The Effects of Oral Combined Nutraceuticals on Skin Health in Post-menopausal Women

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ใบรับรองวิทยานิพนธ์

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หัวข้อวิทยานิพนธ์ The Effects of Oral Combined Nutraceuticals on Skin Health in Post-Menopausal Women

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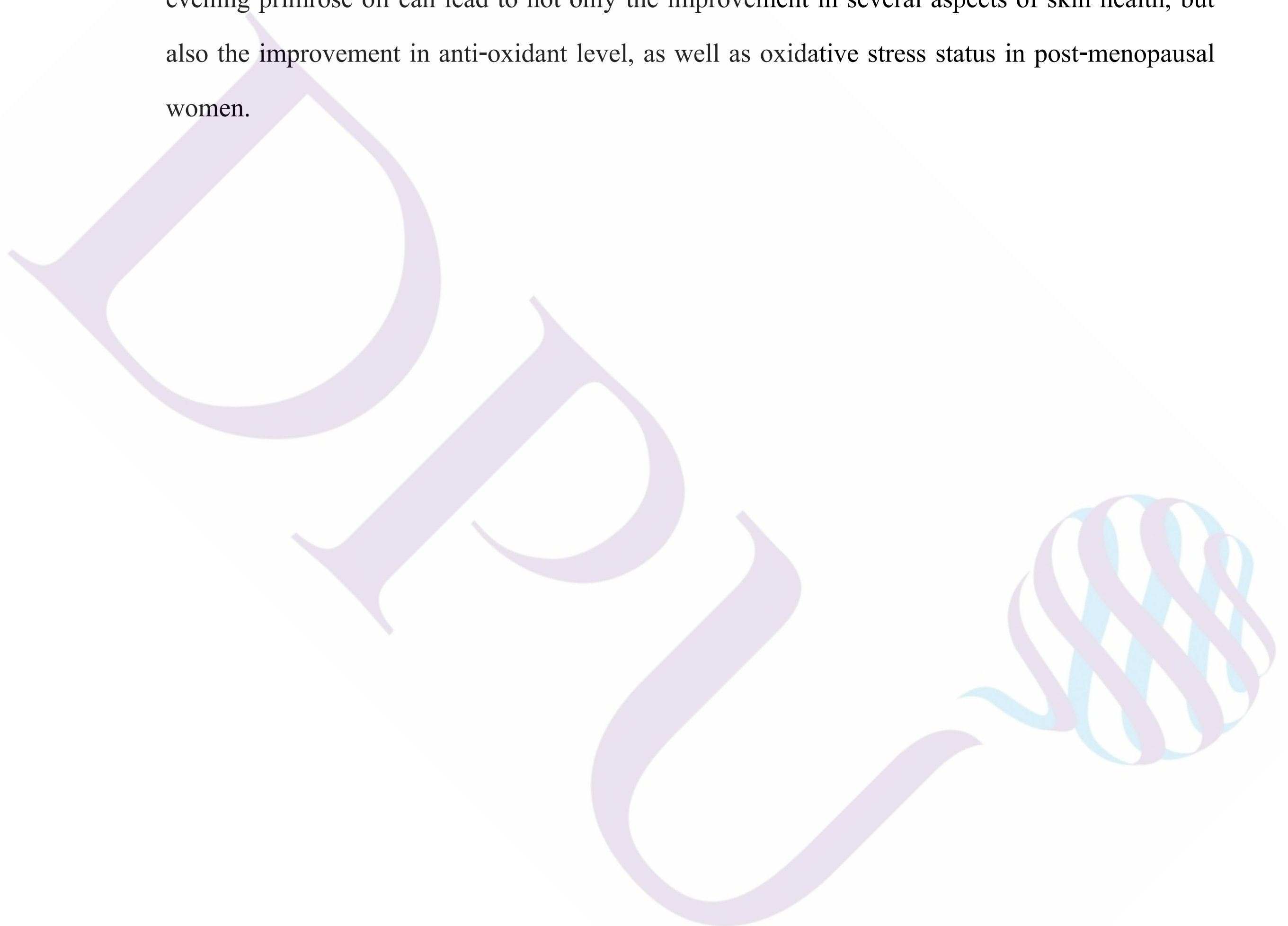
ABSTRACT

Skin aging is one of the most concerned issues that occur during post-menopausal period, mainly due to estrogen deficiency and reactive oxygen species. Despite the promising effects of hormonal replacement therapy on improving skin quality, a handful amount of post-menopausal women are still concerned about the long-term adverse outcomes those could occur following the treatment. Therefore, nutraceuticals that contain estrogenic and anti-oxidative effects have gained a lot of attention as a possible alternative therapy for reversing skin aging in post-menopausal women. The objective of this study was to evaluate the efficacy and the safety of combined nutraceuticals in improving skin health, anti-oxidant level, and oxidative stress status in post-menopausal women.

The study design was randomized, double-blinded, placebo-controlled clinical trial. One hundred and ten post-menopausal women aged 45-60 years old were enrolled and randomly assigned into treatment group and placebo group, equally. Combined nutraceuticals containing 100 mg of soy isoflavones, 80 mg of black cohosh, 40 mg of chasteberry, and 500 mg of evening primrose oil, were administered 1 capsule per day for 12 weeks in the treatment group, while the another group received a placebo. Skin elasticity, hydration, transepidermal water loss (TEWL), gloss, melanin index, smoothness, roughness, scaliness, and wrinkles were evaluated at baseline, week 6, and week 12 of the study. Additionally, reduced glutathione (GSH) and malondialdehyde (MDA) level were measured at baseline and week 12 to evaluate the improvement in anti-oxidant and oxidative stress status.

