CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals

1. Ethanol (Labscan Asia, Ireland)
2. Methanol (Labscan Asia, Ireland)
3. Tween80 (VIDHYASOM Co., Ltd., Thailand)
4. Propylene glycol (VIDHYASOM Co., Ltd., Thailand)
5. Glycerin (VIDHYASOM Co., Ltd., Thailand)
6. Mineral oil (VIDHYASOM Co., Ltd., Thailand)
7. Jojoba oil (VIDHYASOM Co., Ltd., Thailand)
8. Citiol HE (VIDHYASOM Co., Ltd., Thailand)
9. Conc. Paraben (VIDHYASOM Co., Ltd., Thailand)
10. Gentamicin (Schering-Plough., Ltd., Indonesia)
11. Benzoyl peroxide (Sigma Chemical Co., USA)
12. Gallic acid (Sigma Chemical Co., USA)
13. Trolox (Sigma Chemical Co., USA)
14. Quercetin (Sigma Chemical Co., USA)
15. 2,2'-Diphenyl-1-picrylhydrazyl (DPPH, Sigma Chemical Co., USA)
16. 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Wako Pure Chemical Industries, Japan)
17. Thiobarbituric acid (TBA, Sigma Chemical Co., USA)

18. 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH, Wako Pure Chemical Industries, Japan)
19. Coomassie Brilliant blue G-250 (Sigma Chemical Co., USA)
20. Bovine serum albumin (BSA) (Sigma Chemical Co., USA)
21. Trifluoroacetic Acid (TFA, Sigma Chemical Co., USA)
22. α-L-Rhamnose (Sigma Chemical Co., USA)
23. D (+)-Xylose (Merck KGaA, Darmstadt, Germany)
24. L (+)-Arabinose (Merck KGaA, Darmstadt, Germany)
25. D (+)-Galactose (Merck KGaA, Darmstadt, Germany)
26. HCl (Sigma Chemical Co., USA)
27. NaOH (Sigma Chemical Co., USA)

3.2 Algal materials

Rhizoclonium hieroglyphicum (C.Agardh) Kützing was collected from the Nan River, Nan Province, Thailand

