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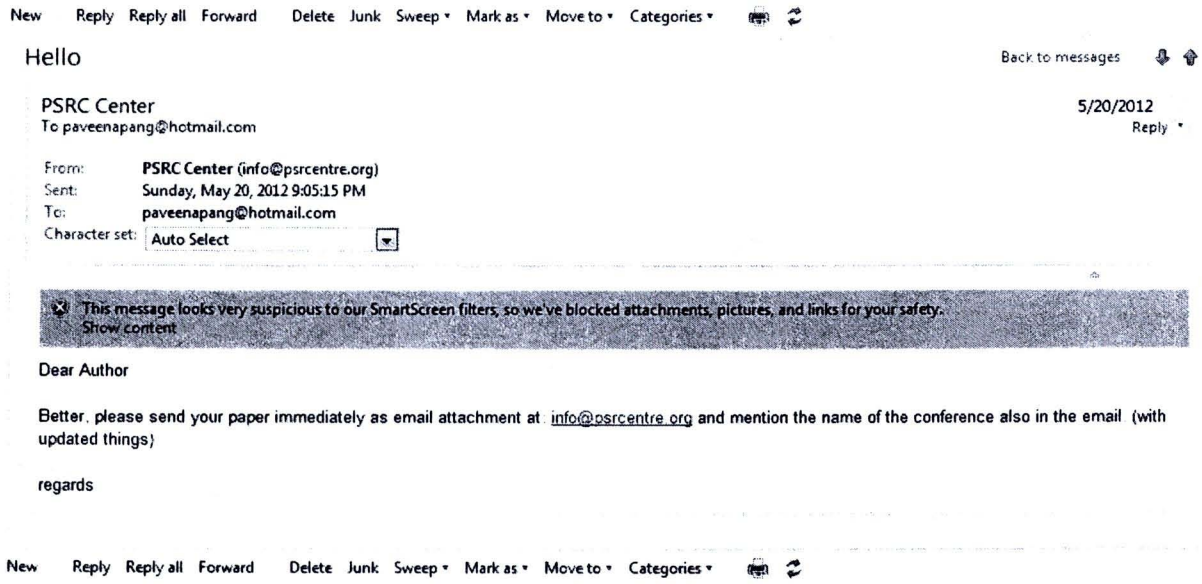

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Effects of *Hesperethusa crenulata*'s bark extract on production of pro-collagen type I and inhibition of MMP-1 in fibroblasts irradiated UVB

Paveena Amornnopparattanakul[1], Nantaka Khorana[2], and Jarupa Viyoch[3]

***Abstract*—** Ultraviolet B (UVB) radiation induces photoaging by upregulating the expression of matrix metalloproteinase (MMP) and decreasing collagen synthesis in human skin cells. This study evaluated the effects of *Hesperethusa crenulata*'s bark extract on cytotoxicity and phototoxicity in human skin cells, MMP-1 and type I pro-collagen levels in UVB-irradiated human skin fibroblasts by ELISA. Powdered *H.crenulata* extracted with dichloromethane. The toxicity showed in term of percentage of cell viability and the morphology of cells was observed under microscope. Percent of cell viability was compared with control (non-treat). The results indicated that this extract didn't show both cytotoxicity in human skin fibroblasts and phototoxicity in human skin keratinocytes at the range of dose that used in this study. For morphology, both of cells didn't change after were treated with this extract. It was found that *H.crenulata* extract reduced MMP-1 expression and increased type I pro-collagen production.

***Keywords*—*Hesperethusa crenulata*,** pro-collagen type I, MMP-1, fibroblasts irradiated UVB.

I. Introduction

SKIN aging is influenced by several factors; including genetics, environmental exposure (ultraviolet (UV) irradiation, xenobiotics, mechanical stress), hormonal changes, and metabolic processes (generation of reactive chemical compounds such as activated oxygen species, sugars, and aldehydes). The influence of the environment, especially solar UV irradiation, is of considerable importance for skin aging. Skin aging due to UV exposure (photoaging) is superimposed on chronological skin aging. Historically, photoaging and chronological skin aging have been considered to be distinct entities. Although the typical appearance of photoaged and chronologically aged human skin can be readily distinguished, recent evidence indicates that chronologically aged and UV-irradiated skin share important molecular features including altered signal transduction pathways that promote matrix-metalloproteinase (MMP) expression, decreased pro-collagen synthesis, and connective tissue damage. This concordance of molecular mechanisms suggests that UV irradiation accelerates many key aspects of the chronological aging process in human skin [1].

Ultraviolet B (UVB) irradiation results in photodamage and premature skin aging by inducing the synthesis of matrix metalloproteinases (MMPs); the enzyme MMP-1 is primarily responsible for degradation of the extracellular matrix (ECM) [2]. In addition, UVB-induced oxidative stress mediates phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 kinase, leading to the phosphorylation of c-Jun. Phosphorylated c-Jun increases the transcriptional activity of activator protein-1 (AP-1), a heterodimer composed of c-Jun and c-Fos, which in turn upregulates MMP-1 transcription [1],[3].

Hesperethusa crenulata is in Rutaceae family, synonyms are *Naringi crenulata* and *Limonia acidissima* L. Common names are Thanaka and wood apple. It is a common tropical plant species in the Indian subcontinent and Southeast Asia. The chemical constituents of *H.crenulata* have revealed 2-quinolone, 2-hydroxyquinoline [4], N-acetyl-N-methyltryptamine, tanakine and tanakamine from the stem bark [5], sitosterol, suberosin, suberenol, 7-methoxy-6-(2,3-epoxy-6-methylbutyl) coumarin, 4-methoxy-1-methyl-2-quinolone and marmesin from the organic extracts of the root bark [6]. The stem bark of *H. crenulata*, when ground to a pale yellow powder, has been commonly applied to the face by Myanmar women for more than a thousand years as a skin care. The previous study found that *H. crenulata* had many activities such as antioxidant, tyrosinase inhibitory, anti-inflammatory and anti-bacterial activities [7].

The purpose of this paper was to determine cytotoxicity and phototoxicity of this stem bark extract to the human skin fibroblasts and keratinocytes. And to evaluate the effects of *H.crenulata*'s bark extract on MMP-1 and type I pro-collagen levels in UVB-irradiated human skin fibroblasts.

II. Methodology

A. Extraction

Hesperethusa crenulata's stems were collected from Mae-

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sod, Tak, Thailand. The stems were peeled off and ground to powder. The powder was macerated with dichloromethane for a week. Then, the solution was evaporated by rotary evaporator to remove solvent.

B. Cytotoxicity in human skin fibroblasts

In this study XTT assay was used to test cytotoxicity. The principle of this assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells. Therefore, this conversion only occurs in viable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a spectrophotometer [8]. The extract was prepared in 0.1%dimethyl sulfoxide (DMSO) and sterilized by 0.2μm filter. Primary fibroblasts isolated from human skin were seeded into 96-well plate (10^4 cells/well) and incubated in incubator at 37°C, 5% CO_2 for 24 hours. After incubation, the extract in various concentrations (5-100 μg/ml) were treated to cells and incubated in the same condition for 24 hours. Then, the XTT solution and medium were treated in each well and incubated in the same condition for 4 hours. After incubation, the absorbance was measured at 490 nm. The absorbance was compared with control (non-treated cells) and calculated in term of percentage of cell viability. And morphology of cells was observed under microscope.

$$\% \text{ Cell viability} = [A\text{sample}/A\text{control}] \times 100$$

C. Phototoxicity in human skin keratinocytes

The extract was prepared in 0.1%dimethyl sulfoxide (DMSO) and sterilized by 0.2μm filter. HaCaT (human keratinocyte cell lines) were seeded into 96-well plate (10^4 cells/well) and incubated in incubator at 37°C, 5% CO_2 for 24 hours. After incubation, the medium was removed and the monolayer was washed with DMEM without FBS. After washing, various concentration of extract (5-100 μg/ml) were added to each well and incubated at the same condition. Twenty-four hours later, the extract was removed and the cells were washed with phosphate buffer saline (PBS). After washing, PBS was added to each well. The cells were irradiated with UVB (56 watt) for 30 minutes. After irradiation, PBS was removed and medium were added to each well. Then, the XTT solution and medium were treated in each well and incubated in the same condition for 4 hours. After incubation, the absorbance was measured at 490 nm. The absorbance was compared with control (non-irradiated and non-treated cells) and calculated in term of percentage of cell viability. And morphology of cells was observed under microscope.

