of cream product expect for the odor.

The product efficacy was measured every week using SELS program calculation from Visoscan® VC 98. Twenties volunteers aged between 20 and 50 years old were asked to use the product daily twice a day.

The results of the skin images were processed by SELS program calculation and the outputs are indicated in skin wrinkles or SEw and skin scaliness or SEsc values. The SEw refers to the wrinkles of the skin and the small value means less wrinkles on the skin. It was found that, after 1 month applied, 90% of the volunteers showed a decrease in SEw value. The SEsc refers to the scaliness of the skin and the small value means less scaliness on the skin. The results after using product for 1 month indicated that 85% of the volunteers have a decrease SEsc value. In conclusion, the research results indicated that *P. amaryllifolius* extract might be useful in cosmetic industry.

บทสรุปผู้บริหาร

เตย (Pandanus) เป็นพืชใบเลี้ยงเดี่ยว ซึ่งพบประมาณ 600 สายพันธุ์ โดยสายพันธุ์ที่พบในประเทศไทยได้แก่ เตยหอม และเตยทะเล ใบของเตยหอมเป็นที่รู้จักกันดีโดยนิยมนำมาใช้แต่งกลิ่นอาหารทั้งอาหารคาวและหวาน ให้กลิ่นหอมสดชื่น นอกจากนี้ใบเตยยังให้คุณสมบัติทางยาที่สำคัญได้แก่ ยาขับปัสสาวะ ยารักษาโรคผิวหนัง ยารักษาโรคเบาหวาน และยาบำรุงหัวใจ อีกทั้งยังช่วยให้ร่างกายรู้สึกสดชื่น ลดอาการไข้ และบรรเทาอาการท้องอืด อีกด้วย

ใบเตยหอมประกอบไปด้วยสารกลุ่ม alkaloids เช่น pyrrolidine alkaloids และ pandamarilactonines นอกจากนี้ยังประกอบไปด้วยโปรตีน pandamin ซึ่งมีคุณสมบัติในการต้านเชื้อไวรัสเริม ชนิดที่ 1 (HSV-1) และเชื้อไข้หวัดใหญ่ H1N1 นอกจากนี้ยังพบสารจำพวก quercetin, carotenoids, tocopherols, tocotrienols และ essential oil

เตยทะเล พบได้ทั่วไปบริเวณภาคใต้ของประเทศไทย โดยผลสุกของเตยทะเลให้กลิ่นคล้ายใบเตยหอมเนื่องจากมีสารจำพวก ester essential oil ใบมีฤทธิ์ยับยั้งการอักเสบ รากประกอบไปด้วยสารเคมีหลากหลายชนิด เช่น steroidal compounds, lignin, benzofuran derivatives และ phenolic compounds เตยทะเลเป็นหนึ่งในพืชที่ใช้เป็นยาต้านมะเร็ง นอกจากนี้สารสกัดจากรากเตยทะเล ยังประกอบไปด้วย 3,4-bis(4-hydroxy-3-methoxybenzyl) tetrahydrofuran มีคุณสมบัติเป็นสารต้านอนุมูลอิสระซึ่งให้ค่าสูงใกล้เคียงกับ BHA

ในตำรายาแผนโบราณได้รวมพืชทั้งสองชนิดนี้ โดยใช้เป็นหนึ่งในส่วนประกอบของยาโดยความพยายามของนักวิทยาศาสตร์ที่จะสกัดและทดสอบสารสำคัญในพืชทั้งสอง ในส่วนของเครื่องสำอาง ยังมีการศึกษาจากนักวิทยาศาสตร์ค่อนข้างน้อย ดังนั้น โครงการวิจัยนี้จึงมุ่งศึกษาวิธีการสกัดเตยเพื่อให้สารสกัดที่ได้อยู่ในรูปแบบที่เหมาะสมสำหรับการนำไปประยุกต์ใช้เป็นสารต้านอนุมูลอิสระจากธรรมชาติที่ใช้เป็นส่วนประกอบในเครื่องสำอาง ซึ่งตัวทำละลายที่เลือกศึกษาคือ ethanol และ propylene glycol เนื่องจากเป็นตัวทำละลายที่นิยมใช้ในเครื่องสำอาง มีความปลอดภัย และง่ายต่อการจัดเตรียม

สารสกัดจากเตยหอมซึ่งสกัดด้วย propylene glycol มีค่า DPPH radical scavenging activity และ total phenolic content ที่สูงกว่าการสกัดด้วย ethanol อีกทั้งใบยังแสดงฤทธิ์ที่ดีกว่าราก โดยมีค่า IC_{50} ของใบและรากเป็น 0.810 mg/ml และ 2.340 mg/ml ตามลำดับ ถึงแม้ว่าค่าความสามารถในการต้านอนุมูลอิสระของสารสกัดหยาบ ยังคงน้อยกว่าสารมาตรฐาน (vitamin C และ BHT) แต่อย่างไรก็ตาม solution extract ของเตยหอมแสดงค่าการยับยั้งใน linoleic acid peroxidation ที่สูงถึง 90.1% ซึ่งสูงกว่าสารมาตรฐาน vitamin C (57.1%) และ BHT (71.1%) ที่ความเข้มข้น 200 ppm โดยทั่วไป อิมัลชัน (emulsion) เป็นสารผสมที่ประกอบด้วยของเหลวต่างชนิดกันตั้งแต่ 2 ชนิดขึ้นไป เช่น น้ำมันและน้ำผสมกันโดยมีตัวประสาน (emulsifier) ดังนั้นการศึกษาฤทธิ์ต้านอนุมูลอิสระโดยวิธี linoleic acid emulsion จึงเป็นการจำลองแบบอีมัลชันหรือตามระบบชีววิทยาของไขมัน โดยผลการศึกษาพบว่า สารสกัดจากเตยหอมและเตยทะเลสามารถทำให้ปฏิกิริยา peroxidation ของ linoleic acid ในระบบอิมัลชันช้าลง อีกทั้งยังพบว่าสารสกัดมีความคงตัวสูงภายใต้อุณหภูมิที่สูงขึ้น

ในการวิจัยนี้ได้พัฒนาครีมบำรุงผิวอิมัลชัน ซึ่งมีส่วนประกอบคือ cyclopentasiloxane (4 %), jojoba oil (5 %), dimethicone (2 %), shea butter (2.5 %), jojoba ester (3.5 %), Span-80 (1.7 %), Tween-80 (1.3 %), Sepigel-305 (2.5 %), propylparaben (0.15 %), propylene glycol (3 %), methylparaben (0.15 %) และ deionized water โดยใส่สารสกัดใบเตยหอมที่ 1, 3 และ 5 % ซึ่งพบว่าครีมบำรุงผิวที่ใส่สารสกัดใบเตย 3% มีลักษณะเนื้อครีมเหมาะสม มีกลิ่นหอมของเตยอ่อนๆ แต่ถ้าใส่มากเกินไปถึง 5% เนื้อครีมจะมีความเหลวเกินไป จากนั้นได้ทดสอบความคงตัวของครีมโดยการปั่นเหวี่ยงที่ 6000 rpm นาน 30 นาที พบว่าไม่มีการแยกชั้น และทดสอบความคงตัวของครีมที่สภาวะเร่ง 4°C, 45°C และ heating-cooling cycle (4°C, 24 h, 45°C, 24 h) เปรียบเทียบ กับสภาวะอุณหภูมิห้อง นาน 1 เดือน พบว่าครีมมีความคงตัวดี มีการเปลี่ยนแปลงของสีน้อย โดยสภาวะที่มีการเปลี่ยนแปลงมากที่สุดคือ 45°C และ heating-cooling นอกจากนี้ได้ทดสอบการแพ้เบื้องต้นในอาสาสมัคร โดยวิธี single patch test และไม่พบอาการแพ้ใดๆ อีกทั้งยังได้ทำการประเมินคุณภาพของครีม โดยการทำ sensory test และให้อาสาสมัครทดลองใช้ครีมโดยทาที่ผิว วันละ 2 ครั้ง แล้วประเมินผลโดยการถ่ายภาพผิวด้วยเครื่อง Visoscan® VC 98 ซึ่งพบว่า อาสาสมัครมีความพอใจต่อเนื้อครีม คุณสมบัติของครีมในระดับสูง แต่มีความต้องการให้ปรับปรุงกลิ่นโดยต้องการให้ใส่น้ำหอม และผลการถ่ายภาพผิวด้วยเครื่อง Visoscan® VC 98 พบว่าอาสาสมัครมีค่า SEw และ SEsc ลดลง ซึ่งแสดงถึงการลดลงของ wrinkles และ scaliness ดังนั้นผลการวิจัยนี้ได้แสดงให้เห็นว่าสารสกัดจากเตยหอมอาจเป็นตัวเลือกที่สามารถนำมาใช้ประโยชน์ทั้งในผลิตภัณฑ์เครื่องสำอางต่อไป

1. Introduction

1.1 Background and rationale

Pandanus (Pandanaceae) is a genus of monocots with about 600 known species (Takayama et al., 2002). *Pandanus amaryllifolius* Roxb. (Toei-hom) and *Pandanus odoratissimus* Linn. (Toei-talay) are of example species found in Thailand. *Pandanus amaryllifolius* Roxb., commonly known as pandan, is widely cultivated in southeastern Asia such as Thailand, Malaysia, and Philippines. Pandan leaves are often used to give a refreshing, fragrant flavor to both sweet and savory of South-East-Asian dishes. Pandan leaves are also used in cooking ordinary non-aromatic rice to imitate the more expensive aromatic Thai jasmine rice. The pandan leaves emit a pleasant aroma, mainly due to the presence of 2-acetyl-1-pyrroline (Buttery, 1982). Use of *P. amarylifolius* leaves as coloring and flavoring agents in culinary application arouses one's curiosity and motivation to study the chemical constituents of this plant. Hot water extracts of pandan root illustrate hypoglycemic activity, and 4-hydroxybenzoic acid has been isolated as an active principle (Peungvich, 1998). The antioxidative activities of methanolic *P. amaryllifolius* leave extracts show lower activity than that of BHT (Mohd Nor, 2008). Additionally, chemical component analysis of the root part of *P. odoratissimus* led to the isolation of 3,4-bis(4-hydroxy-3-methoxybenzyl) tetrahydrofuran which shows strong antioxidative activities when compared to BHA (Jong, 1998). Traditional folk medicine has incorporated pandan as one of the component in the formula and scientists have been trying to isolate active compounds and prove for their activities. In view of cosmetic application, there has been less scientific study. This project thus has focused on the preparation of Thai pandan extract as natural antioxidant ingredient for cosmetics. The extraction of leave and aerial root of *P. amaryllifolius* were performed using alcohols and polyols in various ratios. The total phenolics and antioxidative activities of the extracts were evaluated. In addition, topical emulsion containing the extract was developed and the physiochemical properties, the stability and efficacy of the emulsion were investigated and reported.

1.2 Objectives

1. To perform the extraction of aerial root and leaf of *P. amaryllifolius* using alcohols and polyols.
2. To determine the total phenolics and study the antioxidative activities of the extracts.
3. To prepare the standard extracts of *P. amaryllifolius* as natural antioxidant for cosmetics.
4. To develop skincare emulsion containing the extract.

1.3 Expected results

1. Suitable extraction method of aerial root and leaf of *P. amaryllifolius* using alcohols and polyols.
2. Suitable form of aerial root and leaf extract of *P. amaryllifolius* for cosmetic use.
3. Practical information for utilization of *P. amaryllifolius* in cosmetic industry

1.4 Conceptual framework

Cosmetics are commercially available products that are used to improve the appearance of the skin. The skin is the largest organ; as our primary external barrier, it is on the forefront of the battle with external causes of damaging free radicals. Free radicals are highly reactive molecules with an unpaired electron that result in damage to surrounding molecules and tissues. The most significant damage by free radicals is to biomembranes and to DNA. It is thought that additional, topical use of antioxidants in cosmetics can better protect and possibly correct the damage by neutralizing these free radicals. Antioxidants may be synthetics, such as BHA and BHT, or of natural origin, such as phenolics as well as polyphenolics. Phenolic compounds are found in all foods of plant origin, and rich sources are tea, coffee, nuts, and chocolate, but especially fruit, including preserved fruit, such as wine, juices, and dried fruits. Recently, there has been an increase in the use of polyphenolic compounds in cosmetics. For example, catechin, a green tea extract, has been found to be protective against photocarcinogenesis. Such data suggest a need for future study and possible incorporation of similar compounds into cosmetic formulation. Natural antioxidative substances from the polyphenols of edible herbs are believed to be safer and may provide additional health benefits, compared to synthetic antioxidants. It is an area worth investigating due to current consumer concerns about health.

Clearly, pandan plants have long been used in food industry. Traditional folk medicine has incorporated pandan as one of the component in the formula and scientists have been trying to isolate active compounds and prove for their activities. In view of cosmetic application, there has been less scientific study. This project thus focused on the preparation of *P. amaryllifolius* extract as natural antioxidant ingredient for cosmetics. The extraction of leave and aerial root has been performed using alcohols and polyols in various ratios. The total phenolics and antioxidative activities of the crude extracts were evaluated. Application of the pandan extract as natural antioxidant in skincare topical emulsion was explored and the physiochemical properties, the stability and efficacy of the emulsion were examined and reported.

1.5 Scope of Research

1. Perform extraction of aerial root and leaf of *P. amaryllifolius* using alcohols and polyols in various ratios.

2. Determine the total phenolics and antioxidative activities of the extracts.

4. Prepare the standard extracts of *P. amaryllifolius* as natural antioxidant ingredient for cosmetics.

5. Develop skincare emulsion product containing the extract and study the possible application of the extract as natural antioxidant in cosmetics.

1.6 Timeline of Research

Year 1

Activities	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
1. Sample collection and preparation	←	→										
2. Extraction		←	→									
3. Total phenolic content of the extract				←	→							
4. Antioxidant activities of the extract					←	→						
5. Preliminary study on the use of extract in skincare emulsion product									←	→	→	→
6. Report												← →

Year 2

Activities	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
1.Antioxidant activity of emulsion containing the extract		←	→	→	→	→						
2.Preparation of the standard extract				←	→	→						
3. Preparation of the prototype of the skincare emulsion containing the extract.						←	→					
4. Sensory evaluation of the skincare emulsion containing the extract.						→	→	→	→	→		
6. Manuscript preparation									←	→	→	→
7.Final report												← →

2. Literature Review

2.1 Pandanus

Pandanus is a genus of monocots with about 600 known species. Plants vary in size from small shrubs less than 1 m tall, up to medium-sized trees 20 m tall, typically with a broad canopy and moderate growth rate. The plant grows prolifically in tropical areas, including the pacific islands, Africa, South Asia and South East Asia. *Pandanus amaryllifolius* Roxb. (Toey-hom) and *Pandanus odoratissimus* Linn. (Toey-Talay) are of example species found in Thailand. *P. amaryllifolius* (synonym: *P. odorus)* is a tropical plant in the screwpine genus which is known commonly as pandan and used widely in Southeast Asian cooking. It is an erect green plant with fan-shaped sprays of long, narrow, bladelike leaves and woody aerial roots (Figure 1). The plant is sterile, flowers only very rarely, and is propagated by cuttings. *P. odoratissimus* (synonym: *P. fascicularis*) is erect branched small tree, growing 3-5 m, the trunk bearing many support roots. Leaves are spirally crowded toward the ends of the branches, linear lanceolate, slenderly long-acuminate, up to 1.5 meters long, 3-5 cm wide, the margrins and midrib armed with sharp spiny teeth pointing toward the apex of the leaf. Fruit is solitary, pendulous, ellipsoid to globose-ellipsoid, about 20 cm long, composed of 50-75 obovoid, angular, fibrous and fleshy drupes, 4-6 cm long, narrow below and truncate at the apex (Figure 2).

Figure 1 *Pandanus amaryllifolius* Roxb. (Toey-hom)

Figure 2 *Pandanus odoratissimus* Linn. (Toey-Talay)

2-Acetyl-1-pyrroline (ACPY) is the major compound responsible for unique pleasant aroma present in *P. amaryllifolius.* ACPY was isolated from *P. amaryllifolius* by Buttery (Buttery, 1982) and Laksanalamai and Ilangantileke (Laksanalamai, 1993). Furthermore, ACPY is also the principle aroma component of aromatic rice varieties such as Thai jasmine rice (Buttery, 1983). ACPY has been of great interest in food favoring industry and was chemically synthesized and is now patented (Buttery, US 500049, 1984 and US 4522838, 1985). The chemical structure of ACPY is shown in Figure 3.

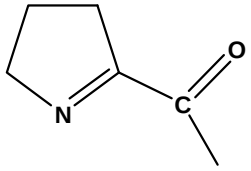
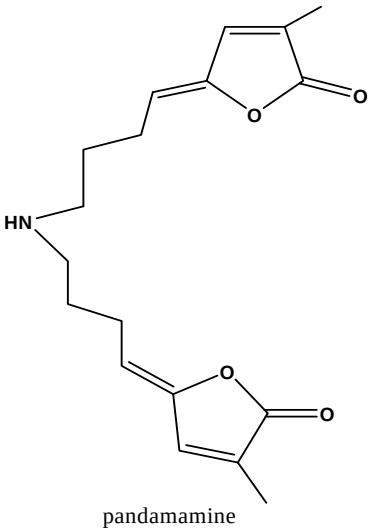


Figure 3 Structure of 2-Acetyl-1-pyrroline (ACPY)

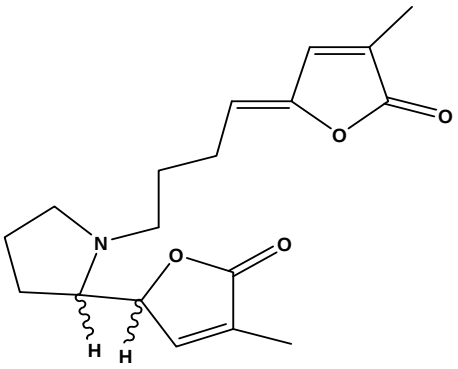
Information posted on the http://www.uni-graz.at/~katzer/engl/Pand_ama.html claimed that additional aroma components found in *P. amaryllifolius* are 3-methyl-2-(5H)-furanone, 3-hexanol, 4-methylpentanol, 3-hexanone and 2-hexanone. Furthermore, very small amount of the following components are also found in *P. amaryllifolius*: Styrene 0.62 ppb, Formylthiphene 0.76 ppb, Linalool 0.29 ppb, β-caryophyllene0.55 ppb, β-farnesene 0.18 ppb, 1,2-dimethoxybenzene 0.15 ppb and β-selinene 1.24 ppb (MacLeod, 1982). Various alkaloids, such as pyrrolidine alkaloids, pandamamine and pandamarilactones have been isolated from pandan leaves (Takayama, 2001; Salim, 2004) and the structures are shown in Figure 4.

P. amaryllifolius leaves also contain pandamin protein which is classified as unglycosylated protein and possesses antivirus activities against human viruses, herpes simplex virus type-1 (HSV-1) and influenza virus (H1N1) (Ooi, 2004).

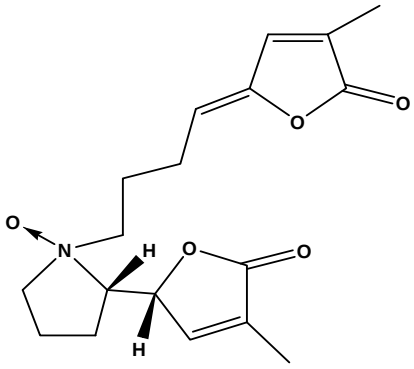
The decoction of the *P. odorus* root and rhizome has been traditionally used in treating diabetic patients without much scientific evidence. Recently, 4-hydroxybenzoic acid has been identified from *P. odorus*. This compound showed a hypoglycemic effect in normal rats after the oral administration of 5 mg/kg. Additionally, the compound increased serum insulin levels and liver glycogen content in normal rats (Peungvich, 1998). The structure of 4-Hydroxybenzoic acid is shown in Figure 5.



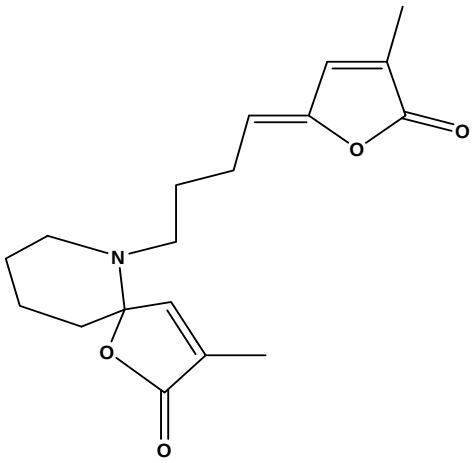
pandamamine



pandamarilactone A,B



pyrrolidine alkaloids



pandamarilactone -1

Figure 4 Structures of pyrrolidine alkaloids, pandamamine, pandamarilactones

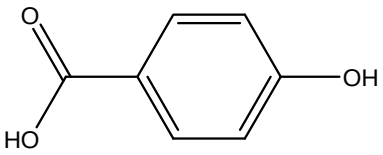


Figure 5 Structure of 4-Hydroxybenzoic acid

P. odorus leaves are used medicinally in South East Asia to refresh the body, reduce fever, and relieve indigestion and flatulence (Cheeptham, 2002). The leaf contains essential oils, carotenoids, tocopherols and

tocotrienols (Lee, 2004), quercetin (Miean and Mohamed, 2001), alkaloids (Busqué, 2002), and non-specific lipid transfer proteins (Ooi, 2006). Occasionally, bunches of pandanus leaves were also found to use to ward off cockroaches in taxi or bus (Li and Ho, National University of Singapore).

The study by researchers from Mahasarakarm University revealed that the ethanolic extract of *P. amaryllifolius* root of about 5 mg/kg tended to be able to be applied for the purpose of lowering the blood glucose level and aortic disorder in diabetic patients (Kongsodsup, 2006).

Recently, the study shows that an ehanolic extract of *P. amaryllifolius* leaves cultivated in Malaysia, which had a polyphenol content of 102 mg/g, exhibited an excellent heat-stable antioxidant property. This result may lead to the use of *P. amaryllifolius* leaves as a good natural alternative to existing synthetic antioxidants in industry (Mohd Nor, 2008). Mohd Nor et al. also tested the oxidative stability index (OSI) of the extract compared with BHT and the results showed that the OSI of pandan leave extract is significantly higher than those of both control and BHT, Figure 6.

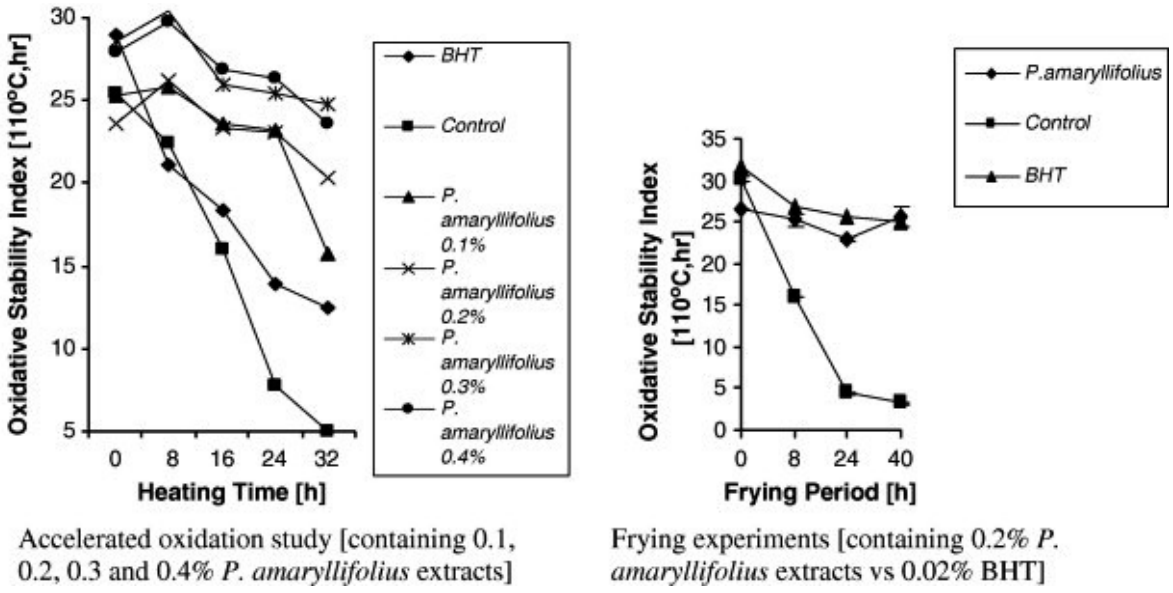


Figure 6 Changes in oxidative stability index (OSI) over time in bleached and deodorized palm olein

Other related study of Pandanaceae plant is an antioxidative activities of constituents isolated from *P. odoratissimus* Linn. It has been found that methanolic extract of *P. odoratissimus* flourishes in southern Taiwan shows great antioxidative activities in study of the crude drugs used in Taiwan (Jong, 1998). Chemical component analysis of the root parts of *P. odoratissimus* led to the isolation of phenolic compounds and lignan

type compounds plus a new benzofuran derivative. Among them, pinoresinol and 3, 4-bis(4-hydroxy-3-methoxybenzyl) tetrahydrofuran showed strong antioxidative activities when BHA was used as a standard in the thiocyanate method. Figure 7 shows the structure of 3,4-bis(4-hydroxy-3-methoxy-benzyl)-tetrahydrofuran.