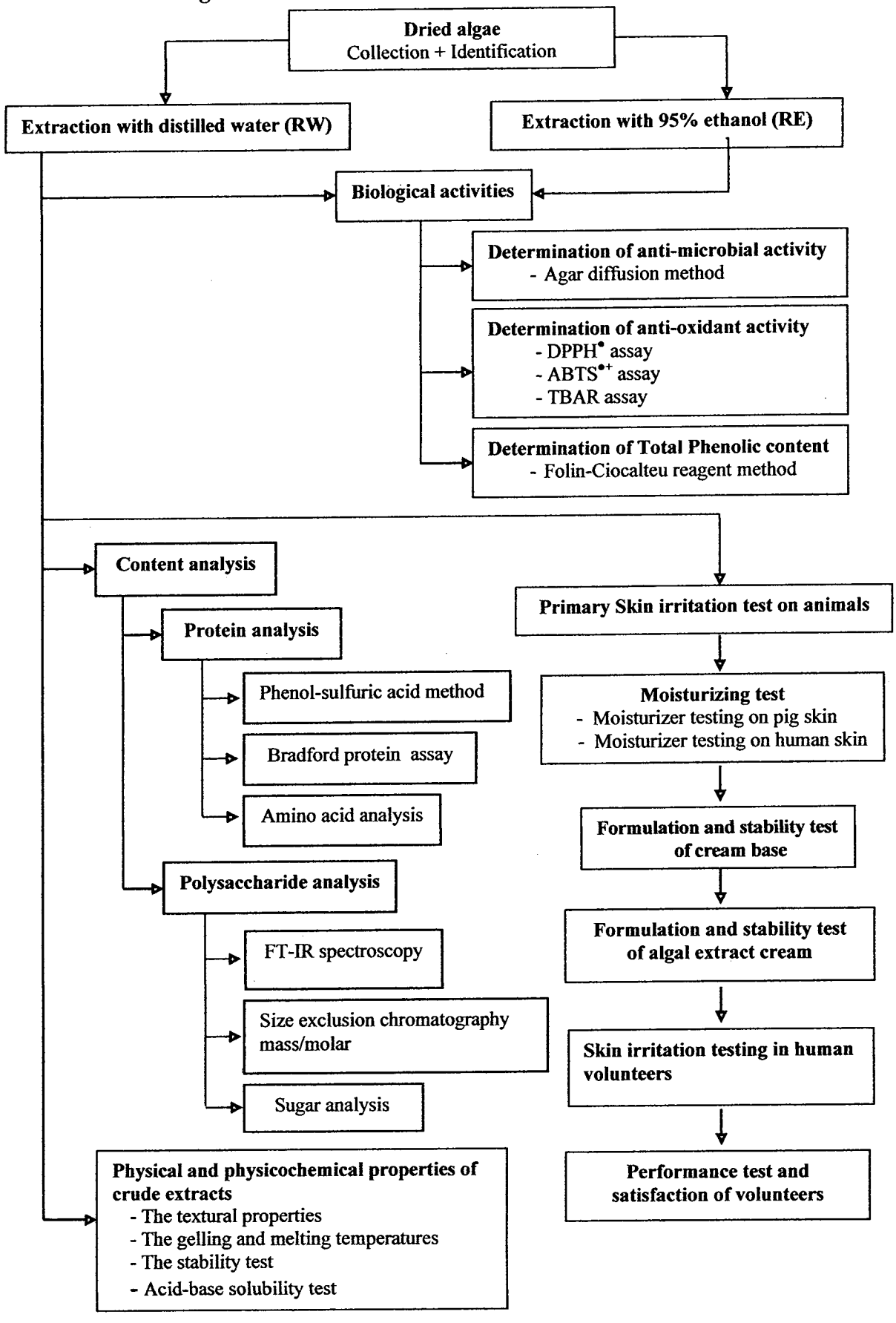
Spirulina platensis was purchased from Boonsom Farm, Chiang Mai, Thailand and its ethanolic extracts (SE) was used as a positive control for the antimicrobial and antioxidant activity assays.

3.3 Instruments

1. pH meter (Horiba Model EX-20, Korea)
2. Corneometer® (MPA580, CK Electronic GmbH, Germany)
3. Freeze dryer (Christ / Alpha 2-4, Germany)
4. TA.XT *Plus* Texture Analyzer (Stable Micro System, UK)

5. Water bath (Memmert®, CMbH Co., Ltd., Germany)
6. Microtiter plate reader (Beckman Coulter®, DTX 880 multimode detector, Australia)
7. Microplate 96 well (Nunc®, USA)
8. Spectrophotometer (Shimadzu UV-Vis 2450, Japan).
9. Evaporator (Buchi, Switzerland)
10. Fourier transform infrared (FT-IR) spectroscopy (Thermo nicolet NEXUS 470 FTIR,USA)
11. Rotary evaporator (Rotavapor R-210, Buchi Labortechnik AG, Switzerland)
12. Analytical balance 4 position (Precisa XT220A, Switzerland)
13. Vortex mixer (Scientific Industry, USA)
14. Autoclave (HICLAVE® HVE-50, Hirayama Manufacturing Co., Ltd., Japan)
15. Rheometer (Brookfield Model DV-III, Brookfield Co., Ltd.)
16. Auto pipett 1-200 l, 1-1000 l (pipetman®, Gilson Co., Ltd., France)
17. Incubator (Napco® Model 332, National Appliance Co., Ltd., USA)
18. Laminar air flow hood (Nuaire®, USA)
19. Anaerobic jar (ENVIMED Co. Ltd., Bangkok)
20. Anaerobic generator, Gaspak®, Mitsubishi Anaeropak® (ENVIMED Co. Ltd., Bangkok)
21. Sonicator (Elma®, Elma GmbH & Co KG, Germany)
22. Heating mantle (KIKA®, Yellow line, Germany)
23. Hot air oven (Memmert®, CMbH Co., Ltd., Germany)
24. High speed homogenizer (Model : Yellow line DI 25 basic, IKA Werke GmbH & Co, KG Germany)