D. UVB irradiation and cell treatment

The *H. crenulata* extract was prepared in 0.1% DMSO (5, 10, 25, 50 and 100 μg/ml). Primary fibroblasts were seeded in 96-well plate (10^4cells/well) in DMEM with 10% fetal bovine serum (FBS); 24 hours later, the medium was removed and the monolayer was washed with DMEM without FBS. After washing, various concentrations of extract were added to each well. 24 hours later, the extract was removed and the cells were washed with phosphate buffer saline (PBS). After washing, PBS was added to each well. The cells were irradiated with UVB 56 watt for 30 minutes. After irradiation, PBS was removed and medium were added to each well. The cells and supernatants were harvested for 4 and 24 hours. Control cells were cultivated without the extract. L-ascorbic acid (vitamin C) was used as a positive control. After incubation, the supernatant was collected and stored at -80°C

E. Measurement of MMP-1 and type I pro-collagen content

The MMP-1 and type I pro-collagen concentration in the medium will be determined using an ELISA kit (RayBio®, USA and TaKaRa, Japan). The level of MMP-1 and type I pro-collagen will be normalized against a standard dose-response curve based on the absorption at the wavelength of 450 nm using a microplate reader.

III. Results

A. Cytotoxicity in human skin fibroblasts

Percentage of cell viability of control, 0.1%DMSO, extract 5, 10, 20, 40, 80 and 100μg/ml are 100.00±2.77, 104.94±7.00, 96.51±10.82, 101.43±1.92, 104.69±4.12, 100.12±3.13, 101.82±2.12 and 100.27±6.67, respectively. Cytotoxicity of *H.crenulata*'s bark extract in human skin fibroblasts is showed in Fig. 1. This graph showed percentage of cell viability of the extract at various concentrations (compared with control). At every concentrations of extract showed no significant difference at *p-value* < 0.01. And cells morphology didn't change at every concentration, showed in Fig. 2.

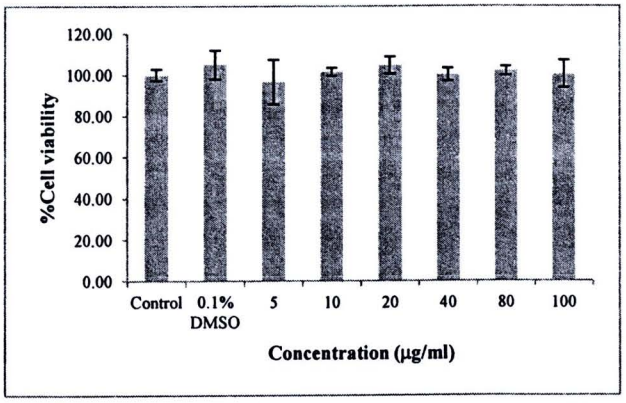

Fig.1 showed percentage of cell viability of primary fibroblast was treated with the *H. crenulata* extract in various concentrations

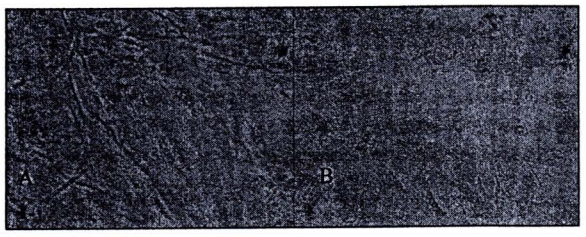

Fig.2 showed cell morphology of primary fibroblasts after treated with DMEM and *H. crenulata* extract at the highest concentration (A: DMEM, B: 100 μg/ml)

B. Phototoxicity in human skin keratinocytes

Percentage of cell viability of control, 0.1%DMSO, extract 5, 10, 20, 40, 80 and 100μg/ml are 100.00±2.66, 100.16±2.38, 103.23±0.77, 105.09±0.64, 104.81±0.24, 105.00±0.21, 105.05±0.49 and 104.35±0.45, respectively. Phototoxicity of *H.crenulata*'s bark extract in human skin keratinocytes is showed in Fig.3. This graph showed percentage of cell viability of the extract at various concentrations (compared with control). At every concentrations of extract showed no significant difference at *p-value* < 0.01. And cells morphology didn't change at every concentration, showed in Fig.4.

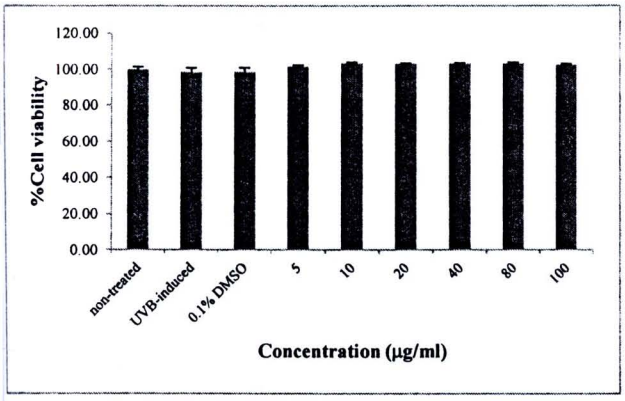

Fig.3 showed percentage of cell viability of HaCaT was treated with the *H. crenulata* extract in various concentrations and treated with UVB

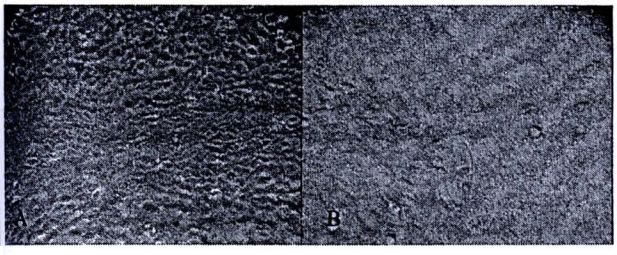

Fig.4 showed cell morphology of HaCaT after treated with of *H. crenulata* extract and UVB irradiated (A: non-treated and non irradiated, B: 100 μg/ml)

C. Effect of H. crenulata extract on MMP-1 expression in UVB-induced fibroblasts

To examine UVB-induced MMP-1 expression in cultured cells. Cells were pretreated with various concentrations of *H. crenulata* extract for 24 hour followed by irradiation with UVB (56 watt, 30 min). Cells were further incubated for 4 hour (Fig. 5a) and 24 hour (Fig. 5b). After 4 h incubation period, the reduction of MMP-1 content with 25, 50 and 100 μg/ml of extract showed significant difference when compared with UVB-induced (p<0.05). After 24 h incubation period, MMP-1 content with every concentration of extract showed slightly reduced but no significant difference when compared with UVB-induced (p<0.05).

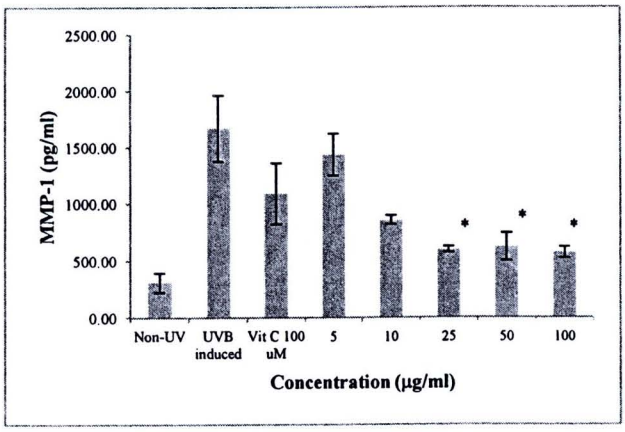

Fig. 5a showed quantities of MMP-1 in fibroblasts after treated with *H. crenulata* extract in various concentrations and induced with UVB for 4 hour

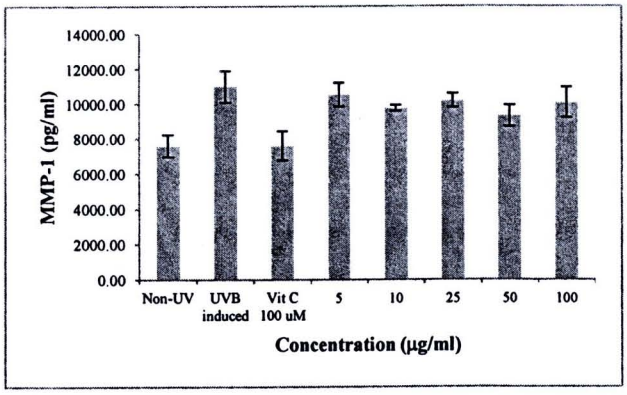

Fig. 5b showed quantities of MMP-1 in fibroblasts after treated with *H. crenulata* extract in various concentrations and induced with UVB for 24 hour

D. Effect of H. crenulata extract on type I pro-collagen production in UVB-induced fibroblasts

To examine type I pro-collagen production in UVB-induced cells. Cells were pretreated with various concentrations of *H. crenulata* extract for 24 hour followed by irradiation with UVB (56 watt, 30 min). Cells were further incubated for 24 hour (Fig. 6). After incubation period, the production of type I pro-collagen was increase at every concentration of extract. They showed significant difference at 10, 25, 50 and 100 μg/ml of extract and 100 μM of vitamin C when compared with UVB-induced (p<0.05).

