CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

1. 2,2'-Azobis(2-methylpropionamidine) dihydrochloride 97 % (AAPH) (Sigma-Aldrich Inc., USA)
2. 2,2'-Diphenyl-1-picrylhydrazyl 85 % (DPPH) (Sigma-Aldrich Inc., USA)
3. 2-Thiobarbituric acid 98 % (TBA) (Sigma-Aldrich Inc., USA)
4. Absolute ethanol (Merck KGaA., Germany)
5. Acetic acid (Lab-Scan Ltd., Ireland)
6. Alpha-tocopherol acetate (Sigma-Aldrich Inc., USA)
7. Butylated hydroxytoluene (BHT)
8. Cholesterol from lanolin 99 % ($C_{27}H_{46}O$) (Sigma-Aldrich Inc., USA)
9. Dipotassium hydrogen phosphate (K_2HPO_4) (Sigma-Aldrich Inc., USA)
10. Ethyl acetate (Lab-Scan Ltd., Ireland)
11. Formic acid (Fisher Scientific UK Ltd., UK)
12. Gallic acid (Sigma-Aldrich Inc., USA)
13. Hexane (Lab-Scan Ltd., Ireland)
14. Phosphatidylcholine (Lucas Meyer GmbH, Germany)
15. Potassium dihydrogen phosphate (KH_2PO_4) (Sigma-Aldrich Inc., USA)
16. Quercetin (Sigma-Aldrich Inc., USA)
17. Resveratrol (Merck KGaA., Germany)
18. *t*-Octylphenoxypolyethoxyethanol (Triton X-100)

3.2 Natural extracts

All of the extracts that used in this study were commercially claimed for their anti-aging/anti-wrinkle activities, and provided by Rubia Industries Limited.

1. French maritime pine bark extract (PBE) (Horphag Research Ltd., Switzerland) (*orange powder*)
2. Red wine extract (RWE) (Seppic Inc., France) (*red brown powder*)
3. Grape seed extract (GSE) (Mibelle AG Biochemistry, Switzerland) (*brownish clear liquid*)
4. Pomegranate extracts (PME-1 & PME-2) (*yellowish clear liquid*)
    a. PME-1 (Green Tech Ingenierie Biologique, France)
    b. PME-2 (Berli Jucker Public Co. Ltd., Thailand)
5. Soy bean extract (SYE) (Mibelle AG Biochemistry, Switzerland) (*light yellowish clear liquid*)
6. Dulse algae extract (DSE) (Parc Technologique Ltd., Canada) (*clear liquid*)

3.3 Instruments

1. Analytical balance (Model: BP610) (Sartorius Mechatronics Co. Ltd., Germany)
2. Centrifuge (Model: Sigma 2-6) (Sigma Laborzentrifugen GmbH, Germany)
3. Clear 96-well microplate (Nunc®) (Thermo Fisher Scientific Inc., USA)
4. Climatic chamber (Model: F-115) (Binder Ltd., UK)
5. Finn chamber® (Allerderm Laboratories Inc., Finland)
6. Homogenizer (Model: Yellow line DI 25 basic) (IKA Werke GmbH & Co. KG, Germany)

7. Microcentrifuge tube 1.5 ml (Hycon Plastics Inc., USA)
8. Micropipette (Rainin®) (Mettler-Toledo Ltd., USA)
9. Microplate reader (Model: DTX-880 multimode detector) (Beckman Coulter Inc., USA)
10. pH meter (Model: EX-20) (Horiba Ltd., Japan)
11. Rheometer (Model: R/S-CPS) (Brookfield Engineering Laboratories, USA)
12. Rotary evaporator (Model: Rotavapor R-210) (Buchi Labortechnik AG, Switzerland)
13. Silica gel 60 F_{254} plate (Merck KGaA., Germany)
14. Skin-Visiometer (Model: SV 600 FW) (CK Electronic GmbH, Germany)
15. Water bath incubator (Model: WNB 14) (Memmert GmbH & Co. KG, Germany)

