CHAPTER II

REVIEW OF RELATED LITERATURE AND RESEARCH

This part contains 9 main parts which are related to this study including skin structure, skin properties related to the measurement outcomes, facial cleansing lotion, the instruments for evaluating skin properites, alpha-hydroxy acids, tamarind, study products (interventions), clinical research methodology, and relevant studies of the efficacy and safety of facial cleansing product containing extract of tamarind's fruit pulp. The details of them were given below.

1. Skin structure

The skin is the largest organ of human body, covering an area of about 16 square feet. Generally, it is classified into 4 types, based on composition of skin hydration and lipid, including oily, mixed, normal, and dry skin type. Normal skin pH is 4.5-5.5 [32-33]. The skin is responsible for protecting human body from heat, cold, water loss, microoraganism, and physical and chemical injuries. It provides human with sensory information and is also an excretory organ, removing toxins from the body via perspiration. The skin consists of tree layers including epidermis, dermis, and hypodermis or subcutaneous tissue as show in figure1 [34]. Each layer has particular functions but works in coordination with the next layer.

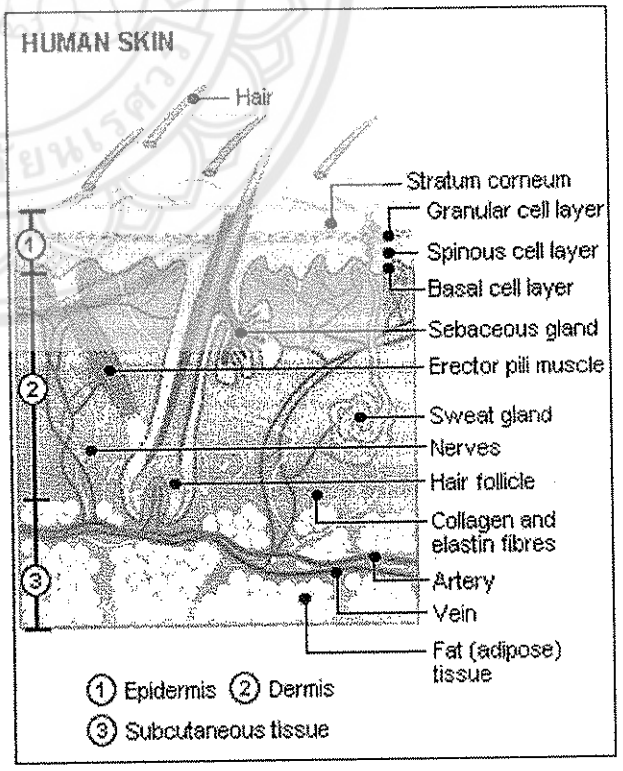


Figure 1 Skin structure

1.1 Epidermis

The epidermis is the outermost layer of the skin. Therefore, it is the first barrier between human skin and environments. Generally, the thickness of this layer is 75-150 micrometers. However, it varies in age, sex, and body region. The epidermis consists of different five layers including stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. Stratum basale is the lowermost of the epidermis and it contains the important cell as keratinocytes. It is constantly undergoing mitosis and producing new cells. Within 2 to 4 weeks, new cells migrate to the surface, then passing the stratum lucidum out to the stratum coneum. Besides keratinocytes, stratum basal also contains melanocytes, which produce melanin or skin pigmentations and transport them to the superior epidermal cells.

The Langerhans and the Merkel cells are found in the epidermis layer. The Langerhans cells function as macrophages, it catches invading antigens and delivers them to the immune system. The Merkel cells function as epidermal nerve cells

1.2 Dermis

The dermis is the middle layer of the skin located between the epidermis and subcutaneous tissue. A structure characteristic of this layer is fibrous network of protein. It is the thickest of the skin layers and comprises many types of cells including fibroblasts, which synthesize collagen, elastin, and other structural molecules, sebaceous glands, sweat glands, hair follicles as well as a relatively small number of nerves and muscle cells (Figure1). This layer is responsible for the skin's structural integrity, elasticity and resilience. In addition, wrinkles arise and develop in the dermis. Therefore, an anti-wrinkle treatment product has a chance to succeed if it can reach as deep as the dermis.

1.3 Subcutaneous tissue (Hypodermis)

Subcutaneous tissue is the lowermost layer of the skin. It is a layer of fatty tissue that provides nourishment to the dermis and upper layers of skin. It also conserves body heat and cushions internal organs against trauma. Blood vessels, nerves, and deeper hair follicles are found in this layer.

2. Skin propertied related with outcome measurement in this study

In general, skin property is defined by a combination of surface texture, color, and physiologic properties including hydration, sebum content, and surface acidity. In addition, the characteristics of skin condition are affected by endogenous and environmental factors including aging, exposure to sunlight, chemicals, and mechanical damage [32-33]. The detail of each skin property is given below.

2.1 Skin color

Skin color is important parameter to evaluate the efficacy of whitening products. Skin color or skin pigmentation in human results from the synthesis and distribution of melanin in the skin. Melanin is the pigment that mainly gives the color to the skin. This pigment is produced in the melanocytes of epidermis and migrates in small particles to the epidermal cells within 24-48 hours after sun exposure. The differences in racial skin pigmentation depend on the quantity of melanin pigments produced and the deposition of these pigments throughout the epidermis. In addition, melanin plays a crucial role in the absorption UV light or radiation, thus it protects the skin against sunburn. The over strain of the protection mechanisms of the body by radiation will lead to erythema. The erythema is a critical reaction to radiation, the border at which a burn develops. It looks red because the arterial vessels extend under the damaged part of the skin to allow transportation of nutritive and moisturizing substances for the healing.

Several factors have influence on skin color. The genetic and sunlight are mainly factors for appearance of skin color. The other factors are melanocyte stimulating hormone, health status such as pregnancy, and drug such as contraceptive [35].

2.2 Skin hydration or water content of skin

Water is the main substance of the body. A newborn's water content is approximately 75% of their weight while an adult is approximately 50-60%. The water content of the skin surface is important parameter because it provides appearance of a soft, smooth, and healthy skin.

Many factors affect the value of skin moisture. Normally, body temperature of the human is higher than the environment's temperature so body balances this difference by evaporation. Under normal climatic conditions (20° C, 40-60% air humidity), the

temperature regulation is mainly done by evaporation. The measured body site also influences the moisture value, especially due to the thickness of the skin and the activity of the sweat glands. For example, the moisture content is very high on the forehead and the palms while it is mostly very dry on the arm. The higher of air humidity and the room temperature can induce the higher of the skin's moisture level. In addition, the life style of a person, the food, the regular consumption of nicotine and alcohol, often washing the skin, and the use of pharmaceutical and cosmetic products influence the moisture level. Moreover, hormones, emotion stress, physical exercise may also lead to the increased transpiration and changes of the value of skin moisture so the test persons should rest at least 10-20 minutes before measure the moisture level [36].