The results of this study showed that at week 6, the skin roughness was the only parameter that significantly improved in the treatment group. At the end of the study, however, there were significant differences between the treatment group and the control group, and also a significant

intragroup improvement in the treatment group, regarding effects on skin elasticity, skin roughness, skin smoothness, skin scaliness, and wrinkles. Meanwhile, no significant improvement in skin hydration, TEWL, melanin index, and skin gloss was observed in both groups. For the evaluation of anti-oxidant and oxidative stress status, both GSH and MDA levels were found to be significantly improved in the treatment group at the end of the study. In conclusion, this study suggests that the intake of these combined nutraceuticals containing soy isoflavones, black cohosh, chasteberry, and evening primrose oil can lead to not only the improvement in several aspects of skin health, but also the improvement in anti-oxidant level, as well as oxidative stress status in post-menopausal women.



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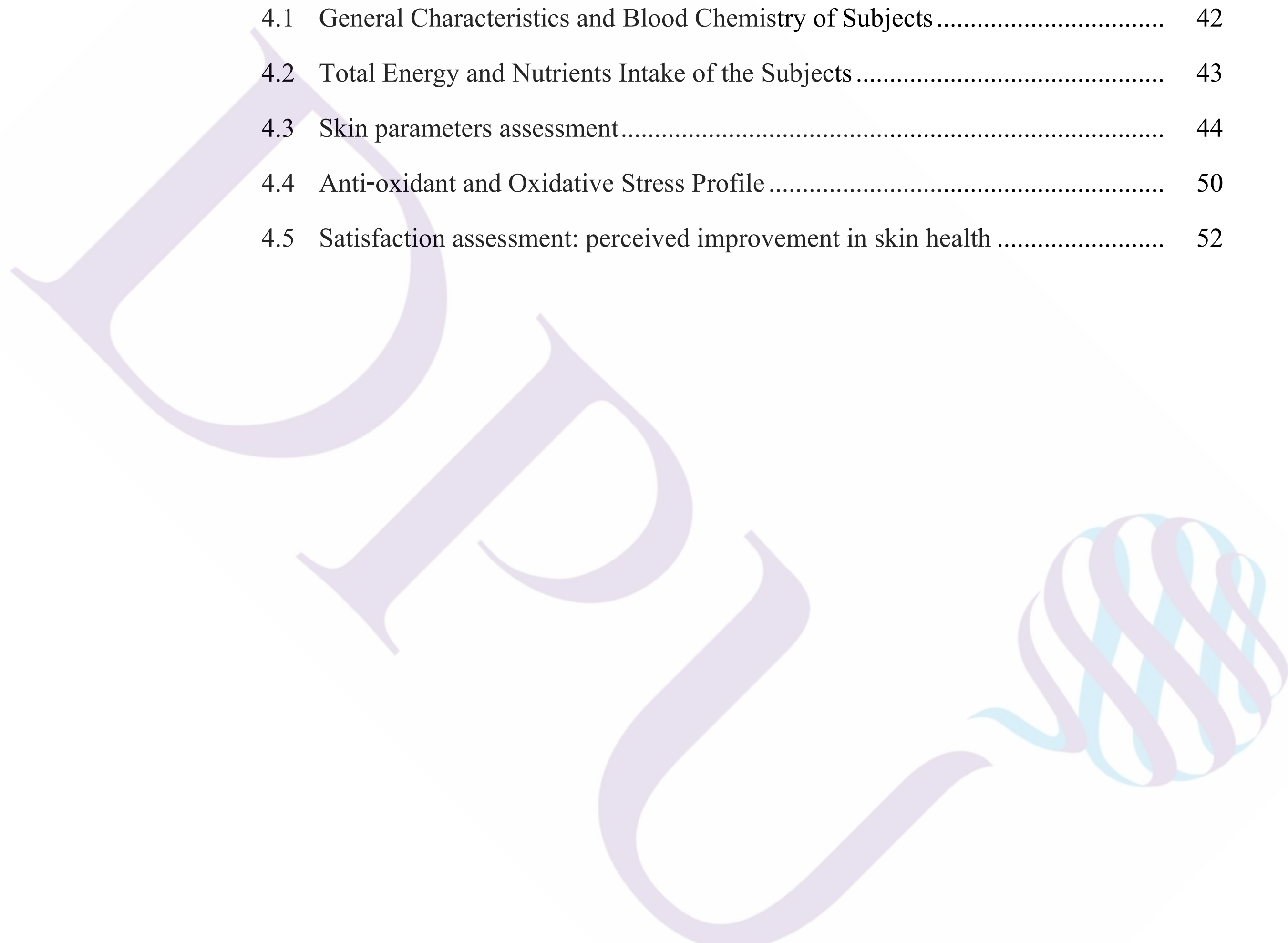

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Chapter 1

Introduction

1.1 Background and Significance of the Research Problem

Menopause is defined as a period of 1 year without menstruation as a result of the progressive failure of the ovaries to produce estrogens. This failure often begins in the late 30s, and most women experience near-complete loss of production of estrogens by their mid-50s (Goodman et al., 2011). It is estimated that in 2030, the at-risk population of peri- and post-menopausal women will reach 1.2 billion, globally (Gold et al, 2006). Although some of these women may not experience any symptoms, estrogen deficiency is often associated with hot flushes, sweating, insomnia, and vaginal dryness in up to 85% of post-menopausal women.