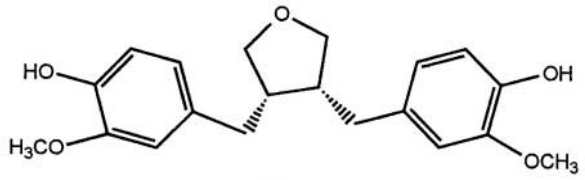


Figure 7 Structure of 3,4-bis(4-hydroxy-3-methoxy-benzyl)-tetrahydrofuran

2.2 Free radical and antioxidant activity

Free radical is an atom or group of atoms that have one or more unpaired electrons. Radicals can have positive, negative or neutral charge. They are formed as necessary intermediates in a variety of normal biochemical reactions, but when generated in excess or not appropriately controlled, radicals can wreak havoc on a broad range of macromolecules. A prominent feature of radicals is that they have extremely high chemical reactivity, which explains not only their normal biological activities, but how they inflict damage on cells. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols.

A variety of *in-vitro* chemical methods are being used to determine the antioxidant activity of products and ingredients, but questions regarding whether the results have any bearing on effectiveness in the human body are leading to development of additional methods that may be more appropriate for screening potential antioxidant ingredients (Mermelstein, 2008).

2.2.1 2,2-diphenyl-1-picrylhydrazyl (DPPH)

A simple method that has been developed to determine the antioxidant activity of foods utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The structure of DPPH and its reduction by an antioxidant are shown in Figure 8. The odd electron in the DPPH free radical gives a strong absorption maximum at 517 nm

and is purple in color. The color turns from purple to yellow as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with a hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured.

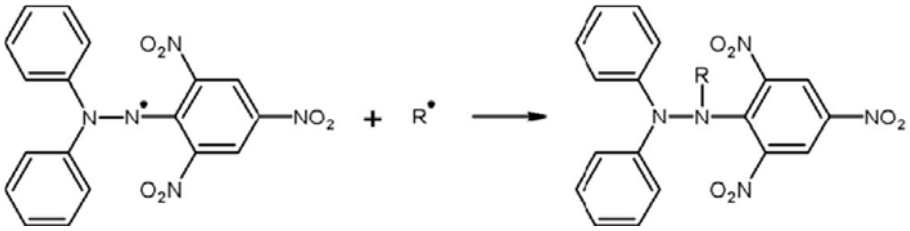


Figure 8 Oxidation reaction of DPPH radical (purple) to DPPH-H (yellow)

2.2.2 Folin-Ciocalteu method

The Folin-Ciocalteu method is an electron transfer based assay and measures reducing capacity, which has normally been used to estimate phenolic contents of biological materials. The Folin-Ciocalteu reagent (FCR) or Folin's phenol reagent or Folin-Denis reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent. However, this reagent dose not only measure total phenols and will react with any reducing substance. The reagent therefore measures the total reducing capacity of a sample, not just the level of phenolic compounds. Phenolic compounds are a class of chemical compounds consisting of a hydroxyl functional group (OH^-) attached to an aromatic hydrocarbon group. They are diverse in structure but are characterized by hydroxylated aromatic ring (e.g., flavan-3-ols) and categorized as secondary metabolites. Furthermore, phenolic compounds may occur in food plant as esters or glycosides conjugated with other natural compounds such as flavonoids, alcohols, hydroxyl fatty acid, sterols and glucosides. Phenolic compounds are plant metabolites widely spread throughout the plant kingdom. Some phenolic compounds in plants are polymerized into larger molecules such as the proanthocyanidins and lignins. Significantly, phenolic compounds have high antioxidant capacity, are excellent free radical scavengers and have been used in processed foods in recent years.

2.2.3 Ferric thiocyanate method (Scheirs, 2000)

The Ferrous/ferric methods for the determination of hydroperoxides exploit the transition metal ion-catalysed decomposition of hydroperoxides as shown in the following reaction: [ROOH + $2Fe^{2+}$ + $2H^{+}$ ------> $2Fe^{3+}$ + H_2O + ROH]. The concentration of Fe^{3+} ions formed during the hydroperoxide decomposition can be determined by either: (i) monitoring the decrease in the concentration of Fe^{2+} ions by measuring the decrease in the absorbance of the complex formed between these ions and 1, 10-phenanthroline, or (ii) by the complexation of the Fe^{3+} ions with thiocyanate ions (SCN^{-}), with this latter reaction giving $[Fe(SCN)_6]^{3-}$. The method assumes that hydroperoxides are stoichiometrically consumed in the oxidation of Fe^{2+} to Fe^{3+}; the Fe^{3+} ions are then quantitatively complexed with SCN^{-} ions. The concentration of hydroperoxides can be determined spectrophotometrically by measuring the coloured $[Fe(SCN)_6]^{3-}$ complex at its maximum absorbance wavelength, or by titration of the hydroperoxides with a ferrous ammonium thiocyanate solution in benzene.

2.3 Topical oil-in-water emulsion

Emulsions are by far the most popular delivery form for skincare cosmetic. Most emulsions consist of droplets, which form the internal or dispersed phase, that are uniformly distributed into a continuous phase. Generally, the dispersed phase is composed of oil or oil soluble ingredients and the continuous phase is composed of water or water soluble ingredients. This is considered an oil-in-water (O/W) emulsion. Since water soluble and oil soluble ingredients do not mix, an emulsifier is incorporated to reduce the interfacial tension between the oil and water phase by adsorbing to the oil/water boundary and thus acting as a barrier to coalescence. The properties that are most important in a cosmetic emulsion are appearance, feel and odor on application, and effectiveness and having these properties the same each time the product is purchased and used. For any given formula, these properties will depend on the components or ingredients and their properties, the type of emulsion, and the ratio of the major phases. The appearance and feel of a cosmetic depends on a variety of more specific properties such as viscosity or consistency and stability, especially under application conditions (Barel et al., 2001)

2.4 Stability study of cosmetic emulsion

Stability test was performed to ensure that the products meet the intended physical, chemical and performance characteristics when they stored under various conditions. Stability prediction is usually performed by accelerated storage conditions (Tadros et al., 2004). Before initiating the stability studies, the product should be submitted to a centrifugation test at 3,000 rpm for 30 minutes (Anchisi et al., 2001). Signs of instability of product would be observed and if the product does not change, it may be stable. The stability study is divided into 1) preliminary stability test 2) accelerated stability test, and 3) shelf test. Preliminary

stability test is known as the screening test, or short term test. It aims to assist the choosing of formula with a reduced duration. It uses extreme temperature conditions to accelerating reactions on the components and appearance for observed the characteristics of product. The formulations under test are submitted to stress conditions: heating in ovens and cooling in refrigerators. The duration of the study is generally fifteen days. Accelerated stability test is known as normal or exploratory stability test. It provides the data to foresee the stability of the product and used to estimate the expired date of the product. It generally uses less extreme conditions than the preliminary test. The samples can be submitted to heating in ovens, cooling in refrigerators, exposure to light radiation and to the environment. The duration is generally of ninety days, some cases can be extended for six months or even one year. Shelf test is known as the long-term test which validates the stability limits of the product. This study is carried out over a period equivalent to the time of expiry estimated to evaluate the behavior the product under normal storage conditions.

2.5 Safety Test

Clinical trial is the research study conducted to evaluate a new treatment in human. Each study is designed to learn about a potential treatment and its effect on human. Clinical trial is separated into safety test, efficacy test and consumer test. Safety test is the test that performed to ensure the safety of a cosmetic product. The example of safety tests include single patch tests, determination of irritating effect “non-irritation”, human repeat insult patch test (HRIPT), determination of allergic effect “hypoallergenic”.

Single patch testing is determination of irritating effect on the skin, by applying allergens under occlusion on intact skin of patients. This test may be used for new or novel formulations with known raw materials, and for novel formulations that have been shown to be safe to skin in an open patch test (Walker et al., 1996). The test products are applied diluted or undiluted to the skin of, for example, the upper arm or back for periods up to 48 hours under occlusive or semi-occlusive patches and evaluations are performed, for example, l, 24 and 48 hours after removal of the patch. The evaluation is performed visually, assessing, for example, redness, scaling, following the exposure period (Colipa Guidelines, 1997).

2.6 Sensory evaluation

Sensory evaluation is defined as a scientific discipline used to measure, analyze and interpret reactions to those characteristics of products as they are perceived by the senses of sight, smell, taste, touch and hearing. Sensory evaluation may be subjective or objective evaluation. Subjective test is based on consumer expectation and results will express in form acceptance or preference. Objective sensory methods are those controlled test variables such as environment, sample handling and respondent (panelist) selection and training.

Affective test is of one type of sensory evaluation used to measure preference for products or magnitude of like/dislike for a product. It can be used for consumers or trained panelists, for example, Hedonic test. The word 'hedonic' is of Greek origin and relates to degree or magnitudes of like or dislike. So, it is important to conduct the right type of sensory evaluation in order to find out opinions of consumers and panelist in regards to the quality attributes of products.

2.7 Efficacy test

The efficacy test serves the assessment of the skin mechanical properties before and after cosmetic application. Several parameters of skin function and appearance are measured such as whitening effect (Chromameter® measurement, Mexameter ® measurement etc.), moisturizing effect (Corneometer® measurement), anti-wrinkle effect (SIA® measurement or PRIMOS), etc.

Measurement of the Skin Topography by using Visioscan®VC 98

Special UV-A light video camera with high resolution has been developed especially to study the skin surface directly. The images show the structure of the skin and the level of dryness very impressively. With its multi-functional software, the Visioscan® VC 98 (Courage+Khazaka, Cologne, Germany) is a very unique flexible system to characterize skin surface condition easily, accurately and very economically. Interesting studies were done with this measurement method. There are numerous fields of applications. Besides efficacy testing and claim support for cosmetics, pharmaceuticals, detergents and objective clinical diagnosis in dermatology, there are also applications in occupational medicine, medical consultancy and many more fields. The camera features a high resolution sensor and a ring shaped UV-A light source (proven to present no hazard to normal human skin) for uniform illumination of the skin. With its special light it brings out a very sharp, non glossy image. Especially pigmentation underneath the skin is visible with the UV-light. The image processing function, special software permits the calculation of a variety of skin surface parameters: SELS (Surface Evaluation of the Living Skin). This evaluation method was developed by the Institute for Experimental Dermatology, Prof. Tronnier, University of Witten-Herdecke, Germany. The grey level distribution of the image is used to evaluate the skin. There are four clinical parameters to quantitatively and qualitatively describe the skin surface as an index: Skin smoothness (SE_{Sm}), Skin roughness (SE_r), Scaliness (SE_{sc}), Wrinkles (SE_w).

3. Materials and Methods

3.1 Plant materials and reagents

P. amaryllifolius plant was collected from Chiangrai province, Thailand. Leaves and aerial root were used in extraction. It should be noted that criteria of source selection for each plant sample is based on the availability and ease of accessibility. Solvents for plant extraction (95% ethanol and propylene glycol) and vitamin E acetate are of cosmetic grade. All chemicals and reagents for activity study were of A.R. grade.

3.2 Preparation of extracts

Crude extract of P. amaryllifolius

Plant samples were cut into small pieces and dried in hot air oven at 40 ^{o}C for 48 hours. 100 grams fresh pandan resulted in about 16 grams dried plant. *P. amaryllifolius* was thoroughly immersed into solvent (10 grams leaves or 20 grams of aerial root per 250 ml) and macerated and were extracted into ethanol at 50 °C for 8 hours under sonication. The solvent was removed using a rotary evaporator.

Solution extract of P. amaryllifolius

A portion of 10 grams pandan leaves were used per 250 ml solvent and 20 grams of aerial root were used per 250 ml solvent. Plant samples were thoroughly immersed into solvent and macerated for specific extractions' condition. The solution extract was then filtrated and kept in refrigerator at 4 ^{o}C. The solvent systems were ethanol, propylene glycol and mixture of ethanol/propylene glycol at 4:1 and 1:1 volume ratio. *P. amaryllifolius* was extracted at ambient temperature for 1 day and 3 days and at 50 ^{o}C for 8 and 10 hours.

3.3 DPPH radical-scavenging assay

The scavenging activity of the extracts was measured on DPPH radicals according to Que et al. (Que et al., 2006) and Xu et al. (Xu et al., 2008) with some modifications. DPPH radicals in absolute ethanol (0.1 mM), this solution (3 ml) was added to the sample (1 ml). The reaction mixture was mixed at ambient temperature for 30 minutes in the dark and then the absorbance (Abs) was determined at 517 nm with a UV-Vis spectrophotometer. The scavenging activity (%SA) on DPPH radicals was calculated from the following equation: $\%SA = [Abs_{control}-(Abs_{sample} - Abs_{blank})/Abs_{control}] \times 100$. The experiment was performed in triplicate and the results were reported in an average value.

3.4 Determination of total phenolic content

The total phenolic content of the samples was determined by the Folin-Ciocalteau total phenolic assay (Singleton and Rossi, 1965; Waterhouse, 2002) with some modifications. Gallic acid was used as a standard and the range of concentrations (50, 100, 250, 500, 750, 1000 mg/L) was used to create a standard curve. Deionized water (1.58 ml) and Folin-Ciocalteau reagent (100 μl) was added to the sample, the standard, or

blank (20 μl). The reaction mixture was incubated at room temperature for 5 minutes. Sodium carbonate solution (300 μl, 10% Na_2CO_3) was mixed and incubated for 90 minutes. Absorbance of the mixture was measured in UV-visible spectrophotometer at 765 nm. The total phenolic content was calculated from a standard curve and expressed as gallic acid equivalents (GAE), mg. The experiment was performed in triplicate and the results were reported in an average value.

3.5 Linoleic acid emulsion system –thiocyanate method

Antioxidant activity of the extracts was also evaluated using the thiocyanate method according to Suja et al. (Suja et al., 2005) with some modifications. Vitamin E acetate and butylated hydroxytoluene or BHT (200 ppm) were used as positive controls. The reaction mixture (linoleic acid emulsion) consisted of 0.28 grams linoleic acid, 0.28 grams of Tween 20 and 50 ml of phosphate buffer (0.2 M, pH 7.0). The linoleic acid emulsion (2.5 ml) was mixed with 0.5 ml of test sample and 2.5 ml of phosphate buffer and incubated at 40 ^{o}C for 4 days. The mixture prepared without test sample was the control. Then aliqouts (0.1 ml) was taken from the incubation mixture at an interval of 24 hours and mixed with 5.0 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml ferrous chloride (20 mM in 3.5% HCl) and allowed to stand at room temperature for 3 min. Absorbance of the mixture was measured in UV-visible spectrophotometer at 500 nm. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated at 72 hours by the following equation: Inhibition of lipid peroxidation (%) = 100- [(As/Ac) x 100], where, Ac is the absorbance of control reaction which contains only linoleic acid emulsion and sodium phosphate buffer and As is the absorbance of the presence of sample or standard (Gülçin, 2006).

3.6 Preparation of topical emulsion product

The development of oil in water emulsion was formulated at different ratio of oil and water, and various amounts of emulsifiers to achieve a stable product. Physical properties of the developed products were evaluated by both visual observation and equipments. Its color was measured by chromameter, viscosity of product measured by viscometer. Odor and appearance of product were evaluated by visuality.

Microorganism contamination test

Microorganism contamination test of anti-wrinkle product was carried out by using Mikrocount® combi test kits. It is made from plastic that composes of slide coated on one side with TTC-agar (bacterial growth) and on the other side with Rose-bengal-agar (yeast and mould growth). 0.1 grams of product was smeared on each site, and then the slide was placed back in the tube and screw on the top and incubated at 37^{o}C for 3-4 days. After that the slide was evaluated.

Stability test

The emulsion cream was subjected to centrifugation test at 6,000 rpm for 30 minutes and accelerated conditions at ambient temperature, 4 °C, 45 °C and heating-cooling cycle (45 °C, 24 hours; 4 °C, 24 hours) for 1 month. The properties or changes of the products were monitored every week.

Single patch test

The tolerance of skin to irritation of the developed product was determined by a single patch test using Finn Chamber® on 23 healthy subjects. The product (0.01 grams) was applied on forearms area for 24 hours. DI water was used as control. The grading result (0, 0.5, 1, 2 and 3) of irritation on the area applied the product was evaluated by comparing with negative control as shown in Table 1.

Table 1 The grading for irritation zone

Score	Quotation	Criteria	
		Erythema (E)	**Odema (O)**
0	Absent	No erythema	No edema
0.5	doubtful	Very slightly erythema (quiet pinked coloration of tested area)	Very slightly edema (palpable, barely visible)
1	Slight	Slightly erythema (rather visible on tested area)	Slightly edema (palpable, visible)
2	Obvious	Obvious erythema (clear erythema covering all of the tested area)	Obvious edema with or without papules
3	Important	Important erythema (severe erythema covering all of tested area)	Important edema (extended area outside the tested area with or without vesicles or blisters)

Interpretation of the results

The calculation of the acute irritation index (M.I.I) was used to classify the potential irritation of the product. The interpretation of the calculated M.I.I scale is shown in Table 2.

$$M.I.I = \frac{\sum \text{of the grads (erythema + odema)}}{\text{Number of subjects}}$$

Table 2 The potential irritation scales of the product

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

Sensory evaluation

A group of 30 female volunteers' age between 25-50 years old was asked to use the sample of anti-wrinkle product and answer on the questionnaire.

Skin surface evaluation

Effectiveness of anti-wrinkle product was clinically measured by using Visoscan® VC 98 that comprised of UVA light camera with high resolution. A group of 20 female volunteers' age between 25-50 years old was taken photographs of facial skin surface before and after applied the emulsion product. The image of facial skin surface after applied the emulsion product (W1, W1, W4) were assessed and the results were calculated using SELS program and compared with the image of facial skin surface before using the product (W0).

4. Results and Discussion

4.1 *Extraction of P. amaryllifolius*

1. Solution extract

The leaf extracts are dark green with unique, pleasant pandan odor in all conditions. The aerial root extracts are odorless with yellow color. The pH values of the extracts were about 6.0 ± 0.1, Table 3 and Table 4.

Table 3 Physical properties of pandanus leave extracts**.**

Condition			Color	Odor	pH
Solvent	Time	Temp.			
Ethanol	1 day	Ambient	Dark green	Sweet teoy's odor	5.96
Ethanol	2 days	Ambient	Dark green	Sweet teoy's odor	6.00
Ethanol	3 days	Ambient	Dark green	Sweet teoy's odor	6.10
Ethanol	8 hours	50°C	Dark green	Sweet teoy's odor	6.07
Ethanol	10 hours	50°C	Dark green	Sweet teoy's odor	6.17
Ethanol /propylene glycol (4:1)	8 hours	50°C	Dark green	Sweet teoy's odor	5.73
Propylene glycol	8 hours	50°C	Dark green	Sweet teoy's odor	5.93

Table 4 Physical properties of pandanus root extracts.

Condition			Color	Odor	pH
Solvent	**Time**	**Temp.**			
Ethanol	1 day	Ambient	Yellow	Odorless	6.02
Ethanol	2 days	Ambient	Dark yellow	Odorless	5.85
Ethanol	3 days	Ambient	Dark yellow	Odorless	5.94
Ethanol	8 hours	50°C	Dark yellow	Odorless	6.09
Ethanol	10 hours	50°C	Dark yellow	Odorless	5.76
Ethanol/ propylene glycol (4:1)	8 hours	50°C	Dark yellow	Odorless	5.43
Propylene glycol	8 hours	50°C	Yellow	Odorless	6.01

2. Crude extract

The ethanolic crude extracts were prepared at 50 °C, 8 hours under sonication-assisted condition. The extracts were evaporated to dryness and percent yield was calculated by comparing the mass of crude to the amount of plant used and are shown in Table 5. It was found that dried aerial root gave 14.05% yield which is higher than that of dried leaf (9.24%). The pandan leaf and root crude extracts are dark green and yellow color, respectively.

Table 5 General information of *P. amaryllifolius* crude extracts.

Plant sample	% Yield	Color
Dried leaf	9.24	Dark green
Fresh leaf	3.47	Dark green
Dried aerial root	14.05	Yellow
Fresh aerial root	2.58	Yellow-green

4.2 DPPH radical-scavenging assay

4.2.1 Effect of time and temperature

In order to find optimum time and temperature in extraction, the *P. amaryllifolius* leaf was first extracted into ethanol solvent. The antioxidant activity of the extract was screened using DPPH radical-scavenging assay. The results showed that the activity of extraction at high temperature (50 °C, 8 hours) was higher than those of ambient temperature conditions, Table 6. Extraction at 50 °C for 8 and 10 hours were also performed

in order to compare activity at different extraction time. It was found that the activity was not significant different.

Table 6 DPPH radical-scavenging activities of dried *P. amaryllifolius* leaf extracted in ethanol solvent.[a]

Extraction time	Extraction temperature	% Activity
1 day	Ambient temperature	64.29 ± 2.41
3 days	Ambient temperature	74.65 ± 2.62
8 hours	50 °C	96.37 ± 4.50
10 hours	50 °C	95.65 ± 1.84

[a] Solution extract, solvent was removed until the volume of the extract reached 20% of starting volume. Values are express as mean ± (n =3).

4.2.2 Effect of solvent system

Ethanol and propylene glycol were selected as solvent in extraction because they are normally used as ingredient in cosmetic formulation. The solvent systems were ethanol, propylene glycol and mixture of ethanol/propylene glycol at 4:1 and 1:1 volume ratio. The DPPH radical-scavenging activities of leaf extracted at 50 °C for 4 hours (performed twice, 4 hours for each extraction) in different solvent systems are shown in Table 7. It was found that activity was highest in propylene glycol solvent followed by ethanol/propylene glycol at 1: 1 volume ratio, 4:1 ratio and ethanol.

Table 7 DPPH free radical–scavenging activity of dried *P. amaryllifolius* leaf extracted at 50 °C, 4 hours under sonication assisted condition.

Solvent system	% Activity	
	1st extraction	2nd extraction
Ethanol	86.36	69.61
Ethanol/Propylene glycol (4:1)	93.06	79.41
Ethanol/Propylene glycol (1:1)	93.32	90.37
Propylene glycol	94.56	92.06

Additionally, DPPH radical-scavenging activities of both pandan leaf and root extracts were determined in order to select for the most suitable part for cosmetic application. Propylene glycol was used as solvent and the resulted showed that the activity of dried leaf (93.56%) is higher than dried root (29.55%).

4.2.3 Determination of IC_{50} value

IC_{50} value is used to determine effectiveness of antioxidant activity. Vitamin C and butylated hydroxytoluene (BHT) were used as standards. It was found that the antioxidant activity of *P. amaryllifolius* crude extracts were much lower than activity of vitamin C and BHT. The IC_{50} values of vitamin C, BHT, dried leaf and dried root crude extract were 0.012 mg/ml, 0.290 mg/ml, 0.810 mg/ml and 2.340 mg/ml, respectively.

4.2.4 Effect of sonication

Sonication is generally used for mixing, solubilizing and driving chemical reaction where water can transfer the sonic energy from transducer to the sample. Sonication was indirectly applied to the extraction by placing the flask containing the plant-solvent mixture into a sonication cleaning bath (Crest Ultrasonics). DPPH assay showed that sonication-assisted condition helps increase antioxidant activity of pandan extracts. As can be seen from Figure 7, there is a significant increase in activity of ethanolic leaf extract about 2 times (20% concentration). The results showed that sonic energy provided is of great help in the extraction.