3.4 Research designs



3.5 Collection and identification

R. hieroglyphicum was collected from the Nan River, Nan Province, Thailand (latitude 19°05′12.12″ N and longitude 100 ° 47′14.91″ E) between November and March. The samples were identified using the morphological features of its macroscopic and microscopic structures [119-121]. The fresh algae were washed and dried at 60 °C for 48 hours, then ground into powder and stored in a vacuum desiccator at room temperature for further study.

3.6 Extraction of the algae

Extraction was performed on 50 g dry weight of the alga. The dried powder was produced in 1 liter each of two different solvents, distilled water and 95% (v/v) ethanol, at 50 °C. The solutions were separated from the residual alga by filtration using a No.1 Whatman filter and then dried to obtain aqueous extract (RW) and ethanolic extract (RE), respectively. These were done in triplicate. The aqueous extracts were freeze-dried while the ethanolic extracts were evaporated under vacuum until dryness. The dry extracts were stored in a vacuum desiccator at room temperature for further study. The percentage yield of the dry weight of each extract was then calculated.

3.7 Determination of antimicrobial activities

The algal extracts (RE, RW) were examined for their antimicrobial activities by Agar diffusion method [133]. Trypticase soy agar (TSA) was inoculated with the test organisms – *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* (MRSA) and *Propionibacterium acne* ATCC 6919 (obtained from the Microbiology

Laboratory, Faculty of Medicine, Chiang Mai University) (0.5 McFarland turbidity standards) and allowed to solidify. Twelve millimeter-diameter wells were punched into the agar and then filled with 200 µl of the extract. Two and 5 % (w/v) of the extracts were used. Distilled water was used as a negative control and two standard antimicrobial agents (gentamicin and benzoyl peroxide) were used as positive controls. *S. aureus* and MRSA plates were incubated at 37 °C for 24 hours and the *P. acne* plate was incubated at 37 °C for 72 hours under anaerobic conditions. The antimicrobial activity was determined by observation and measuring the inhibition zone diameter. These were compared with the positive control, *S. platensis* extract (SE).

3.8 Determination of antioxidant activities

Antioxidant activity of *R. hieroglyphicum* extracts was determined by three different methods, scavenging effects on 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) assays as well as inhibition on lipid peroxidation (thiobarbituric acid-reactive substance, TBARS) assay. The antioxidant activities were compared with the extract from a well-known antioxidative alga, *S. platensis* (SE) and 3 reference standards, namely gallic acid, trolox and quercetin.

3.8.1 Determination of antioxidant activity by free radical scavenging activity on 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) The antioxidant activity in term of radical scavenging ability based on hydrogen donating capacity was measured using the DPPH method adapted from Brem *et al.* [134]. Twenty microlitres of each extract with different dilutions were added to 180 µl of 167 µM $DPPH^{\bullet}$/ethanol

solution in a 96-well microtiter plate. After incubation at room temperature in the dark for 30 minutes, the absorbance of the mixtures was measured spectrophotometrically at 540 nm with a multimode detector (DTX 880, Beckman Coulter). Gallic acid served as the reference standard. The antioxidant activity of each sample was then calculated in terms of its Gallic Acid Equivalent Antioxidant Capacity (GEAC) and IC_{50} value (mg/ml). The inhibition percentage of the sample was calculated by the following equation:

$$\% \text{ Inhibition} = [\ (A_0 - A_1) / A_0\ ] \times 100$$

A_0 is the absorbance of the control; A_1 is the absorbance of the test compound.