The skin, one of the largest organs in the body, is also significantly affected by the aging process and menopause. The estrogen receptor has been detected on the dermal cellular component; thus, the changes in dermal cellular metabolism is thought to be influenced by the reduction of the estrogens during menopause, which leads to changes in the collagen content, the concentration of glycosaminoglycans, and most importantly, the water content. Changes in the skin collagen leads to reduction in skin elasticity and skin strength. Decreased glycosaminglycans leads to a direct reduction in water content, which further affects the skin turgor. Changes in the cutaneous vascular reactivity can also be noted following menopause. Capillary blood flow velocity also decreases significantly in post-menopausal women. Consequently, the changes in these basic components leads to skin aging. Evidences suggest that these changes may be reversed with the administration of topical or systemic estrogen (Raine-Fenning & Brincat, 2003). Apart from the decreased level of estrogens, oxidative stress from UV exposure and inflammation also play a significant role in skin aging process.

Hormone replacement therapy (HRT) is the current gold standard for the treatment of menopause symptoms and for delaying skin aging process. However, many considerable

evidences support that HRT may increase the risk of cancer in estrogen receptor α-rich tissues, for example, uterus, breast and ovaries (Zhou et al., 2008). Nutraceuticals containing phytoestrogen effects are promising alternative therapy which have been used for the treatment of menopause symptoms and skin aging for many years.

Phytoestrogens are heterocyclic phenolic compounds produced naturally in legumes, soybeans, beans, nuts, cereals, flax seeds, sesame seeds, hops and other plants that may exert estrogenic actions due to their structural similarities to estrogens. They bind to estrogenic receptors (ERs) α and β, with a higher affinity for ERβ, and act as agonists, partial agonists or antagonists (Mostrom & Evans, 2018).

Soy is one of the richest natural sources of isoflavones; these include genistein, daidzein and glycitein. Isoflavones, particularly genistein, have a higher affinity for the β subtype of the estrogen receptor, which can be found in bones, skin and the cardiovascular system, as opposed to the α subtype, which can be found in the uterus and breasts. Soy isoflavones are considered to contain beneficial effects on the skin by preventing lipid oxidation of the skin tissue, stimulating fibroblast proliferation, reducing collagen degradation, and inhibiting 5α-reductase, and have been widely used as ingredients in cosmetic products. Numerous studies of the mortality of hormone-dependent cancers (e.g. breast cancer and prostate cancer) have revealed a negative correlation between soy isoflavones intake and the incidence of these cancers (Izumi et al, 2007). Asian populations, such as those in Japan, Taiwan and Korea, are estimated to consume approximately 20–150 mg/day of isoflavones, with a mean amount of approximately 40 mg from tofu and miso (Murkies, Wilcox, & Davis, 1998).

Evening primrose oil (EPO) is extracted from the seeds of evening primrose (*Oenothera biennis*). It is a rich source of essential fatty acids, including gamma linolenic acid and various phytosterols. It has been reported to restore a defective epidermal barrier and normalize excessive transepidermal water loss (TEWL). The skin-improving effects have been found to be related with gamma-linolenic acid (GLA) which is inherently required in the skin for optimal structure and function (Muggli, 2005).

Black cohosh is a medicinal root containing potent phytochemicals. It has been widely used to treat hormone-related symptoms including PMS, menstrual cramps and menopause symptoms. It also possibly contains antioxidant activities (Poinier et al, 2018).

Chasteberry contains many phytochemicals which are found to be effective in treating irregular cycles and relieving PMS symptoms. More studies are required to determine the effects of black cohosh and chasteberry on skin health. However, the antioxidant properties of both nutraceuticals may play a role in improving the skin in menopausal women.

The clinical studies of these combined nutraceuticals in menopausal women have not yet been conducted. Therefore, in this study, we aimed to evaluate the effects of the combination of these natural compounds (soy isoflavone, evening primrose oil, black cohosh and chasteberry) which may have a potential to improve skin health in post-menopausal women as an alternative therapy.

1.2 Objectives

1. To study the effects of the combined nutraceuticals (the combination of soy isoflavone, evening primrose oil, black cohosh and chasteberry) on skin parameters including wrinkles, skin smoothness, skin roughness, skin elasticity, skin hydration, transepidermal water loss (TEWL), melanin index and skin gloss in post-menopausal women.
2. To study the effects of the combined nutraceuticals on anti-oxidants and oxidative stress level, which may also affect the skin health. The biomarkers used to evaluate were malondialdehyde (MDA) and reduced glutathione (GSH).

1.3 Hypothesis

1. The combined nutraceuticals have beneficial effects on improving skin health in post-menopausal women, comparing to placebo.
2. The combined nutraceuticals have beneficial effects on improving anti-oxidants and oxidative stress biomarkers level in post-menopausal women, comparing to placebo.

1.4 Expected benefits

1. The effects of the combined nutraceuticals on skin health in post-menopausal women will be observed.

2. If the effects of the combined nutraceuticals on skin health are observed to be beneficial, the combination of the nutraceuticals may be suggested to menopausal women as an alternative therapy for delaying skin aging process.
3. The effects of the combined nutraceuticals on anti-oxidant and oxidative stress biomarkers level, which may be related with the skin health, will be determined.