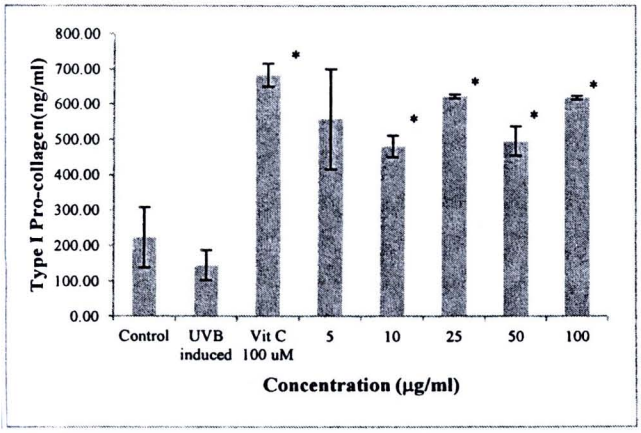

Fig.6 showed quantities of type I pro-collagen in fibroblasts after treated with *H. crenulata* extract in various concentrations and induced with UVB for 24 hour

IV. DISCUSSION

Cytotoxicity of *H.crenulata* extract to human skin fibroblasts showed no toxicity at the highest concentration is 100 µg/ml and cell morphology didn't change.

UVB-irradiation upregulates MMP-1 and reduces type I pro-collagen levels in human skin [1]. However, it was found that treating fibroblasts with *H.crenulata* extract before UVB-induced. MMP-1 expression was reduced and type I pro-collagen was upregulated.

ACKNOWLEDGMENT

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(4) S. D. Gershon, M. A. Goldberg, and M. M. Rieger, "Permanent Waving," in *Cosmetics, Science and Technology,* 2nd ed., M. S. Balsam and E. Sagarin. Eds. (Wiley Interscience, New York, 1972), Vol. 2, pp. 167-250.

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Title;

Efficacy evaluation of combination of thanaka bark extract-loaded solid lipid particles and aloe gel extract-loaded liposomes in sun protection

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Summary

This study was aimed to evaluate sun protection efficacy of the product containing *Naringi crenulata* (thanaka) bark and *Aloe vera* (aloe) gel extracts. *N. crenulata* bark was extracted by using methanol and a yield of 3.24% was obtained. Aqueous solution of *A. vera* gel was precipitated with 35% ammonium sulfate and a yield of 0.06% of the lyophilized extract was obtained. The thanaka bark extract was then determined the antioxidant activity based on DPPH assay. The obtained EC_{50} value was 146.1 ± 1.1 μg/ml while vitamin E provided EC_{50} of 11.1 ± 1.1 μg/ml. The aloe gel extract was determined antiinflammatory activity based on inhibitory TNF-α production from RAW 264.7 macrophage cell lines with LPS activating. The extract inhibited production of TNF-α with IC_{50} of 66.7 ± 1.5 μg/ml. Solid lipid particle loading thanaka bark extract and liposome loading aloe gel extract were successfully prepared by using hot melt emulsion and reverse phase evaporation techniques, respectively. Consequently, the loaded particles and liposomes were homogeneously mixed with gel-forming polymer. The final concentrations of thanaka bark and aloe gel extracts in the formulation was 3% and 0.03%, respectively. By using SPF 290S optometric, the prepared product showed SPF value of 1.06. For *in vivo* study in 10 subjects with skin type III and IV, the prepared product showed no difference in times to protect first perceptible erythema with the commercial product with SPF 13.40. These obtained results indicate the potential of the prepared product for application as sun protection for Asian skin type.

Keywords: *Naringi crenulata* bark extract, *Aloe vera* gel extract, Solid lipid partilces, Chitosan-coated liposomes, Sun protection

Introduction

In the recent years, the risks associated with harmful effects of sunlight or UV radiation on the skin, such as sunburn, photoaging and carcinogenic are well considered. Therefore, the demanding prevention of harmful reactions of UV radiation to skin increases and then leads to improve photoprotection by topically applied sun protection agents. In general, sunscreen products in market can only protect against UV radiation, but may be insufficient to prevent harmful reactions of UV radiation, including erythema and sunburn.

Besides synthetic compounds, the several natural extracts from plant sources such as *Camellia sinensis* (green tea), lichens, rutin (buckwheat), *Passiflora incarnata* L. (maypop) and *Plantago lanceolata* (ribwort plantain) are applied in cosmetic products. This is because these extracts show various biological activities which can be applied as sun protection agent. Such activities include antiinflammation, antioxidation and antityrosinase (1-3). In addition, they are mostly free from side effects. In this reason, the idea of development of product from the natural extracts having potential UV protection combination with antioxidation and antiinflammatory actions attracts our attention.

Naringi crenulata (thanaka) belongs to Rutaceae family and grows in tropical regions. The bark and heartwood of *N. crenulata* have been applied as cosmetic ingredients in skin care products. Recently, several researches have reported that *N. crenulata* extract consists of coumarin derivatives such as 6-formyl-7-methoxycoumarin, 6-methyl-7-methoxycoumarin, 7-methoxycoumarin, 7-phenylacetoxycoumarin, umbelliferone, geranylumbelliferone, suberosin, suberenol, suberosin epoxide and marmesin (4-6). These compounds show ability of UV absorption and can also inhibit oxidative stress (6, 7). For medicinal plant *Aloe vera* (aloe), it belongs to Asphodelaceae family and grows in arid climates. The inner gel of *A. vera* leaves is well known having an antiinflammatory

property. Recently, it has been reported that the molecular sizes of proteins in range of 20 - 100 kDa of *A. vera* gel can inhibit the production of inflammatory mediators (8). According to the actions of the thanaka and aloe gel extracts, they are possibly used as the active ingredients for sun protection purpose. However, as far as we know, *in vivo* efficacy on sun protection of these extracts has never been reported. We theorize that *in vivo* actions of such extracts will be as expected if they are formulated into the proper forms. For examples, the lipophilic extract of thanaka is formulated into solid lipid particle for enhancing attachment of the UV absorption and/or antioxidant compounds on skin, thus increasing efficacy of UV protection (9). On the other hand the aqueous extract of the aloe gel is formulated into liposome for enhancing penetration of antiinflammatory compounds through skin (10).

For this reason, the sun protection product from the natural sources was formulated. The solid lipid particle and chitosan-coated liposome forms were selected for preparing thanaka bark extract-loaded lipid particles and aloe gel extract-loaded liposomes, respectively. Consequently, the loaded particles and liposomes were homogeneously mixed with sodium polyacrylate gel. The sun protection efficacy of the prepared product was determined both *in vitro* and *in vivo*, as compared to the commercial product containing synthetic sunscreen agents.

Materials and methods

Materials

Analytical grade (AR) of chloroform, ethyl acetate, ethyl ether and methanol was purchased from LabScan Asia Co. Ltd., Bangkok, Thailand. Hexane (AR grade) was purchased from RCI Labscan Ltd., Bangkok, Thailand. Ammonium sulfate was purchased from Merck, Darmstadt, Germany. 2,2-Diphenyl-picrylhydrazyl (DPPH, AR grade) was purchased from Sigma-Aldrich Chemie

GmbH, Steinheim, Germany. Lipopolysaccharide (LPS), Dulbecco's Modified Eagle's Medium (DMEM) and cholesterol (95%) were purchased from Sigma-Aldrich Corp., St. Louis, Missouri, USA. Fetal bovine serum (FBS) was purchased from GIBCO, California, USA. TNF-α enzyme-linked immunosorbent assay kit was purchased from eBioscience, Inc., California, USA. Cell proliferation kit (sodium 3'-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonicacid hydrate, XTT) was purchased from Roche Diagnostics GmbH, Mannheim, Germany. Cetyl alcohol and cetyl palmitate were distributed by S. Tong Chemicals Co., Ltd., Bangkok, Thailand and Namsiang Company Limited, Bangkok, Thailand, respectively. Almond oil was distributed by Srichand United Dispensary Co., Ltd, Bangkok, Thailand. Rheodol TW-O120 (polyoxyethylene sorbitan monooleate) was distributed by Hong Huat Co., Ltd., Bangkok, Thailand. Disodium laureth sulfosuccinate was distributed by Water Doctor Co., Ltd, Bangkok, Thailand. Lipoid® S 75 (75% phosphatidylcholine from soybean) was purchased from Lipoid GmbH, Darmstadt, Germany. Chitosan (derived from crab shells with molecular weight in range of 100,000–1,000,000 Da, degree of deacetylation > 90%) was purchased from Aqua Premier Co., Ltd., Chon Buri, Thailand. Sodium polyacrylate (Cosmedia SP) was supported from Cognis Thai Ltd., Bangkok, Thailand.