3.8.2 Determination of antioxidant activity by free radical scavenging activity on 2,2'-azino-bis3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$) The antioxidant activity in term of radical scavenging ability by electron donation was measured using a modified version of the method described by Re *et al.* [105]. The stock solutions included 7 mM $ABTS^{\bullet+}$ solution and 2.45 mM potassium persulfate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hours at room temperature in the dark. The solution was diluted by mixing 1 ml $ABTS^{\bullet+}$ solution with 30 ml of absolute ethanol to obtain an absorbance of 0.7-0.9 units at 734 nm using a spectrophotometer. Fresh $ABTS^{\bullet+}$ solutions were prepared for each assay. Each extract (70 µl) was allowed to react with 630 µl of the $ABTS^{\bullet+}$ solution for 5 minutes in the dark. The absorbance was then measured at 734 nm using a UV-2450 spectrophotometer (Shimadzu UV-Vis 2450, Japan). Trolox was used as the reference standard. The antioxidant activity of each sample was expressed in terms of its Trolox

Equivalents Antioxidant Capacity (TEAC) and IC_{50} value (mg/ml). The percentage inhibition was calculated as previously described in DPPH assay.

3.8.3 Determination of antioxidant activity by inhibition of lipid peroxidation using the thiobarbituric acid reaction substances (TBARS) assay

The antioxidant activity in terms of inhibition on lipid peroxidation was measured using a modified TBARS assay [135-136]. The liposome suspension (600 µl) and 100 µl of each sample was mixed with 60 µl of 0.07 M AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) into the tube and incubated at 60 °C for 24 hrs. Then 80 µl of the mixture was mixed with 24 µl of 0.02% w/v BHT, 100 µl of 3% v/v Triton X-100, 500 µl of 20% v/v acetic acid pH 3.5 and 250 µl of 0.2% w/v TBA in a microtiter plate. The reaction mixture was incubated for 30 minutes at 90 °C and then cooled to room temperature. The absorption of the mixture was measured at 540 nm with a multimode detector (DTX 880, Beckman Coulter). Quercetin was used as the positive control. The inhibition of lipid peroxidation was expressed as IC_{50} value (mg/ml). The percentage inhibition was calculated as previously described in DPPH assay.

3.9 Determination of total phenolic content in *R. hieroglyphicum* extracts

The total phenolic content of the extracts was determined using the Folin-Ciocalteu reagent method [135]. Each sample was mixed with Folin-Ciocalteu reagent for 3 minutes and followed by addition of 7.5% (w/v) sodium carbonate. The solutions were mixed using a vortex mixer. The reaction mixture was incubated for 30 minutes in the dark. The absorption of the mixture was measured at 765 nm using a UV-2450 spectrophotometer (Shimadzu UV-Vis 2450, Japan). The concentration of

total phenolic compounds in all samples was expressed as milligrams of gallic acid equivalents (GAE) per gram of the extract.

3.10 Content analysis of RW extract

3.10.1 Phenol-sulfuric acid method

Phenol-sulfuric acid method was employed to determine the total quantity of sugar in RW. Fifty µl of sample was added in a 96-well microplate, followed by 150 µl of concentrated sulfuric acid and the mixture was shaken for 30 min. Then, 30 µl of 5% phenol in water was added and the mixture was heated for 5 min at 90 ^{0}C in a water bath, then cooling at room temperature. The absorbance was measured at 490 nm using a multimode detector. The results are expressed in terms of a glucose. [138].

3.10.2 Protein analysis

3.10.2.1 Bradford protein assay

The protein content was determined by Bradford assay [139]. One hundred µl of each extract was added to 1 ml of Coomassie reagent and incubated for 2 minutes at room temperature. The absorbance was measured at 595 nm using spectrophotometer (Beckman DU-62, Backman Coulter). Protein determination was done by comparison to protein assay standards, usually prepared as a series of known dilutions of bovine serum albumin (BSA).