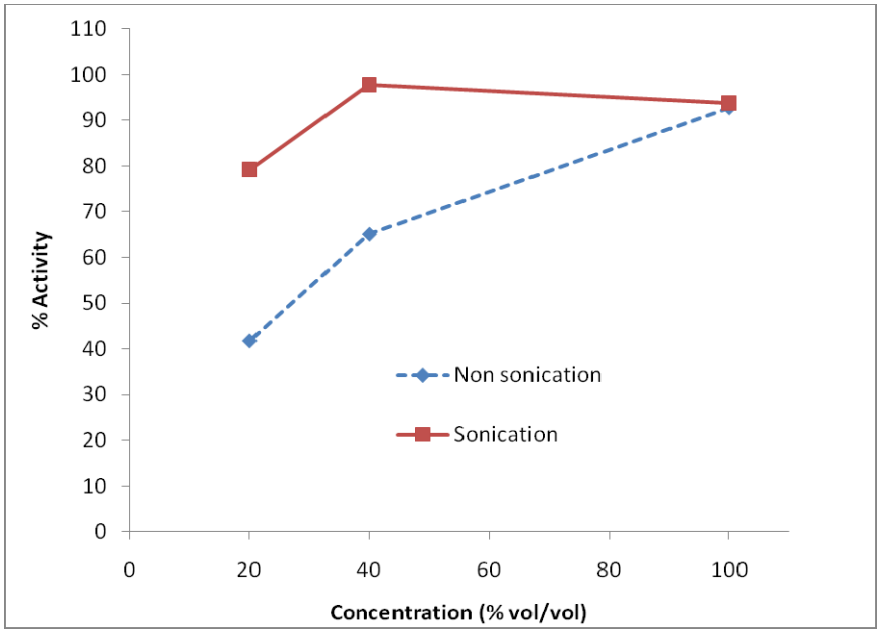


Figure 7 DPPH radical-scavenging activity of concentrated ethanolic *P. amaryllifolius* leaf extract (50°C for 8 hours); concentrated extract is prepared by evaporation of ethanol solvent until the volume reached 20% of starting volume. Absorbance of the reaction was measured at 517 nm.

4.3 Determination of total phenolic content

The Folin-Ciocalteu method has normally been used to estimate phenolic contents of biological materials. The method is based on the fact that phenols ionize completely under alkaline conditions, and can be readily oxidized by the Folin-Ciocalteu reagent. The oxidation causes a color change from yellow to blue. Total phenolic content of ethanolic extracts was determined and it was found that the dried leaf extract showed higher total phenolic content value (319.17±15.91 mg GAE/100g plant) than dried root (28.75±1.09 mg GAE/100g plant). Furthermore, extraction under sonicated-condition resulted in about 2 to 4 times higher phelolic content in ethanolic extracts, Figure 8. Additionally, total phenolic content of pandan leaf extracted in ethanol/propylene glycol (1:1) were higher than those of ethanolic extracts. Sonication increased the phenolic content of dried leaf extracted in ethanol/propylene glycol mixture from 360.83 ± 10.78 to 815.83 ± 28.76 mg GAE. It can be seen that the extract in the solvent system containing propylene glycol possessed higher phenolic content which related to a higher in DPPH radical scavenging activity.

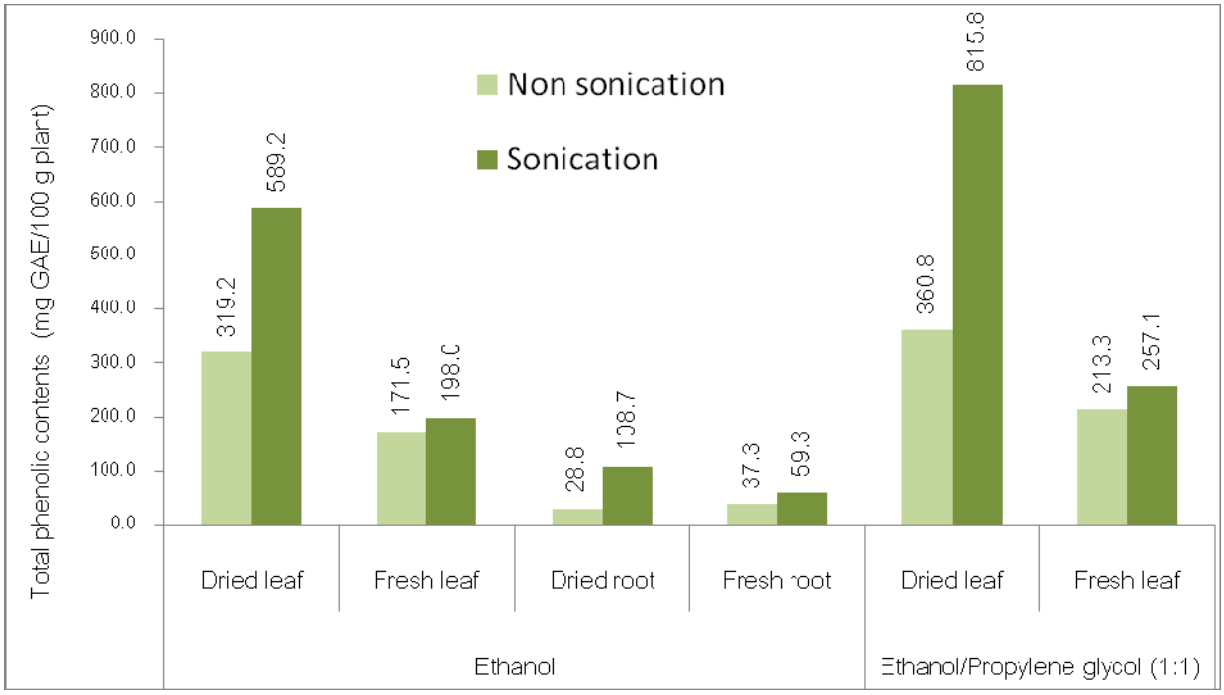


Figure 8 Total phenolic content of the *P. amaryllifolius* solution extracts. Absorbance of the reaction was measured at 765 nm.

4.4 Linoleic acid emulsion system–thiocyanate method

The ferric thiocyanate method evaluated the antioxidant activity in heterogenous system. In this method, hydroperoxide was produced by linoleic acid emulsion system. Ferrous chloride was added into the reaction mixture which will be oxidized by the peroxide and resulted in ferric (Fe^{3+}) in which will further reacts with thiocyanate to produce ferric thiocyanate (red color). Vitamin C and BHT were used as standard references. The inhibitions of the crude extracts at different concentrations are shown in Figure 9. Increase in absorbance indicates less inhibition in lipid peroxidation. As can be seen from the figure, absorbance of vitamin C, BHT and the extracts is lower than that of control signify the ability to slow down the peroxidation of linoleic acid. The inhibition percentages of lipid peroxidation of the crude extracts are as follows: dried leaf 49.3%; dried root 45.2%, which were lower than that of vitamin C 57.1% and BHT 71.1% at the same concentration (200 ppm, calculated at 72 hours). When doubled the extract's concentration from 200 to 400 ppm the lipid peroxidation inhibition increased about 10%.

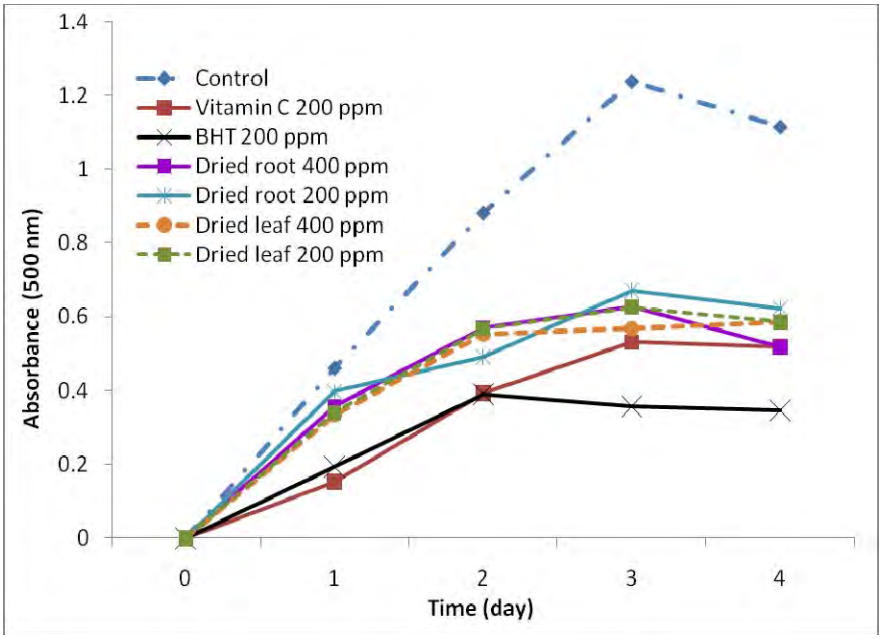


Figure 9 The determination of antioxidant activity by the ferric thiocyanate method of vitamin C and BHT (200 ppm), *P. amaryllifolius* root and *P. amaryllifolius* leaf (200, 400 ppm).

The peroxidation inhibition of solution extracts was also investigated and the results are shown in Figure 10. The extract used in the test is a neat concentrated solution of the extract potentially prepared for topical emulsion formulation. The inhibition percentages of *P. amaryllifolius* extracted in different solvents are about

90.1 ± 1.2%. The solution extracts showed higher activity than positive control (200 ppm, vitamin C 57.1% and BHT 71.1%). Consequently, these results clearly indicate that the pandan solution extract has an effective antioxidant activity by ferric thiocyanate method. Topical emulsion systems generally consist of multiple phases in which lipid and water coexists with some emulsifiers. Thus an antioxidant assay, using a linoleic acid emulsion can be simulated with heterogeneous cosmetic emulsion or with the biological lipid system. However, it must be noted that the concentration of the solution extract in the linoleic acid emulsion system is quite high (about 9%), accordingly further investigation in bulk oil-in-water emulsion system containing various percentages is recommended.

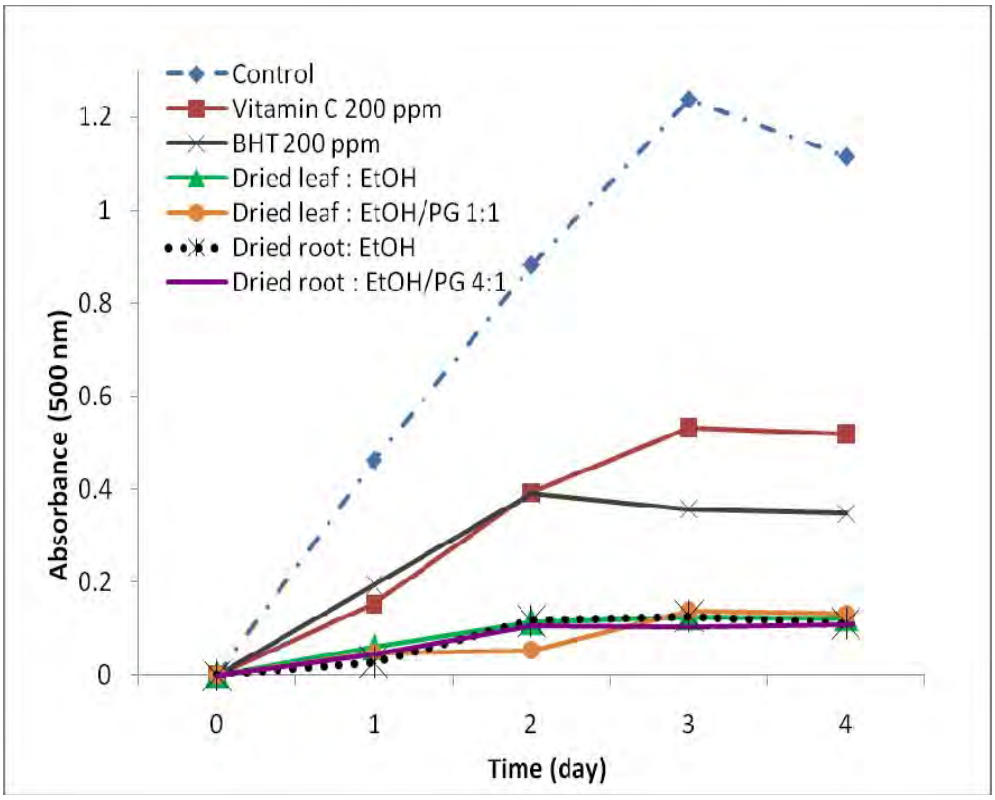


Figure 10 The determination of antioxidant activity by the ferric thiocyanate method of positive control (Vitamin C and BHT, 200 ppm) and neat solution extracts of *P. amaryllifolius* leaf and root (solvent of solution extract was removed until reached 20% of starting volume in ethanol and ethanol/propylene glycol 4:1 mixture, and 50% of starting volume in ethanol/propylene glycol 1:1 mixture).

4.5 Preparation of the standard extracts of P. amaryllifolius

According to the antioxidant activity studies, it is sensible to propose the conditions for preparation of the standard extracts as shown follows:

Conditions	**Select suitable condition**
Plant part	Dry leaves (10 g plant sample: 250 ml solvent)
Temperature	50 °C
Sonication	Sonication-assisted condition
Extraction time	4 hours x 4
Solvent systems	Ethanol/Propylene glycol (1:1)

After extraction, the extract was collect and ethanol was removed by evaporator. The physio-chemical properties were measured as the following:

Item	Properties
Appearance	Dark green viscous liquid
pH	6.52 ±0.50
Solubility	Soluble in ethanol, propylene glycol, water

4.6 Heat stability of the solution extract

P. amaryllifolius leaf solution extract (EtOH/PG 1:1) was subjected to 70 °C heating and the DPPH assay was collected at 1, 3 and 5 hours heating time. The stability was also tested at ambient temperature, 4 °C and 45 $^{\circ}$C for 4 weeks in order to check the stability at different storage conditions. The DPPH assay was evaluated each week for 4 weeks. The heat stability of the extract is graphically shown in Figure 11. It can be seen that the DPPH activity of the extract stored at ambient temperature and 4 °C were comparatively constant but decreased after subjected to high temperatures.

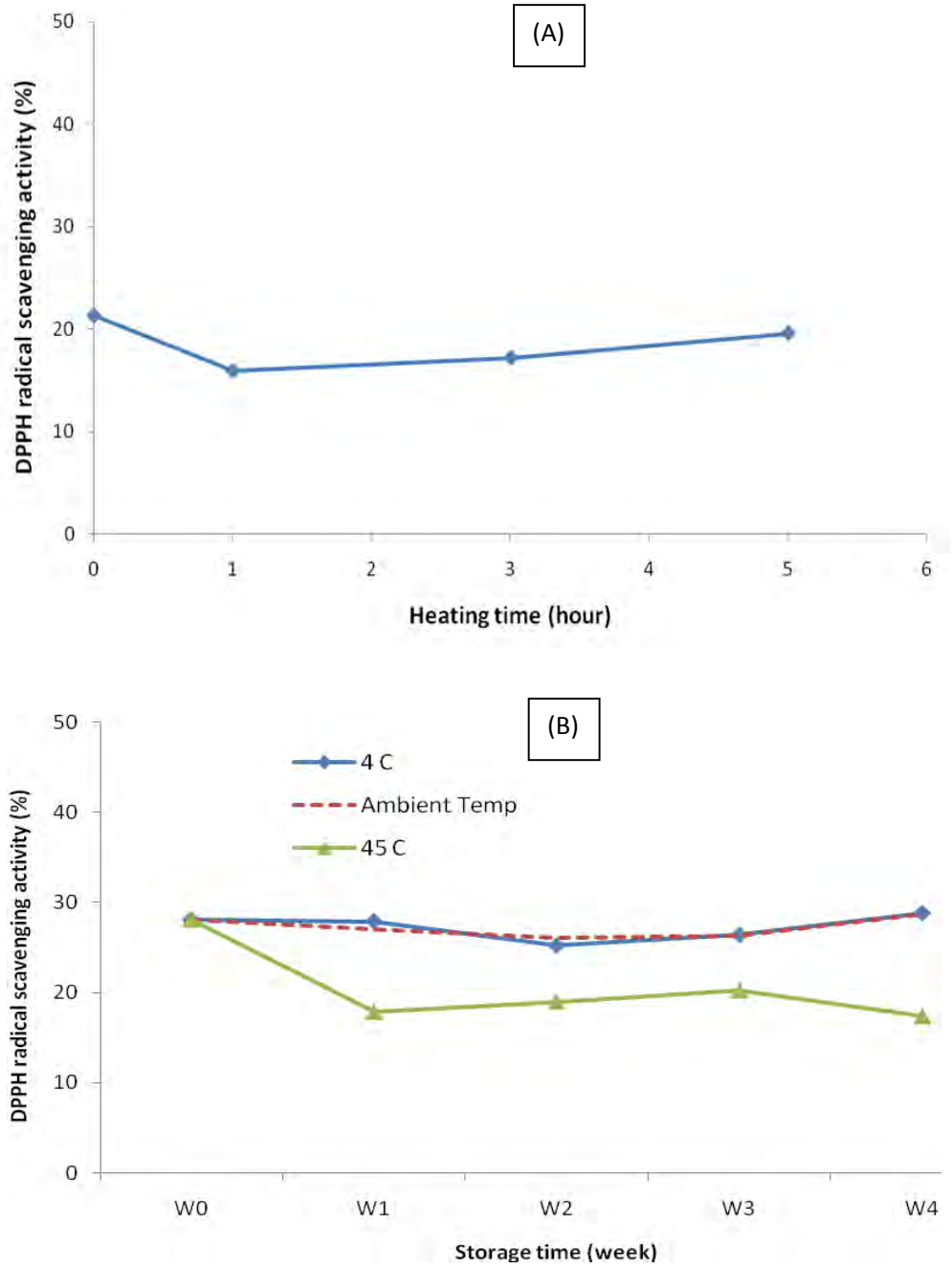


Figure 11 DPPH free radical–scavenging activity of *P. amaryllifolius* extracted in EtOH: PG (1:1 and absorbance of 10% extract was measured at 517 nm, (A) the solution extract was subjected to 70 °C and (B) the solution extract was stored at ambient temperature, 4 °C and 45 °C.

4.7 Antioxidant activity of emulsion containing the extract

Antioxidant activity of emulsion containing the extract was investigated by modified lipid hydroperoxide method. A series of emulsions was prepared as shown in Table 8. The products were stored at 70 °C for 3 days and antioxidant activity was determined every day interval and the results are shown in Figure 12.

Table 8 Emulsion composition for antioxidant activity measurement

Ingredient	Control	BHT	Vit E acetate	Toey extract	Extract+ Vit E acetate
Linoleic acid	10.0	10.0	10.0	10.0	10.0
Propylparaben	0.2	0.2	0.2	0.2	0.2
Water	86.6	86.3	86.3	81.6	81.3
Methylparaben	0.2	0.2	0.2	0.2	0.2
Extract	0	0	0	5.0	5.0
Vit E Acetate	0	0	0.3	0	0.3
BHT	0	0.3	0	0	0
Sepigel 305	3.0	3.0	3.0	3.0	3.0
Total	100.0	100.0	100.0	100.0	100.0

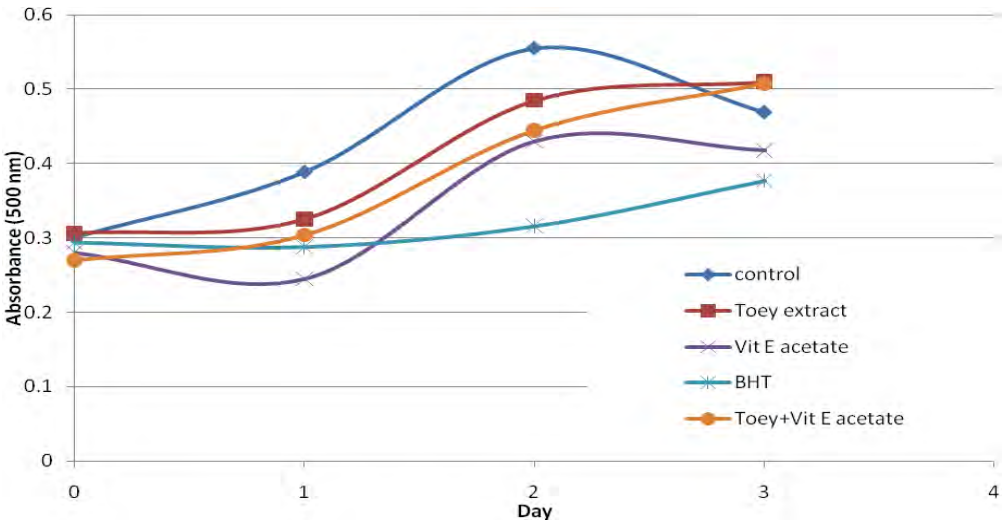


Figure 12 Antioxidant activity of emulsion by lipid hydroperoxide method at 70 °C

As it can be seen in Figure 12, an emulsion containing only the extract (5%) showed lower absorbance than that of control (at 2 days) and this pointed out that the extract added in to the emulsion can help slow down the oxidation of the lipid or polyunsaturated oils in the system at high temperature. It was also found that in an emulsion that contains the extract together with the commercial vitamin E acetate has higher efficacy in slowing down the oxidation of the system.

4.8 Development of topical emulsion containing the extract

1. Selection and preparation of the extract

According to the activity study, it is sensible to select dried pandan leaf extracted in solvent system containing propylene glycol as antioxidant ingredient in cosmetic application due to its highest values in DPPH assay and total phenolic content. However, the neat propylene glycol system might not be the most suitable choice for cosmetic application due to various issues such as high percentage needed in formula and sensory feeling. Therefore, owing to the possibility of concentrated extract can be arranged which is more suitable for cosmetic, trials on the use of ethanol/propylene glycol 1:1 mixture and neat ethanol have been performed. The ethanol solvent of the ethanol/propylene glycol solution extract was removed by evaporation until the solution volume reduced to 50% volume (labeled as EP50). The ethanolic extract was prepared by evaporation of the ethanol solvent until the solution volume reached 20% volume (labeled as ET20).

2. Preparation of emulsion product

The oil in water (O/W) emulsion cream was developed as general moisturizing cream containing antioxidant. The oil phase consisted of cyclopentasiloxane (4%), jojoba oil (5%), dimethicone (2%), shea butter (2.5%), jojoba ester (3.5%), Span-80 (1.7%), Tween-80 (1.3%), Sepigel-305 (2.5%) and propylparaben (0.15%) while the aqueous phase was comprised of propylene glycol (3%), methylparaben (0.15%) and deionized water. *P. amaryllifolius* extract was added at 40-45 $^{\circ}$C after an emulsion is formed. The concentration of the extract in the preparation was varied as 1, 3 and 5%. It was found that the products containing up to 3% extract had superior texture and no phase separation observed under centrifugation at 6000 rpm, 30 minutes. The developed product is emulsion cream with light pandan odor. The product containing ET20 was greener in color and slightly less viscous than that containing EP50 as shown in Figure 13, and other physio-chemical properties are tabulated in Table 9.

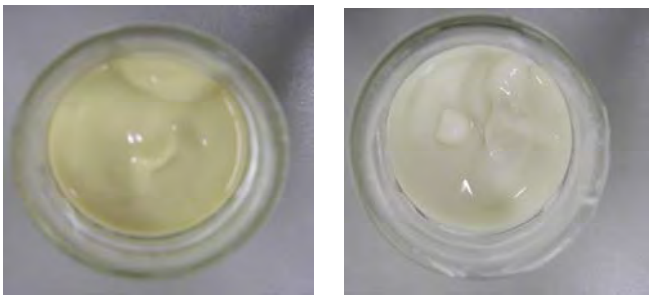


Figure 13 Topical emulsion contaioning *P. amaryllifolius* extract, (A) Emulsion cream containing ethanolic extract (ET20) and (B) Emulsion cream containing ethanol/propylene 1:1 extract (EP50)

Table 9 Properties of emulsion cream containing 3% *P. amaryllifolius* extract.