3.4 Determination of antioxidation activities

The antioxidation activities of the commercially claimed extracts were evaluated by two *in vitro* methods, i.e. the DPPH radical scavenging method and the lipid peroxidation assay (TBARS method) [66, 67]. The absorbance was measured by using Beckman Coulter Multimode Detector DTX-880, and calculated for the percentage of inhibition by the following equation:

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

where A_C is the absorbance of the control (consisting of all reagents without the test antioxidant), and A_A is the absorbance of the test antioxidant. The curve between the

percentage of inhibition and the concentration had been plotted, and calculated for the half maximal inhibitory concentration (IC_{50}). The antioxidant activities of the extracts were compared with quercetin, gallic acid, resveratrol, oligomeric procyanidin [*appreciated to Dr. Nasapon Povichit for OPC supply*] and alpha-tocopherol as reference standards.

3.4.1 DPPH radical scavenging method

All test antioxidants were initially prepared into the ethanolic dilution series, and the DPPH in ethanol was prepared in the concentration of 167 μM. The antioxidant solution was mixed with the DPPH solution at ratio of 9:1 in microplate. The mixture was shaken well, and incubated in the dark at room temperature for 30 min [68, 69]. The absorbance of the resulting solution was measured at 520 nm.

3.4.2 TBA-reactive substances (TBARS) method

Liposome suspension, consisting of cholesterol, phosphatidylcholine and 0.2 M potassium phosphate buffer solution (pH 7.2), was mixed with the antioxidant solution. 0.07 M AAPH solution in water was added to induce lipid peroxidation [67]. The mixture was then incubated for 24 hr at 50 °C. After incubation, 0.2 % w/v BHT solution in ethanol (to terminate lipid peroxidation), 3 % v/v Triton X-100 solution in ethanol, 20 % v/v acetic acid solution and 0.6 % w/v TBA solution were added. The resulting mixture was then heated at 90 °C for 30 min. After cooling at room temperature, the absorbance was measured at 540 nm.

3.5 TLC chromatograms of selected extracts

The extracts with high antioxidant activities were selected, and their TLC chromatograms were performed on Merck Silica gel 60 F_{254} plates. Some standards and chemical were also spotted in the same plates as references, i.e. Merck resveratrol standard (RS), Sigma-Aldrich alpha-tocopherol acetate standard (α-E), and oligomeric procyanidin (OPC) [*from Dr. Nasapon Povichit*]. The solvent systems were adapted from Medic-Saric M et al. (2004) [70], and the proper solvent system was the mixture of hexane/ethyl acetate/formic acid (4:2:1, v/v/v).

After developing with mobile phase, TLC plates were observed under visible light, short wavelength UV (254 nm) and long wavelength UV (365 nm). Furthermore, the chromatograms were sprayed with 167 μM ethanolic DPPH solution to confirm the antioxidant spots or bands.

3.6 Solubility of selected extracts

Solubility of selected extracts was investigated. The descriptive terms of solubility were also displayed in Table 3.1.

Table 3.1 The descriptive terms of solubility [71]

Descriptive Term	**Parts of Solvent Required for 1 Part of Solute**
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to100
Slightly soluble	From 100 to 1,000
Very slightly soluble	From 1,000 to 10,000
Practically insoluble, or Insoluble	Greater than or equal to 10,000

In this study, the selected extracts were dissolved in four commonly cosmetically used solvents including propylene glycol, 5 % v/v tween 80, 2 % v/v PEG-400 and mineral oil at ratio of 30:1.

3.7 Stability of selected extracts

The selected extracts were conducted at the accelerated stability test: heating-cooling cycling, which defined as alternation of storage conditions from 45 °C for 48 hr to 4 °C for 48 hr (one cycle), for six cycles. Afterwards, the extracts were analyzed by DPPH radical scavenging method.