1.5 Definition of terms

Menopause: the point in time when menstrual cycles permanently cease due to the natural depletion of ovarian oocytes from aging. The diagnosis is typically made retrospectively after the woman has missed menses for 12 consecutive months.

Post-menopause: the period of life after menopause.

Nutraceutical: a substance that may be considered food or part of food which provides medical or health benefits, encompassing prevention and treatment of diseases.

Phytoestrogen: non-steroidal substances of vegetal origin that exhibit agonistic and antagonistic estrogen activities due to their structural similarities to the estrogens.

Transepidermal Water Loss (TEWL): the evaporation rate of the water from the skin ($g/h/m^2$). High values of TEWL reflect a loss of skin integrity or protective barrier function.

Malondialdehyde (MDA): An endogenous genotoxic product of enzymatic and oxygen radical that is able to induce lipid peroxidation. It is one of the most frequently used indicators of lipid peroxidation.

Reduced Glutathione (GSH): Reduced glutathione (GSH), a ubiquitous tripeptide thiol, is a vital intracellular and extracellular protective antioxidant.

1.7 Research framework

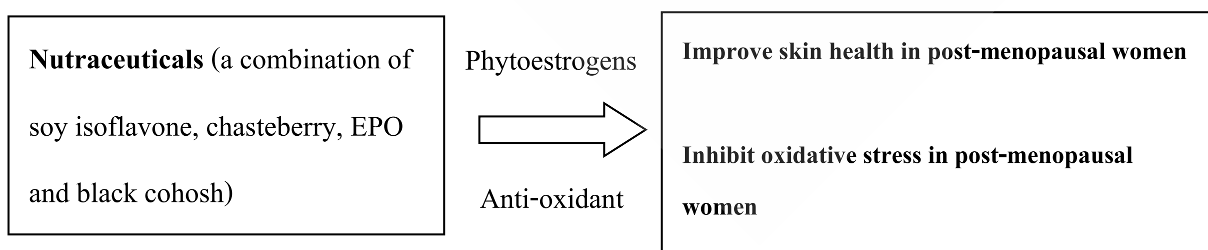


Figure 1.1 Research framework

Chapter 2

Related Concept, Theories and Literature Review

2.1 Estrogen Biosynthesis and Sites of Actions

Estrogens are the group of steroid compounds, named after their significance in the estrous cycle (cyclical changes induced by reproductive hormones in most mammals; in human called menstrual cycle). All estrogens are hydrocarbon estrange derivatives with aromatic ring and 18-carbon molecule. Their production is far higher in women and they function mainly as the female sex hormones, but also play important role in male fertility. Although more than 30 various forms of estrogens have been recognized, only three of them are the most important ones: estrone (E1), estradiol (E2) and estriol (E3). Estradiol (17β-estradiol, E2) is the major form of estrogens in non-pregnant females, whereas estrone (E1) is the main form during menopause, and estriol (E3) is the main estrogen during pregnancy (Thomas & Potter, 2013).

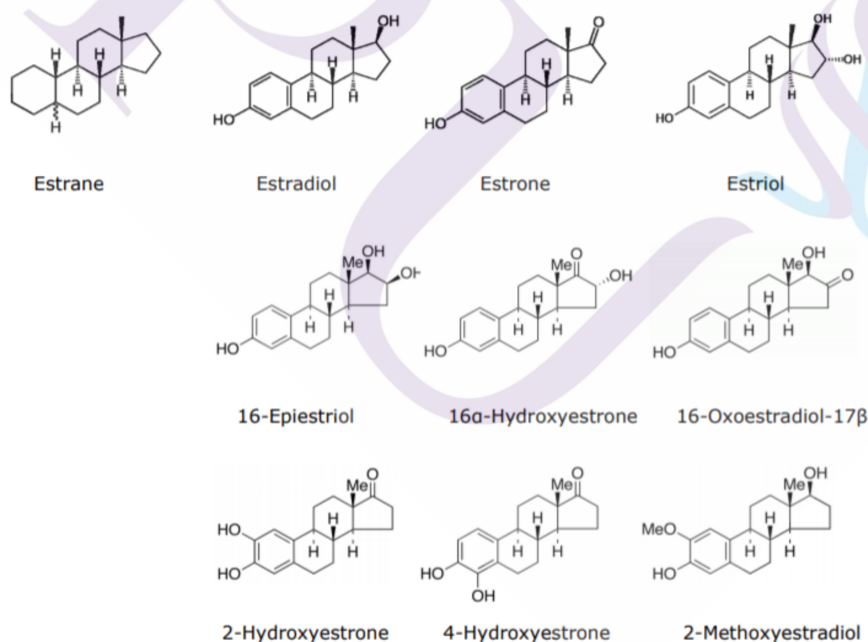


Figure 2.1 The molecular structures of estrone, estradiol, estriol, and some other estrogens

Source Beckman Coulter reproductive estrogens analytes information

The ovary (ovarian follicles as well as corpus luteum) is the main source of estrogen biosynthesis in females of reproductive age. The main source of estrogen production during pregnancy is the placenta. In men, small amounts of estrogens are produced in the peripheral tissues by the actions of aromatase on androstenedione and testosterone. In postmenopausal women, peripheral conversion of adrenal dehydroepiandrosterone (DHEA) is the main source of estrogen production; therefore, estrone becomes the main form of estrogens, while estradiol significantly decreases comparing to premenopausal period. However, when aging, DHEA production can be decreased to the level as low as 10–20% of the peak concentrations; thus, peripheral estrogen biosynthesis is also greatly reduced (Thornton, 2013).