2.3 Skin pH

The pH-value of the skin results from water-soluble substances contained in the horny stratum and the secretion of perspiration and sebum as well as the exuded carbonic acid. Normal skin pH is 4.5-5.5 [32-33]. However, this value varies depending on many factors including the tested skin area (pH of the cheek is higher than the forehead), age (pH-value increases with age), hormones, emotion stress, and physical exercise. Therefore, the participants should rest at least 10-20 minutes before measurement [36].

2.4 Skin elasticity

Improving skin elasticity is one of the efficacy claims for cosmetic products. Therefore, measurement skin elasticity should be performed. Physiological skin aging starts at the age of thirty. Indications of this are flabbiness, wrinkles, dehydration, blotchy pigmentation and loss of elasticity. The values of skin elasticity vary depending on many factors. The detail of each factor is provided below.

First, age has a very big influence on skin elasticity. With increasing age, the numbers of collagen and elastine fibres are reduced and elasticity decrease significantly. The elasticity values are very different depending on the body sites. Stretchiness decreases from head to foot. The most important factor is the thickness of the skin. The ability to return to the original state after strain is quite independent of the body site. Second, the UV radiation of the sun has a very big influence on the skin

elasticity because it penetrates the skin to the dermis and damages the elastine and collagen fibres. Sunlight is important cause of premature aging. In addition, sex, skin diseases and internal diseases as well as healing of wounds and scars, lifestyle, e.g. smoking, alcohol, drugs, food and sleep, pharmaceuticals products, cosmetic products, and room condition also influence skin elasticity [37].

2.5 Transepidermal water loss (TEWL)

Transepidermal water loss (TEWL) is a parameter, which describes ability of living skin cells for evaporation of water molecules into the surrounding environments. Thus, measurement of TEWL is important. The TEWL value express in g/hm^2. The lower the TEWL defines that the better of the barrier function of the skin and the lower natural moisture loss. The values of TEWL vary depending on many factors. The detail of each factor is provided below.

The temperature regulation mechanism of the body influences the TEWL. The higher the room temperature, the higher TEWL-value (at 30° C it is almost twice the 20° C-value). This value strongly depends on age. Newborn have a higher water vapour permeability of the skin because the barriers develop in the course of the first 2 to 4 weeks. Therefore, TEWL level decreases with increasing age. The measured body regions have influence on the TEWL. Graduations are: palms > sole of the foot > forehead = ear region = nail = back of the hand > forearm = upper arm = thigh = chest = abdomen = back. In addition, air humidity, airflow and direct light radiation cause changes of the measuring value of TEWL [38-39].

3. Facial cleansing lotion

Facial cleansing lotion is a skin care product which is used to remove dirty materials such as oily residues from cosmetics. Normally, this cleanser is applied by hand to remove oily deposits of colors or pigments from the skin, and then wiped out with tissues or cloth. Oil-in-water (o/w) emulsions work satisfactorily for this purpose because the water phase can wash the dirt without leaving any oily feeling. In the last decade, there has been increasing interest in adding ingredients for other cosmetic purposes. For

example, alpha hydroxyl acids (AHAs) are added to facial skin cleanser formula to provide skin lightening effects.

Moisturizers, both emollients and humectants, within cleansers can maintain skin hydration as well as maintaining and restoring barrier function [40]. Emollients impair evaporation of skin moisture by forming a film on the skin surface to impede water loss. Humectants attract and bind water, drawing it up from the dermis into the epidermis. The acid mantle of the skin plays an integral role in skin barrier function as well as regulating bacterial flora [41]. Studies have shown that skin barrier regeneration proceeds more slowly at neutral pH (7.2) than at physiological pH 4.5-5.5 [32-33].

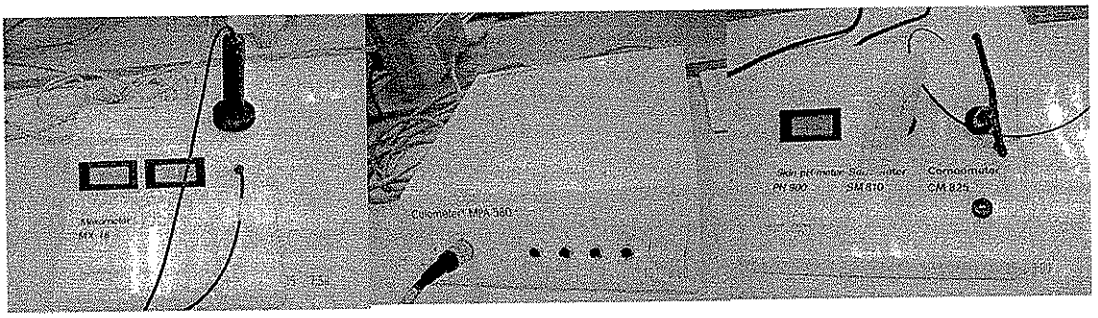
4. The instruments for measuring skin properties

In this study, we use five instruments including Mexameter, Corneometer, pH meter, Tewameter, and Cutometer for measuring 6 skin properties. Figure 2 shows the picture of each instrument which uses in this study. The detail of each instrument is given below.

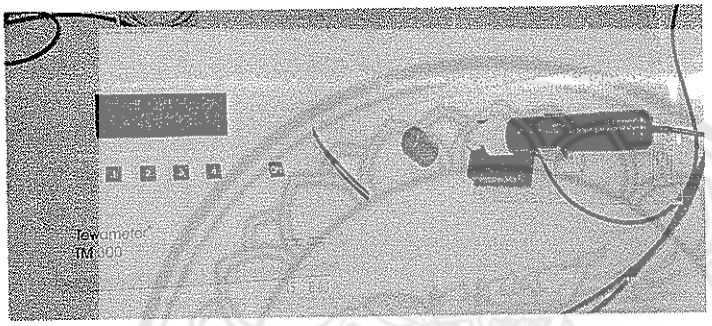
4.1 Mexameter® MX 18

Measurement of skin color and eryhtema can be made using two difference principle including spectrophotometric and tristimulus methods [42]. Spectrophotometric method uses either a broad band or selected wavelengths in the visible range with measurement of absorbance and reflectance. Tristimulus method analyzes three colors including blue, red, and green of light reflected from skin structure.

In this study, we use Mexameter® MX 18 (Courage and Khazaka Electronic GmbH, Cologne, Germany) to measure the skin color, and skin erythema (Figure2a.). Mexameter is an industry standard spectrophotometer designed to measure melanin and hemoglobin (erythema) content in the skin. The melanin value represents index of whitening effect of product while erythema value represents index of irritating effect of product [42]. This measurement is based on absorption and reflection. A measuring probe with a measuring area of 5 mm diameter emits light of three pre-defined wavelengths including green (wavelength=568$\pm$ 3 nm), red (wavelength=660$\pm$ 3 nm), and infrared (wavelength=880$\pm$ 10 nm) and measures the light reflected by skin.



a) Mexameter MX 18 b) Cutometer MPA 580 c) Skin-pH-Meter PH 900 and Corneometer CM 825



d) Tewameter TM 300 and its probe

Figure 2 The instruments for measuring skin properties in this study

The melanin value is measured using 2 wavelengths (660 and 880 nm) to achieve different absorption rate by the melanin granules. The erythema value or hemoglobin measurement is also measured using two wavelengths (568 and 660). The wavelength of 568 nm corresponds to the spectral absorption peak of hemoglobin. The other wavelength (660 nm) is chosen to avoid other color influences (e.g. bilirubin). The range of value is 0-1000, with higher value representing more melanin or erythema [35].