3.8 Development of serum base

3.8.1 Formulation of serum base

Serum bases were o/w emulsions which developed from various compositions; cetyl alcohol, cyclomethicone, jojoba oil, avocado oil (Lipovol®), cremophor A-25, xanthan gum, carbopol ETD 2020, tween 20, propylene glycol, EDTA disodium, triethanolamine (TEA), concentration parabens and deionized water.

The preparation method was conventional hot process (beaker method). All ingredients were divided in two parts – oil phase (part A) and aqueous phase (part B). Part A were melted and mixed in approximately 65 °C. Part B were also mixed and heated to about 65 °C. Then, all heated components were combined by adding part B into part A, and TEA was also added. The emulsion was homogenized, and other additives were then added after cooling down.

The preparations were determined for their physical properties, pH, spreadability, feel on skin and short-term stability within a week. And serum base formulation with the best performance was selected for the further stability test before developed into active serum (incorporated with selected extracts).

3.8.2 Stability test of selected serum base

The stability of selected serum base was tested by heating-cooling cycling for six cycles. Moreover, pH and visually physical changing along with color, smoothness and unstable conditions (creaming and cracking) were also investigated.

3.9 Development of active serum

3.9.1 Formulation of active serum

After serum base formulation had been chosen, selected extracts in proper solvent were combined. Then, active serums were determined for their physical properties, pH, viscosity, spreadability and feel on skin.

The stability of active formulations was conducted at heating-cooling cycling likewise serum base. And their pH, viscosity and visually physical changing were also investigated.

3.9.2 Rabbit skin primary irritation test

The safety of active serums was examined by rabbit skin primary irritation. Three healthy young adult male albino rabbits – *Oryctolagus cuniculus* (strain: New Zealand white; NZW) were used as test animals, and kept separately in the 20±3 °C

experimental animal room. Approximately 24 hr before the test, fur had been removed from the test area by clipping from the dorsal area. Then, 1 ml of test substances, i.e. serum base, active serums, 1 % w/v sodium lauryl sulfate (SLS) as positive control and deionized water as negative control, were applied to 25x25 mm^2 areas and covered with gauze patches. After 4 hr of exposure, gauze patches were removed and the areas were cleaned with water. The test areas were observed at 1, 24, 48 and 72 hr after removal for erythematous and edematic reactions. The reactions were scored based on Draize scoring system. The primary irritation index (PII) was calculated and categorized for type of irritation [72, 73].

Table 3.2 Draize scoring system

Reactions	Value
Erythema and Eschar Formation:	
No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beef 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 approximately 1 mm)	3
Severe edema (raised > 1 mm and extending beyond area of exposure)	4

Table 3.3 Classification of skin irritation

Primary Irritation Index (PII)	Classification of skin reaction
0 – 0.4	No irritation
0.5 – 1.9	Slightly irritation
2.0 – 4.9	Moderately irritation
5.0 – 8.0	Severe irritation

3.9.3 Selection of active serum

The active serum with the best performance toward physical appearances, pH, spreadability, feel on skin, stability and without irritation reaction was selected and used for the further studies.

3.10 Stability and antioxidant activities of active serum

3.10.1 Stability test of active serum

Stability of serum base and selected active formula was evaluated in four stability test conditions, i.e. six cycles of heating-cooling cycling and four months of room temperature (RT), cool place (4°C) and hot place (45°C).

3.10.2 Antioxidant test of active serum

Serum base and selected active serum were extracted by absolute ethanol, and centrifuged at 4,000 rpm for 30 min. The antioxidant supernatant was determined by DPPH radical scavenging method and TBARS method at before (t=0) and after four stability test conditions.

3.11 Skin compatibility test of active serum in human volunteers

Skin compatibility, as defined in Walker AP et al. (1996) [74], was evaluated with single application closed patch epicutaneous test under occlusion. Initially, the test protocol was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. The volunteers received the information (Appendix A) regarding the nature of study, timetable, limitation and possible risks, and gave their written informed consent (Appendix B) prior participation.