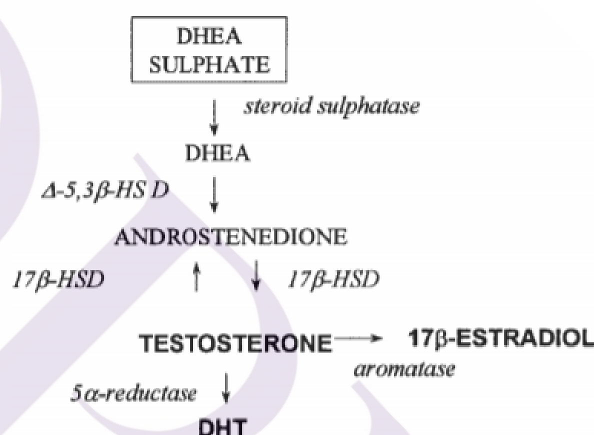


Figure 2.2 The peripheral conversion of active androgens and estrogens from adrenal precursors

Source Thornton, 2013

Estrogens are mandatory for the normal female physical maturation. Together with other hormones, estrogens play a part in processes of ovulation and implantation of the fertilized ovum, and also stimulate the development and maintain the growth of accessory organs.

Estrogens exert their actions through two distinct tissue-dependent intracellular estrogen receptors, ERα and ERβ, which are members of the superfamily of nuclear hormone receptors. The ERα and ERβ proteins are encoded by separate genes on different chromosomes. ERβ is located on chromosome 14, whereas ERα can be found on chromosome 6 (Enmark et al., 1997). They share approximately 97% homology in the DNA binding domain, with only a few different in amino acids in this region. However, their ligand binding domain only share 59% homology, and share little homology in other domains (Gustafsson, 1999*)*. The binding of

estrogens to the estrogen receptors triggers specific responses in terms of proliferation activation and apoptosis arrest. Some actions of the estrogens require the collaboration of other hormones, such as progesterone and androgens. Both estrogen receptors have a wide distribution in women. ERα can be found mainly in endometrium, breast cancer cells, ovarian stromal cells and the hypothalamus, while expression of the ERβ protein has been detected in ovarian granulosa cells, kidneys, brain, bone, heart, lungs, intestinal mucosa, prostate gland, and endothelial cells (Clarkson et al., 2011).

Previous study using the RT-PCR technique successfully detected mRNA of both ERs in the skin fibroblast cultures with greater mean level of ERβ mRNA expression than ERα mRNA. In human culture, skin fibroblasts ERβ also co-expresses with ERα. The cultured female skin fibroblasts has shown dominant expression of ERβ; therefore, it suggested that ERβ may play a dominant role in the regulation of estrogen actions in human skin in collaboration with ERα, and suggested a strong involvement of ERβ in maintaining skin homeostasis (Haczynski et al, 2002). Furthermore, ERβ is found to be strongly expressed in the stratum basale and stratum spinosum of the epidermis, whereas the expression of ERα is quite low (Thornton, 2002).

2.2 Effects of Estrogens on Skin

It has been recognized that estrogens have significant effects on skin physiology and pathophysiology, although there have been limitations in studies on estrogen actions in the skin. However, estrogens clearly have an important role in many skin components including the epidermis, dermis, vasculature, hair follicles, sebaceous glands, eccrine glands and apocrine glands. Estrogens also have significant roles in skin aging, pigmentation, hair growth, sebum production and skin cancer (Thornton, 2002). Cultured human epidermal keratinocytes have been revealed to express an estrogen receptor and causing human melanocytes to enlarge and become dendritic following a 2-day incubation with estradiol (Maeda et al., 1996). Some studies have reported that estradiol significantly increases melanin synthesis and tyrosinase activity in human skin (McLeod, Ranson, & Mason, 1994). It has been reported that cultured mouse dermal fibroblasts can increase collagen production by approximately 76% in response to estrogens (Hosakawa et al., 1981).

Therefore, estrogens are important in the maintenance of human skin. They improve content and quality of skin collagen, increase thickness of skin, enhance vascularization (Brincat, 2000) and have been recognized to increase mitotic activity in the epidermis (Punnonen, 1972). The estrogen receptors in the skin vary in different parts of the body; receptor levels are higher in facial skin and lower in the skin from thigh or breast. However, these studies did not distinguish between the different components within the skin (Hasselquist, 1980).

Some study has provided evidence that estrogens may play a role in all phases of wound healing by modifying the inflammatory response, accelerating re-epithelialization, stimulating granulation tissue formation and regulating proteolysis (Thornton, 2013).

In the inflammatory phase, estrogen receptors have been identified in human leucocytes, monocytes, macrophages and megakaryocytes. There is an evidence to suggest that estrogens affect the function of inflammatory cells (Ashcroft et al., 1999). Estrogens have been reported to down regulate the expression of macrophage migration inhibition factor (MIF), which is a pro-inflammatory cytokine released by monocytes, T lymphocytes, endothelial cells and keratinocytes (Ashcroft et al., 2003).

The proliferative phase of wound healing involves re-epithelialization, angiogenesis, formation of granulation tissue and wound contraction. The dermal fibroblast is the key cell involved in wound healing, expressing both ERα and ERβ (Stevenson et al., 2008). Previous study showed that estrogen stimulates the migration of dermal fibroblasts derived from scalp, breast (Stevenson et al., 2008), and abdominal skin (Stevenson, Sharpe, & Thornton, 2009). However, the increase of migration only occurred in response to 17β-estradiol and an ERα agonist, while an ERβ agonist had no effect. Further observation has reported that migration in the presence of the ERα agonist was higher than that seen with 17β-estradiol alone. This observation may highlights the importance of ERα (Stevenson et al., 2009).