4.2 Cutometer® MPA 580

Currently, there are four techniques to measure elasticity of skin. These include indentation, extension, torsion, and suction techniques. However, the suction technique is the popular for measuring the skin elasticity.

In this study, we use Cutometer® MPA 580 (Courage and Khazaka Electronic GmbH, Cologne, Germany) to measure the elasticity of skin (Figure2b.). The measuring principle of this instrument is based on the suction and elongation method [43-44]. It is connected to a computer for control and display of the measurement. It measures the

vertical deformation of the skin in millimeters when the skin is pulled by means of a controlled vacuum into the circular aperture (standard is 2 mm in diameter) of the probe [44]. This instrument offers four different modes but mode one is generally selected and is recommended. In this mode, built-in database file can calculate the reading R0-R8 automatically. Then a time curve (Figure3) of skin deformation can be obtained. The typical skin deformation curve is composed of the following parts: Ue, immediate distention or the extent of skin stretching within the first 0.1 s of the vacuum period; Uv, delayed distention; Uf, final distention or the maximal skin extension is the deformation at the end of the vacuum period; Ur, immediate retraction or relaxation of skin within the first 0.1 s after ending of the vacuum.; and Ua, final retraction (Figure3).

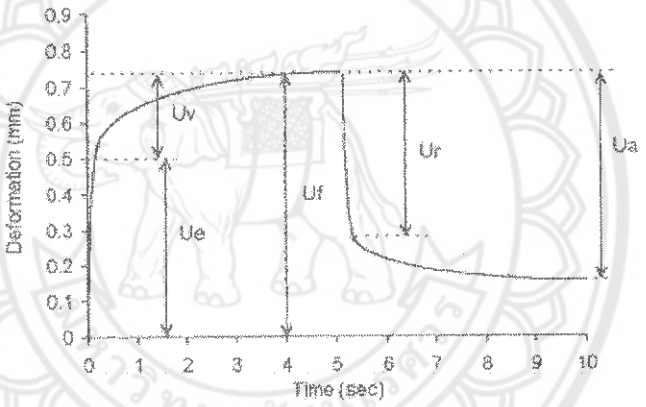


Figure 3 Time-curve of skin deformation obtained with Cutometer [45]

The Uf and Ue are linked to the stretching of collagen and elastic fibers [45-47]. The Ur is an indicator of skin ability to return to its initial position after deformation and is related to the function of elastic fibers [45-47]. Therefore, R2 (Ua/Uf), and R5 (Ur/Ue) are measured in this study. R2 or gross elasticity is the ratio of total retraction to total distention. This value is important for evaluating skin elasticity. R5 or net elasticity is the ratio between immediate recover and immediate deformation. This value is independent of skin thickness, thus it is considered to be a biologically important factor for the characterization of elasticity of the skin [43-48]. If these values close to 1, the skin elasticity is good.

4.3 Skin-pH-Meter® PH 900

The measurement of the pH-level on the skin surface is an important for evaluating the skin condition especially in developing soaps, cleanser or detergents. This value is determined by the concentration of hydrogen ions (H^+) and hydroxide ions (OH^-). The pH value from this device is given rang 0 (strongly acidic) to 14 (strongly alkaline), with a value of 7 representing neutrality.

In this study, Skin-pH-Meter® PH 900 (Courage and Khazaka Electronic GmbH, Cologne, Germany) is used to measure the skin pH (Figure2c). The measurement of the pH-value is preferably done with a glass electrode. It is very important that a reproducible potential is developed at this electrode which is not influenced by the measuring solution. The glass electrode is filled with an inner buffer (mercurous/calomel: Hg/HG_2Cl_2 or silver/silver chloride Ag/AgCl). This inner buffer is separated from the measuring solution by a special glass membrane and carries away the potential of the internal side of the glass membrane (one of the metals contained in the internal buffer). A reference electrode carries away the potential of the external side of the glass membrane, which contacts the measuring solution. As skin with its excretion is almost an aqueous solution pH-measurement can be performed directly on the skin surface.

For healthy skin and normal room conditions (20° C and 40-60% air humidity), the pH value in women and men shows following table 2. Based on the pH-value, we can detect the skin condition of each person including acidic, normal, and alkaline conditions [36].

Table 1 The range of skin pH-value in women and men [36]

pH-value	<3.5	3.8	4.0	4.3	4.5	5.0	5.3	5.5	5.7	5.9	6.2	6.5	>6.5
Women	+ acidic range -				Normal				- alkaline range +				
Men	+acidic range-				Normal				- alkaline range +				

4.4 Corneometer® CM 825

It has been known for a long time that the electrical properties of the skin are related to the water content of the horny layer. The Corneometer is one of instruments that its measuring principle is based on electrical property of skin. In this study, the Corneometer® CM 825 (Courage and Khazaka Electronic GmbH, Cologne, Germany) is

used for measuring stratum corneum hydration (Figure2c). This instrument measures the electrical capacitance of skin surface as an indicator of stratum corneum hydration [49]. One unit represents a water content of stratum corneum of 0.02 mg/cm^2, at a measuring depth of 20 nm [50]. Corneometer units rang from 0 to 120. These units are related to the hydration of the upper parts of the epidermis. The ranges of measurements are rather large: values of 30-40 units for very dry skin to 120 units for very hydrate skin. The sensitivity and reproducibility of the measurement are very high while the measurement time is very short, about 1 second [36].

4.5 Tewameter® TM 300

The measurement of the transepidermal water loss (TEWL) is used to evaluate the efficiency of the skin barrier function. An increase in TEWL reflects an impairment of water barrier of skin. In this study, Tewameter® TM 300 is used to measure the TEWL of skin (Figure2d). This instrument is included in guidelines that have been published about proper measurements of TEWL [39, 47]. Its measuring principle is based on the diffusion principle in an open chamber by measuring the pressure gradient of boundary layer resulting from the water gradient between the skin surface and ambient air. After completion of the measurement, the cursor remains automatically at the lowest average deviation of the curve [38, 51]. TEWL measurements can be affected by the anatomical site, sweating, skin surface temperature, inter-and intra-individual variation, air convection, and ambient air temperature [39, 51].

5. Alpha-hydroxy acids (AHAs)

5.1 General information of AHAs

Alpha-hydroxyacids (AHAs) are a group of naturally occurring organic acids with a hydroxyl group alpha to the carbonyl carbon of the carboxylic acid group [3] as shows in figure 4. There are several types of AHAs for example glycolic acid derieved from sugar cane juice, lactic acid derived from soured milk, tartaric acide derieved from tamarind and grape fruits, and malic acid derived from apple [52]. The primary action of AHAs is to exfoliate dead skin cells by weakening bonds that hold dead skin cells together, thus resulting in skin that looks bright [7-9]. By normalizing corneocyte

cohesion, the stratum corneum is thin, smooth and more flexible [6]. In addition, it also has a moisturizing effect by induced increased mucopolysaccharide content, in particular dermal glycosaminoglycans (GAGs) in the skin [7-9, 53]. The overall result is skin which looks and feels better [9].