3.10.2.2 Amino acid analysis

Amino acid residues were analyzed as described by AOAC [140]. Approximately 2-3 mg of the RW extract was added to 6 N HCl 15 ml, then heated at 100-110 ^{0}C for 72 hours, followed by methyl chloroformate derivatization, which

could be identified and quantitatively determined by the gas chromatography equipped with a mass spectroscopic detector (GC-MS).

3.10.3 Polysaccharide analysis

3.10.3.1 Fourier transform infrared (FT-IR) spectroscopy

A FT-IR spectrum of the alga extract was recorded in the 4,000-400 cm^{-1} range using a Nicolet Nexus Model 470 FT-IR instrument with KBr pellet technique.

3.10.3.2 Determination of the molecular weight by size exclusion chromatography (SEC) mass/molar

The RW extract was separated on a SEC system consisting of two columns filled with Sephacryl S200 (100 x 1.5 cm) and Biogel P2 (120x1.5 cm) using bidest water as an eluent, flow rate : 0.6 ml min^{-1}; injection volume : 5mL (304 mg crude extract/5ml bidest water); mass detector (RI-detector); recorded by analog recorder with 0.2mm/min (Pharmacia Biotech REC 102); fractions (3 ml) were collected by means of a fraction collector FRAC-100 (Pharmacia). Each fraction was freeze-dried and stored in a vacuum desiccator at room temperature for further study. The molecular weight distributions were obtained from the refractive index detection according to Praznik and Huber [141]. For each fraction, 3 mg of the sample was dissolved in 1 ml of 0.05 M NaCl. The flow rate was set to 0.6 ml/min by a LKB2150 HPLC-pump. Lag-molecular of refractometer- and fluorimeter-profiles was attained by prior determined retention-shift for methanol.

3.10.3.3 Sugar analysis

Approximately 2-3 mg of each SEC fraction was mixed with 0.5-1.0 ml of a 2M TFA solution in a glass vial sealed with a screw cap and left for hydrolysis to be completed for two hours at 100-110 °C. The solution was dried under a stream of

nitrogen at 60 °C until the TFA was totally evaporated. One ml of absolute methanol was added to the solution and dried under nitrogen. This process was repeated until the solution was neutral and then re-dissolved in 1 ml of distilled water. The hydrolyzed solutions were stored in a refrigerator (2-8°C).

Approximately 30 µl of the standard sugar solutions (Merck Chemical Co.) and the fractions were applied to the bottom of the TLC plates using Linomat 5 with a syringe. The spots on the TLC plates were detected by spraying Thymol reagent. The TLC plates were dried using a hair dryer and then heated in an oven for 5-10 minutes at 105-110 °C.

For the analysis, standard solutions for the comparison of monosaccharide constituents were prepared and the contents of monosaccharide were determined with the aid of the Un-SCAN-IT program. Two, 4 and 6 µl of sample and 1, 2 and 3 µl of standard solution A and B were applied (The concentration of standard B is 50% of that of standard A) see Table 3.1.

Table 3.1 Preparation of standard sugar solutions A and B

monosaccharide	mg/ml MeOH	
	Standard A	Standard B
Rhamnose	0.5	0.25
Xylose	1	0.5
Arabinose	1	0.5
Galactose	1	0.5

The TLC plates were developed with acetonitrile:water (17:3). The spots on the chromatograms were detected with the thymol reagent. The plates were dried

with a hair dryer and heated in the oven for 7 min at 100°C for producing the color of the spots.

3.11 Physical and physicochemical properties

3.11.1 Morphology and observation of algal extracts using scanning electron microscope (SEM)

The algae extracts were mounted on scanning electron micrograph stubs, sputter-coated with gold and observed with a JSM5910LV (JEOL Ltd., Japan) scanning electron microscope.

3.11.2 Textural properties

Textural properties were determined with a Texture Analyzer equipped with a 10 mm in diameter cylindrical plunger operating at a cross-head speed of 1.0 $mm.s^{-1}$ [13, 33]. The measurements were performed on a 10% aqueous solution of *R. hieroglyphicum* gel after stabilization for 24 hours at room temperature. The gel strength, rupture force, deformation, cohesiveness and flexibility of the gels were determined. These were compared with kappa-carrageenan.