Properties	Emulsion cream containing ethanolic extract (ET20)	Emulsion cream containing ethanol/propylene 1:1 extract (EP50)
Appearance	Emulsion cream	Emulsion cream
Color	Green color L* = 86.67 ± 0.30 a* = -7.09 ± 0.07 b* = 20.78 ± 0.08	White color with very light green tint L* = 92.88 ± 0.20 a* = -3.05 ± 0.03 b* = 6.12 ± 0.19
Odor	Light pandan odor	Light pandan odor
pH	6.11	6.26
Viscosity	35000 cP (71% , RV#04, 4 rpm)	36600 cP (73% , RV#04, 4 rpm)

3. Microbial contamination test

Mikrocount® combi test kits were used to determine the microbial contamination of the products. The results showed that all the products had no colonies on both bacteria and yeast sides.

4. Stability of the developed cream containing 3% extract

Stability test was performed to ensure that the products meet the intended physical, chemical and performance characteristics when they stored under various conditions. The developed creams containing 3% *P. amaryllifolius* extract were subjected to preliminary stability test by centrifugation at 6000 rpm for 30 minutes and there was no sign of phase separation observed. Therefore, the product was further studied for accelerated stability test at different storage conditions. The product was divided into 4 samples. They were kept in different conditions for 1 month, i.e. at 4°C in refrigerator, ambient temperature, 45°C in hot air oven

and heating-cooling cycle (4°C, 24 hours; 45°C, 24 hours). These samples were monitored every week with respect to changes in appearance, color, odor, pH and viscosity.

i) Physical properties

Appearance, texture, odor and color of the developed products were visually observed. At initial, the product was creamy texture, medium viscosity with pandan odor. After subjected to stability test under various conditions, it was found that the viscosity of the products in hot air oven and heating-cooling cycle conditions were decreased over time, but there is no phase separation observed in all conditions. The color of the product changed to slightly lighter-green compared with the initial. The odor of the product was somewhat oily and the pandan odor was less significant. Additionally, pH was practically unchanged except for at 45 °C where it decreased from 6.11 to 5.70 in ET20 emulsion and from 6.26 to 5.90 in EP50 emulsion.

ii) Viscosity

The viscosity of the developed products was measured by viscometer. The viscosity of both products changed over time, as can be seen from Figure 14, there was not much different in viscosity at ambient temperature but more noticeable under accelerated 4°C and 45°C condition (20- 30 % changed). Moreover, the change was much more significant when the product were kept under heat-cooling condition (up to 50% changed). The change of the product texture was be able to notice by eyes when the viscosity value changed about 30% or more However, there was no phase separation observed at all conditions. It has been noted that the tolerance of stability after 1-2 heating/cooling cycles is considered as: stable if the change in viscosity is less than 10%; acceptable if the viscosity is higher than 10% but not more than 20%; unstable if higher than 20% (Cheng et al. 2009). The viscosity values obtained show that the developed products were fairly stable. Moreover, the results are very useful in selecting optimal storage condition for the product over time.

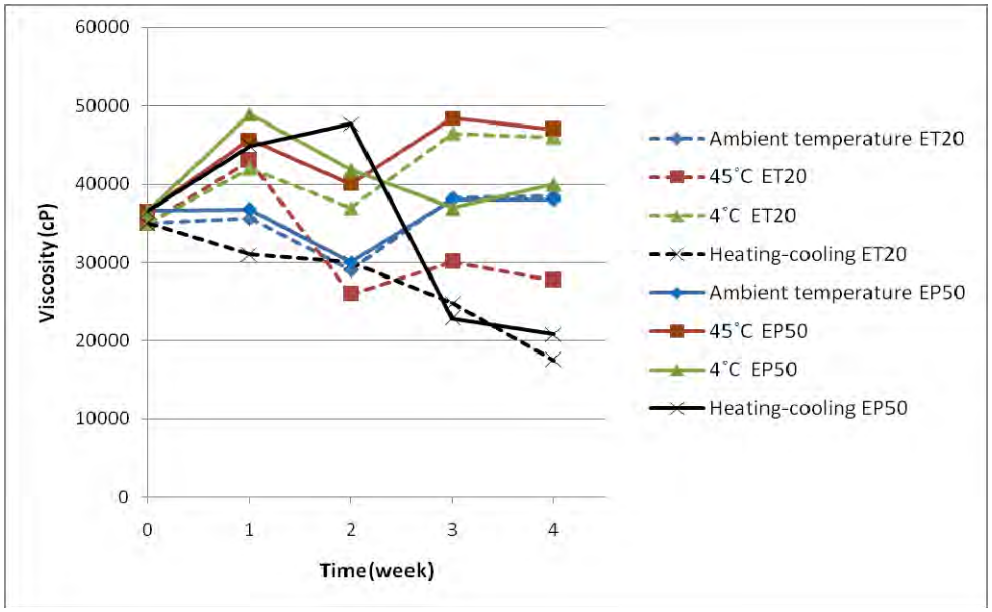


Figure 14. The viscosity of the developed cream containing 3% leaf extract (spindle RV#04, 4 rpm; 29.0 ± 1.0 °C) at various temperatures for 1 month.

iii) Color

The color of the product was measured by colorimeter. The results were recorded in CIELAB or L* a* b* values (CIE, 1986). The color difference of L* a* b* values was calculated and shown in Table 10. The color difference in L* values (ΔL*) showed a decline tendency for all conditions. The greatest value (7.44) is of the product containing ET20 under heating-cooling cycle (from 86.61 to 79.17). The decrease in value meaning that the lightness is diminished, however the change is not significant by visual observation. The color difference in a* values (Δa*) increased over time which means the green hue is minimized. The greatest Δa* raise was 1.8 and exhibited in the product containing ET20 stored at ambient temperature and 45 °C conditions. The change was not being able to differentiate by visual observation. The color difference in b* values (Δb*) decreased over time. It is interesting to see that the greatest change is found in the product containing ET20 at ambient temperature. The decrease in b* meaning the blue hue is dominated and the yellow hue is minimized, however there was not much difference in color when observed by eyes.

The total color differences (ΔE*) between initial (W0) and each week (W1, W2, W3 and W4) were calculated using the following equation: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (Ayala et al., 1997: Gonnet, 2001). The results are graphically shown in Figure 15. It can be seen that the total color difference is highest when the product was kept under heating-cooling condition followed by elevated 45 °C, ambient temperature and 4

°C. The product containing ET20 showed a greater ΔE* value. The greatest total color difference value is 7.6 which is of heating-cooling condition after 1 month.

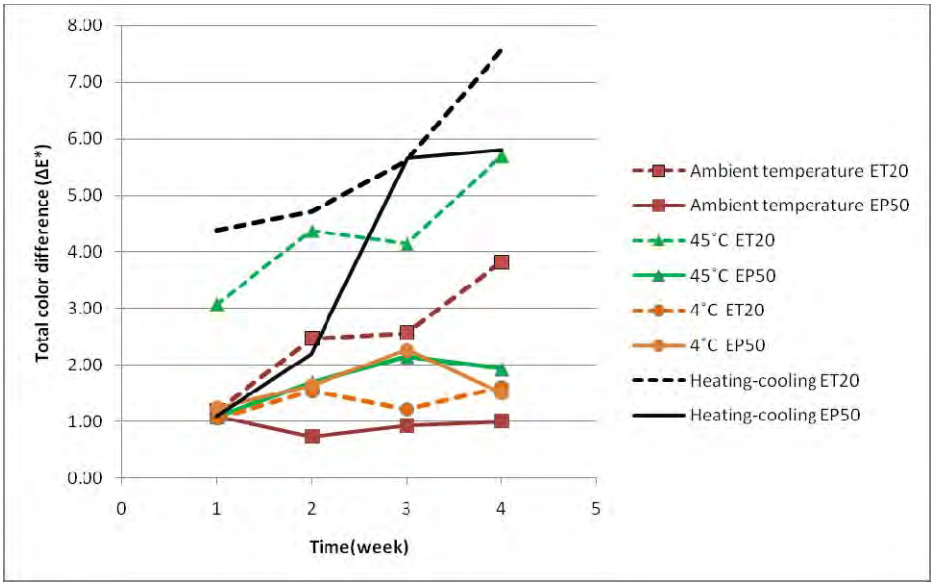


Figure 15 The total color difference ((ΔE*) of developed cream containing 3% extract at various temperature

Table 10 Color variation of emulsion products under various storage conditions.

Colour Difference	Emulsion containing ET20											
	ΔL*				Δa*				Δb*			
	AT[a]	45°C	4°C	HC[b]	AT[a]	45°C	4°C	HC[b]	AT[a]	45°C	4°C	HC[b]
W0/W1	0.38	-2.49	-0.98	-4.08	0.73	0.77	0.09	0.48	-0.86	1.62	0.38	1.55
W0/W2	0.66	-4.08	-1.40	-4.51	1.30	1.17	0.28	0.90	-2.00	1.02	0.59	1.03
W0/W3	0.34	-3.80	-1.00	-5.42	1.51	1.64	0.52	1.37	-2.05	-0.15	0.44	0.45
W0/W4	0.94	-5.28	-1.41	-7.44	1.84	1.82	0.75	1.26	-3.21	-1.14	-0.23	0.48
Colour Difference	Emulsion containing EP50											
	ΔL*				Δa*				Δb*			
	AT[a]	45°C	4°C	HC[b]	AT[a]	45°C	4°C	HC[b]	AT[a].	45°C	4°C	HC[b]
W0/W1	1.00	-1.05	-1.21	-1.07	0.17	0.24	-0.01	0.18	-0.42	0.10	-0.28	0.09
W0/W2	0.61	-1.62	-1.61	-2.18	0.40	0.37	0.16	0.28	-0.11	0.33	0.25	-0.01
W0/W3	0.31	-2.03	-2.23	-5.64	0.79	0.70	0.35	0.39	-0.37	0.07	-0.19	-0.15
W0/W4	0.68	-1.88	-1.50	-5.77	0.61	0.42	0.06	0.18	-0.44	-0.09	-0.09	-0.64

[a] AT = ambient temperature (28-32 °C) , [b] HC= heating-cooling cycle

ΔL* is the color difference between initial (W0) and subsequent week (Wt), (L^*_{Wt} - L^*_{W0})

Δa* is the color difference between initial (W0) and subsequent week (Wt), (a^*_{Wt} - a^*_{W0})

Δb* is the color difference between initial (W0) and subsequent week (Wt), (b^*_{Wt} - b^*_{W0})

5. Preliminary sensory evaluation

Thirties volunteers aged between 18-30 years old were selected for sensory evaluation. Volunteers were asked to use the product and fill out their opinion regarding the properties of the product in sensory evaluation form. Emulsion cream containing 3% extract was first prepared without addition of any fragrances. The sensory evaluations were preliminary surveyed by 10 volunteers and it was found that all volunteers had less preference in odor of the product. Therefore, the formula with 0.1% fragrance was prepared and surveyed in 30 volunteers. The results showed that volunteers had more preference in odor than those without fragrance. However, percentage of the volunteers who did not like the product's odor was still higher than other attributes, Figure 16. Moreover, it can be seen that preference on appearance and odor of moisturizing cream containing ET20 and moisturizing cream containing EP50 were pretty similar. However, moisturizing cream containing ET20 had higher preference in spreadability, penetration and lightness feeling after product application than moisturizing cream containing EP50. These might be an effect from ethanolic active ingredient which was less oily and therefore resulting in lighter feeling on the skin. Finally, it was found that most volunteers preferred the overall feature of moisturizing cream containing ET20 more than that containing EP50.

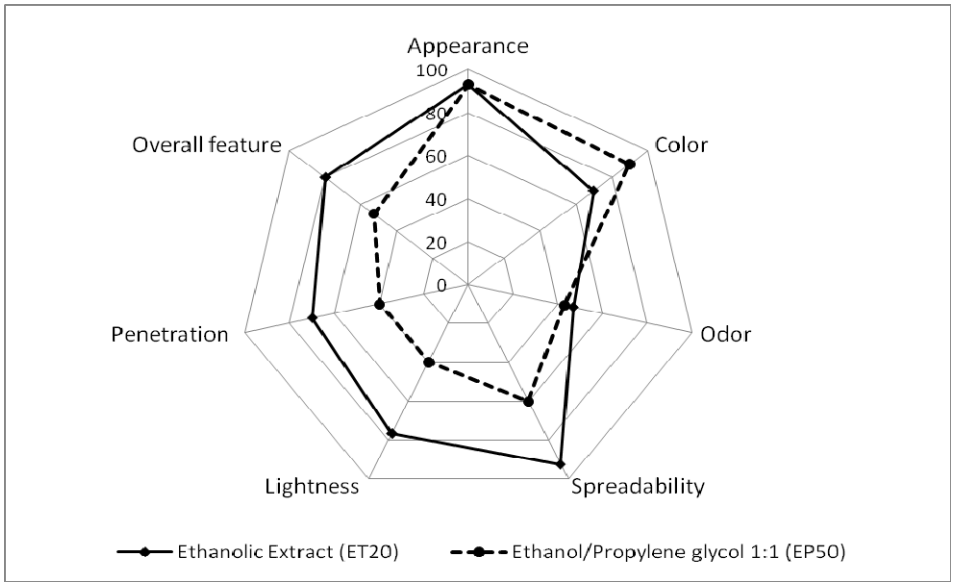


Figure 16 Percentages of the volunteers preferred on each attribute of the developed products.

6. Single patch test

Single patch test was performed on 23 volunteers using occlusive patch test to determine the potential irritation of the product. The acute irritation index or mean irritation index (M.I.I) was calculated according to an equation below and the results are shown in Table 11. The M.I.I. values of all volunteers is 0, 0, 0 and 0 after removed, at 24 h, 48 h, and 72 h., respectively. Therefore, the product may be classified as non-irritation on the skin.

$$M.I.I = \frac{\sum \text{of the grade (erythema + odema)}}{\text{Number of subjects}}$$

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

Table 11 The mean irritation index (M.I.I) of the product.

	Erythema								Odema							
	sample				control				sample				control			
Subject NO.	0	24	48	72	0	24	48	72	0	24	48	72	0	24	48	72
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11																
12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
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24	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

7. Skin surface evaluation

The product efficacy was measured every week using SELS program calculation from Visoscan® VC 98. Twenties volunteers aged between 20 and 50 years old were asked to use the product daily twice a day. The results are presented in Table 12.

Table 12 The effectiveness of emulsion product by SELS program calculation.

Subject No.	SEw (wrinkles)				SEsc (Scaliness)			
	W0	W1	W2	W4	W0	W1	W2	W4
1	41.96	41.96	37.97	41.47	0.69	0.54	0.61	0.37
2	39.22	38.62	40.29	41.86	0.40	0.59	0.43	0.23
3	51.83	39.41	44.04	37.09	0.70	0.54	0.37	0.48
4	37.94	37.94	34.4	37.94	0.83	0.61	0.49	0.66
5	40.60	42.55	36.59	39.93	1.75	0.66	0.56	0.91
6	40.54	34.74	34.40	36.33	0.69	0.50	0.36	0.62
7	38.62	37.50	38.62	40.52	0.42	0.49	0.38	0.53
8	35.46	39.56	36.33	33.97	0.55	0.81	0.74	0.43
9	40.00	40.00	40.00	36.59	0.52	0.48	0.48	0.37
10	60.00	45.38	45.80	46.00	0.76	0.33	0.36	0.36
11*	*	*	*	*	*	*	*	*
12	38.98	43.75	43.73	40.60	0.79	0.73	0.74	0.63
13	41.93	38.36	35.29	38.65	0.76	0.48	0.59	0.69
14	37.94	40.29	38.00	37.94	0.76	0.34	0.52	0.51
15**	**	38.65	37.94	40.29	**	0.39	0.42	0.65
16	48.63	41.47	37.37	37.90	0.24	0.44	0.45	0.62
17	45.00	40.91	39.08	40.75	0.54	0.57	0.46	0.43
18	40.60	37.94	40.52	33.44	0.92	0.64	0.59	0.56
19	48.63	41.54	40.29	42.35	1.31	0.65	0.45	0.51
20	49.09	34.35	36.40	38.14	0.25	0.43	0.37	0.33
21*	*	*	*	*	*	*	*	*
22	46.30	37.91	38.14	37.94	0.99	0.88	0.86	0.75
23	39.04	46.96	32.73	31.99	0.49	0.49	0.63	0.48

* leave the project, ** No week 0 data

The SEw refers to the wrinkles of the skin and the small value means fewer wrinkles on the skin.

The SEsc refers to the scaliness of the skin and the small value means less scaliness on the skin.

The results of the skin images were processed by SELS program calculation and the outputs are indicated in skin wrinkles or SEw and skin scaliness or SEsc values. The SEw refers to the wrinkles of the skin and the small value means less wrinkles on the skin. It was found that, after 1 month applied, 90% of the volunteers showed a decrease in SEw value (Table 12). The SEsc refers to the scaliness of the skin and the small value means less scaliness on the skin. The results after using product for 1 month indicated that 85% of the volunteers have a decrease SEsc value. However, about 10-15 % of the volunteers had higher SEw and SEsc values which were not as expected. These errors might be contributed from unclear images taken during the experiment. The macroimages of the volunteer's skin before and after applied the product are shown in Figure 17.

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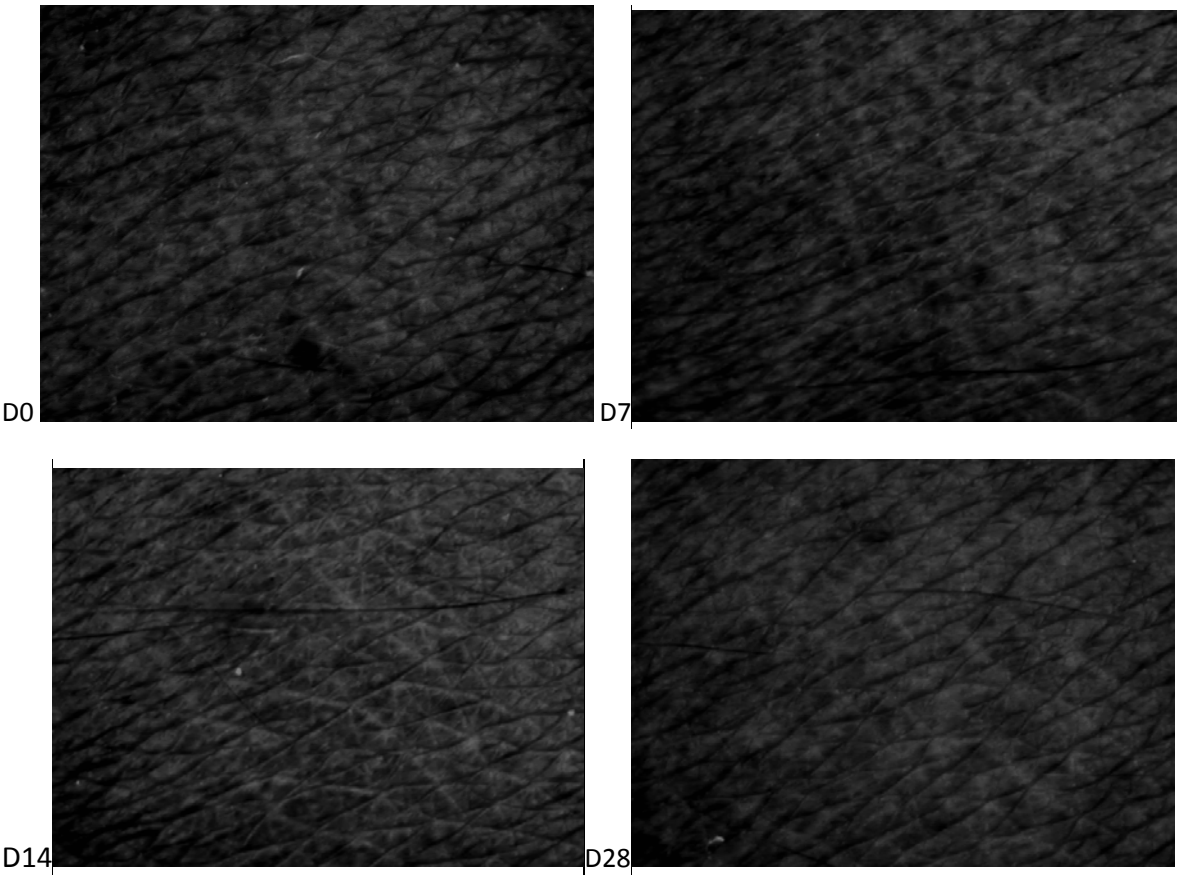

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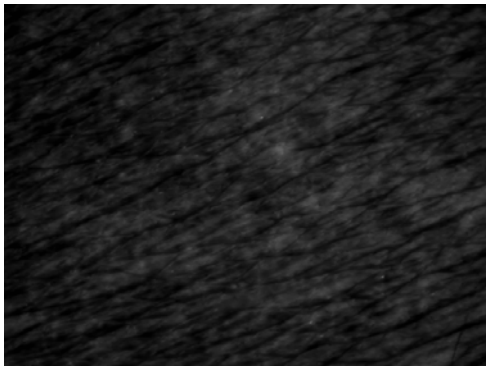
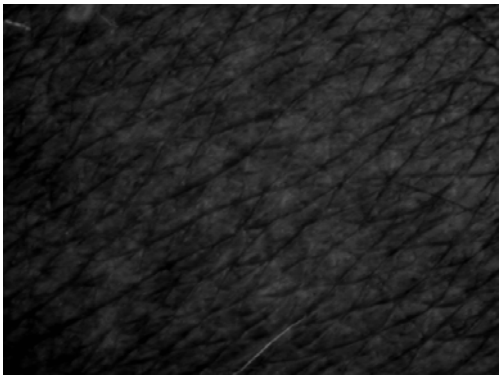
D0 

D7 

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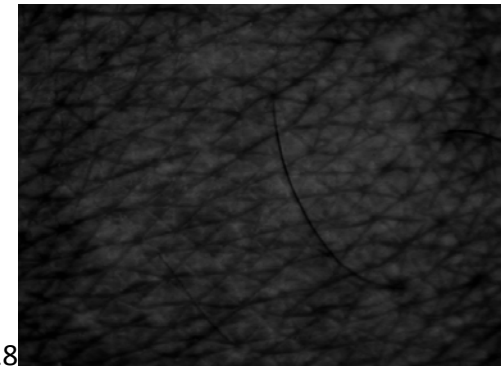
D28 

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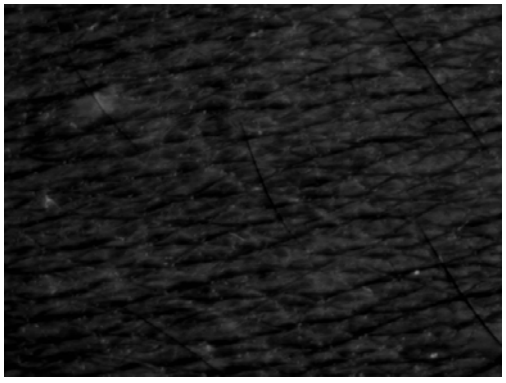
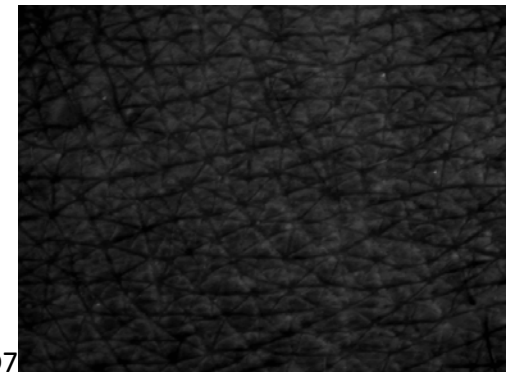
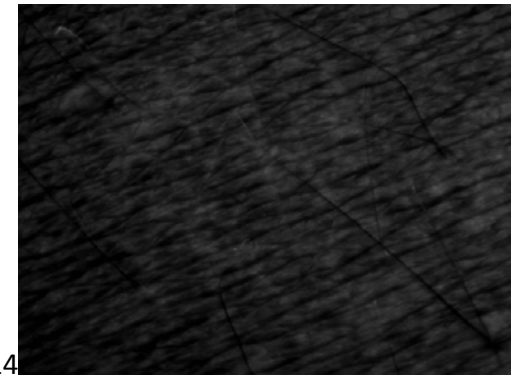
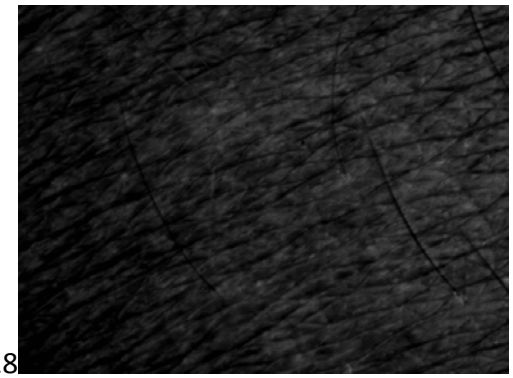
D0 