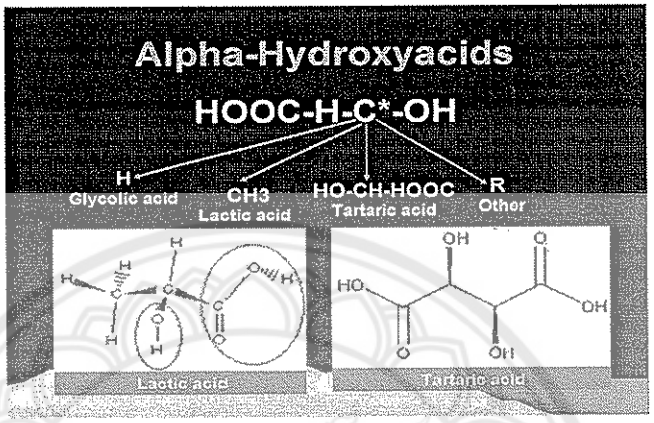


Figure 4 The structure of AHAs

The efficacy of AHAs is directly related to the amount of free acid that presents in the product. A low pH (pKa) means more free acid, and greater penetration into the dermis, but with a greater potential for adverse reactions. Many products are prepared with a high concentration of an AHA, but are "buffered" with an alkaline solution such as Sodium hydroxide or Ammonium hydroxide, decreasing the acidity (raising the pH) and irritation potential of the product. This is why some products containing as little as 2% AHA can be more effective than products containing 20% or even more AHA [54].

AHAs are commonly used in low concentrations (2-10%) in cleansers, moisturizers, and toners, and in higher concentrations as light peel solutions. Normally, daily cosmetic products in the market contain 2-10% of glycolic or lactic acids and their pH range from 3.5-4.0 [10-12]. It has been used for several decades by dermatologists to correct skin disorders including ichthyosis, acne, solar lentigos, melasma, xerosis, actinic keratosis, warts, seborrheic keratosis, and psoriasis [55-57]. Moreover, one commonly promoted use of AHA products is to correct photoaged skin [10, 22, 44-45, 51-54]. Due to the irritation effect, FDA recommends that the concentration of AHAs for daily products was 10% or less than that and pH of product should not less than 3.5 [54-56].

5.2 The consequence of AHAs in each skin layer

5.2.1 Stratum corneum

The initial response of the stratum corneum to an AHA is reduced corneocyte cohesion, thus it increases skin renewal rate and provides skin lighter [7-9, 55]. Histologically, separation is found to occur at the innermost level of the stratum corneum just above the stratum granulosum, resulting in a temporary thinning of the stratum corneum. This effect can found in application of both low and high concentrations of AHA products.

5.2.2 Epidermissss

The application of AHA containing products to human skin results in increased epidermal thickness and skin elasticity, and decreased skin wrinkles [8, 22, 25-26, 58-59]. However, these effects can found in the application of high concentration of AHAs (more than 10%). The possible mechanisms are the dispersal of melanin pigmentation, and increased glycosaminoglycan and collagen synthesis [25].

5.2.3 Dermis

Detectable dermal responses appear more slowly than epidermal responses, first appearing within 2 to 3 months and becoming more apparent thereafter [25, 63]. An initial clinical response to sustained applications is a distinct increase in skin thickness. Increased skin thickness is associated with increased stainable accumulations of dermal glycosaminoglycans, including hyaluranic acid [58, 64].

5.3 Adverse effects of AHAs

Possibilities of adverse effects when using AHAs products, especially in high concentration, are severe redness, swelling, burning, blistering, bleeding, rash, itching, and skin discoloration [54, 64-65]. No long-term effect use of AHAs has been reported to date. Furthermore, removal of the upper skin layer has been proven to increase photosensitivity in subjects [54, 66]. Thus, the FDA has recommended that cosmetic manufacturers should inform consumers about the possibility of increase photosensitivity. For example, "this product contains an alpha hydroxyl acid (AHA) that may increase your skin's sensitivity to sunburn", "use a sunscreen and limit sun exposure while using this product and for a week afterwards."

For people who will use AHAs, they should avoid sunlight as much as possible, wear hats that protect the ears and have a brim of at least 4 inches. It is helpful to wear gloves, long-sleeved shirts, and long pants, they should choose a broad-spectrum sunscreen with the SPF value of 15 or higher and should apply to areas that are still exposed, such as the lips and face. In addition, they should also attempt to see the exact AHA concentration on the product label and the product's acidity level (pH) before use on the skin.

5.4 Comparisons of synthetic and natural AHA

Many of AHA substances can be derived from natural sources and often referred to "fruit acid". However, a number of synthetic source provides access to compounds with structure analogous. Nowadays, the AHAs used in cosmetic products are usually produced by chemical synthesis because the synthetic products offer higher purity and quality in a dependable consistency [52].

Despite the increased use of AHA in cosmetic products, the debates over the potential differences of natural versus synthetic AHAs have still been ongoing for several years. The synthetic AHAs have a high ability to stimulate cell renewal and high irritation too while these properties are reduced in natural AHAs. It has been found that the therapeutic index (the ratio of skin stimulation to irritation) of the natural AHAs surpasses that of the synthetic AHAs. The possible reason for support this finding is natural AHAs contains natural soothing agents that can reduce their irritation potential, and not interfere with the stimulating activity [10]. Therefore, in the clinical viewpoint, using naturally derived AHAs in cosmetic products will provide much more benefit comparing to synthetically derived AHAs.

6. Tamarind

6.1 Overview of tamarind

Nowadays many kinds of botanical extract are playing an increasingly important role in cosmetics and tamarind extract is one of them. Tamarind *(Tamarindus indica L.)*, which belongs to the Leguminosae family, is a common tree which usually is found in humid tropical areas including Thailand. Its fruit pulp with acidic taste has been used for

centuries as skin-scrubbing material to smoother and lighter skin appearance. The improving visible effect on skin raises the question about the action of the components contained in its fruit pulp. Several studies revealed that the fruit pulp of tamarind contained naturally occurring organic carboxylic acids, alpha hydroxyl acids (AHAs) including tartaric acid (8-23.8%), ascorbic acid (0.7-3%), lactic acid (2%) and malic acid [13-21]. The main action of tamarind pulp's extract was to exfoliate dead skin cell as action of AHAs [22-24]. In addition, lactic acid was also a highly effective moisturizer [56]. Citric acid when topically applied, stimulates collagen synthesis while both tartaric and malic acids boost skin elasticity [25-28]. Besides AHAs, pectin (2.5%) and invert sugar (30-41%) were also found in tamarind fruit pulp. Both possess hygroscopic property and can improve the better looking of the skin by their moisturizing action [29].