3.11.3 Gelling and melting temperature

Gelling and melting temperatures were determined by a modified method of Rodriguez *et al.* [33]. The gelling temperature was measured on 10 ml of a 10% (w/v) hot solution of the agar. The gels were placed in glass test tubes at a cooling rate of 0.5 °C $minutes^{-1}$. A glass rod was periodically introduced in the agar solution and the temperature at which a permanent hole developed was considered as the gelling temperature.

The melting temperature was determined by heating the 10% w/v gel with a temperature increase of about 0.5 °C minutes^{-1}. The temperature at which a glass bead (on the surface of the gel) dropped to the bottom was recorded as the melting temperature.

3.11.4 Solubility test

The solubility of the RW extract in various solvents was tested. DI water, mixtures of DI water:ethanol (ratio of 1:1, 1:2, 2:1), propylene glycol, tween80 (1%), glycerin, mineral oil, jojoba oil and PEG-7 glycerylcocoate were used as solvent. The RW extract was added to each solvent at the ratios of 1:10, 1:15, 1:20, 1:30 and 1:50. These were mixed with vortex mixer and kept at room temperature and 60 ^{0}C for 10 min. Then, the tested solutions were observed for their solubility and compatibility.

3.11.5 Acid-base solubility test

The solution of HCl (1N) or NaOH (10% w/v) was added to the RW extract solution to adjust the pH to 2-9. Then, their physical changes were observed immediately and after storage in various conditions; at room temperature, 4°C and 45°C for 1 month and the 8 cycles of heating/cooling condition (45°C 48 hr alteration with 4°C 48 hr for 1 cycle).

3.12 Primary skin irritation testing on animal [142-144]

The Draize model and its modification are commonly performed to assay skin irritation. In this study, three albino rabbits were used for the skin irritation test by a modified Draize model. Approximately 24 hr prior to the test, three rabbits were removed of their fur at dorsal area. Each test substance was applied to a gauze patch (1x1 inch) then it was covered over the assigned test site. After occlusion period

(4 hr), patches were removed and the test sites were cleaned with water. The erythema and edema formations on rabbits'skin were observed and scored at 1, 24, 48 and 72 hrs after removing the patches using Draize scoring system (Table 3.2). The primary dermal irritation index (PDII) was calculated and classified for their irritation reactions (Table 3.3). The PDII was obtained by the sum of the average erythema and edema scores at each time obtained and dividing by 4 time intervals. (1, 24, 48 and 72 hrs)

Table 3.2 Draize-FHSA Scoring System [144]

Topics	Score
Erythema and eschar formation	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Edema formation	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised > 1mm)	3
Severe erythema (raised > 1mm and extending beyond the area of exposure)	4

Table 3.3 Classification of skin irritation

Primary dermal irritation index (PDII)	Classification of skin reaction
< 0.5	Non-irritating
0.5-2.0	Slightly-irritating
2.1-5.0	Moderately-irritating
> 5.0	Severe-irritating

3.13 Skin moisturizing test

Since Kai are mainly composed of polysaccharides with arabinose, rhamnose, xylose and galactose as a sugar unit) and amino acids which possess a moisturizing property, the moisturizing effect of RW extract, therefore, should be further studied.

3.13.1 Moisturizing test on pig skin

The moisturizing capacity of the RW extract (0.1%) was examined and compared with 5% glycerol (5G), 5% propylene glycol (5PG), 0.1% of hyarulonic acid (0.1 HA). The pig skins were prepared from abdomen of the pig; aged 6 months, and removed off the fat layer and cut into 1x1 inch. Each sample (0.2 ml) was applied on the skin surface. The skin without any substances was used as a control. The moisture content had been measured before applying on sample and after application at 5, 15 and 30 min intervals using Corneometer®. Before applying the sample and recording the parameter, the pig skins were kept at room temperature for 30 min. This method had been adapted from O'Goshi *et al.* [145].