3.11.1 Study population

Thirty-two healthy Thai volunteers (aged 20-50) were selected on the basis of inclusion and non-inclusion criteria.

Inclusion criteria

- Informed volunteers
- Subjects agreeing to follow the conditions in the study information sheet

Non-inclusion criteria

- Any active skin disease that may interfere with the study
- Blemishes or marks (e.g. tattoos, scars, sunburn) on the test sites
- Irritated skin on test sites
- Pregnancy or nursing condition
- Medication that may affect skin response (e.g. using antiallergic drugs), or past medical history
- Participation in another clinical study

Withdrawal criteria

- Having any severely adverse effects
- No longer wish to participate in the study

3.11.2 Test materials

Test substances were serum base, selected active serum, 1 % w/v sodium lauryl sulfate (SLS) as positive control and deionized water as negative control. Test sites were both left and right of upper back (duplication). And the devices were Finn Chambers® as an occlusive closed patch epicutaneous test devices.

3.11.3 Test method

The test sites were firstly cleaned by gently swabbing with water. Then, 0.1 ml of test substances, with the sequence according to Figure 3.1, was applied to upper back skin using Finn Chambers® for 48 hr. The test sites were also cleaned suddenly with water after patch removal, and skin compatibility was then assessed at 1 hr, 24 hr and 7 days for the irritation responses based on Draize scoring system.

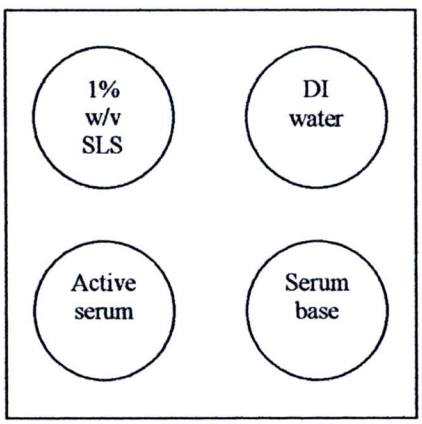

Figure 3.1 Sequence of application on skin compatibility test

3.12 Performance test of wrinkle reducing capacity of active serum

This clinical study protocol was adapted from Lee KK et al. (1999) [75]. The protocol was approved by the Committee on Human Rights Related to Human Experimentation of Chiang Mai University. The volunteers were informed and agreed with the consent before participation in the study.

3.12.1 Study population

A total of twenty-five informed volunteers (aged 20-50) were recruited on the basis of inclusion and non-inclusion criteria which paralleled to the criteria for the skin compatibility test. Prior to the measurement, they were left in the 20±2 °C room for at least 30 min in order to allow full skin's adaptation [76].

3.12.2 Test method

This clinical study was designed as a randomized, single-blinded, placebo controlled, trail. The purpose was to evaluate the wrinkle reducing effects of active serum, compared with serum base (as placebo) and untreated skin (as control), at the beginning of the study (day 0) and after 6 weeks of applications.

The subjects were instructed to apply approximately 0.1 g of test formulations twice daily, once in the morning and once in the evening, for 6 weeks. The test sites were the upper part (1 inch below the elbow) and the lower part (1 inch above the wrist) of lower forearms, left and right (Figure 3.2). The orders of application were shown in Table 3.4. Skin-Visiometer SV 600 FW was used as device measuring four parameters – Ra, Rz, surface and volume. And the percentage of efficiency was calculated by the following equation:

$$\% \text{ Efficiency} = [(V_M - V_0) / V_0] \times 100$$

where V_0 is the value at initial point (day 0), and V_M is the value at measuring point (6 week).