D7 

D14 

D28 

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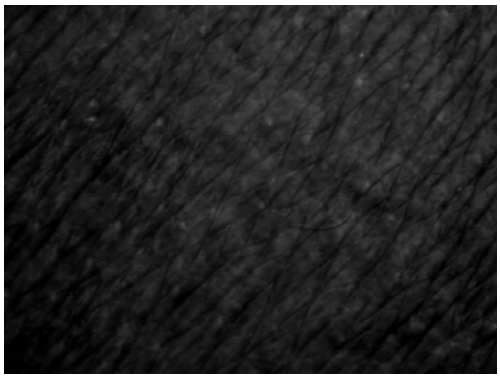
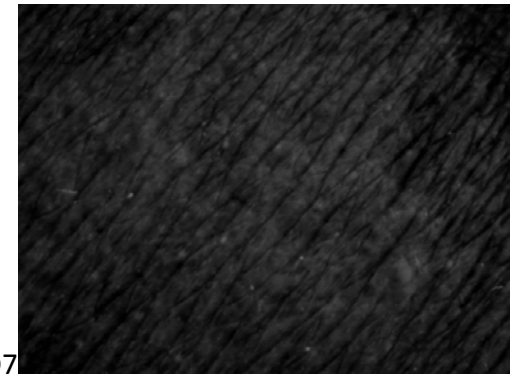
D0 

D7 

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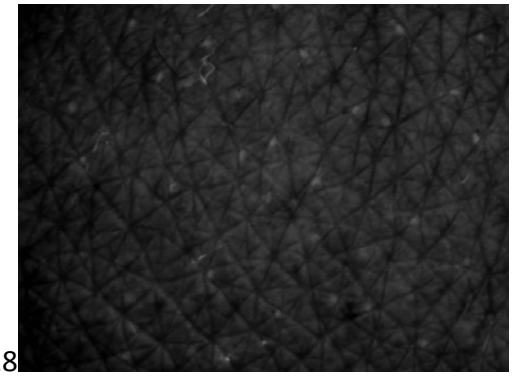
D28 

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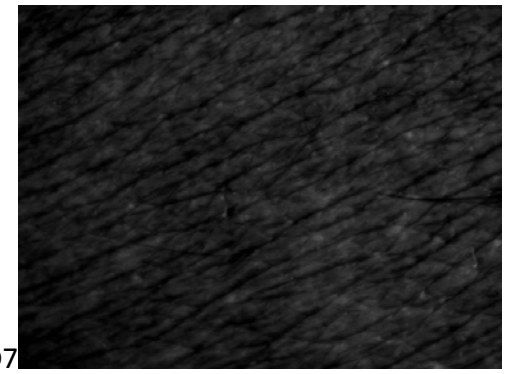
D7 

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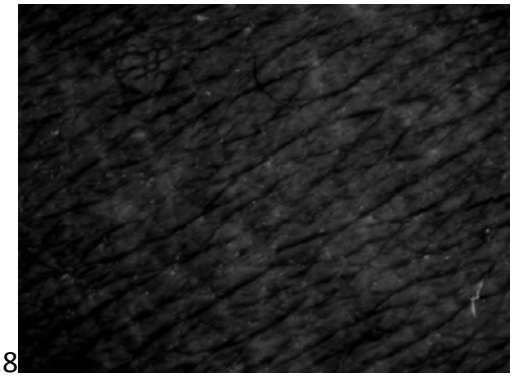
D28 

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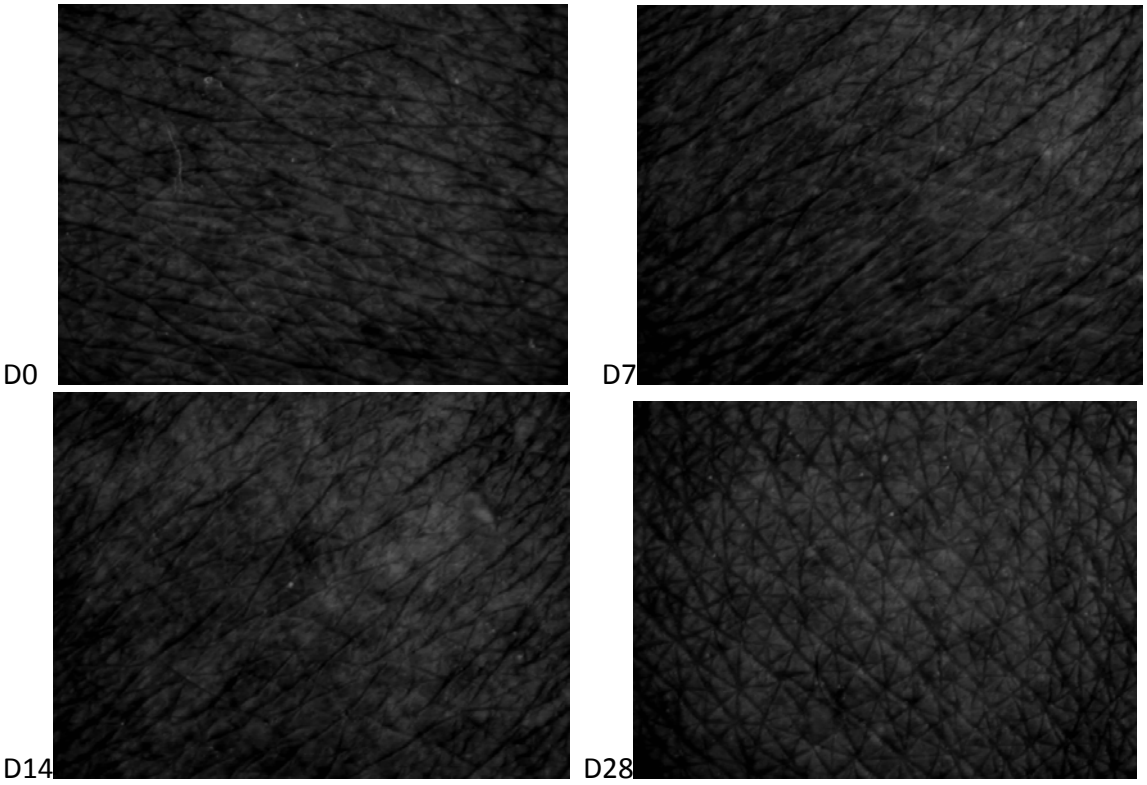

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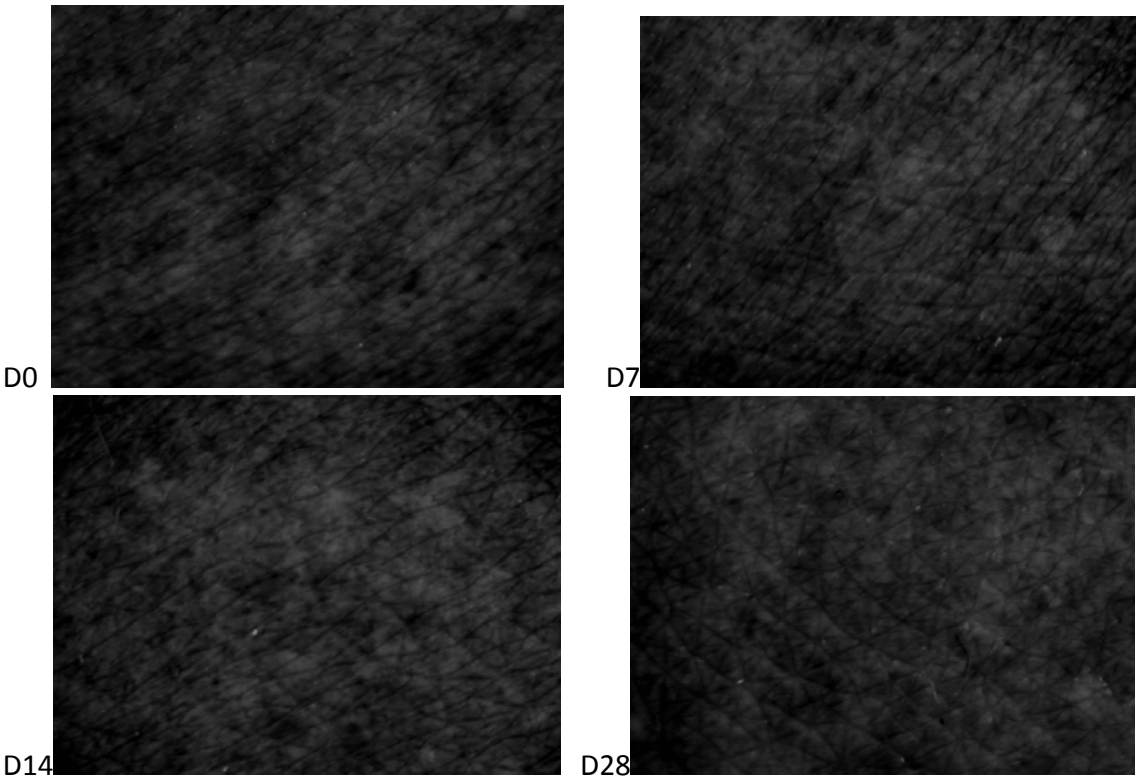

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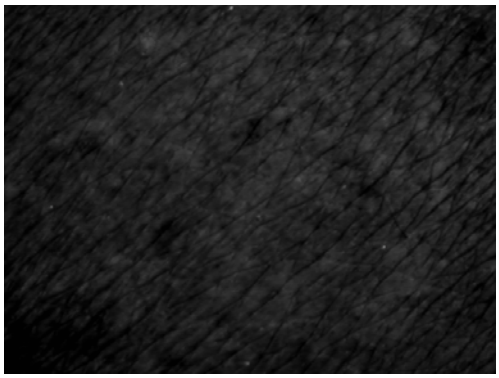
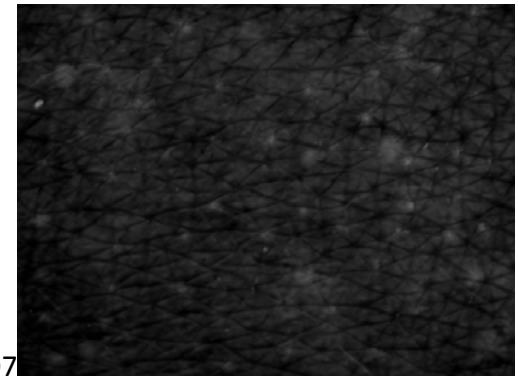
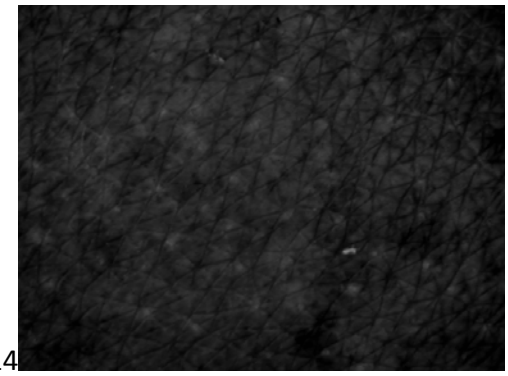
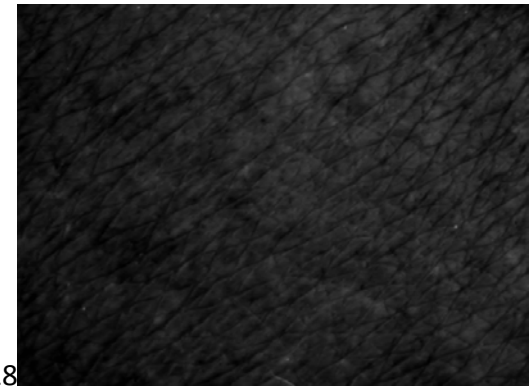
D0 

D7 

D14 

D28 

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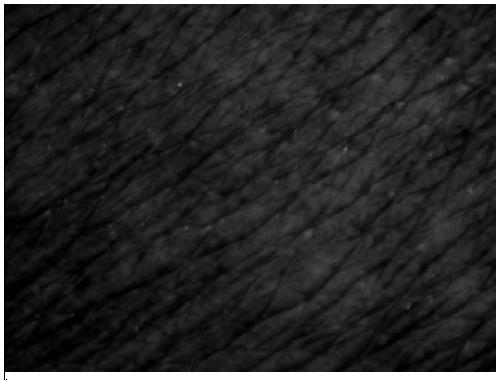
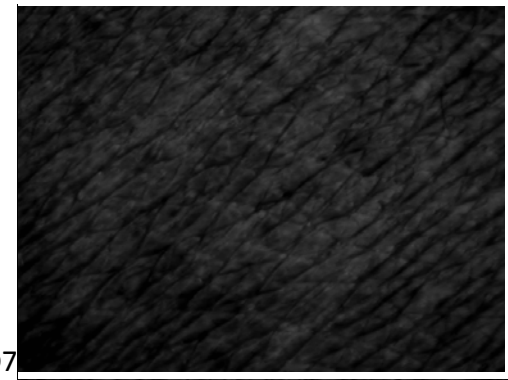
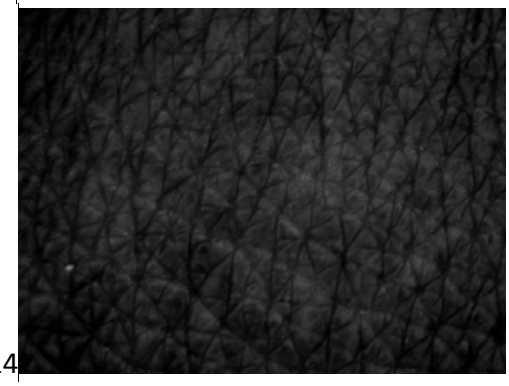
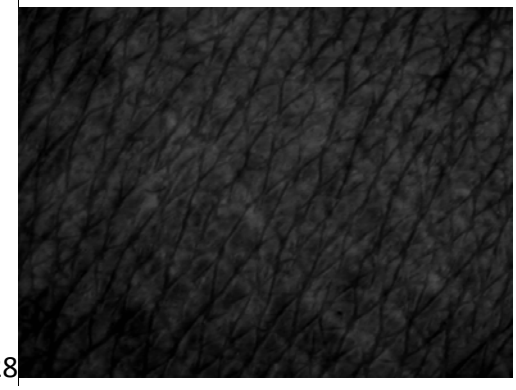
D0 

D7 

D14 

D28 

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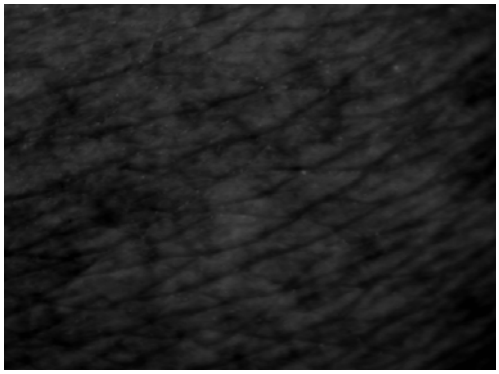
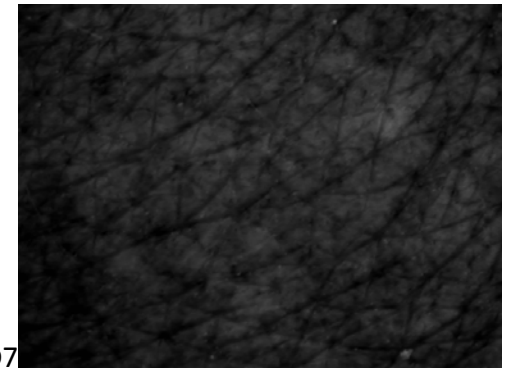
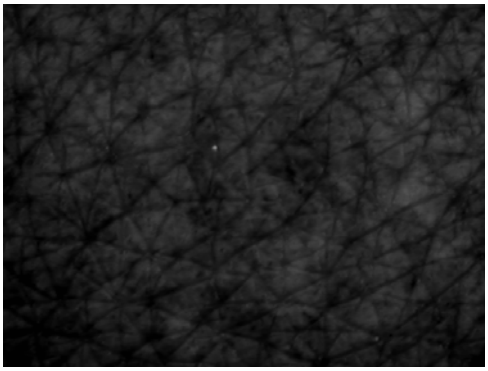
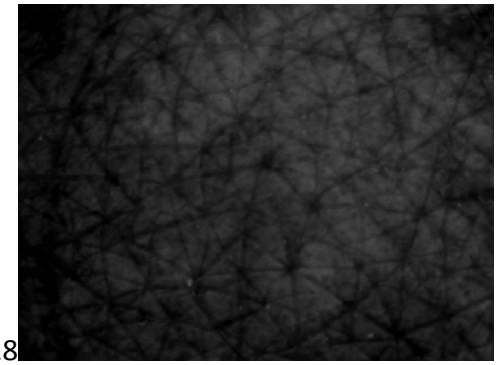
D0 

D7 

D14 

D28 

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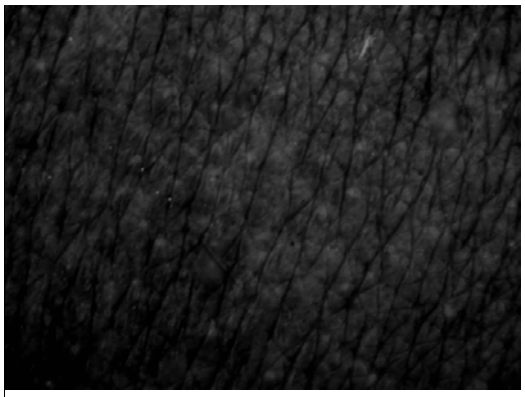
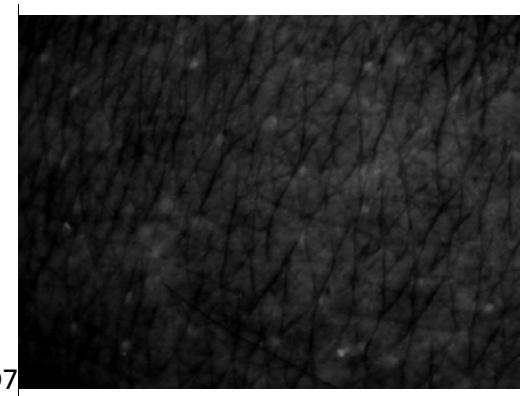
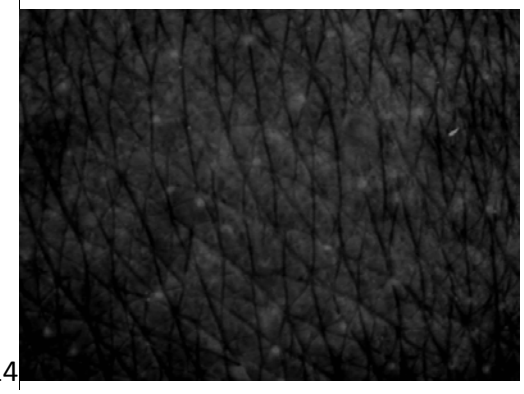
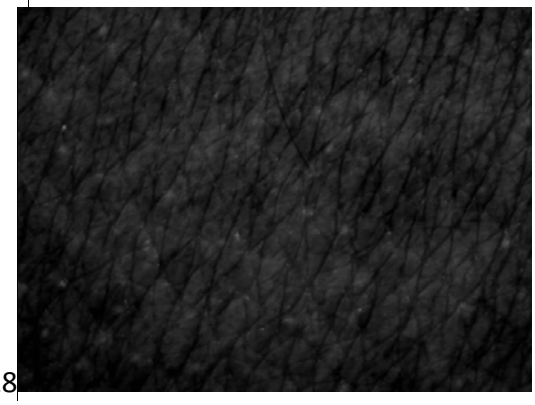
D0 

D7 

D14 

D28 

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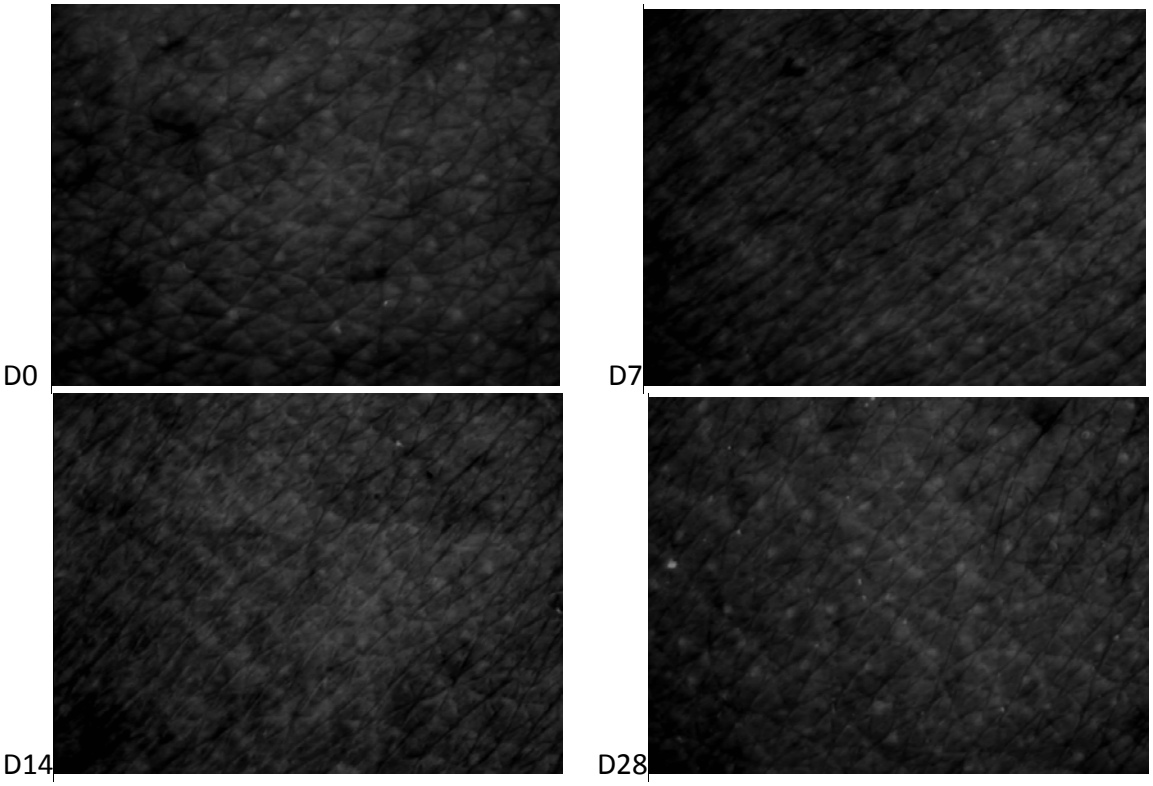