Preparation of the crude extract from *N. crenulata* bark

The stems of *N. crenulata* were collected from Mae Hong Sorn Province, Thailand. The barks were separated, chipped and dried at room temperature. One kilogram of dried barks was macerated with methanol at room temperature for one week. The supernatant solution was filtered and concentrated with a vacuum evaporator to remove organic solvent. Then the extract was dried in a desiccator. The obtained crude extract (namely, thanaka bark extract) was kept in a tight container at -20°C for further studies.

Preparation of the lyophilized extract from *A. vera* gel

The leaves of *A. vera* were collected from Bang Krathum District, Phitsanulok Province, Thailand. The fresh leaves were cut in half and the colorless gels were carefully separated by scraping the green cortical layer. One kilogram of colorless gel was minced and filtered through a woven cloth. The aqueous of aloe gel was centrifuged at 12,100 x g for 30 min at 4°C. The supernatant was collected and 35% ammonium sulfate was then added to precipitate protein compositions. After standing overnight at 4°C, the supernatant was centrifuged at 12,100 x g for 30 min at 4°C. The obtained precipitate was dialyzed against deionized water for 48 h at 4°C (8) and the dialyzed precipitate was then dried by lyophilization. The lyophilized portion (namely, aloe gel extract) was kept in a tight container at -20°C for further studies.

Qualification of the extract

Thin layer chromatography (TLC) profile of the thanaka bark extract

One-hundred and fifty milligrams of the extract was redissolved in 5 ml methanol. Then the extract solution was diluted with 95 ml deionized water. The obtained suspension was partitioned with ethyl acetate in ratio of 1:1 and incubated at room temperature overnight, according to the method from previous study with modification (11). The fraction of ethyl acetate was spotted on the TLC sheet and developed with hexane and ethyl acetate in ratio of 1:1. The TLC profile was observed under UV illumination.

SDS-PAGE profile of the aloe gel extract

The lyophilized extract was weighed in amount of 10 mg and redissolved in 1 ml deionized water. The obtained solution was mixed with loading dye in ratio of 5:1. Then 25 µl of the mixture was added into each well of SDS-PAGE gel and separated protein profile by utilizing 12% Bis-Tris gel. The running voltage was set at 120 volt. After running for 2 h, the SDS-PAGE gel was stained with

coomassie blue overnight. The staining gel was then destained with destining solution (50% methanol and 10% acetic acid in deionized water) until to obtain clear gel.

Determination of *in vitro* biological activities of the extract

Free radical scavenging activity of the thanaka bark extract by using DPPH assay

The sample solution of the extract or vitamin E, a positive control, was prepared by dissolving in methanol. The reaction mixtures consisted of 75 µl sample solution and 150 µl of 0.2 mM DPPH. The mixtures were mixed using a vortex for 15 sec and incubated at room temperature for 30 min. The absorbance of the mixtures containing DPPH was measured at wavelength of 515 nm (12) using spectrophotometer (Model Spectra Count®, Perkin Elmer, Connecticut, USA).

The radical scavenging activity was calculated as a percentage of DPPH discoloration by using the following equation:

$$\% \text{ Radical scavenging activity} = [1 - (A_S/A_B)] \times 100$$

where

A_S is an absorbance of DPPH with test sample, and

A_B is an absorbance of DPPH without test sample.

EC_{50}, the concentration that produces half maximal effective concentration, of the extract or control was determined by plotting between log concentrations of the extract or control and percent free radical scavenging.

Antiinflammatory activity of the aloe gel extract by using LPS-activated macrophage model

The antiinflammatory activity of the extract was determined by measuring the percent inhibition of tumor necrosis factor-α (TNF-α), inflammatory mediator,

released from LPS-activated macrophages, according to the modified method (13). Briefly, murine macrophage cell lines (ATCC Name RAW 264.7, Lot No. 300319-Pr410, Cell Lines Service, Eppelheim, Germany) were maintained in DMEM containing 10% FBS and incubated at 37°C in a humidified 5% CO_2 atmosphere. The passage numbers of 6 to 8 were used in the present study. To determine the antiinflammatory activity, the cell suspensions (1 x 10^4 cells per well) were firstly seeded in 96-well plates and cultured for 18 h. Next, the maintenance medium were replaced with serum-free DMEM containing various concentrations of the extract. Cells were further incubated for 15 min and subsequently activated with 1 μg/ml LPS for 24 h. The cell-free supernatants were then collected and TNF-α content was determined using TNF-α enzyme-linked immunosorbent assay kit.

The percent inhibition of TNF-α released was calculated by using the following equation:

$$\% \text{ TNF-}\alpha \text{ inhibition} = [1 - [A_{(treatment)}/A_{(control)}]] \times 100$$

where

$A_{(treatment)}$ is absorbance intensity of supernatant of cells treated with the extract and activated with LPS, and

$A_{(control)}$ is absorbance intensity of supernatant of cells activated with LPS.

EC_{50} of the extract was determined by plotting between log concentrations of the extract and percent inhibition.

Additionally, viability of cells was observed by using XTT-colorimetric assay. This assay is based on the conversion of the water soluble XTT to an orange formazan produced by actively respiring cells. The mean absorbance value of the non-treated cells (control group) was adjusted as 100% cell viability (14).

Preparation of solid lipid particles and chitosan-coated liposomes

Preparation of solid lipid particle loading thanaka bark extract (SLPTE)

The SLPTE was simply prepared by hot melt emulsion technique (9). Briefly, the oil-rich part containing 1% (w/w) extract, 2% (w/w) cetyl palmitate, 3% (w/w) cetyl alcohol, 3% (w/w) almond oil and 6% (w/w) rheodol TW-O120 was heated at 60°C. At the same temperature, the hot aqueous solution of emulsifier including 3% (w/w) disodium laureth sulfosuccinate in deionized water was added into oil-rich part. The hot emulsion was then formed by continuous stirring. Finally, the dispersion of the extract-loaded solid lipid particles was obtained by immediate cooling down at 4°C with a magnetic stirrer at speed of 300 rpm for 5 min.

Preparation of chitosan-coated liposome loading aloe gel extract

Liposomes were simply prepared by reverse phase evaporation, according to our previous study (14). The mixture of Lipoid® S 75 and cholesterol in 2:1 molar ratio was dissolved in the solvent mixture of chloroform and diethyl ether (1 : 1 by volume). The aqueous solution of 0.06% extract was then added into lipid solution. The mixing solution was ultrasonicated until w/o emulsion formed and such emulsion had to be stable for minimum of 30 min. The emulsion was then evaporated by using a rotary evaporator under vacuum set at 45°C until gel film appeared around the evaporating flask. Finally, the gel film was agitated with a vortex until to obtain the free-flowing liposomes. The obtained liposomes were then coated with chitosan. The purpose of coating was to improve their stability during application. By following method described in our previous study (14), 1% chitosan was dissolved in acidic solution adjust to pH 4 and 1 ml of the prepared liposomes was then added to 2 ml of the chitosan solution. The resulting dispersion was gently agitated at room temperature for 2 h and the dispersion of the extract-loaded liposomes coated with chitosan was finally obtained.

Characterization of the solid lipid particles and chitosan-coated liposomes

Morphology

The morphological shape of lipid particles was observed using a SEM (Model LEO 1455VP, LEO, Cambridge, England). The sample was evenly spread over a cover slide on a stub and then coated with gold in a cathodic evaporator. For the chitosan-coated liposomes, their morphological shape was observed using a TEM (Model Technai F20, Philips, Eindhoven, the Netherlands). A drop of the sample was attached on a carbon-coated grid. After 2 min later, the excess of the liposomes was carefully removed with filter paper. An aqueous solution of saturated uranyl acetate was used as a staining agent.

Particle size and zeta potential analysis

The mean particle size, size distribution and zeta potential of the samples were determined using a zeta potential analyzer (Model ZetaPALS, Brookhaven Instruments Corporation, New York, USA). The concentrated particles were diluted with deionized water. Then the measurements of mean particle size and size distribution were performed at a fixed angle of 90° to the incident light and data were collected over period of 3 min. The particle size and zeta potential of particles were analyzed using the hydrodynamic diameter and laser doppler electrophoresis, respectively.

Preparation of the sun protection product

The sun protection product was prepared into gel form by homogeneous mixing the extract-loaded solid lipid particles and –loaded chitosan-coated liposomes with the prepared gel (4.5% Cosmedia SP and 5% propylene glycol). The final concentration of the thanaka bark extract in the gel formulation was 3% (w/w) while that of the aloe gel extract was 0.03% (w/w). The pH and rheological behavior of the formulation was determined by a pH meter (Model delta 320, Mettler-Toledo Instruments (Shanghai) Co., China) and a rheometer (Model DV-III, Brookfield Engineering Laboratories, Inc., Massachusetts, USA) equipped

with a cone and plate (plate diameter 40 mm, cone angle 4°), respectively. Rheogram measurement was made under the following experimental conditions: 25°C, 2 g samples of the product and CP 14 spindle. To obtain the ascendant curve, rotation speeds were progressively higher (10-150 rpm) and the procedure was repeated in reverse with gradually decreasing speeds (150-10 rpm) for the descendant segment.