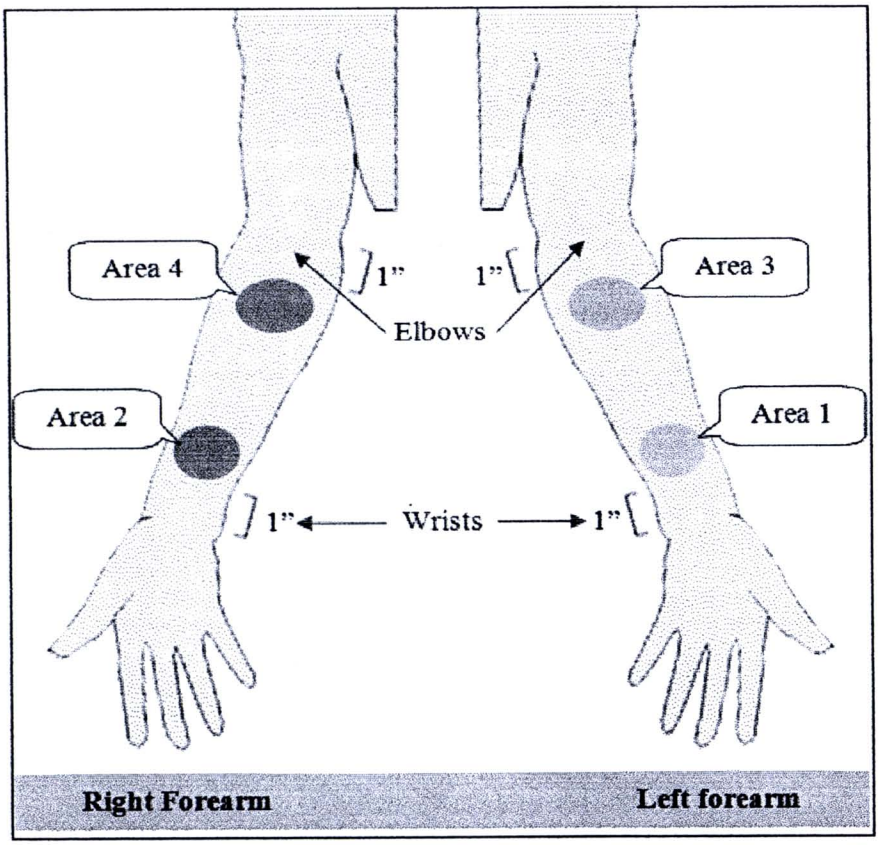

Figure 3.2 The test sites of clinical study

The paired *t*-test was used for comparison of multiple measured data points (untreated skin, placebo and active serum) using statistical software, SPSS version 14.0 for Windows. Differences were accepted as statistically significant at $p < 0.05$.

Table 3.4 The application orders for clinical study

No.	Test site application			
	Area 1	**Area 2**	**Area 3**	**Area 4**
1	Active serum	Serum base	Control	-
2	Active serum	Serum base	-	Control
3	Active serum	Control	Serum base	-
4	Active serum	Control	-	Serum base
5	Active serum	-	Serum base	Control
6	Active serum	-	Control	Serum base
7	Serum base	Active serum	Control	-
8	Serum base	Active serum	-	Control
9	Serum base	Control	Active serum	-
10	Serum base	Control	-	Active serum
11	Serum base	-	Active serum	Control
12	Serum base	-	Control	Active serum
13	Control	Active serum	Serum base	-
14	Control	Active serum	-	Serum base
15	Control	Serum base	Active serum	-
16	Control	Serum base	-	Active serum
17	Control	-	Active serum	Serum base
18	Control	-	Serum base	Active serum
19	-	Active serum	Serum base	Control
20	-	Active serum	Control	Serum base
21	-	Serum base	Active serum	Control
22	-	Serum base	Control	Active serum
23	-	Control	Active serum	Serum base
24	-	Control	Serum base	Active serum
25	Active serum	Serum base	Control	-

Note: The symbol (-) refers to the unused site.

3.12.3 Satisfaction on test serums

After finishing the clinical test, volunteers were questioned on the product satisfaction. The questionnaire (Appendix C) was regarding product appearances (color, odor, and texture/smoothness), skin feel characteristics (softening, viscosity, spreadability, skin absorption, greasiness, tackiness, skin moisture, and film forming) and overall satisfaction.