The remodeling phase of wound healing relies on a balancing between synthesis and degradation of the extracellular matrix, with estrogens thought to be the factor that influences both. Estrogens are associated with an overall increase in collagen deposition during the remodeling phase (Ashcroft et al., 1999).

Estrogens also play a role in skin pigmentation. An increase in cutaneous pigmentation due to increased estrogens is common during pregnancy with regression after

parturition. Melasma is a form of hyperpigmentation in the facial area commonly seen in pregnant women and is often accompanied by increased pigmentation in other areas such as areolae, linea alba and perineal skin, all of which usually fade after parturition (Thornton, 2002). There is also an evidence that fluctuations of estrogens during the menstrual cycle may affect epidermal pigmentation in some cases. Some studies also reported that estrogen-containing oral contraceptives can cause hyperpigmentation of the face in 8–29% of women (Wade et al., 1978).

2.3 Menopause and Hormonal Changes

Menopause is defined as cessation of the menstrual cycle, which occurs on average at 45-55 years old as part of natural aging. It results from a loss of ovarian production of estrogens and progesterone, although the adrenal glands production of their precursor hormone, testosterone, continue. Peri-menopausal stage occurs at the onset of menopause. During this stage, there is an increase of irregularity of circulating ovarian hormones, which causes irregular periods and spotting. This process can last as long as 10 years (McNamara, Batur, & DeSapri, 2015).

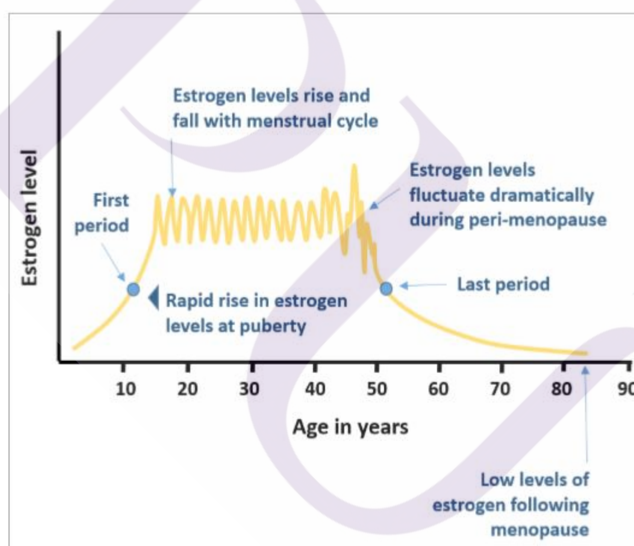


Figure 2.3 The ovarian production of estrogen

Source www.maxbiocare.com

Estrogens and Progesterone have their actions in endometrial thickening, preparation for implantation, regulating breast tissue, bone density, vasomotor function and neurological

activity in cycling women. The symptoms of menopause are directly related to lack of estrogens. Some of the most common symptoms of menopause include: hot flushes, night sweats, vaginal dryness and atrophy, anxiety, depression, urinary incontinence, mood changes, breast tenderness, insomnia, headaches, general aches and pains, weight gain, decreased libido, osteoporosis and fatty liver (Santoro, Epperson, & Mathews, 2015).

2.4 Skin Changes During Menopause

During menopausal period, many skin alterations can be observed. Some of these changes seem to be associated with the extrinsic process of reactive oxygen species generated mainly by UV exposure; while others occur as a consequence of intrinsic or chronological process which results from estrogen deficiency. The mechanisms involved originate from the dermal and epidermal depletion of cellularity and collagen quantity and from skin thinning. The decrease in levels of collagen in the skin occurs at a rapid rate immediately after menopause; it reaches around 2.1% per year, causing a total cumulative loss of about 30% of skin thickness in the first five years of the menopausal period (Brincat et al., 2005).

Following menopause, skin becomes thinner with decreased collagen content, decreased elasticity, increased wrinkling, and increased dryness. Most of these effects can be modulated by estrogen replacement which increases epidermal hydration, skin elasticity and skin thickness as well as reducing skin wrinkles and augmenting the content and quality of collagen and the level of vascularization. Previous study has shown that while there is no difference between the expression of ERα and ERβ in male and female skin, the expression of ERβ is significantly decreased in the epidermis of those above 70 years old (Inoue et al., 2011).

The recognition of the effects of estrogen on aging human skin have been derived by the comparison between post-menopausal women who have received estrogen replacement therapy with women who have not (Punnonen, 1972). A report has shown an increase in epidermal thickness in human female skin following six months after oral estrogen administration with an increase in keratinocyte volume and more well-defined rete ridges (Maheux et al., 1994). Administration of topical estrogen can increase keratinocyte proliferation and epidermal thickness after only two weeks (Son et al., 2005). In women who lack estrogen, there is a reduction of skin thickness by 1.13% and collagen content by 2% per postmenopausal

year. Type I and III skin collagen can decrease by as much as 30% in the first five years following menopause, which parallels the reduction in bone mass observed in post-menopausal women. This skin thickness and collagen content reduction in elderly females correlates with the period of estrogen deficiency rather than chronological age (Brincat et al., 1987). An alteration in collagen subtypes has also been reported in post-menopausal women; compared with pre-menopausal women, post-menopausal women demonstrate a decrease in collagen types I and III and a decrease in the type III/type I ratio within the dermis, also thought to be correlating with the period of estrogen deficiency rather than chronological age (Affinito et al., 1999).