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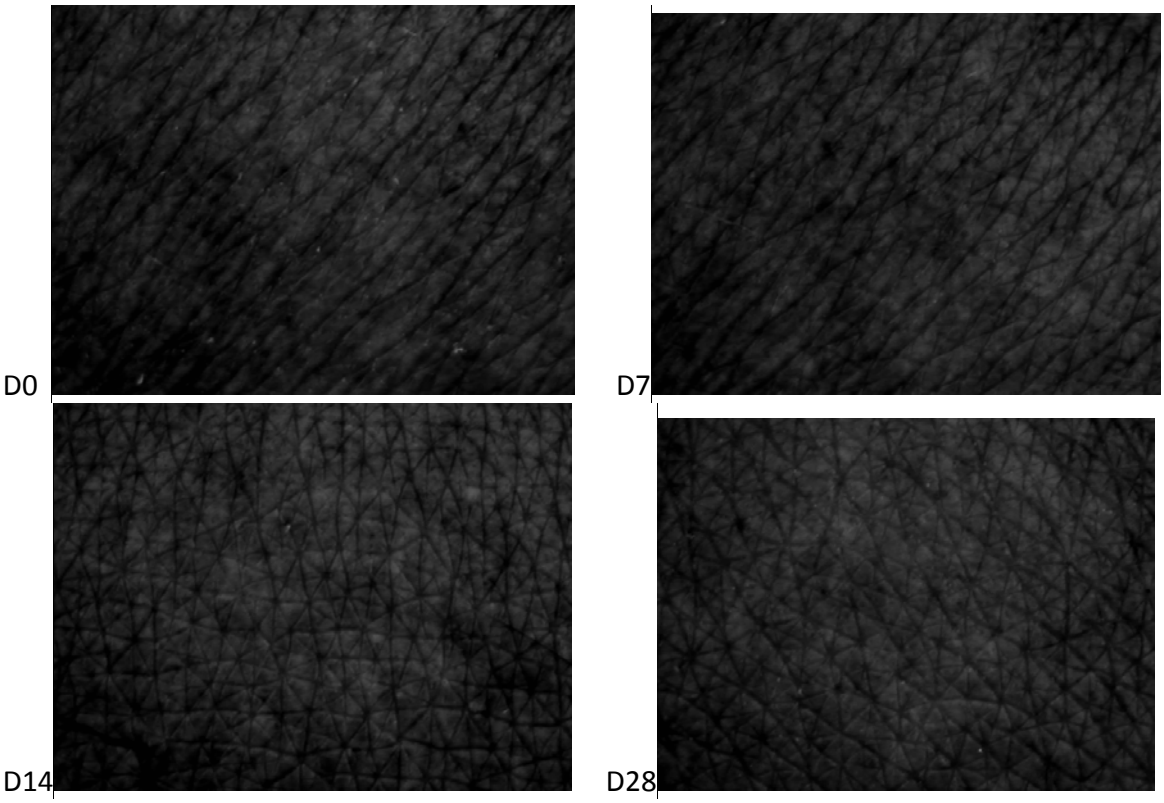

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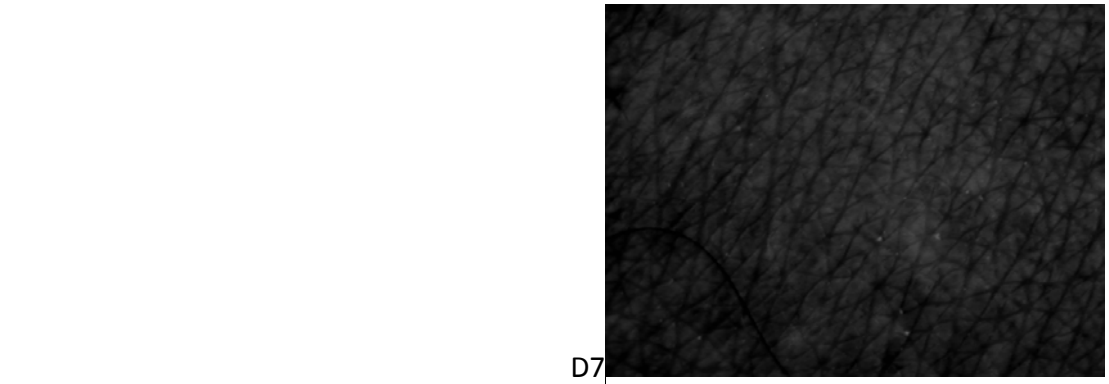

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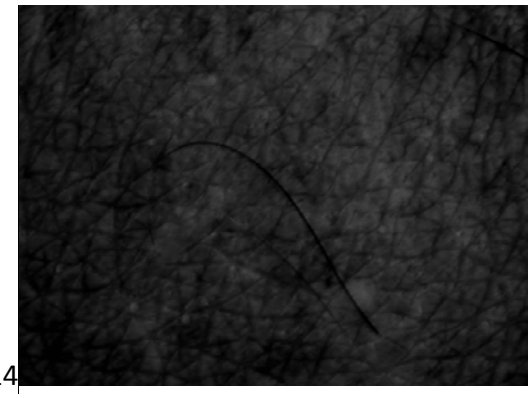

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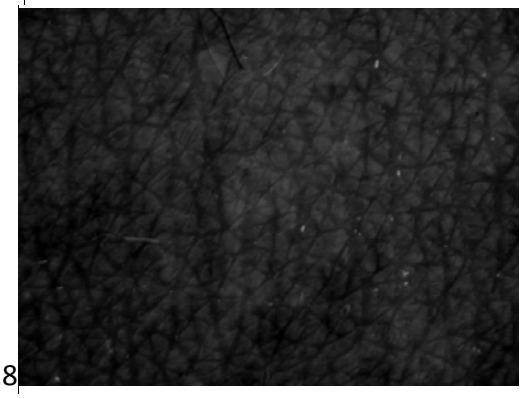

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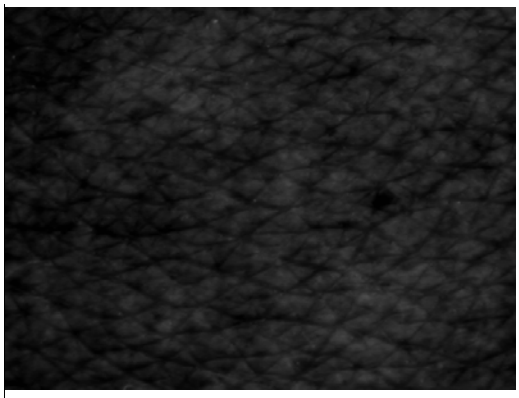

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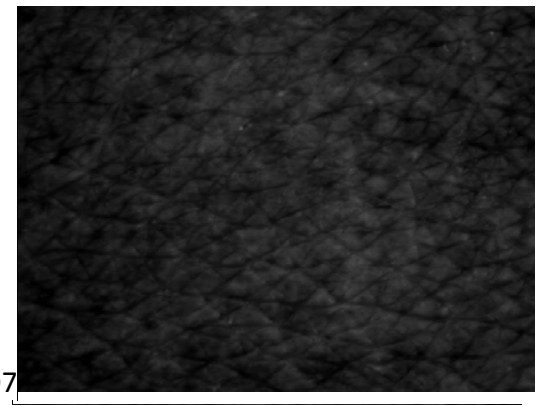

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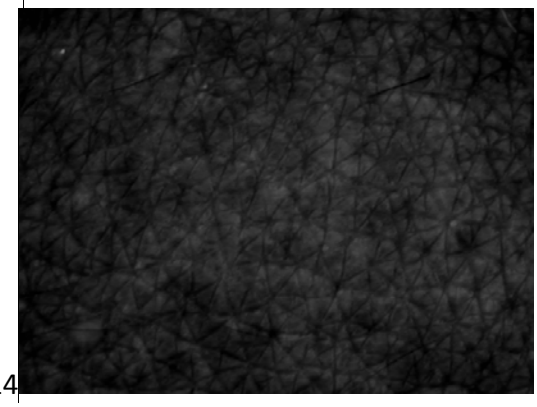

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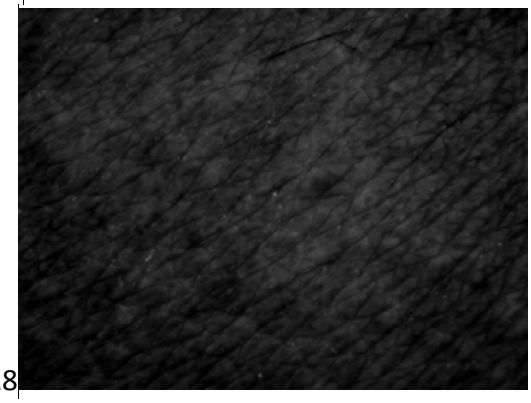

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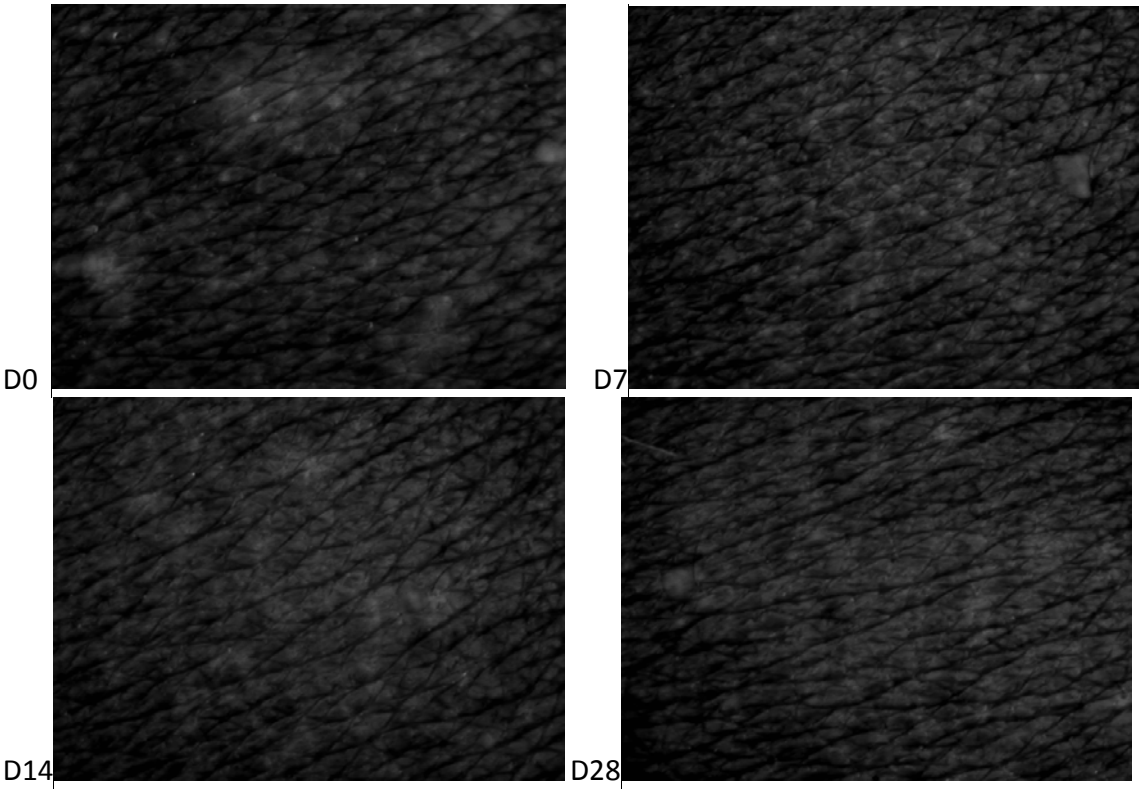

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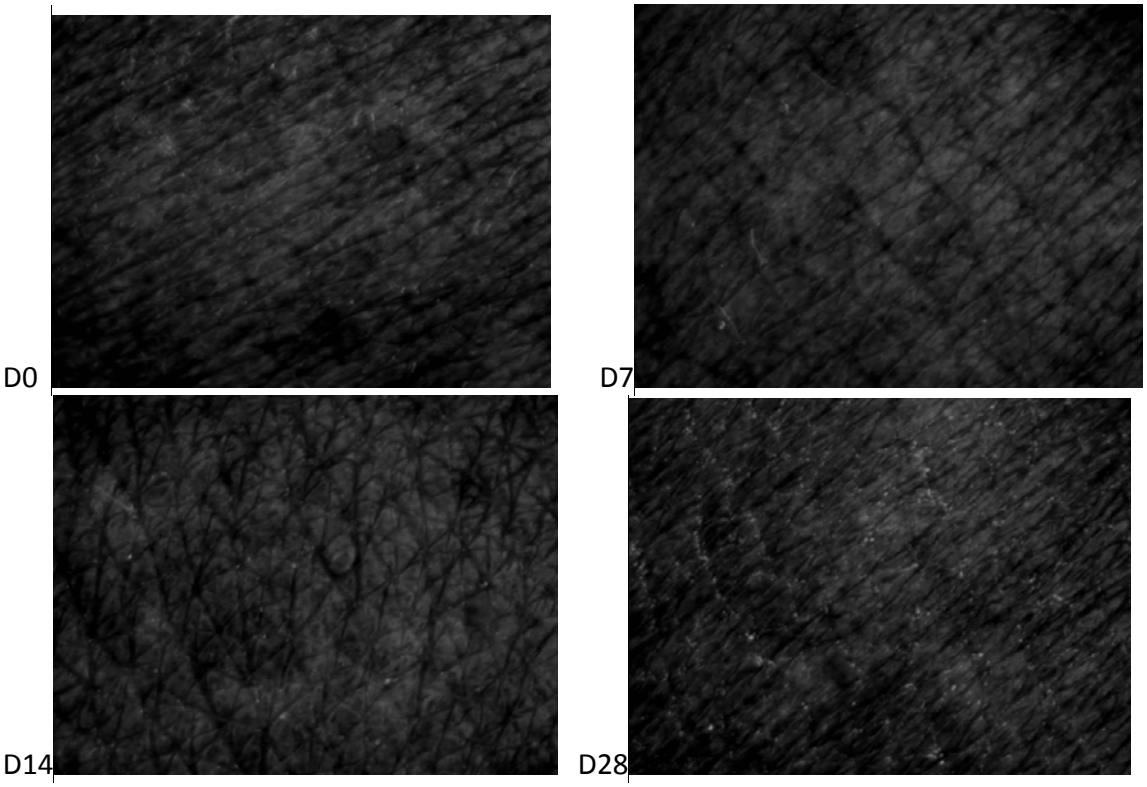

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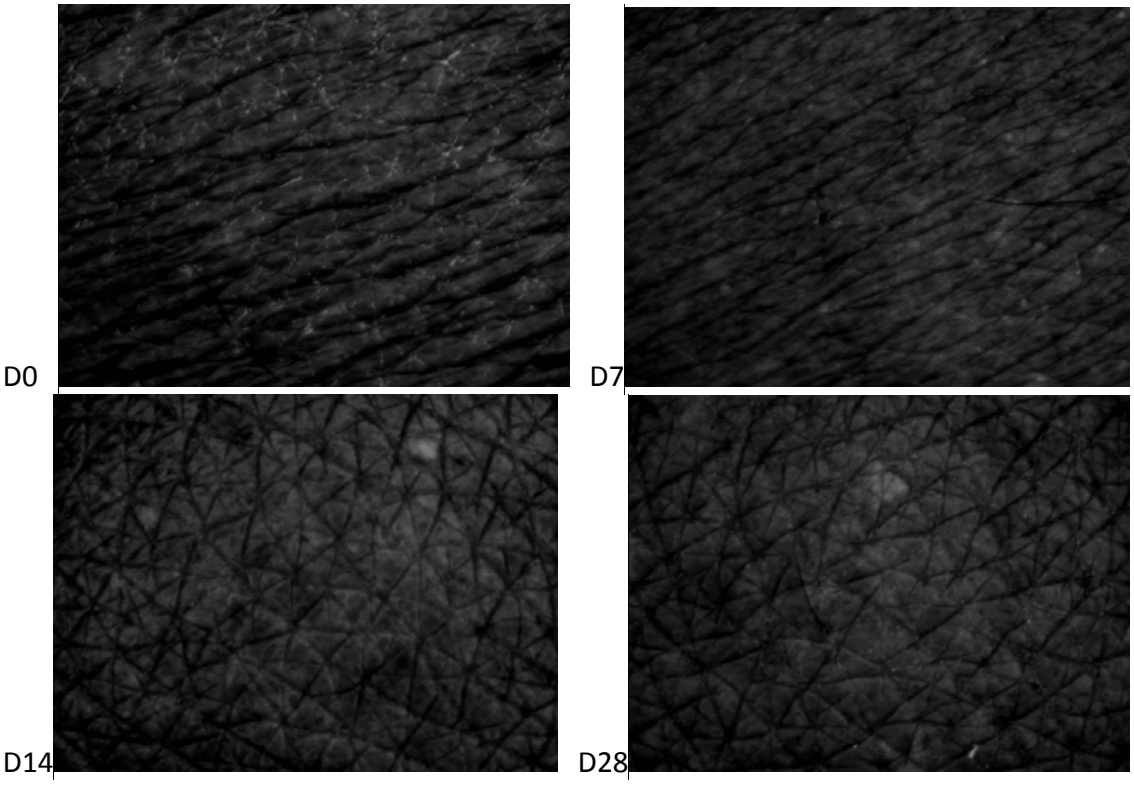

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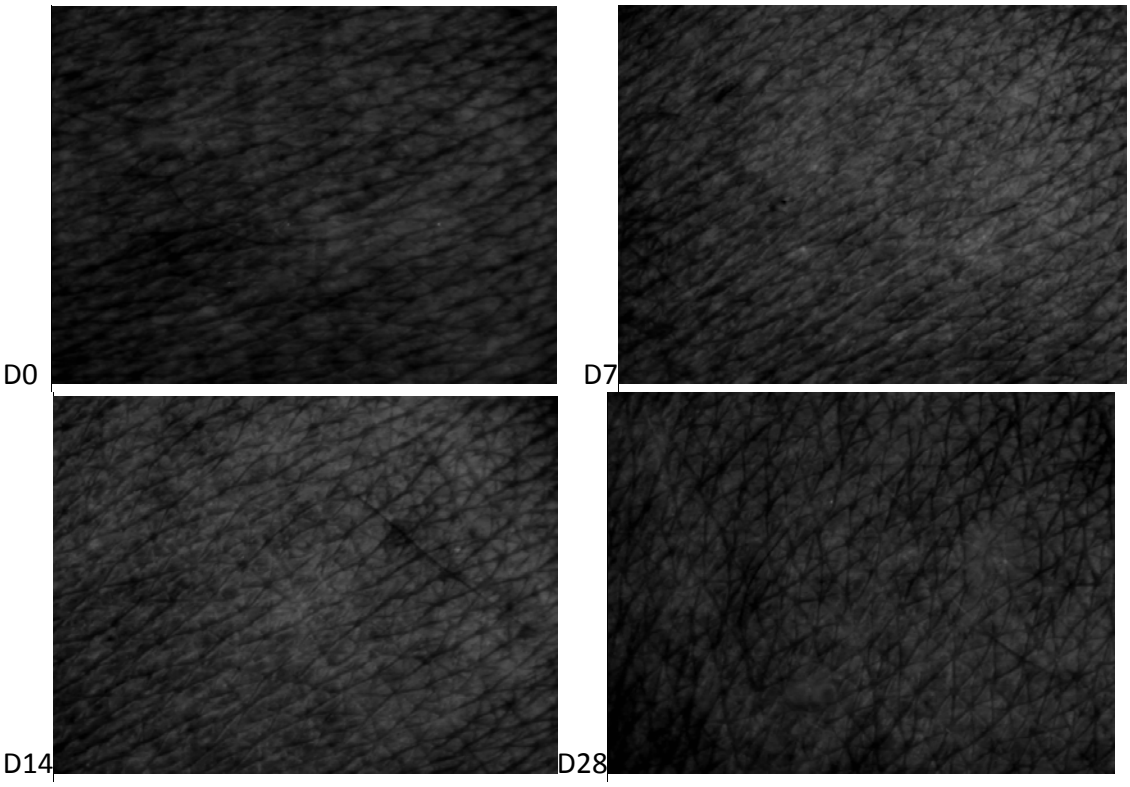

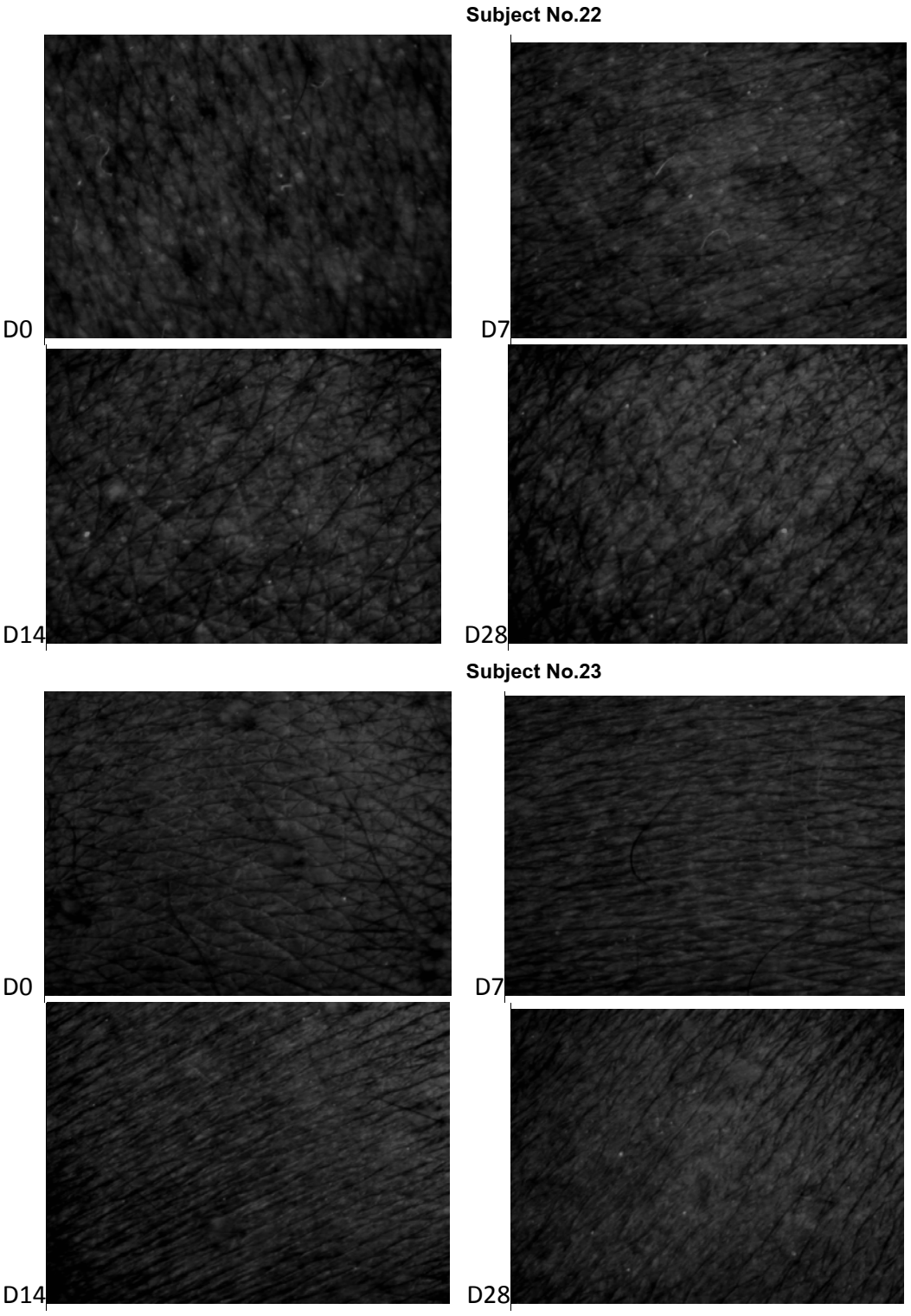

Figure 17 Macroimages of the volunteer's skin before (D0) and after (D7, D14 and D28) applied the product

5. Conclusions

P. amaryllifolius has been developed as natural antioxidant for topical emulsion. Ethanol and propylene glycol were selected as solvent in extraction because they are normally used in topical cosmetic formulation and therefore they are safe and easy to use in the preparation. Extraction in propylene glycol solvent gave higher DPPH radical scavenging activity and total phenolic content than that of extracted in ethanol. Pandan leaves possessed a higher antioxidant activity than aerial root. The *P. amaryllifolius* crude extracts show lower antioxidant activities than standard vitamin C and BHT after tested by DPPH radical scavenging activity and thiocyanate method. However, a neat solution extract of pandan showed higher inhibition in linoleic acid peroxidation than vitamin C and BHT (200 ppm). Moreover, the solution extract of *P. amaryllifolius* showed good heat stability. Topical emulsion product containing the extracts were developed and showed acceptable properties in cosmetic point of view. The products possessed good stability and have a fairly small change of color which mainly caused by the extract. The product is non-irritant to the skin as performed by single patch test. The sensory test showed that most volunteers liked the overall feature of the emlusion cream expect for the odor. The product efficacy measured by Visoscan® VC 98 illustrated that 85-90% of the volunteers possessed a decrease in wrinlkes and scaliness of the skin. Thus, the results obtained indicated that *P. amaryllifolius* extract might be useful in cosmetic industry.

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Output of Research

Publication

Ampa Jimtaisong* and Panvipa Krisdaphong “Antioxidant Activity of *Pandanus amaryllifolius* Leaf and Root Leaf and Root Extract and its Application in Topical Emulsion” Tropical Journal of Pharmaceutical Research, Accepted (expected to be published on June 2013).