Tartaric acid is one type of AHAs that is a key substance of tamarind for improvement the skin appearance [13-21]. Tartaric acid has a dicarboxylic acid with two hydroxyl groups at the alpha positions of the acid, similar to the compound formed from two molecules of glycolic acid. It is nontoxic, and is present as a carbohydrate metabolite in the skin. This acid is a fruit acid derived from tamarind and grape fruits, and is also found in the fermentation mixture for wine. Owing to the nonsymmetrical nature of the molecule, tartaric acid has isomeric forms such as O-tartaric acid, L-tartaric acid, DL-tartaric acid, and meso-tartaric acid [17]. All four isomers of tartaric acid are commercially available as white crystalline compounds and in soluble in water and alcohol.

6.2 Clinical trial of product containing tamarind's fruit pulp extract

There has not been any published reports of a clinical trial of products containing tamarind fruit pulp extract. Nowadays, the information of effects of them used based on evidence of general AHAs that did not specific with AHAs of tamarind pulp extract. However, the preliminary study[1] of oil-in-water (o/w) emulsion containing tamarind fruit pulp extract or tamarind cleansing lotion for 1 week with forearm of 12 subjects shows that the trends of this cleansing lotion was less irritation than the control product.

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[1] Preliminary study of oil-in-water emulsion containing tamarind fruit pulp extract by Jarupa Viyoch. Faculty of pharmacy, Naresuan University, Thailand.

Moreover, the trends of skin conditions including skin moisturizing, skin lightening, skin pH, and skin elasticity are improved with this lotion than control product.

7. Interventions or study products

Interventions of this study are two different products including the test and the control products for cleansing facial skin. Both study products are oil in water (o/w) emulsion and they are the same physical characteristics excepting test product contains the extract of tamarind's fruit pulp, which consists of natural AHAs. The proper formula and stability of these products have been conducted in previous study [30]. According to this study [30], the product did not show any sign of incompatibility or precipitation after standing at the room temperature for 1 month. The stability test using heat-cool cycling method shows that this product did not show any sign of creaming and coalescence, and was not significant different in the viscosity, color, smoothness of texture, pH, and tartaric acid concentration of the emulsion between before and after the heat-cool cycling. The viscosity of it was also comparable to that of the commercial product. Moreover, the pH of product was 4 that was close to the normal pH range of the skin (4.5 to 5.5) [32] and could maintain AHAs activity [10]. The formula of each product and basic function of each ingredient are provided in table 2.

The study products are freshly prepared before start clinical study. All materials are cosmetic grade and all of them are supplied by Namsiang International Co.,Ltd, Thailand. Tamarind powder is prepared using fresh fruit pulp of tamarind with a brownish red color and acid flavored. One kilogram of fruit pulp without seed is extracted with 4.5 L water for overnight at room temperature. After that, the resultant paste is filtered through a cloth to remove rubbish. Then, the aqueous solution of fruit pulp was dried by lyophilization (FTS system Dura dry type FD 95C12, USA). In addition, the amount of tartaric acid in the extract is determined, as a marker of AHA concentration in the extract, by isocratic HPLC technique (Model CM3200, Sitronic Co., USA). Tamarind powder contains approximately 24% of tartaric acid.

Table 2 The formula of each product and basic function of each ingredient [28]

Ingredients	Placebo product (%w/w)	Test product (%w/w)	Basic function
Oil phase			
Span®80	1.51	1.51	Nonionic surfactant
Isopropyl myristate	5.00	5.00	Emollient
Stearyl alcohol	1.00	1.00	Stiffening agent, emollient
Stearic acid	1.50	1.50	Stiffening agent
Liquid paraffin	3.00	3.00	Occlusive emollient
Water phase			
Tween 60	5.50	5.50	Nonionic surfactant
Propylene glycol	5.00	5.00	Humectant
Glycerin	5.00	5.00	Humectant
Disodium EDTA	0.15	0.15	Chelating agent
Carbopol aqua SF	0.20	0.20	Gelling or thickening agent
Triethanolamine (TEA)	0.70	0.70	Neutralizing agent
Tartaric acid	-	2.0	Active ingredient
Methyl paraben	0.20	0.20	Preservative
Propyl paraben	0.02	0.02	Preservative
Deionized water added to	100.00	100.00	

The preparation procedure of test product is as following. Water phase ingredients including Tween 60, Propylene glycol, Glycerin, and Disodium EDTA were added to the Carbopol aqua SF solution. The solution mixture of water phase is heated to 70 degree Celsius while oil phase ingredient is heated to 75 degree Celsius. The water phase is constantly added to the oil phase with rapid agitation. Agitation is continued until the emulsion was cooled down to 40-45 degree Celsius. The extract powder was dissolved in part of deionized water and then added to emulsion. After addition of methyl and propyl paraben, a certain amount of TEA is added to neutralized Carbopol aqua SF and the viscosity of the preparation was then increased [30]. For the placebo product the preparation procedure is the same as test product except it is not added powder extract to emulsion. According to these products have been tested the stability, thus we test only physical characteristic including pH, color, and texture of study products.

For the safety issue, material safety data sheet (MSDS) of each ingredient is provided in appendix J. In addition, the irritation test of study products is performed and the detail of it is provided in appendix M.

8. Clinical research methodology

8.1 Overview of clinical trials/study

A clinical trial is defined as a prospective study comparing the effect or value of intervention(s) against a control in human beings [67]. At baseline, the control group must be similar in relevant respects to the intervention group to ensure that differences in outcome come from an action of the intervention. The clinical trial is also called clinical study. It is often classified into four phases of experimentation. The detail of each phase is given below.

Phase I trials are the first trials in human and usually conducted in a small number (No more than 50 -100 subjects) of healthy volunteers and limited to single dose or a few repeated doses. It is also called pharmacology and toxicity trials. The objective of this phase is to define initial safety, identifies toxicity of drugs or product in human, and also to establish pharmacokinetic and pharmacodynamic profiles of the drug.

Phase II trials are also called initial clinical investigation for treatment effect trials. The objective of this phase is to evaluate efficacy and short-term safety in prime clinical conditions in selected population and to establish efficacy, side effect, clinical toxicities of drug. Usually studies in this phase provide the information of optimal dose or therapeutic dose range of drug or product.

Phase III trials are sometime called full-scale evaluation of treatment trials. This phase is a large-scale pivotal study which is designed to evaluate both efficacy and safety of an intervention. It is conducted in specific subject populations for which the drug is intended and conducted before regulatory submission and provides most of the information required for labeling.

Phase IV trials or post marketing surveillance trials are conducted after local regulatory approval or after the product available in market. Trials in this phase are designed to differentiate the drug from others in its class, compare its efficacy against

similar marketed compounds, and demonstrate health economic benefits in "real world" settings.

8.2 Types of clinical controlled trial designed

In general, randomized controlled trials designed are classified into two types including parallel design, and crossover design trials.