Evaluation of *in vitro* sun protection properties

The extract-free gel, the prepared product and the commercial product were evaluated sun protection factor (SPF), star rating and critical wavelength values by using SPF 290S optometric (Model SPF 290S optometric, Optometrics Company, Massachusetts, USA). Transpore 3D, a surgical tape, was used as artificial human skin. The sample was applied on the Transpore to obtain a sample film thickness of 2 mg/cm^2, evenly distributed by rubbing the whole surface placed on a holder with an ungloved finger for 20-30 strokes and let air drying for at least 15 min. The prepared samples were exposed to Xenon arc lamp with UV range 290 nm to 400 nm for determining the SPF. The SPF-290 software used Trapezoidal Approx. calculating technique to approximate the integral for *in vitro* SPF, critical wavelength and UVA/UVB ratio (15). The following equations were used for;

- Calculation of SPF

$$SPF_{vitro} = \frac{\int_{290nm}^{400nm} E(\lambda) \bullet s(\lambda) \bullet \delta\lambda}{\int_{290nm}^{400nm} E(\lambda) \bullet s(\lambda) / \tau(\lambda) \bullet \delta\lambda}$$

where

$E(\lambda)$ = Spectral irradiance of the used light spectrum at wavelength λ

$s(\lambda)$ = Erythemal action spectrum at wavelength λ

$\tau(\lambda)$ = Spectral transmittance of the sunscreen

$\delta(\lambda)$ = Wavelength step (1 nm)

- Calculation of critical wavelength

$$\int_{290nm}^{\lambda_c} A(\lambda) \bullet \delta\lambda = 0.9 \bullet \int_{290nm}^{400nm} A(\lambda) \bullet \delta\lambda$$

where

$A(\lambda)$ = Absorbance at wavelength λ

λc = the wavelength, where the area under the absorbance spectrum reaches 90% of the whole area in the range of 290 to 400 nm

$\delta(\lambda)$ = Wavelength step (1 nm)

- Calculation of UVA/UVB ratio

$$R = \frac{\int_{320nm}^{400nm} A(\lambda) \bullet \delta\lambda \,/\, \int_{320nm}^{400nm} \delta\lambda}{\int_{290nm}^{320nm} A(\lambda) \bullet \delta\lambda \,/\, \int_{290nm}^{320nm} \delta\lambda}$$

where

$A(\lambda)$ = Absorbance at wavelength λ

R = the ratio of the mean absorbances from two distinct wavelength ranges: UVA (320–400 nm) and UVB (290 – 320 nm)

$\delta(\lambda)$ = Wavelength step (1 nm)

UVA star rating (one to four stars) relates to UVA/UVB ratio value; where 0, UVA/UVB ratio 0.0 to <0.2; 1, UVA/UVB ratio 0.2 to <0.4; 2, UVA/UVB ratio 0.4 to <0.6; 3, UVA/UVB ratio 0.6 to <0.8; 4, UVA/UVB ration ≥ 0.8.

Evaluation of *in vivo* sun protection efficacy

To determine the efficacy of the prepared product on sun protection, a study assessing the perceptible erythema after sunlight exposure was conducted. The study protocol was approved by the Ethics Committee for Human Research of Naresuan University, Phitsanulok, Thailand. Ten subjects with skin type III (intermediate) and IV (tan) were recruited in the present study. Written informed

consent was obtained from all participating subjects. Before studying, subjects were measured level of skin color (melanin and erythema values) on accessing region (back) prior testing one week by using Mexameter® (Model MX 18, Courage and Khazaka Electronic GmbH, Cologne, Germany) and also level of wart, wound, injury, melasma, freckle or acne. At the study day, back of each subject was divided from shoulder to waist in an area of 5 x 5 cm^2. The gel base, prepared product and commercial sunscreen were randomly applied on test sites (2 mg/cm^2). Subjects were then ordered to wait for 15 min prior exposed to sunlight at noon (11.00-13.00 o'clock, summer time). During the period of sun exposure, subjects were accessed the first perceptible unambiguous erythema on test sites and the least times that appeared such perceptible unambiguous erythema were recorded. The subjects were asked to terminate exposing to the sunlight when the erythema had appeared. After 24 h of termination, the test sites of each subject were measured skin color including melanin and erythema values using a Mexameter® and the results are shown in percent changes calculated by using the following equation:

$$\% \text{ Changes} = \frac{(\text{value measured after} - \text{value measured before})}{\text{value measured before}} \times 100$$

Statistical analysis

All quantitative data are expressed as means ± S.D. of the samples for each experiment. Wilcoxon rank-sum test was used for comparison between two groups. $p < 0.05$ was considered significant.

Results and discussion

Plant extract

The extraction of thanaka bark with methanol provided a dark brown paste and a yield of 3.24% (w/w). The yield of the extract in this experiment coincides with

that obtained in previous study using the mixtures of methanol and chloroform as solvent (6).

The extraction of aloe gel with 35% ammonium sulfate provided a white residue and a yield of 0.06% (w/w). As the structural components of the inner gel of aloe generally compose of about 99.5% water, a small yield of extract was obtained.

Qualification of the extract

TLC profile of the thanaka bark extract

The TLC profile of the methanolic extract of thanaka bark exhibited dark spot under short wavelength (254 nm) illumination. This implies the presence of UV absorbing organic compounds. In addition, strong purple fluorescent spots at R_f of 0.24, 0.62, 0.78 and 0.85 under long wavelength UV (366 nm) light were observed, as shown in Figure 1. These compounds might be coumarin derivatives as previous study reported the identification of coumarins with the purple fluorescent spot on TLC under UV light at 366 nm (16).

SDS-PAGE profile of the aloe gel extract

The SDS-PAGE profile revealed 4 major bands including estimated protein molecular weights of 15, 19, 63 and 74 kDa, as shown in Figure 2. The obtained results coincide with the previous study indicating the molecular weight of the precipitated protein from aloe gel in range of 20-100 kDa (8).

Biological activities of the extract

Free radical scavenging activity of the thanaka bark extract

It has been reported that coumarin derivatives are composed of the *N. crenulata bark* (4-6) and can suppress the reactive oxygen species (ROS) (7). For this reason, we expected that the extract obtained in the present study might exhibit a favorable free radical scavenging. By plotting between log concentrations of the extract or vitamin E and percent free radical scavenging

(Figure 3), we found that the extract had scavenging activity with EC_{50} of 146.1 ± 1.1 μg/ml while vitamin E showed EC_{50} of 11.1 ± 1.1 μg/ml. The obtained results indicate that the extract had weaker activity than vitamin E. However, as comparison to the reported free radical scavenging activity of the extracts from mulberry seed (*Morus alba*, EC_{50} of 0.15 ± 0.01 mg/ml) (17) and ginseng (*Panax ginseng*, EC_{50} of 561.9 ± 2.7 mg/ml) (18), antioxidant ingredients used in recent cosmetics for sun protection purpose, the thanaka bark extract shows a stronger activity. In addition, the resulting of DPPH test only recognizes free radical scavenging effects and not pro-oxidant. Therefore, other antioxidant properties, such as reducing of the extract should be taken into consideration and further clarified in the future.

Antiinflammatory activity of the aloe gel extract

In the skin, inflammatory responses to UV exposure appear to be mediated by the production of inflammatory cytokines from epidermis and dermis (19). Among many inflammatory cytokines, we especially paid attention to TNF-α as it is a major proinflammatory cytokine involving the acute inflammatory response that causes erythema and sunburn. LPS is well known that can activate mononuclear phagocytes (monocytes and macrophages) to secrete TNF-α and other types of cytokines. Therefore, in the present study we determined the effect of aloe gel extract on the inhibitory production of TNF-α in LPS-activated RAW 264.7 macrophage cell lines. We found that the extract inhibited the production of TNF-α with IC_{50} of 66.7 ± 1.5 μg/ml (Figure 4). This inhibitory activity is stronger as compared to the activity of catechin, which has been reported inhibition of TNF-α production by LPS induced macrophages with IC_{50} >100 μg/ml (20). Focusing on cell viability study, we found that the aloe gel extract at concentrations used in the present study (10 – 3,000 μg/ml) did not affect on viability of RAW 264.7 macrophage cell lines, as shown in Figure 5. This implies

that the reduction of TNF-α production did not cause from cell death. It is possible that antiinflammatory activity of the aloe gel, at least partially, resulted from the presence of protein components with molecular weight in range of 20 – 100 kDa and/or glycoprotein components with molecular weight about 14 kDa as antiinflammatory activity of these components has been reported (8, 21).