Skin wrinkling is synonymous with skin aging. It can be a result of environmental or hormonal factors, or both. Wrinkling is caused by a reduction in skin elasticity due to elastic degeneration and loss of connective tissue (Shah & Maibach, 2001). In an initial period of menopause, skin elasticity can decrease by 1.5% per year, in contrast to women using topical estrogen which elastic fibers are thickened, the number of fibers increased and the orientation of fibers in the papillary dermis improved (Henry et al., 1997).

2.5 Menopause and Oxidative Stress

One of the characteristics associated with skin aging is an increase in inflammation. In normal skin, reactive oxygen species (ROS) are produced by cellular mitochondrial metabolism. The presence of antioxidant enzymes such as superoxide dismutase (SOD), reduced glutathione (GSH) and catalase (CAT) preserve normal levels of ROS homeostasis and reduce the level of cellular stress. Both UV exposure and inflammation cause elevated ROS and oxidative stress, increasing damage of DNA, lipids and proteins and lead to premature aging (Masaki, 2010).

Lipid peroxidation also increases during postmenopausal period. It can be described as a process under free radicals attack lipid molecules containing double bonds, especially polyunsaturated fatty acids (PUFAs). Among the many different secondary products which can be formed during lipid peroxidation, malondialdehyde (MDA) appears to be the most studied and the most mutagenic product. It has been widely used for many years as a biomarker for the degree of lipid peroxidation (Ayala et al., 2014). A study which evaluated the degree of lipid peroxidation

in 57 postmenopausal women compared to 31 premenopausal women has revealed that MDA was significantly higher in the postmenopausal group (Onvural et al., 1998).

Another study conducted in a total of 186 reproductive, perimenopausal and postmenopausal women has observed that total antioxidant status (TAS) and GSH levels were significantly decreased in perimenopausal and postmenopausal women. The erythrocyte activities of glutathione peroxidase (GSH-Px), SOD and CAT were also significantly decreased in perimenopausal and postmenopausal women compared to women in their reproductive phase (Ogunro et al., 2014).

Estrogens have been discovered to have cytoprotective effects, although their precise mechanism of action is unclear. A previous study has revealed that estrogens can protect against oxidative stress induced in fibroblasts, but the mechanism appears to be unrelated to the intracellular receptors ERα, ERβ (Richardson et al., 2011). There is increasing evidence to support that the antioxidant property of estrogen is ascribed to the presence of the A-ring phenol which can reduce ROS created by the Fenton reaction by a cyclic phenol-quinol mechanism (Prokai et al., 2003).

A recent study conducted in 34 oophorectomized female rats has measured MDA directly on the vascular extract to determine lipid oxidative levels between a group receiving Estradiol Valerate subcutaneous 2.5 mg/kg/week and a control group. After 10 weeks, vascular MDA levels in the experimental group was found to be significantly lower than the control group. TAS was also measured and found to be decreased with oophorectomy in all groups but decreased less in the group that received estrogen therapy compared to the control group (Escalante et al., 2017). Another study conducted in rats using a daily pharmacological dose of 0.4 μg of estradiol-17β has shown that GSH level in the uteri of the treatment group was significantly higher than the untreated group (Suojanen et al., 1980).

Therefore, one of the mechanisms of estrogens involved in delaying the process of skin aging may be due to its antioxidant property.

2.6 Estrogen Therapy for Improving Skin Health in Menopausal Women

A randomized, double blind, controlled trial evaluated in 41 post-menopausal women who were allocated to receive either systemic hormonal replacement (valerate estradiol 2 mg/day

for 21 days and cyproterone acetate 1 mg/day for 10 days) or placebo, both in a cyclic scheme for 6 months, has reported that the collagen content of the hormonal group significantly increased after 6 months of treatment (Sauerbronn et al., 2000). In another study, 12 postmenopausal women, aged 52 to 76 years were assigned to receive topical estradiol treatment on the skin of lower abdomen and the vehicle only on the contralateral site; once a day for 3 months. The content of skin hydroxyproline; the levels of the carboxyterminal propeptide of human type I procollagen (PICP) and of the aminoterminal propeptide of human type III procollagen (PIIINP) and the number and quality of collagen and elastic microfibrils were measured. It was observed that the amount of hydroxyproline in the skin significantly increased (38%) during estradiol treatment and the PICP level was significantly higher on the treated site than on the control site after treatment (Varila et al., 1995).

A randomized, double-blind, placebo-controlled trial, has demonstrated that one year of oral estrogen replacement therapy can increase dermal thickness by 30% in post-menopausal women (Maheux et al, 1994), while a similar trial showed that six months of treatment with oral estrogen increases skin collagen by 6.49% (Sauerbronn et al., 2000). Other studies have stated an increase specifically in collagen type III (Savvas et al., 1993). The topical administration of estrogen can also increase skin collagen as measured by an increase in levels of type I and type III procollagen. However, with topical administration, the effect of estrogen is limited to the area where it was applied (Varila et al., 1995). Topical estradiol applied to the skin of buttock of elderly males and females significantly increased type I procollagen in both sexes. The same study also demonstrated that estrogen can increase tropoelastin and fibrillin, which may be associated with increased elastic fibers. Furthermore, estrogen can also increase TGF-β and TGF-β type II receptor expression, which may be related to dermal fibroblast proliferation and extracellular matrix (ECM) secretion, in contrary, it down regulated the expression of matrixmetalloprotease-I, which may explain the increased collagen content seen in skin treated with estrogen (Son et al., 2005).