A fundamental requirement for the use of the crossover design is that the condition being studied must be stable, so that it will return to the baseline state when a test or treatment product is stopped, allowing subsequent assessment of the control product under the same conditions. This design is defined that the study is permitted the comparison of different treatment in the same subject (Figure5) while in parallel design each patient receives only one product or intervention (Figure6). The problems of parallel design are many intersubject variations and a large number of patients required while the subject variation between groups is eliminated and a few numbers of required patients are advantages of crossover design. One problem of crossover design is that the administration of the first treatment may influence the response to the second, called carry-over effect [67-69], however it can be minimized by designing the trials with a suitable wash-out period between treatments or conduction the within patient design.

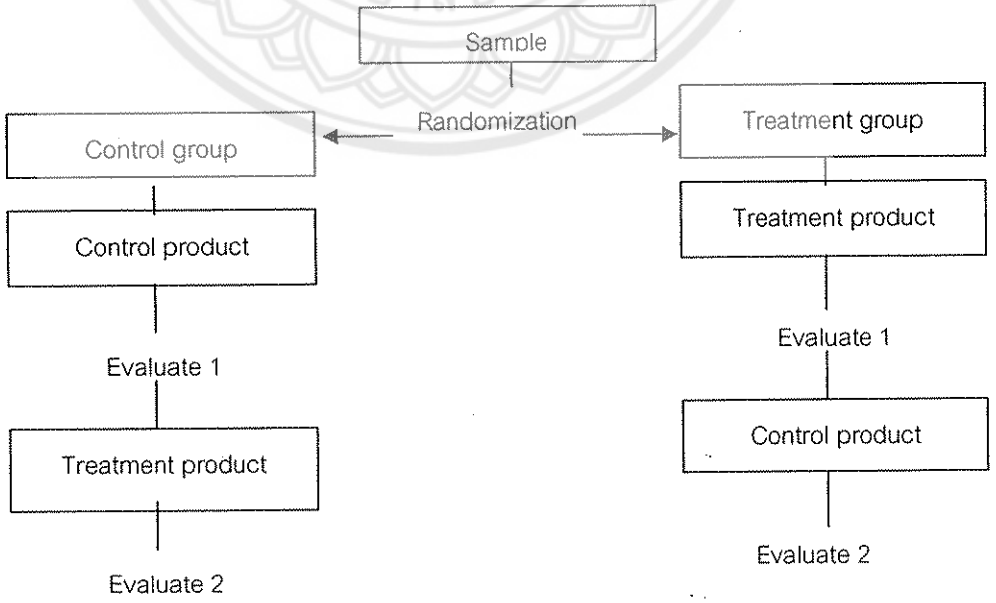

Figure 5 The crossover design

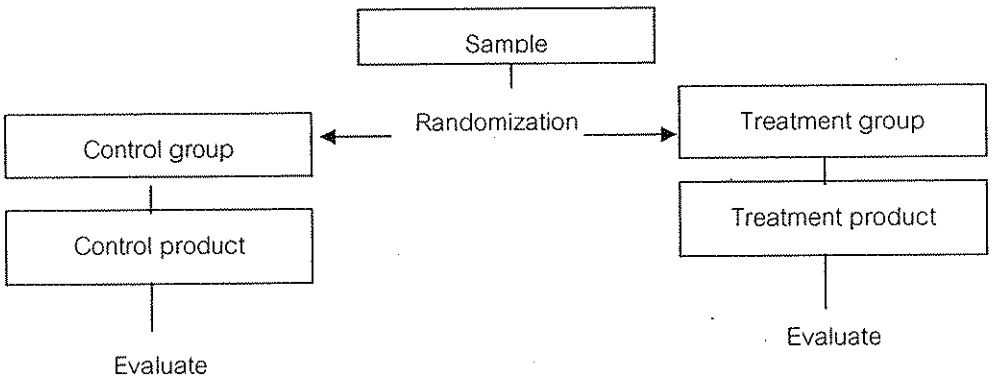

Figure 6 The parallel design

For clinical trial in cosmetic product, within patient design trial was well known design because it can eliminate the limitations of parallel design and crossover design trials. Within patient design is defined that the study with experimental and control treatments are applied to different locations on each patient (Figure7). This design is very similar to crossover design; however, two interventions in this design were used and evaluated in the same time. Therefore, subject variation between groups is eliminated and a few numbers of required patients are advantages of this design like the crossover design while the problem of the carry-over effect is eliminated.

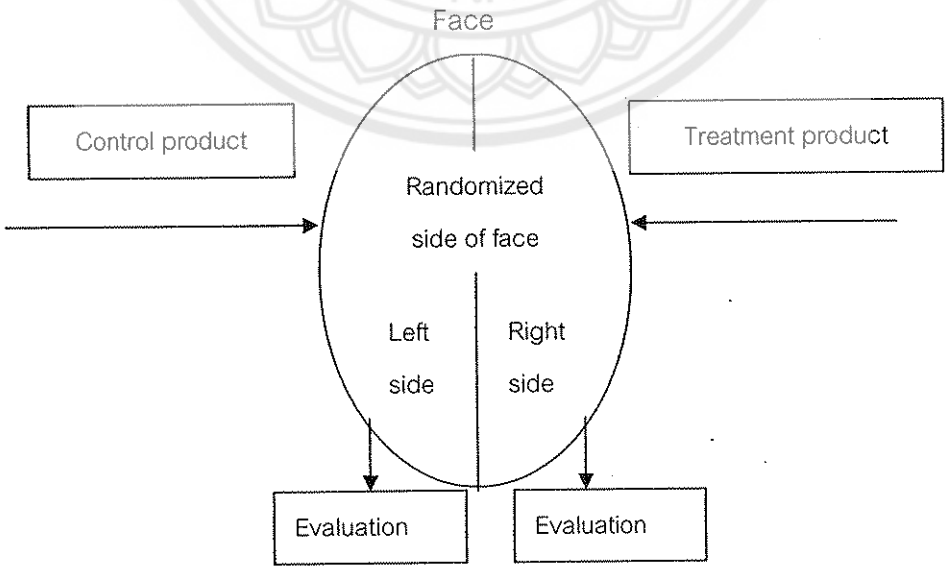

Figure 7 The within person design

8.3 Randomization in clinical trial

Randomization is the process of assigning trials subjects to either treatment or control groups by chance to reduce potential bias. Although, the variation of subject between groups was eliminated by within patient design, the variation between sites to use product still exists. Therefore, the randomization should be performed to reduce selection bias of each site to use each product, and balance all prognostic factors and others characteristic of each study area [70]. Whatever the randomization process is used, the report of the trial should contain a brief but clear description of randomization method. The report of the trial should clearly indicate the type of randomization method and how the randomization is implemented [67, 71].

Generally, there are two types of randomization method including fixed allocation randomization, and adaptive randomization procedures. Fixed allocation randomization assign the intervention to subjects with a pre-specified probability, and this probability is not altered as the study progress. In contrast, the adaptive randomization procedures will be change the allocation probability as the study progress. In this study, three methods of fixed randomization including simple, blocked, and stratified randomization, and overall of adaptive randomization are reviewed.