Characteristics of the solid lipid particles and chitosan-coated liposomes

Solid lipid particle loading thanaka bark extract (SLPTE)

The production of SLPTE was achieved by using hot melt emulsion technique. Table I presents mean particle size, polydispersity index (PI, indication of uniformity of particle size) and zeta potential of the extract-free solid lipid particles (extract-free particles) and SLPTEs. It is interested to see that the size of particle decreased from 391 nm to 179 nm and PI also decreased from 0.36 to 0.29 when the extract was loaded. In general, the particle sizes of lipid particles prepared from hot melt emulsion depend on several factors including content of each component in the particles. It is possible that the excess oils dissolving thanaka bark extract might suddenly separate from melted solid lipid during cooling process, leading to the appearance of the smaller particle size (22). Figure 6 shows a spherical shape and uniformity of particle size of the SLPTEs observed under SEM. For the zeta potential value, both extract-free particles and SLPTEs provided value closed to absolute 30, that is a required value to get an optimal electrostatic stabilization (23). In general, the zeta potential is a measure of electric potential in the interfacial double layer at the location of the slipping plane versus a point in the bulk fluid away from the interface, so it indicates the electronic stability of formulation.

Chitosan-coated liposome loading aloe gel extract

The production of chitosan-coated liposomes loading the aloe gel extract were achieved by using reverse phase evaporation technique. According to our previous

study (14), phosphatidylcholine and cholesterol in an optimal proportion were used to improve the fluidity and stability of the bilayer membrane. However, the structure of liposomes is easily degraded under both oxidation and hydrolysis conditions. Therefore, to improve the stability, the surfaces of liposomes were coated with the polymer material including chitosan. In the present study, the formula and procedure of coating liposomes with chitosan were taken from our previous study (14).

Table II presents mean particles size, PI and zeta potential of uncoated liposome without the extract (extract-free liposomes), uncoated liposome loading the extract (extract-loaded liposomes) and chitosan-coated liposome loading the extract (extract-loaded coated liposomes). The results revealed that the particle size and size distribution had no difference between before and after loading the extract. However, after coating liposomes with chitosan, the mean particle size increased from 510 nm to 1,360 nm whereas the PI was no difference. The zeta potential value changed from negative charges to be positive charges. The previous study described that the cationic character of chitosan could interact with the negative surface charge on liposomes via ionic interaction that reversed the negative values to be positive values. In addition, the thickness of chitosan around surfaces of liposomes caused larger size of particle (14, 24). The morphology of chitosan-coated liposome loading aloe gel extract observed under TEM is shown in Figure 7. The liposome vesicles appeared spherical in shape and the black filaments of chitosan covering liposomes were also observed.

Characteristics of the prepared product

In order to determine the efficacy of the particles and liposomes loading extract on sun protection, the final product containing 3% thanaka bark extract and 0.03% aloe gel extract was prepared in the gel formulation. The concentration of the thanaka bark extract used in the present study corresponded to the common

concentration of chemical sunscreens in markets (3-15 %) (25). For the aloe gel extract, the used concentration was more than value of the IC_{50} obtained from the present *in vitro* antiinflammatory study by about 5 times. The pH of the gel formulation was 6.86 ± 0.11. By plotting between shear rate and shear stress, as shown in Figure 8, the prepared product performed pseudoplastic behavior (thixotropy). This behavior can break down for easy spreading and the applied film can gain viscosity instantaneously to resist running. Therefore, the product with a pseudoplastic flow would produce a coherent protective film covering the skin surface with evenly distributed UV filters (26).

In vitro sun protection properties

Table III presents *in vitro* sun protection properties including SPF, star rating, critical wavelength and UVA/UVB ratio of the extract-free product (gel base), gel containing SLPTEs and chitosan-coated liposome loading aloe gel extract (the prepared product), and the commercial product.

SPF represents the efficacy of UVB protection. UVA/UVB ratio and star rating imply the ratio of the total absorption in the UVA to that in the UVB. These parameters reveal the balance in the protection between UVA and UVB. Critical wavelength is the wavelength at which the sunscreen allows 10% of the rays to penetrate. For the prepared product, its SPF value was found to be 1.06 while that of the commercial product was 13.40. This indicates low UVB protection of the prepared product and it cannot be claimed as UVB protection. However, it is possible to be claimed as UVA protection with star rating of 3 (UVA/UVB ratio, 0.6). The UVA absorption property of the prepared product corresponds with the previous study indicating UVA-absorbing chromophores of coumarin components contained in the *N. crenulata* bark extract (6).

In vivo sun protection ability

The average times of the first appearance of erythema of different products are shown in Figure 9. From the results, the protected test sites with the prepared or commercial product showed times longer to protect first perceptible unambiguous erythema compared to unprotected test sites or test sites applied with the gel base. In addition, the prepared product provided no difference in times to protect first perceptible erythema with commercial sunscreen formulation (SPF 13.40). At 24 h after study termination, the increasing in skin erythema (Figure 10A) and melanin (Figure 10B) of the test sites protected with the prepared product or the commercial sunscreen was significantly lower than that of the unprotected sited. In addition, the percent increase in erythema and melanin values were no difference between commercial sunscreen and the prepared products.

From our study, it is interested to see that the prepared product containing SLPTE and chitosan-coated liposome loading aloe gel extract showed low SPF value in *in vitro* test (SPF, 1.04). However, in *in vivo* test, the sunburn protection efficacy of the prepared product was a similar manner to that of the commercial product with SPF 13.40. This is proof of usefulness of the thanaka bark and aloe gel extracts for protection of the skin form sunlight exposure when each extract is formulated into the proper form. As we known, in the skin, acute or chronic exposure to sunlight or UV ray increases production of free radicals and consequently enhances inflammatory symptoms including erythema and/or sunburn (27-29). Therefore, the application of combination of antioxidant and anti-inflammatory ingredients may minimize or even protect the harmful effects of sunlight exposure. In addition, the adherence and/or absorption of the ingredients by the skin may be a synergistic factor for *in vivo* sun protection efficiency of the product. It is possible that after application of the prepared product, thanaka bark extract loaded in lipid particle well adhered on the skin and acted against the free radicals which are involved in inflammatory reactions. Concurrently, aloe gel

extract penetrated into lower epidermis and reduced the skin cell production of inflammatory mediators, thus consequently slowing an appearance erythema and lowering skin redness or sun burn. Such inflammatory mediators possibly included TNF-α, which is an important key mediator cause erythema (30). In addition, UVA absorption properties of the thanaka bark extract's components may also prevent UV-induced immunosuppression that is a cause of contact hypersensitivity (31).

A limitation of the present study was the inclusion criteria that limited the ability to extrapolate the results to the group of skin types II. In the present study, the subjects with skin type III and IV were recruited. However, the international guideline for *in vivo* SPF test method requires fair skin volunteers with skin type II. This criteria is not applicable in Southeast Asia including Thailand as the majority of population belongs to skin types III, IV and V (32). Skin pigmentation is an important factor for minimal erythemal dose (33-35). A minimal erythemal dose for unprotected skin of type III and IV are expected to be large since the protection is a result of the epidermal melanin. This might cause no large difference in average times of the first appearance of erythema between the formulas with various *in vitro* SPF values. In addition, the results from a small number of subjects within the present study may also not coincide with a larger study with variety of population and skin types. Future studies with a larger number of subjects and variety of skin types should be performed to ensure the beneficial effects of the prepared formulation for sun protection.

Conclusions

In the present study, the prepared extract from *N. crenulata* (thanaka) bark showed ability of free radical scavenging activity while that from *A. vera* (aloe) gel revealed the potential of antiinflammatory activity. The gel formulation containing thanaka bark extract-loaded solid lipid particles and aloe gel extract-

loaded liposomes exhibited low SPF value. *In vivo* study revealed that the sunburn protection of the prepared formulation was a similar manner to that of commercial product with SPF 13.40. All findings indicated the potential of the product containing thanaka bark extract-loaded solid lipid particles and aloe gel extract-loaded liposomes for application in sun protection product.

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Table I The characteristics of the thanaka bark extract-free solid lipid particles (extract-free particles) and thanaka bark extract-loaded solid lipid particles (SLPTEs). Data present as mean ± S.D of three separately prepared batches.

Formulations	**Mean particle size (nm ± S.D.)**	**Polydispersity index (PI ± S.D.)**	**Zeta potential (mV ± S.D.)**
Extract-free particles	391 ± 24	0.36 ± 0.01	-29 ± 3
SLPTEs	179 ± 57	0.29 ± 0.03	-28 ± 1

Table II The characteristics of the uncoated liposome without the aloe gel extract (extract-free liposomes), uncoated liposome loading the aloe gel extract (extract-loaded liposomes) and chitosan-coated liposome loading the aloe gel extract (extract-loaded coated liposomes). Data present as mean ± S.D of three separately prepared batches.