3.13.2 Moisturizing test on human skin

The moisturizing capacity of the 0.1% RW extract (0.1RW) was examined on the skin of normal human volunteers and compared with 5% glycerol (5G), 5%

propylene glycol (5PG), 0.1% hyarulonic acid (0.1HA), 0.1% carrageenan (0.1CA), 0.1% sodium alginate (0.1AL). The skin without any substances was used as a control. Prior to applying the sample and recording the parameters, the volunteers were rested for 30 min at room temperature. Each sample was applied on the forearm of the volunteers (aged 30-60, n=30) in area of 1.5x1.5 inch. To area of testing, an amount of approximately 0.2 ml of each assigned test formulation was applied. The moisturizer content was measured before and after applying the sample at 10, 15 and 30 min. intervals using Corneometer®. This method had been adapted from Keng *et al.* [146].

3.14 Formulation and stability test of the cream base

3.14.1 Formulation of the cream base

Five cream bases were developed from various compositions by conventional hot process. In the oil phase, glyceryl monostearate, stearic acid, cetyl alcohol, cyclomethicone, jojoba oil and ceteareth-25 were heated at 70°C while triethanolamine (TEA), parabens and deionized water were heated together at 75°C in water phase. These were then mixed and homogenized until the homogeneous emulsion was obtained and cooled down to be a cream. The preparation was determined for their physical properties, pH, spreadability, viscosity (Pas) and feel on skin.

3.14.2 Stability test of the cream base

The stability of the three cream bases was investigated in various conditions; room temperature, room temperature in the dark, 4°C and 45°C for 1 month and the heating/cooling condition of 8 cycles (1 cycle defined as 45°C for 48 hrs to 4°C for 48

hrs). Then the physical changes (such as odor, color, pH, appearance, smoothness and rheological changes) of the cream bases were observed immediately and after storage. The most stable cream base was then selected to incorporate with tested moisturizers [147].

3.15 Formulation of moisturizing cream and stability test of alga extract cream

Each test substance: 5% glycerol (5G), 5% propylene glycol (5PG), 0.3%, 0.5% hyaluronic acid (0.3HA, 0.5HA) and 0.3%, 0.5% *R. hieroglyphicum* extract (0.3RW, 0.5RW) was incorporated into the selected cream base to obtain glycerin cream (CG), propylene glycol cream (CPG), 0.3%, 0.5% hyaluronic acid cream (CHA0.3, CHA0.5) and 0.3%, 0.5% *R. hieroglyphicum* extract cream (CRW0.3, CRW0.5), respectively. The stability of each cream was carried out as previously described in 3.14.2. Their pH and visually physical changing along with color, smoothness and unstable conditions (creaming and cracking) were investigated compared with freshly prepared [147].

3.16 Clinical evaluation

Clinical evaluation on normal human volunteers for skin irritation and moisturizing efficacy were performed after it had been approved by the Ethical Review Committee of the Faculty of Pharmacy, Chiang Mai University.

3.16.1 Skin irritation testing in human volunteers [142, 148-149]

The skin irritation test was carried out on 30 healthy volunteers, both male and female (aged 30-60 yrs) which has been modified from Bashir and Maibach [142]. Draize scoring system was used to calculate the primary dermal irritation index

(PDII). Prior to participating in the clinical study, all of the volunteers received the information of this study and signed a written informed consent that contained all the basic elements outlined.hur

3.16.2 Subjects of the study

Thirty Thai volunteers aged 30-60 years were selected by using the following inclusion and exclusion criteria.