8.3.1 Simple randomization

Simple randomization is a simple and the most basic method of random treatment assignment. The classic technique of this method is the tossing an unbiased coin for each trial participant. In practice, for small studies, instead of tossing a coin to generate a randomization schedule, a random digit table on which the quality likely digits 0 to 9 are arranged by rows and columns is usually used to accomplish simple randomization. The advantage of this method is easy to practice. However, there are important limitations including risk of imbalance number of subject and imbalance prognosis between groups, especially for small sample size.

8.3.2 Blocked randomization

This method is also called permuted block randomization. It is used to aviod serious imbalance in the number of participants assigned to each group as could occur in simple randomization. The problem of this method is that assignment to the last person

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in each block can be known if the treatment was not blind. However, this problem can be solved by randomly varying block size. The detail of this method is provided below.

"Blocks" having equal numbers of As and Bs (A = intervention and B = control, for example) are used, with the order of treatments within the block being randomly permuted. This process is repeated for consecutive blocks of participants until all participants are randomized [72-73]. For example, a block of four has six different possible arrangements of two As and two Bs (Figure 8). A random number sequence is used to choose a particular block, which sets the allocation order for the first four subjects. Similarly, treatment group is allocated to the next four patients in the order specified by the next randomly selected block.

1. ABAB	2. BABA	3. AABB	4. BBAA	5. ABBA	6. BAAB

Figure 8 The example of block of four

8.3.3 Stratified randomization

Stratification can add to the credibility of a trial, as it ensures treatment balance on these known prognostic factors, allowing easy interpretation of outcomes without adjustment. Stratified randomization requires that the prognostic factors be measured either before or at the time of randomization. For example, in a trial of chemotherapy for breast cancer, suitable stratification factors might be menopausal status and estrogen-receptor status. Each factor was divided into two groups or strata (i.e., premenopausal or postmenopausal). Within each stratum, the randomization process itself could be simple randomization, but in practice most clinical trials use some blocked randomization strategy. As an example of stratified randomization with a block size of four, suppose an investigator wants to stratify on estrogen receptor status (ER+ or ER-) and menopausal status (premenopausal or postmenopausal). Thus, the design has 2x2 = 4 strata. The randomization for this example appears in table 3.

Table 3 Stratified randomization with block size of four

Strata	estrogen receptor status	menopausal status	Group of assignment
1	ER-	+	ABBA, BABA, …
2.	ER+	+	BABA, BBAA, …
3.	ER-	-	AABB, AABB, …
4.	ER+	-	Etc.

Where ER+ and ER- are estrogen receptor positive-and negative, respectively, + and - are pre- and postmenopausal, respectively.

8.3.4 Adaptive randomization

Adaptive randomization method is divided into two types including baseline adaptive, and response adaptive randomization.

Baseline adaptive randomization uses the differences in number of participants, which are greater than pre-specified value to adjust the probability of assigned participants. This method is being used especially in clinical trials of cancer where several prognostic factor need to be balanced. The advantage of this method is protection of a severe baseline imbalance for important prognostic factors. Response adaptive randomization uses information on participant response to intervention during course of the trial to determine the allocation of the next participant. This method is not commonly used because it is complicated and many clinical trials do not have an immediately occurring response variable.

In summary, we choose simple randomization method for this study because it easy to use and refer to within patient study design, it has a small subject variation between groups. In addition, each patient act as the block in permuted block randomization. Therefore, there is no problem of imbalance between groups.

In practice, the trial statistician or others not directly involved in recruiting patients to the trial commonly generates the randomization sequence. A clinical trial report should clarify who generated the sequence, the method used, and how concealment was achieved.

Allocation concealments are methods used to implement the random allocation sequence, clarifying the sequence were concealed until interventions were

assigned. They are important methods to avoid both conscious and unconscious selections of patients into the study. That means the advantages of randomized process still remain if the allocation concealment was conducted. Typically, "Allocation concealment" is the term used to describe this process and underpins successful randomization strategies [74-75]. There are several methods to concealment such as envelopes, numbered containers or central telephone etc.

8.4 Blinding or masking

Blinding or masking is a procedure to reduce bias by preventing observers and/or participants involved in a result of study from knowing the treatment assignment, and preserving symmetry in the observers' measurements and assessments. This bias is usually not due to deliberate deception but is due to human nature and prior held beliefs about the area of study. The accuracy of study's result is increased by this procedure. Normally, blinding is classified into two types including single blind, and double blind.

Single blind usually refers to only the subjects or the investigators being unaware while double blind usually refers to the subjects, investigators, monitor, and, in some cases, data analyst being unaware of the treatment assignment [67, 74]. Because the terms "single blind" and "double blind" are imprecise [67], many researchers prefer to specify who is blinded.

8.5 Outcome assessment

Outcome assessment is a key step in clinical trial. The evaluation of each patient's progress after the start of study needs to be done in an objective, accurate and consistent manner so that the study as a whole provides a meaningful assessment of the treatments' relative merits. The methods for assessing and recording a patient's progress need precise definition in the study protocol [74].

For clinical trials in cosmetic, selection of the proper outcome and the appropriate test method for evaluation cosmetic product depends on the active ingredient of product, objective to development this product and study design. For example, facial cleansing product containing tamarind pulp extract, the objective of this product is not only keeping facial skin clean but also improving the skin whitening.

Therefore, primary outcome of this product is skin whitening effect depending on the active ingredient (tartaric acid from tamarind pulp extract) of product.

Today there are several tools including expert visual, subject self-assessment, and instrumental measurement methods for assessment cosmetic product [74]. Each method has its own strengths and weaknesses. The summary of each evaluation method is provided below.

Instrumental methods or objective methods are ideal at providing objective data to individual aspects of what the product dose for skin. They can provide quantification of invisible aspects such as moisture content within the skin. Instruments can be highly sensitive to small differences suggesting skin benefits that are consumer irrelevant and imperceptible. However, they provide only one-dimensional, which can lead to an incomplete view of product performance. For this limitation, the instrument method must be relevant to the objectives and should be aligned with expert grading. More over, measurement bias may exist from reading, transient environmental condition and techniques of measurement. The procedures of instrumental reading must be defined in the protocol to decrease measurement bias. For example, three replicate readings are being taken and averaged for each measurement. In addition, the protocol should specify the acceptable test conditions for instruments and the same evaluator should be using the same instrument for evaluation each person. Moreover, all evaluator were blinded.

Expert assessment is qualified human judge integrates many aspects into a measurement of the visual appearance of skin. Any deviation from normal skin or healthy appearance can be captured in the evaluation. For decreasing bias of evaluation, the number of expert should be more than one person and each expert must be completely independent of all other aspects of the trial and must be blind to the products being tested. Grading must also be performed absolutely, that is, how it appears at that moment. There should be no reference to prior data for comparison.