Formulations	**Mean particle size (nm ± S.D.)**	**Polydispersity index (PI ± S.D.)**	**Zeta potential (mV ± S.D.)**
Extract-free liposomes	481 ± 107	0.36 ± 0.01	-29 ± 3
Extract-loaded liposomes	510 ± 57	0.33 ± 0.03	-30 ± 3
Extract-loaded coated liposomes	1,360 ± 235	0.27 ± 0.07	33 ± 2

Table III *In vitro* sun protection properties of the extract-free product (gel base), gel containing solid lipid particle loading thanaka bark extract and chitosan-coated liposome loading aloe gel extract (the prepared product) and the commercial sunscreen product.

Formulations	SPF	Star rating[a]	Critical wavelength[b] (nm)	UVA/UVB ratio
Gel base	0.87	1	362	0.1
Prepared product	1.06	3	376	0.6
Commercial product	13.40	2	360	0.4

Note: [a] Boot's star system, 0-4; 0 = too low for UVA claim, 1 = moderate, 2 = good, 3 = superior and 4 = maximum

[b] critical wavelengths; 340 nm $\leq \lambda_c <$ 370 nm = some (UVA/UVB) and $\lambda_c >$ 370 nm = more broad-spectrum

Legends to figures

Figure 1 TLC profile of the methanolic extract of *N. crenulata* bark with a solvent system of hexane and ethyl acetate in ratio of 1:1 under UV illumination at (A) 366 and (B) 254 nm.

Figure 2 SDS–PAGE profile of the lyophilized extract of *A. vera* gel on 12% Bis-Tris gel stained with coomassie brilliant blue. Lane A and B represent molecular weight markers and the lyophilized extract, respectively.

Figure 3 The free radical scavenging activity of the methanolic extract of *N. crenulata* bark (thanaka bark extract) and vitamin E. Free radical scavenging activity was determined by DPPH assay. Each point represents mean ± S.D. of triplicate study.

Figure 4 Effect of the lyophilized extract of *A. vera* gel on inhibition of TNF-α production from RAW 264.7 macrophages cells activated with LPS. The amount of TNF-α released was measured by immunoassay. Each point represents mean ± S.D. of triplicate study.

Figure 5 Effect of the lyophilized extract of *A. vera* gel (aloe gel extract) on viability of RAW 264.7 macrophages cells. Results are expressed as percentage of cell viability as comparison to non-treated (control) cells for which the optical density was adjusted to 100%. Each bar represents mean ± S.D. of triplicate study.

Figure 6 SEM photograph of the solid lipid particle loading thanaka bark extract at a magnification of 10 kx.

Figure 7 TEM photograph of the chitosan-coated liposome loading aloe gel extract at a magnification of 13.5 kx.

Figure 8 Rheograms of (□) the gel formulation containing solid lipid particle loading thanaka bark extract and chitosan-coated liposome loading aloe gel extract. Each point represents mean ± S.D. of triplicate study.

Figure 9 Average times of the first appearance of erythema of the test sites when protected with different products; the extract-free product (gel base), the gel containing solid lipid particle loading thanaka bark extract and chitosan-coated liposome loading aloe gel extract (the prepared product), and the commercial sunscreen with SPF 13.40. Each bar represents mean ± S.D. of ten subjects. Statistically significant difference from unprotected skin, $*p < 0.05$ (Wilcoxon rank-sum test).

Figure 10 Percent increase in (A) erythema and (B) melanin value of the test sites when protected with different products; the extract-free product (gel base), the gel containing solid lipid particle loading thanaka bark extract and chitosan-coated liposome loading aloe gel extract (the prepared product), and the commercial sunscreen with SPF 13.40. Each bar represents mean ± S.D. of ten subjects. Statistically significant difference from unprotected skin, $*p < 0.05$ (Wilcoxon rank-sum test).

Figure 1 (J. Viyoch)

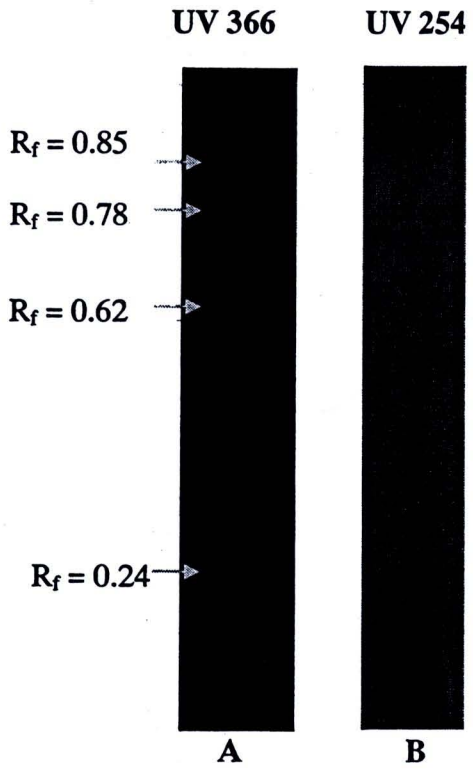


Figure 2 (J. Viyoch)

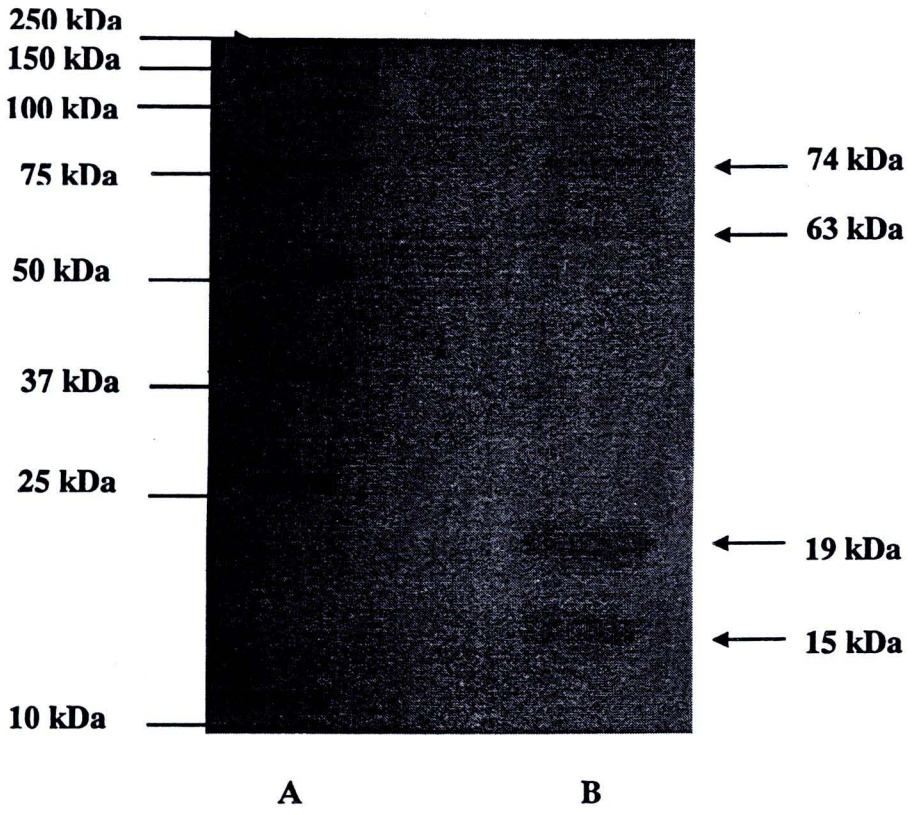
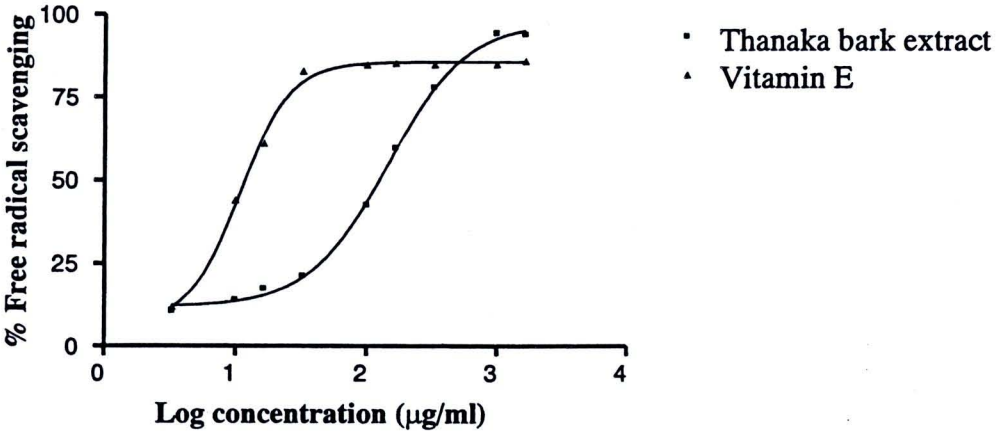