Poster Presentation

Ampa Jimtaisong*, Rungkamol Kraisitthipanich, Waranya Suklim, Panvipa Krisdaphong “Thai Pandan (*Pandanus amaryllifolius* Roxb.) Extract as Natural Antioxidant in Topical Oil-In-Water Emulsion” Poster presentation นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส ครั้งที่ 10, October 14-16, 2010.

Ampa Jimtaisong*, , Panvipa Krisdaphong, Topical Oil-in-Water Emulsion Containing *Pandanus amaryllifolius* Extract: Stability, Safety, Efficiency and Sensory Evaluation. Poster presentation นักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส ครั้งที่ 11, October 19-20, 2011.

Appendix

Antioxidant Activity of *Pandanus amaryllifolius* Leaf and Root Extract and its Application in Topical Emulsion

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Abstract

Purpose*: To develop Thai pandan (Pandanus amaryllifolius) as an antioxidant ingredient for topical emulsion.*

Methods*: Dried leaf and root of P. amaryllifolius (Pandanceae) was extracted by maceration with either ethanol or propylene glycol. The antioxidant capacities were investigated by 2,2-diphenyl-1-picry hydrazyl radical (DPPH) and linoleic acid peroxidation method. The total phenolic content was also measured by Folin-Ciocalteau assay. An oil-in-water topical emulsion containing the extract was prepared and tested for stability.*

Results*: Propylene glycol extract exhibited higher DPPH activity and total phenolic content than the ethanol extract while the DPPH activity of the leaf extract was higher than that of the root. The 50% inhibition concentration (IC_{50}) value of leaf and root extracts was 0.810 and 2.340 mg/ml, respectively. Although the antioxidant activity of the crude extracts was lower than that of vitamin C and butylated hydroxytoluene (BHT), a ethanol/propylene glycol solution extract (ethanol was removed to 50% extract volume) showed higher inhibition (90.1 %) of linoleic acid peroxidation than 200 ppm of vitamin C (57.1 %) and BHT (71.1 %). An oil-in-water emulsion containing 3 % of the ethanol and propylene glycol extract showed creamy texture with medium viscosity and demonstrated good stability under accelerated aging test.*

Conclusion*: The results indicate a potential for the development of P. amaryllifolius leaf extract as an antioxidant ingredient in topically applied formulations.*

Keywords: *Pandanus amaryllifolius, Extraction, Antioxidant, Emulsion, Stability.*

INTRODUCTION

The leaf of *Pandanus amaryllifolius* Roxb., commonly known as pandan, is often used to give a refreshing, fragrant flavor to south-east Asian dishes [1]. Besides its culinary value, pandan leaves are used in the perfume industry and also medicinally as a diuretic, cardio-tonic and anti-diabetic [2].

Furthermore, the leaves are used to refresh the body, reduce fever, and relieve indigestion [3], and are reported to contain various alkaloids [4] and unglycosylated pandamin protein which exhibits antiviral activity against human viruses, herpes simplex virus type-1 and influenza virus [5]. The leaves also contain quercetin [6], carotenoids, tocopherols, tocotrienols and essential oils [7]. The major compound responsible for the unique pleasant aroma of *P. amaryllifolius* is 2-acetyl-1-pyrroline. Additional 30 aroma components have been found of which the main ones are hexanal, 2-hexenal, 3-methyl pyridine, 2-penten-1-ol, nonanal, benzaldehyde and linalool [8]. It has been further reported that the ethanol extract of the leaves cultivated in Malaysia exhibited excellent heat-stable antioxidant property [9]. Also, it has been reported that the decoction of *P. amaryllifolius* root and rhizome has been traditionally used in treating diabetic patients. The compound 4-hydroxybenzoic acid has been identified and it showed a hypoglycemic effect in normal rats after the oral administration of 5 mg/kg [10].

Oil-in-water emulsion is commonly formulated for topical pharmaceuticals and cosmetics. It is thought that inclusion of antioxidants in the products can offer better protection and possibly correct the damage caused by free radicals. Natural antioxidants, such as polyphenols in edible herbs, are believed to be safer than synthetic antioxidants. Recently, there has been an increase in the use of polyphenolic

compounds in cosmetics [11,12]. This trend indicates a need for the study of similar compounds when incorporated in finished products.

Pandan has long been used in food but there have been few studies of its topical pharmaceutical and cosmetic applications. Thus, in this work, an attempt has been made to formulate a topical oil-in-water emulsion containing Thai pandan extract as an antioxidant ingredient and to evaluate its total phenolic content and antioxidant activity.

EXPERIMENTAL

Plant materials and reagents

P. amaryllifolius was collected in August 2009 from Chiangrai province, Thailand and identified by Dr. Tipsuda Tangtragoon (Department of Biology, Maejo University, ChiangMai, Thailand). A voucher specimen (no. QSBG 56488) has been kept at Queen Sirikit Botanic Garden Herbarium, Chiangmai, Thailand for future reference.

Leaves and root were used for extraction. All chemicals and reagents for activity study were of A.R. grade. Solvents for extraction (95 % ethanol and propylene glycol) and other ingredients used in emulsion preparation were of cosmetic grade. All spectrophotometric data were acquired using UV-Vis Spectrophotometer (Libra 522, Biochroms).

Preparation of extracts

Solution extract: *P. amaryllifolius* leaf and root samples were cut into small pieces and dried in hot-air oven (Memment UNE/UFE) at 40 ^{o}C for 48 h. The material (10 g of leaf or 20 g of root) was immersed in solvent (250 ml, ethanol or propylene glycol or ethanol/propylene glycol blends of 4:1 and 1:1 volume ratio) and macerated under

different conditions, i.e. at ambient temperature (28-32 ^{o}C) for 1 and 3 days and at 50 ^{o}C for 8 h. The extract solution was filtered using a filter paper (Whatman No.1) and kept at 4 ^{o}C. Ethanol and propylene glycol were selected as solvent for extraction because they are normally used as ingredient in topically applied formulation. The plant materials (10 g of leaf or 20 g of root) were also extracted with ethanol (250 ml) at 50 °C for 8 h under sonication-assisted conditions (Ultrasonic 690D, Crest) and the solvent removed using a rotary evaporator at reduced pressure to obtain crude extracts.

DPPH radical-scavenging assay

The scavenging activity of the extracts against DPPH radicals was evaluated according to the method Que *et al* [13] with some modifications. A solution of DPPH in absolute ethanol (0.1 mM, 3 ml) was added to the extract (1 ml, as is obtained solution extract). The reaction was allowed to continue at ambient temperature for 30 min in the dark and then the absorbance (Abs) was measured at 517 nm. Scavenging activity (%SA) on DPPH radicals was calculated as in Eq 1. The data are expressed as mean ± SD (n = 3).

$$\%SA = \{Abs_{control} - (Abs_{sample} - Abs_{blank})/Abs_{control}\} \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

Linoleic acid emulsion–thiocyanate method

The antioxidant activity of the extracts was also evaluated using the thiocyanate method [14] with some modifications. Vitamin C and butylated hydroxytoluene (BHT, 200 ppm) were used as positive controls. The reaction mixture (linoleic acid emulsion) consisted of 0.28 g of linoleic acid, 0.28 g of Tween 20 and 50 ml of phosphate buffer (0.2M, pH 7.0). The emulsion (2.5 ml) was mixed with 0.5 ml of the

test sample and 2.5 ml of phosphate buffer, and incubated at 40 ^{o}C for 96 h. The mixture prepared without test sample served as control. Aliquots (0.1 ml) were taken from the incubation mixture at intervals of 24 h, mixed with 5.0 ml of 75 % ethanol, 0.1 ml of 30 % ammonium thiocyanate and 0.1 ml ferrous chloride (20mM in 3.5 % HCl), and allowed to stand at room temperature for 3 min. The absorbance of the mixture was measured spectrophotometrically at 500 nm. The inhibition of lipid peroxidation in linoleic acid emulsion was calculated at 72 h as in Eq 2.

Inhibition (%) = 100 - {(As/Ac)100} ……. (2)

where Ac is the absorbance of the control reaction which contains only linoleic acid emulsion and sodium phosphate buffer, and As is the absorbance of the test sample or standard [15]. All measurements were made in triplicate.

Determination of total phenolic content

The total phenolic content of the extracts was determined by Folin-Ciocalteau total phenolic assay [16]. Gallic acid was used as a standard and a range of concentrations (50, 100, 250, 500, 750 and 1000 mg/L) was used to create a standard curve. Deionized water (1.58 ml) and Folin-Ciocalteau reagent (100 µl) was added to the extract sample, the standard, or blank (20 µl). The reaction mixture was incubated at room temperature for 5 min. Sodium carbonate solution (300 μl, 10% w/v) was then mixed with it and incubated for 90 min. The absorbance of the mixture was measured spectrophotometrically at 765 nm. The total phenolic content was calculated from a standard curve and expressed as gallic acid equivalent (GAE) mg/100 g plant sample. Determinations were made in triplicate.

Preparation of topical oil-in-water emulsion containing the extract

The oil phase consisted of cyclopentasiloxane (4 %), jojoba oil (5 %), dimethicone (2 %), shea butter (2.5 %), jojoba ester (3.5 %), Span-80 (1.7 %), Tween-80 (1.3 %), Sepigel-305 (2.5 %) and propylparaben (0.15 %) while the aqueous phase was comprised of propylene glycol (3 %), methylparaben (0.15 %) and deionized water. The oil phase and water phase were mixed and homogenized (Ultra-Turrax® T25 basic, IKA) at 65 – 70 °C for 5 min. The resulting emulsion was cooled to 40 °C and the extract was added and mixed by using homomixer until homogeneous. Ethanol and ethanol/propylene glycol (1:1) leaf extracts were used as active ingredient and their concentrations in the emulsion varied from 1 to 5 % w/w.

The viscosity of the developed products was measured with a Viscometer (RV#4, 4 rpm, Brookfield, USA). The color of products was measured with a Chromameter (Konica, Minolta). Stability prediction of emulsion product is usually performed by accelerated aging test at different storage conditions [17]. The products are generally tested for gravitational stability under centrifugation (6000 rpm, 30 min) [18] and those with no phase separation will then subjected to accelerated aging test. The product containing 3 % extract was divided into 4 samples in a well-closed 120 ml glass bottle and kept in different conditions, i.e. at 4 °C, ambient temperature (28-32 °C) , 45 °C and heating-cooling cycle (4 °C, 24 h; 45 °C, 24 h). The samples were monitored every week for 1 month.

Statistical analysis

Each experimental data point represents the mean from three independent experiments. The deviation from the mean at the 95% significance level was used to determine the differences in biological activity. The 50% inhibition concentration (IC_{50}) values were calculated from linear regression analysis.

RESULTS

P. amaryllifolius extracts

The solution extracts of leaf were dark green with a unique, pleasant pandan odor while the root extracts were odorless with a light yellow color. Their pH was 6.0 ± 0.1.

The crude extract yield of the root (14.1 %) was higher than that of the leaf (9.2 %).

DPPH radical-scavenging

Extraction at 50 ^{o}C for 8 h produced extract that had higher DPPH radical-scavenging activity (96.37 ± 4.50 %) than those obtained at ambient temperature (28-32 ^{o}C)- Day 1, 64.29 ± 2.41 %; Day 3, 74.65 ± 2.62 %). The DPPH activity of the leaf extract obtained at 50 ^{o}C obtained using various solvents and solvent mixtures are shown in Table 1. It can be seen that DPPH activity was highest for propylene glycol extract followed by ethanol/propylene glycol extract (1:1 and 4:1 solvent ratio), and ethanol extract, in that order. The DPPH activity of the propylene glycol leaf extract (94.56 ± 3.35 %) was three times higher than that of the root extract (29.55 ± 1.21 %).

The data obtained also showed that sonication-assisted conditions increased the antioxidant activity of the extracts approximately two times, which indicates that sonic energy is of great help in extraction.

Table 1: DPPH radical–scavenging activity of *P. amaryllifolius* leaf extracted at 50 °C for 4 h under sonication-assisted conditions.

Solvent	% DPPH activity	
	1st extraction*	2nd extraction*
Ethanol	86.36±2.56	69.61±1.08
Ethanol/Propylene glycol (4:1)	93.06±3.04	79.41±2.39
Ethanol/Propylene glycol (1:1)	93.32±1.89	90.37±3.46
Propylene glycol	94.56±3.35	92.06±3.17

* 1st extraction is a maceration of leaf sample (10 g) in 250 ml solvent.

2nd extraction is a maceration of the leaf sample from the first extraction (collected after solvent was removed) in new portion of 250 ml solvent.

The 50 % inhibition concentration (IC_{50}) was graphically obtained to determine the least extract concentration that can inhibit 50 % of free radicals [19]. The results showed that the IC_{50} values of vitamin C, BHT, as well as *P. amaryllifolius* leaf and root extracts were 0.012 ± 0.001, 0.290 ± 0.007, 0.810 ± 0.009 and 2.340 ± 0.040 mg/ml, respectively.

Linoleic acid emulsion system–thiocyanate inhibition

The inhibitions of the reference standards (vitamin C and BHT) and those of the extracts at various concentrations are graphically shown in Fig 1. Increase in absorbance indicates less inhibition in lipid peroxidation.

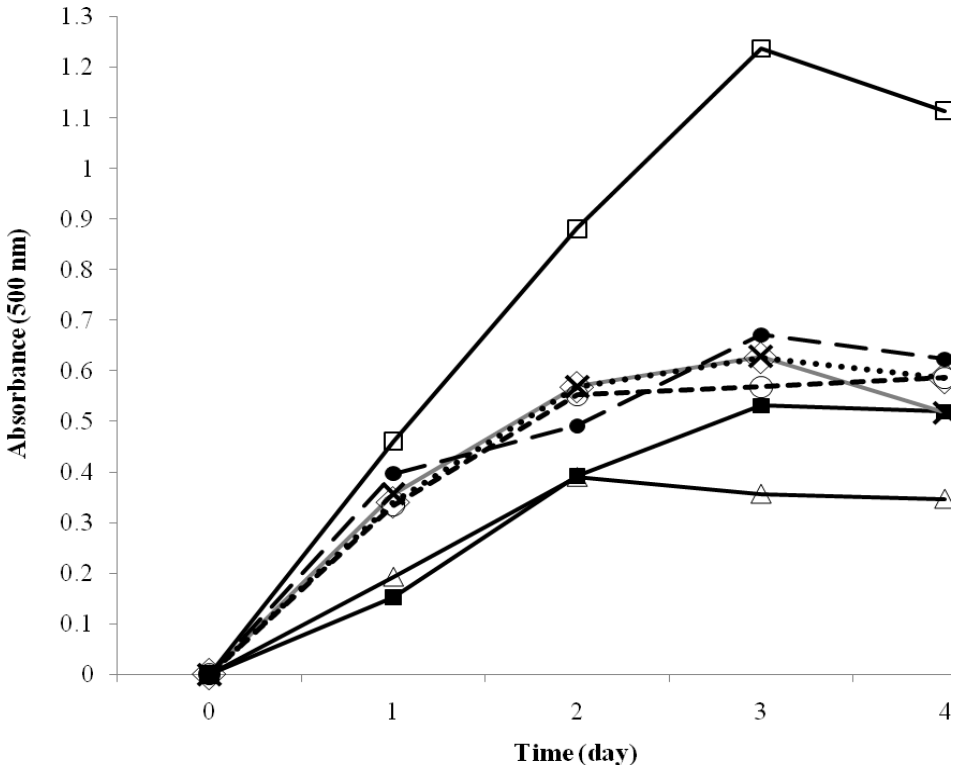

Fig 1: Ferric thiocyanate antioxidant activity of vitamin C and BHT, *P. amaryllifolius* root and leaf crude extracts: Control (□),Vitamin C 200 ppm (■), BHT 200 ppm (Δ), *P. amaryllifolius* root 400 ppm (×), *P. amaryllifolius* root 200 ppm (●), *P. amaryllifolius* leaf 400 ppm (○), and *P. amaryllifolius* leaf 200 ppm (◊).

The absorbance of vitamin C, BHT and the extracts is lower than that of the control signifying the materials' ability to slow down peroxidation of linoleic acid. The inhibition of lipid peroxidation by the extracts was 49.3 ± 1.0 and 45.2 ± 1.2 % for leaf and root extracts while those of vitamin C (57.1 ± 0.9 %) and BHT (71.1 ± 1.0 %) were higher at the same concentration (200 ppm). When the concentration of the extracts was increased, inhibition increased by approximately 10 %.

The peroxidation inhibition of concentrated extract solutions, considered potentially suitable for the formulation of topical emulsion, is shown in Fig 2. The inhibition of the concentrated solution extract (ethanol/propylene glycol blend at 1:1 and ethanol

was completely removed) was 90.1 ± 1.2 %, and is higher than that of the reference standards - vitamin C (57.1 ± 0.9 %) and BHT (71.1 ± 1.0 %).

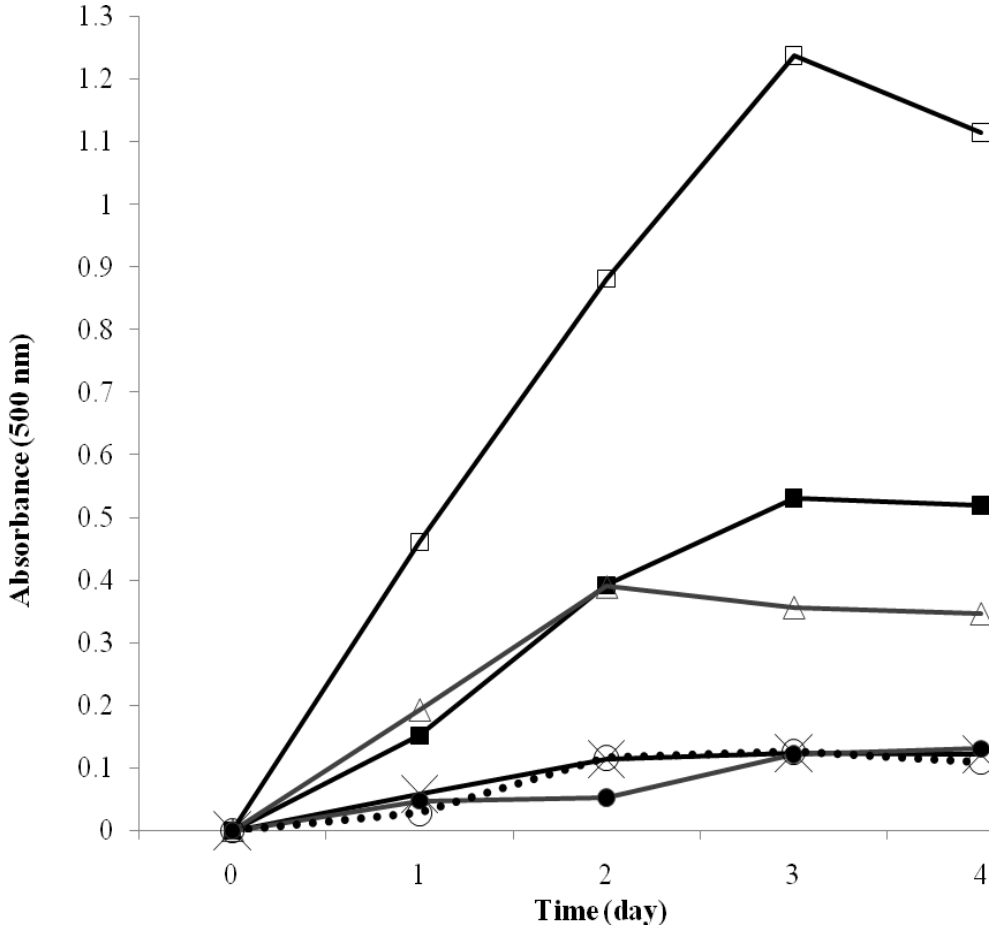

Fig 2: Ferric thiocyanate antioxidant activity of vitamin C and BHT (200ppm each), and concentrated extract solution of *P. amaryllifolius* leaf and root: Control (□), Vitamin C 200 ppm (■), BHT 200 ppm (Δ), *P. amaryllifolius* leaf ethanol extract (solvent was removed until the extract volume remained at 20%) (×), *P. amaryllifolius* leaf ethanol/propylene glycol 1:1 extract (ethanol was completely revoved) (●), and *P. amaryllifolius* root ethanol extract (solvent was removed until the extract volume remained at 20%) (○).

Total phenolic content

The total phenolic content of the ethanol leaf extract (319.2 ± 15.9 mg GAE/100 g plant sample) was much higher than that of the root extract (28.8 ±1.1 mg GAE/100 g plant sample). Furthermore, sonication-assisted extraction resulted in approximately 2 to 4 times higher phenolic content. In addition, propylene glycol extract had higher total phenolic content (360.8 ± 10.8 mg GAE/100 g plant sample) than ethanol extract (319.2 ± 15.9 mg GAE/100 g plant sample).

Topical extract emulsion

The ethanol leaf extract (the extract was concentrated by removing solvent until the extract volume remained at 20% of original volume) , labeled ET20) and the ethanol/propylene glycol (1:1) extract (the extract was concentrated by completely removing ethanol and the volume remained at 50 % of original volume), labeled EP50) were used to prepare separate emulsions. The results indicated that the products containing 1 and 3 % extract had medium viscosity with creamy texture and no phase separation observed under centrifugation. The emulsions were creamy with light sweet, pleasant odor. ET20 emulsion was greener and less viscous than EP50 emulsion (Table 2).

Table 2: Properties of emulsion cream containing 3 % *P. amaryllifolius* leaf extract

Property	**ET20 emulsion**	**EP50 emulsion**
Color	Green	Tinted green
L*, a*, b*	L* = 86.67±0.30	L* = 92.88±0.20
	a* = -7.09±0.07	a* = -3.05± 0.03
	b* = 20.78±0.08	b* = 6.12±0.19
pH	6.11	6.26
Viscosity	35,000 cps	36,600 cps

L***** is the lightness of the color (L*= 0 yields black and L*= 100 indicates diffuse white)

a* indicates red and green hue where negative values indicate green while positive values indicate red

b* is yellow and blue color where negative values indicate blue and positive values indicate yellow

Stability of emulsion product

At the end of the accelerated stability test, the color of the product slightly changed to a lighter green colour and the characteristic pandan odor was less intense. Additionally, pH was practically unchanged except for at 45 °C where it decreased from 6.11 to 5.70 in ET20 emulsion and from 6.26 to 5.90 in EP50 emulsion.

Product viscosity

At initial, the product has creamy texture, medium viscosity. After subjected to accelerated-aging test, as Fig 3 shows, viscosity of the emulsion was largely unchanged after storage at ambient temperature. But the viscosity of ET20 product stored at 45 °C and heating-cooling cycle conditions decreased (20-50 %) while that stored at 4 °C showed a 30 % increase over time. The EP50 product's viscosity at 4 and 45 °C increased ranged from 10 - 30 %. Furthermore, the decrease of viscosity (44 %) was obtained when the EP50 product was subjected to a heating - cooling cycle. Texture change was noticeable when emulsion viscosity varied by 30 % or more.

Product color

The results were recorded as coordinates of CIELAB or L*, a*, b* values in Table 3 with the color difference (Δ) of each parameter also shown. L* represent the lightness of the color (L*= 0 yields black and L*= 100 indicates diffuse white), a* indicates red and green hue (negative values indicate green while positive values indicate red) and b* positions between yellow and blue (negative values indicate blue and positive values indicate yellow. Color difference in L* value ($\Delta L^* = L^*_{wt} - L^*_{w0}$) showed a decline for all conditions except ambient temperature. The greatest change (8% decreased) was found for the emulsion product containing ET20 when subjected to

heating - cooling cycle, and this indicates the lightness is decreased. Δa^* ($\Delta a^* = a^*_{wt} - a^*_{w0}$) increased over time indicating that the green hue decreased. Δb^* ($\Delta b^* = b^*_{wt} - b^*_{w0}$) decreased which indicates the blue hue was dominant and the yellow hue is minimal. The total color difference (ΔE^*) between initial value (W0) and values in week 4 was also calculated as in Eq 3 [20].

$$\Delta E^* = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2} \quad \text{……………………………………………….(3)}$$

The result, (ΔE^*) which incorporates changes in the three components (L*, a*, b*) was highest when the product was subjected to heating - cooling treatment; this was followed by treatment at 45 °C, ambient temperature and 4 ˚C in that order. The product containing ET20 showed the highest ΔE^* value. The greatest total color difference after 1 month storage occurred with the heating - cooling treatment (7.6). Thus overall, the color of the emulsion was remained stable as the ΔE^* is less than 10 [21] and only slightly changed to lighter green compared with the initial color.