Inclusion criteria

1. Healthy skin, no skin diseases such as dermatitis
2. Unnecessary using, receiving or taking any preparation such as antihistamine drug or any other drugs.
3. Non-atopic, with no past or present history of skin diseases
4. No any scar, wound, blemish or any skin diseases
5. No irregular skin color at test site
6. Subjects agreed to sign an informed consent form
7. Comfortable to involve in this study

Exclusion criteria

1. Subjects who did not or could not sign an informed consent form, unable to comply with the requirements of the protocol
2. Subjects who were participating in any other clinical study

Discontinuation criteria

1. Had skin irritation
2. Subjects who wanted to quit from the experiment for any reason
3. Subjects who could not practise following instruction criteria of study

3.16.3 Test substance application protocol

The upper back area of each volunteer was divided into two parts, left and right (duplication). Each side of the upper back was treated by Finn chamber that contained the samples. After 48 hr of application, the patches were removed and the test sites were cleaned suddenly with purified water. The skin irritation was evaluated at 1, 24 and 48 hr after patch removal, based on Draize scoring system. 1% w/v sodium lauryl sulfate (SLS) was used as a positive control and deionized water as a negative control.

3.17 Performance test and satisfaction of volunteers

The short-term moisturizing effect of each test cream was conducted with 30 healthy volunteers. They were instructed to apply each test cream on each site of their forearms. The volunteers were rested at room temperature (21±2°C, 50-60% RH) for 30 min [150]. To each testing area (4cm x 4cm), 0.2 g of each assigned test cream was applied. Untreated area on one site of volunteer forearm was used as a control (Figure 3.1). The data was collected prior to applying the test cream and again after applying the product for 15, 30 min, 1 hour using Corneometer®. In addition, the long-term use for 1 week (twice daily: morning-evening) was also evaluated [146]. At the end of the test, the volunteers finally filled out a questionnaire, presenting their satisfaction about the test creams after using them for 1 week.

3.17.1 Volunteers of the study

Thirty Thai volunteers aged 30-60 years were selected by using inclusion and exclusion criteria, same as the skin irritation testing in human.

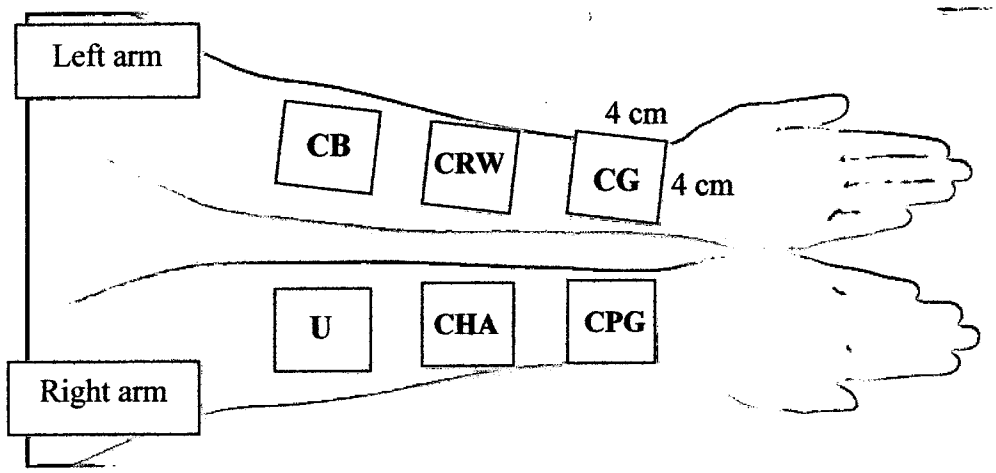


Figure 3.1 Application sites of moisturizing test

CB: Cream base

U: Untreated

CRW: Cream base + RW extract

CHA: Cream base +Hyaluronic acid

CG: Cream base + Glycerol

CPG: Cream base + Propylene glycol

3.18 Statistical analysis

All data are expressed as means ± S.D. Data were analyzed by an analysis of variance ($p<0.05$) and the means compared between group by cluster analysis and ANOVA post hoc Tukey's b Test. Results were processed by computer programs: Excel and SPSS version 17.0.