Subject self-assessment within the context of the clinical trial gives quantitative measure to the perception of skin look and feel. Self-assessment measurements have two roles: they provide a means of measuring sensory attributes that cannot be

measured instrumentally and they demonstrate whether the instrumentally measured changes are resulting in meaningful and perceivable benefits to the user.

8.6 Adherence to the protocol

According to the concept of International Conference on Harmonization of technical (ICH) Harmonized Tripartite Guideline, guideline for Good Clinical Practice or ICH GCP, all investigators should conduct the trials in compliance with the protocol agreed to by the sponsor and, if required by the regulatory, authorities, and which was given approval/favourable opinion by the Institutional Review Board (IRB)/ Independent Ethics Committee (IEC). Adherence to the protocol is important because the trial results can be affected by nonadherence. Nonadherence leads to underestimate the possible therapeutic and toxic effects, and can undermine even a properly designed study. Therefore, investigator should monitor adherence to identify any problems during step of study.

8.7 Patient safety

Clinical safety issues related to products will be reviewed thoroughly and appropriate action taken to ensure the safety of patients or volunteers. This means that on an ongoing basis we should monitor signal generation, review the risk-benefit ratio of products, review product labeling, and provide safety reports to regulatory authorities. In this issue, Data and Safety Monitoring Board (DSMB) is one of committee that we should know. This board is an independent committee, composed of community representatives and clinical research experts that reviews data while a clinical trial is in progress to ensure that participants are not exposed to undue risk. They may recommend that a trial be stopped if there are safety concerns or if the trial objectives have been achieved. They are now considered a critical component of the administration and conduct of large multicenter clinical trials.

For assessing products within short period, no need to have the DSMB. However, one got to find a way to assess this as patient safety is the most paramount thing in clinical trials. Collection safety data of participants is important requirement of ethic, regulation, and ICH-GCP of clinical trials. In this study, we perform several procedures to ensure the safety of patient including procedure of exclusion criteria for high-risk subject,

procedure to test the irritation of product as show in appendix M, monitoring clinical adverse event by subjects themselves and by dermatologist.

9. Statistical issues

9.1 Sample size calculation

Clinical trials should have sufficient statistical power to detect differences between groups considered to be of clinical interest. Therefore, calculation of sample size to adequate levels of significance and power is a necessary part of planning. Statistical analysis can predict the minimum panel size required to show an expected level of change where the level of variability is known. This statistical method is only a guideline as to how many patients are needed. However, it is important now to realize that if the trials had fewer patients than the calculation would automatically decrease the chances of finding a statistically significant of clinical interest.

The formula for calculation depends on question and study design [76]. If N is the sample size, Sd is standard deviation of the result, $\triangle$ is the difference of the expect values of the outcome between each group, $Z_{1-\alpha/2}$ is Z statistic with α, for example α = 0.05 that is Z value as 1.96, $Z_{1-\beta}$ is Z statistic with the considered power, for example power was defined as 80% that is Z value as 0.84. The formula for two independent sample or parallel design, and cross over study or within patient stud are shown as formula A, and B, respectively.

$$N = \frac{2*[Z_{1-\alpha/2} + Z_{1-\beta}]^2 * Sd^2}{\triangle^2} \quad \text{---------- Formula A}$$

$$N = \frac{[Z_{1-\alpha/2} + Z_{1-\beta}]^2 * Sd^2}{\triangle^2} \quad \text{---------- Formula B}$$

9.2 Analysis result

In this study, we analyzed data based on intention to treat method. This method prevented bias due to the loss of participants, which might disrupt the baseline equivalence established by random assignment and which might reflect non-adherence to the protocol [70]. If the missing values are occurred, we used last-observation-carried-forward (LOCF) to impute the data because LOCF was quite common method, easy to

understand, and had been recommending for situation of noncompliance or loss follow-up in several studies [77].

10. Relevant studies of the efficacy and safety of facial cleansing product containing extract of tamarind's fruit pulp

10.1 Clinical trials for evaluation the efficacy and safety skin whitening products contain herbal extract

In general, several clinical trials were conducted to determine the efficacy and safety of skin whitening products. However, most of products which were evaluated products containing synthetic active ingredients including hydroquinone, ascorbic acid, kojic acid, arbutin, azealic acid, vitamin A and C, alphahydroxy acids (AHAs), poly hydroxyl acids (PHAs) [10, 62, 78-86].

According to review literature, there were only two studies that were conducted to determine the efficacy and safety of skin whitening containing herbal extract [62, 87]. One of them was conducted to determine the efficacy and safety of the product containing Chinese herb extract including *Atractylodis Rhixoma, Angelicae Dahuricae Radix, Paeoniae Radix*, and *Typhonii Rhizoma* [87]. The other study was conducted to evaluate the products containing natural AHA including lactic acid from honey extract, glycolic acid from sugar cane, malic acid from apple extract, combination AHAs (mostly lactic acid) from tropical fruit and multifruit extract [62]. For product containing the tamarind pulp extract, there were available in two studies but they were conducted to develop the formulation [30-31]. Based on a review of clinical trial in whitening product contain herb extract, there was no clinical study for evaluated the efficacy and safety of this product.

10.2 Measurement of skin color

The skin color was a primary outcome of this study so measure skin color was important part. In general, the measurement of skin color was divided into two types including visual grading, and instrumental methods. Visual grading was a subjective method that traditionally measurement of skin color. The evaluator may be subjects themselves or other evaluators or both. Advantages of this method were integration

numerous aspects of condition into measurements, and it was direct measure of user perceptions. There were many studies using this method for evaluation the skin color by both subject self-assessment and expert assessment [78, 80-82]. However, color assessment of skin by visual inspection alone may be difficult to classify. Therefore, several instruments were developed and used with or without the visual grading for evaluation the skin color.

Nowadays, the instruments of skin color evaluation had been achieved in many studies using either reflectance colorimetry (Chromameter from Minolta) following the CIE (Commission Internationale de l'Eclariage) recommendations [83, 86-89] or reflectance spectrophotometry (narrow-band simple reflectance meters) including Dermaspectrometer and Mexameter [86-90]. All types of them had proven both sensitive and reliability. That mean they were able to characterize skin color and to measure small skin color changes. Therefore, we can use Chromameter or Dermaspectrometer or Mexameter for evaluation the skin color.

10.3 The duration of study

Duration of study should be enough to capture the different effect of each intervention. Based on a review of clinical study of AHAs, durations of study range from 4 weeks to 26 weeks [10, 62, 78-81, 84, 86]. These studies can identify the difference of each intervention in this range of duration. One study [81], which was conducted to evaluate the effect of an alpha hydroxyl acid-blend skin cream in the cosmetic improvement of symptoms of moderate to severe xerosis, epidermolytic hyperkeratosis, and ichthyosis for 4 weeks, showed that the product containing AHA reduced symptoms and improved cosmetic appearance following 2 weeks of use and continued improvement following the end of study. Smith et al reported that the ability of glycolic and lactic acid to increase cell renewal reduced about 30-40% after 10 weeks of treatment [10]. Thus, we believe that continuous use of this product for 8 weeks is enough to determine its effect.