Figure 3 (J. Viyoch)



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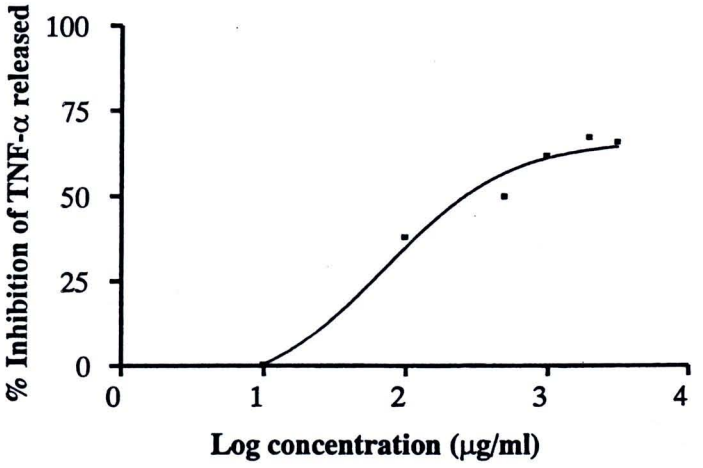

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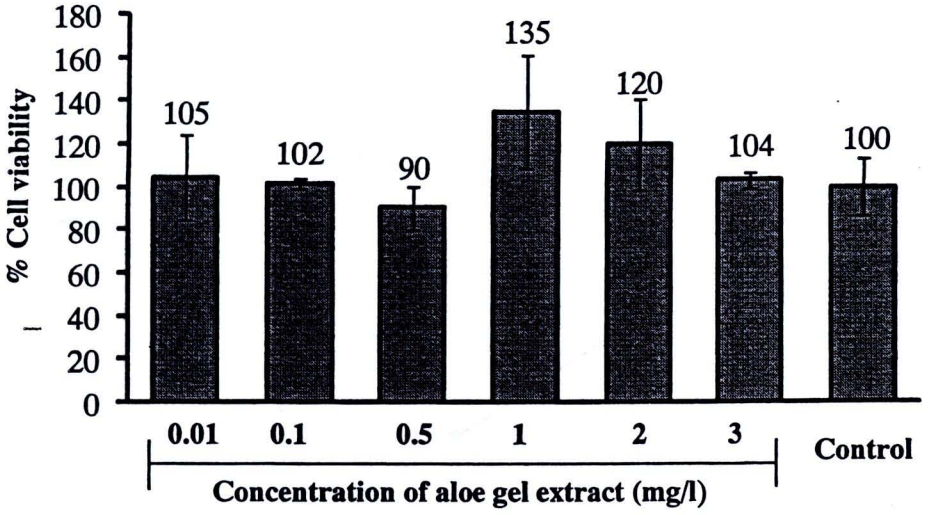

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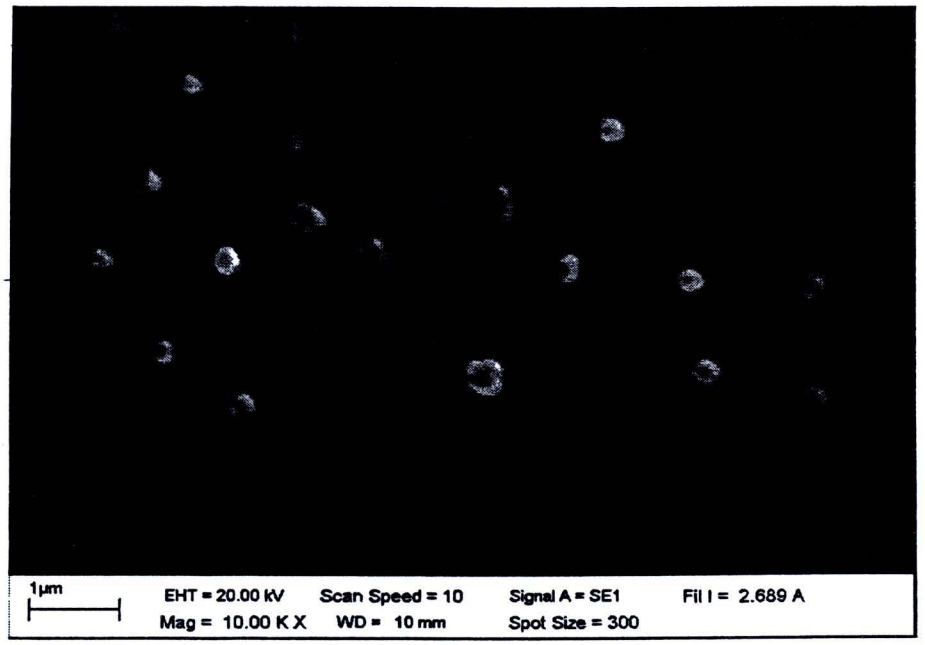

Figure 7 (J. Viyoch)

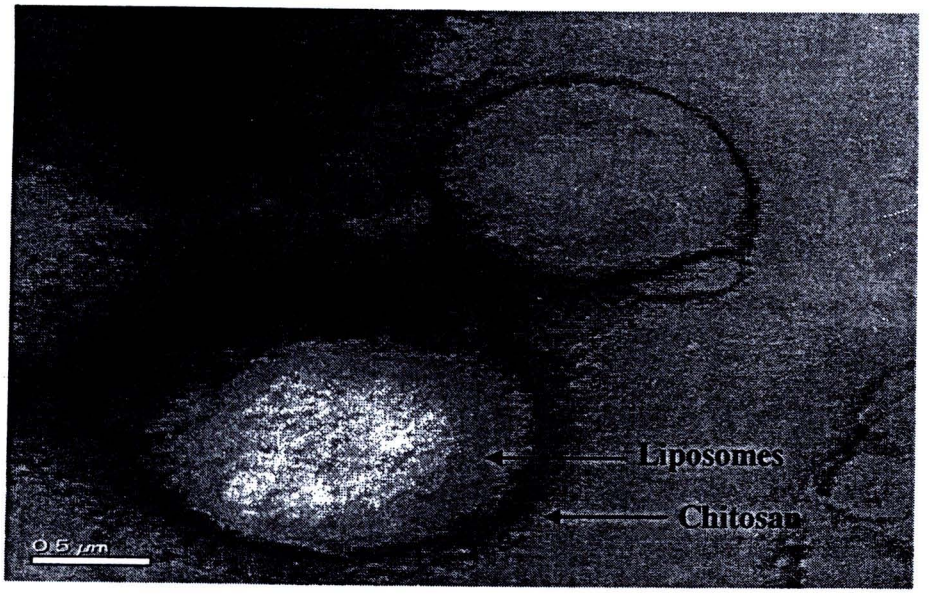

Figure 8 (J. Viyoch)

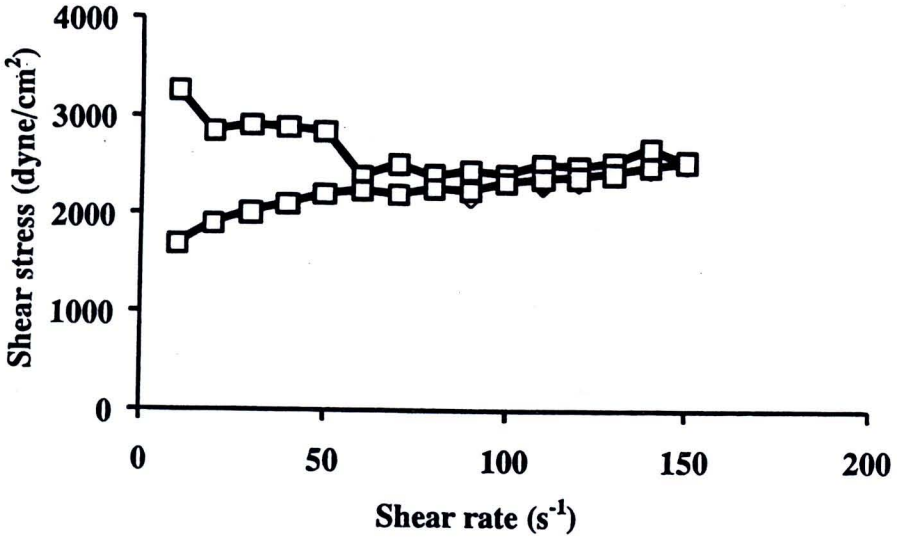

Figure 9 (J. Viyoch)