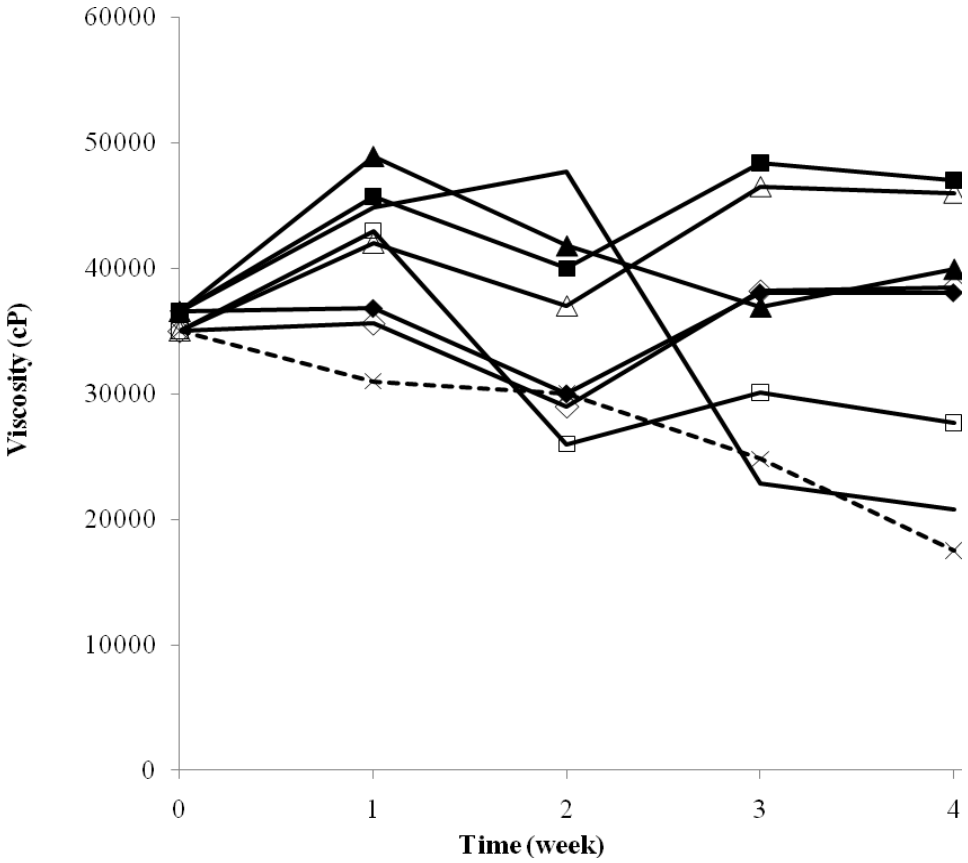

Fig 3: Viscosity of emulsion containing 3 % *P. amaryllifolius* leaf extract following storage at various conditions: Ambient Temp. ET20 (◊), 45 °C ET20 (□), 4 °C ET20 (Δ), Heating-cooling ET20 (×), Ambient Temp. EP50 (♦), 45 °C EP50 (■), 4 °C EP50 (▲), Heating-cooling ET20 (—). ***Note:*** ET20 contains ethanol extract while EP50 contains ethanol/propylene glycol extract.

Table 3: Color variation of emulsion products under various storage conditions.

Colour Difference	Emulsion containing ET20											
	Δ*L**				Δ*a**				Δ*b**			
	AT[a]	45˚C	4˚C	HC[b]	AT[a]	45˚C	4˚C	HC[b]	AT[a]	45˚C	4˚C	HC[b]
W0/W1	0.38	-2.49	-0.98	-4.08	0.73	0.77	0.09	0.48	-0.86	1.62	0.38	1.55
W0/W2	0.66	-4.08	-1.40	-4.51	1.30	1.17	0.28	0.90	-2.00	1.02	0.59	1.03
W0/W3	0.34	-3.80	-1.00	-5.42	1.51	1.64	0.52	1.37	-2.05	-0.15	0.44	0.45
W0/W4	0.94	-5.28	-1.41	-7.44	1.84	1.82	0.75	1.26	-3.21	-1.14	-0.23	0.48
Colour Difference	Emulsion containing EP50											
	Δ*L**				Δ*a**				Δ*b**			
	AT[a]	45˚C	4˚C	HC[b]	AT[a]	45˚C	4˚C	HC[b]	AT[a].	45˚C	4˚C	HC[b]
W0/W1	1.00	-1.05	-1.21	-1.07	0.17	0.24	-0.01	0.18	-0.42	0.10	-0.28	0.09
W0/W2	0.61	-1.62	-1.61	-2.18	0.40	0.37	0.16	0.28	-0.11	0.33	0.25	-0.01
W0/W3	0.31	-2.03	-2.23	-5.64	0.79	0.70	0.35	0.39	-0.37	0.07	-0.19	-0.15
W0/W4	0.68	-1.88	-1.50	-5.77	0.61	0.42	0.06	0.18	-0.44	-0.09	-0.09	-0.64

[a] AT = ambient temperature (28-32 °C) , [b] HC= heating-cooling cycle

Δ*L** is the color difference between initial (W0) and subsequent week (Wt), ($L^*_{Wt} - L^*_{W0}$)

Δ*a** is the color difference between initial (W0) and subsequent week (Wt), ($a^*_{Wt} - a^*_{W0}$)

Δ*b** is the color difference between initial (W0) and subsequent week (Wt), ($b^*_{Wt} - b^*_{W0}$)

DISCUSSION

P. amaryllifolius leaf extracted in propylene glycol had higher DPPH radical-scavenging activity than that extracted in ethanol which may indicate that antioxidant active components in *P. amaryllifolius* are more soluble in a more polar propylene glycol solvent [22]. The propylene glycol extract also showed higher content phenolic compounds and this can be linked to its higher DPPH activity [23].

Incorporation of the extract in a heterogeneous emulsion system resulted in a product with antioxidant characteristics. Topical emulsion systems generally consist of multiple phases in which lipid and water coexists with some emulsifiers [24]. However, it should be noted that the concentration of the extract in the linoleic acid emulsion system used was relatively high (approx. 9 %), and therefore further investigation in bulk oil-in-water emulsion system containing various levels of extract is required.

Propylene glycol extract may not be the suitable choice for topical application due to the fact that a high content in a formulation may result in an oily feeling. Hence, a concentrated mixture of ethanol/propylene glycol (1:1) extract was tested.

Rheological properties of emulsions are important not only for physical characterization but are also parameters indicating system quality. Thus, stability test was performed to ensure that the products meet the intended physical, chemical and performance characteristics when they stored under various conditions. It has been noted that tolerance of stability after 1 - 2 heating/cooling cycles is considered as: stable if the change in viscosity is < 10 %; acceptable, if the viscosity is higher than 10 % but not more than 20 %; and unstable if > 20 % [25]. The viscosity results obtained reveal that the developed products were stable. Moreover, the results are very useful for selecting optimal storage condition of products. Furthermore, the colour stability results obtained also indicate that the color of the products, which is mainly derived from the extract, was basically stable. The results of this study are useful as it may extend the use of *P. amaryllifolius* leaf from traditionally employed in foods [9] into topically applied products.

CONCLUSION

P. amaryllifolius extracted in propylene glycol solvent exhibited higher DPPH radical scavenging activity and total phenolic content than that extracted in ethanol, while the leaf demonstrated higher antioxidant activity than the root. The oil-in-water topical emulsion containing 3 % leaf extract possessed good stability under accelerated aging conditions. Thus pandan leaf extract is a potential suitable natural antioxidant, which

can serve as an alternative to synthetic antioxidants used in topical emulsion. However, further work on the stability and efficacy of the extract in bulk emulsions is required in this regard.

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Article Title: Antioxidant Activity of Pandanus amaryllifolius Leaf and Root Extract and its Application in Topical Emulsion

Names of all Authors: Ampa Jimtaisong and Panvipa Krisdaphong

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Ampa Jimtaisong	Ampa Jimtaisong	Jan 26, 2013

Thai Pandan (*Pandanus amaryllifolius* Roxb.) Extract as Natural Antioxidant in Topical Oil-In-Water Emulsion

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ABSTRACT

The study aimed to develop Thai pandan (*Pandanus amaryllifolius* Roxb.) as natural antioxidant for cosmetic emulsion. Pandan leaf and aerial root were extracted in ethanol and propylene glycol solvent at various conditions. The antioxidant capacities and total phenolic content were investigated. Cosmetic emulsion containing the extract has been prepared and subjected to stability test. Extraction in propylene glycol solvent gave higher DPPH activity and total phenolic content than extraction in ethanol. DPPH radicals scavenging activity of pandan leaf extract is higher than aerial root. The IC_{50} values of leaf and aerial root crude extract is 0.810 mg/ml and 2.340 mg/ml, respectively. The antioxidant capacities of pandan crude extracts by DPPH assay, reducing power and thiocyanate methods were lower than those of vitamin C and BHT. Interestingly, a neat solution extract of pandan showed higher inhibition of linoleic acid peroxidation than vitamin C, vitamin E acetate and BHT (200 ppm). An oil-in-water emulsion cream containing 3% *P. amaryllifolius* extract has been developed. The product showed good characteristics and possessed a fairly good stability under accelerated aging test. The results obtained show that there is a potential in development of pandan leaf extract as cosmetic ingredient.

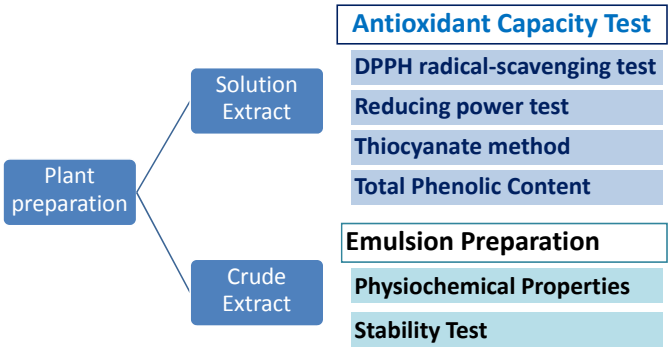
Keywords: Thai pandan, *Pandanus amaryllifolius* Roxb., Antioxidant, Emulsion, Cosmetics

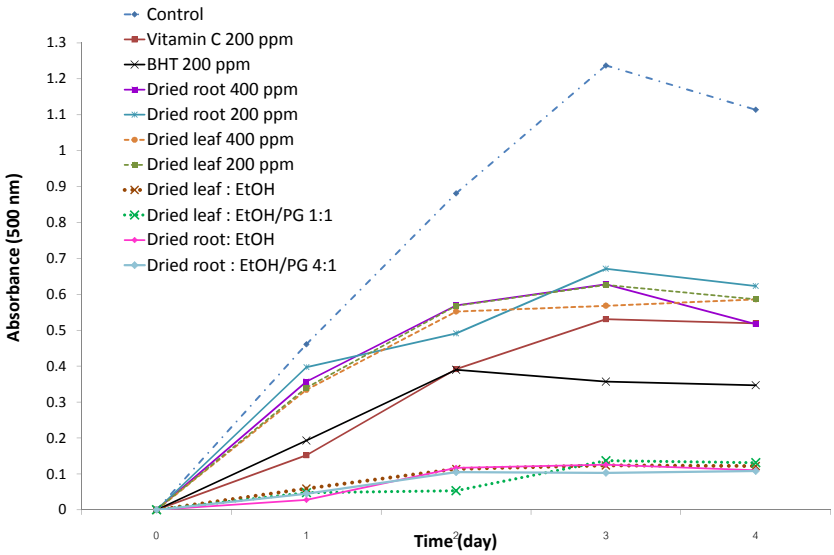
INTRODUCTION

Pandanus amaryllifolius Roxb has long been used in food. Besides its culinary value, pandan leaves are used in perfume industry and also medicinally important as diuretic, cardio-tonic, anti-diabetic and for skin diseasesis. Moreover, *P. amaryllifolius* leaves are used to refresh the body, reduce fever, and relieve indigestion and flatulence. The decoction of *P. amaryllifolius* root and rhizome has been traditionally used in treating diabetic patients. Various alkaloids, such as pyrrolidine alkaloids and pandamarilactonines have been isolated from pandan leaves. The leaves also contain unglycosylated pandamin protein which exhibits antivirus activity against human viruses, herpes simplex virus type-1 (HSV-1) and influenza virus (H1N1).

Moreover, pandan leaves contain quercetin, carotenoids, tocopherols, to cotrienols and essential oils. Additionally, an ethanolic extract of *P. amaryllifolius* leaves cultivated in Malaysia, which had a polyphenol content of 102 mg gallic acid/g extract, exhibited an excellent heat-stable antioxidant property.

METHODOLOGY





The determination of antioxidant activity by the ferric thiocyanate method of vitamin C, BHT (200 ppm), *P. amaryllifolius* root and *P. amaryllifolius* leaf

DPPH RADICAL-SCAVENGING ACTIVITY OF THE EXTRACTS

Extraction time	Extraction temperature	% Activity
1 day	Ambient temperature	64.29 ± 2.41
3 days	Ambient temperature	74.65 ± 2.62
8 hours	**50 °C**	**96.37 ± 4.50**

Solvent system	% Activity	
	1st extraction	2nd extraction
Ethanol	86.36± 2.56	69.61± 1.08
Ethanol/Propylene glycol (4:1)	93.06± 3.04	79.41± 2.39
Ethanol/Propylene glycol (1:1)	93.32± 1.89	90.37± 3.46
Propylene glycol	**94.56± 3.35**	**92.06± 3.17**

The IC50 Value of Vitamin C , BHT and Crude Extract

Sample	IC50 (mg/ml)
Vitamin C	0.012
BHT	0.290
P. amaryllifolius leave crude extract	0.810
P. amaryllifolius root crude extract	2.340

EMULSION PRODUCTS CONTAINING THE EXTRACT

Properties	Emulsion cream containing ethanolic extract (ET20)	Emulsion cream containing ethanol/propylene glycol extract (EP50)
Appearance	Emulsion cream	Emulsion cream
Color	Green color L* = 86.67 ± 0.30 a* = -7.09 ± 0.07 b* = 20.78 ± 0.08	White color with very light green tint L* = 92.88 ± 0.20 a* = -3.05 ± 0.03 b* = 6.12 ± 0.19
Odor	Light pandan odor	Light pandan odor
pH	6.11	6.26
Viscosity	35000 cPs (71% , RV#4, 4 rpm)	36600 cPs (73% , RV#4, 4 rpm)

DISCUSSION AND CONCLUSION

Thai pandan (*Pandanus amaryllifolius* Roxb.) has been developed as natural antioxidant for topical emulsion. Ethanol and propylene glycol were selected as solvent in extraction because they are normally used in topical cosmetic formulation and therefore they are safe and easy to use in the preparation. Extraction in propylene glycol solvent gave higher DPPH activity and total phenolic content than that of extracted in ethanol. Pandan leaves possessed a higher antioxidant activity than aerial root. Pandan crude extracts shows lower antioxidant activities than standard vitamin C and BHT after tested by DPPH radical-scavenging activity, reducing power and thiocyanate method. However, a neat solution extract of pandan showed higher inhibition in linoleic acid peroxidation than vitamin C and BHT (200 ppm). The oil-in-water topical emulsions containing the *P. amaryllifolius* extract at various percentages were prepared and they possessed a fairly good stability under accelerated aging test. The results obtained indicate that pandan leaf extract might be an alternative choice as natural antioxidant in topical emulsion, however more work on stability and efficacy of the extract in bulk emulsion formula are recommended and studies are in progress.

ACKNOWLEDGEMENTS

This project is financially supported by The Thailand Research Fund (TRF)

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Topical Oil-in-Water Emulsion Containing *Pandanus amaryllifolius* Extract: Stability, Safety, Efficiency and Sensory Evaluation

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ABSTRACT

The topical oil-in-water emulsion containing *Pandanus amaryllifolius* extract was developed. The extract was added into a stable oil-in-water emulsion at various concentrations. The product was subjected to accelerated stability test for 1 month at different storage conditions (ambient temperature, 45 degree Celsius, 4 degree Celsius and heating-cooling cycle) and it was found that the viscosity of the products showed slightly changes and the color of products were slightly darker green from the initial. Safety of the product containing 3% extract was tested by single patch test on the forearm area and no sign of irritation was observed. In addition, the efficacy of the product was evaluated from the skin surface by SELS program calculation and the results showed that over 70% of the volunteers indicated a fewer wrinkles and 65% is fewer in scaliness on the skin. Finally, sensory evaluation of the developed moisturizing emulsion cream containing *P. amaryllifolius* extract was collected and it showed that more than 90% of volunteers like overall features of the product. The results obtained indicate that the *P. amaryllifolius* leave extract can be used as a potential antioxidant active in topical emulsion.

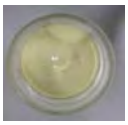
Keywords: *Pandanus amaryllifolius*, Antioxidant, Patch test, Efficiency, Cosmetic Emulsion

INTRODUCTION

P. Amaryllifolius or pandan leaves has long been used in food and also in perfume industry and medicinally important as diuretic, cardio-tonic, anti-diabetic and for skin diseases. The leaves contain alkaloids, pandamarilactonines, unglycosylated pandamin protein, quercetin, carotenoids, tocopherols, tocotrienols and essential oils.

The antioxidant capacities and total phenolic content of *P. amaryllifolius* leaves and aerial root extracted in ethanol and propylene glycol solvent have been investigated. Propylene glycol extract has higher DPPH activity and total phenolic content than that of ethanol extract. DPPH activity of the leaves extract is higher than that of aerial root extract. The IC_{50} values of leaves and aerial root crude extract is 0.810 mg/ml and 2.340 mg/ml, respectively. A neat solution extract of pandan showed higher inhibition (90.1%) of linoleic acid peroxidation than 200 ppm of vitamin C (57.1%) and BHT (71.1%).

Preparation of Emulsion Containing the Extract

Properties	Emulsion cream containing ethanolic extract (ET20)	Emulsion cream containing ethanol/propylene glycol extract (EP50)
Appearance	Emulsion cream	Emulsion cream
Color	Green color L* = 86.67 ± 0.30 a* = -7.09 ± 0.07 b* = 20.78 ± 0.08 	White color with very light green tint L* = 92.88 ± 0.20 a* = -3.05 ± 0.03 b* = 6.12 ± 0.19 
Odor	Light pandan odor	Light pandan odor
pH	6.11	6.26
Viscosity	35,000 cPs (71% , RV#4, 4 rpm)	36,600 cPs (73% , RV#4, 4 rpm)

Stability Test

Table 1 The color difference (ΔL*, Δa*, Δb*) values of the products at different storage conditions.

Color Difference	Emulsion cream containing ET20											
	ΔL*				Δa*				Δb*			
	TR	45°C	4°C	Heating -cooling	TR	45°C	4°C	Heating -cooling	TR.	45°C	4°C	Heating-cooling
W0/W1	0.38	-2.49	-0.98	-4.08	0.73	0.77	0.09	0.48	-0.86	1.62	0.38	1.55
W0/W2	0.66	-4.08	-1.40	-4.51	1.30	1.17	0.28	0.90	-2.00	1.02	0.59	1.03
W0/W3	0.34	-3.80	-1.00	-5.42	1.51	1.64	0.52	1.37	-2.05	-0.15	0.44	0.45
W0/W4	0.94	-5.28	-1.41	-7.44	1.84	1.82	0.75	1.26	-3.21	-1.14	-0.23	0.48
Color Difference	Emulsion cream containing EP50											
	ΔL*				Δa*				Δb*			
	TR	45°C	4°C	Heating -cooling	TR	45°C	4°C	Heating -cooling	TR.	45°C	4°C	Heating-cooling
W0/W1	1.00	-1.05	-1.21	-1.07	0.17	0.24	-0.01	0.18	-0.42	0.10	-0.28	0.09
W0/W2	0.61	-1.62	-1.61	-2.18	0.40	0.37	0.16	0.28	-0.11	0.33	0.25	-0.01
W0/W3	0.31	-2.03	-2.23	-5.64	0.79	0.70	0.35	0.39	-0.37	0.07	-0.19	-0.15
W0/W4	0.68	-1.88	-1.50	-5.77	0.61	0.42	0.06	0.18	-0.44	-0.09	-0.09	-0.64

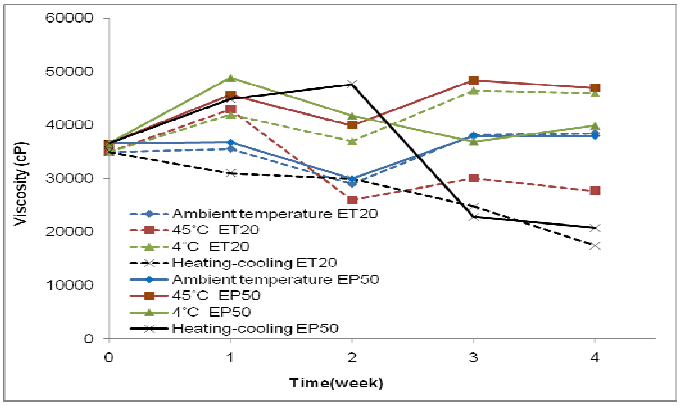
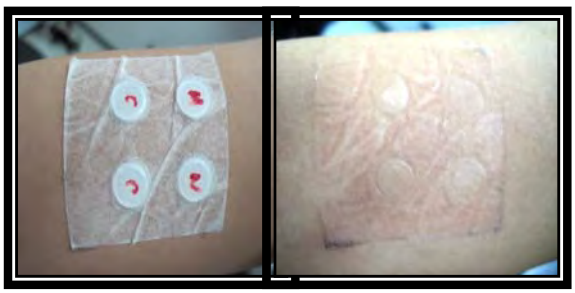
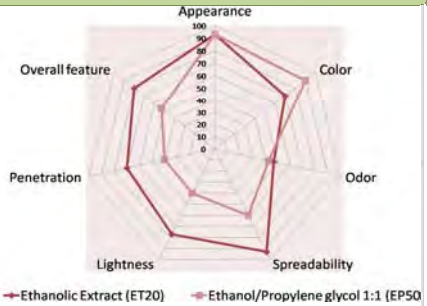


Fig. 1 The viscosity of the developed emulsion containing 3% *P. amaryllifolius* leaves extract (spindle RV#04, 4 rpm; 29.0 ± 1.0 °C) at various storage conditions (ET20 is an emulsion cream containing ethanolic extract; EP50 is an emulsion cream containing ethanol/propylene glycol extract).

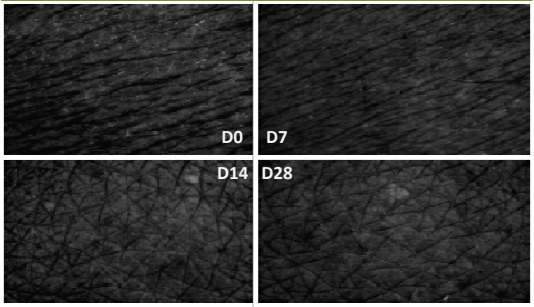
Patch Test



Sensory Test



Efficiency Test



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Thai pandan (*Pandanus amaryllifolius*) has been developed as natural antioxidant for topical emulsion. Extraction in propylene glycol solvent gave higher DPPH activity and total phenolic content than that of extracted in ethanol. Pandan leaves possessed a higher antioxidant activity than aerial root. A neat solution extract of pandan showed higher inhibition in linoleic acid peroxidation than vitamin C and BHT (200 ppm). The oil-in-water topical emulsions containing the *P. amaryllifolius* extract at various percentages were prepared and they possessed a fairly good stability under accelerated aging test. Safety of the product containing 3% extract was tested by single patch test on the forearm area and no sign of irritation was observed. In addition, the efficacy of the product was evaluated from the skin surface by SELS program calculation and the results showed that over 70% of the volunteers indicated a fewer wrinkles and 65% is fewer in scaliness on the skin. Finally, sensory evaluation of the developed moisturizing emulsion cream containing *P. amaryllifolius* extract was collected and it showed that more than 90% of volunteers like overall features of the product. The results obtained indicate that pandan leaf extract might be an alternative choice as natural antioxidant in topical emulsion.

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