However, estrogen therapy may not always be practicable due to its side effects and contraindications. Therefore, many studies have evaluated the possibility of using phytoestrogens as an alternative to hormonal therapy in post-menopause period.

2.7 Phytoestrogens

Phytoestrogens are compounds derived from plants with estrogen-like biological properties. Plant extracts were first reported to exhibit estrogenic activity in 1926. By 1975, several hundred plants had been found to contain estrogenically active compounds. In traditional medicine, the use of certain plants may be ascribed to their estrogenic properties. For example, the pomegranate is associated with fertility, and the Thai vine, *Pueraria Mirifica*, is used as a rejuvenant and aphrodisiac (Murkies et al., 1998).

1. Phytoestrogens Classification and Structures

Based on their chemical structure and biosynthesis patterns, phytoestrogens may be divided into chalcones, isoflavonoids, lignans, coumestans, stilbenoids, and miscellaneous classes. Particular attention should be given to isoflavonoids, the subgroup of flavonoids which includes amongst others the chemical groups of isoflavones, isoflavanones, pterocarpanes, and coumestans (Michel et al., 2013).

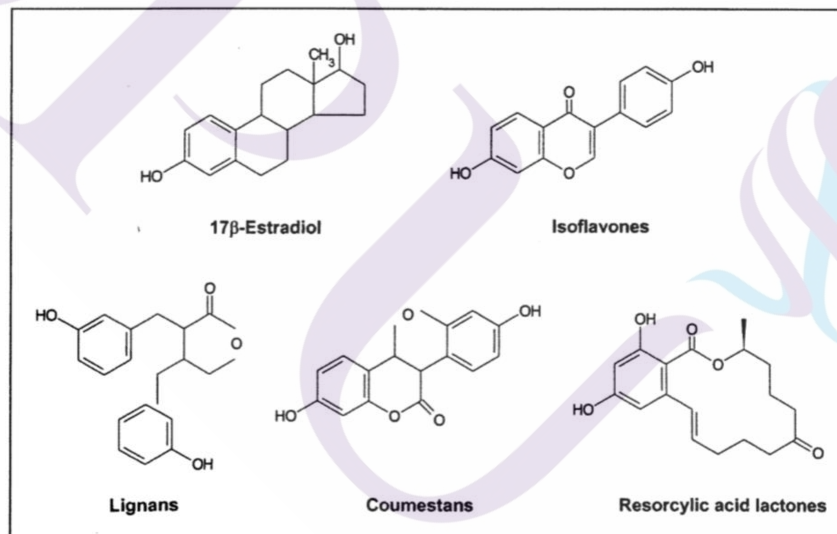


Figure 2.4 Molecular structure of human estrogen and phytoestrogens compounds

Source: Munro et al., 2003

2. Phytoestrogens Sources and Metabolism

Phytoestrogens are known to be presented in vegetables, fruits, and whole grains commonly consumed by humans. They are abundant in several medicinal plants, belonging

mostly to the Leguminosae family. Plant extracts with estrogen-like activities include soy, red clover, kudzu, hops, licorice, rhubarb, yam, and chasteberry (Hajirahimkhan et al., 2013). Isoflavones are found in legumes, mainly soybeans. Several soy foods are made by adding soy ingredients to a wide variety of manufactured foods; thus, the isoflavone content is diminished, for example; tofu, yoghurt and soy noodle. Lignans can be found in cereals, fruit, and vegetables but the main source of lignans is flaxseed. Coumestans are abundantly present in clover, alfalfa and soybean sprouts. 8-Prenyl flavonoids are commonly found in vegetables, hop, and beer (Murkies et al., 1998; Sirotkin et al., 2014).

Table 2.1 Food sources of phytoestrogens

Isoflavones		**Lignans**			**Coumestans**	
Legumes	**Soybean products**	**Whole grain**	**Fruits, Vegetable**	**Alcoholic sources**	**Bean sprouts**	**Fodder crops**
Soybeans Lentils Beans Chick peas	Soy meal Soy grits Soy flour Tofu Soy milk	Wheat Wheat germ Barley Hops Rye Rice Brans Oats	Cherries Apples Pears Stone fruits Linseed Carrot Fennel Onion Garlic Olive oil	Beer from hops Bourbon from corn	Alfalfa Soybean sprouts	Clover

Source: Murkies et al., 1998

Dietary phytoestrogens are metabolized by gut microflora, absorbed, conjugated in the liver, circulated in plasma and excreted in urine (Cassidy, 2003). The biological effect of dietary phytoestrogens is mainly due to their metabolites generated by gut microflora (Branca & Lorenzetti, 2005). The estrogenic activity of phytoestrogens can be enhanced after they are

metabolized into more active compounds such as genistein and daidzein by gut microflora (Zhengkang et al., 2006). The effects of daidzein is variable depending on ability of individuals to convert daidzein into more active equol (Gil-Izquierdo et al., 2012). Bioavailability of isoflavones requires intestinal beta-glucosidases for an initial hydrolysis of the sugar moiety to allow the following uptake by enterocytes and enter the peripheral circulation. After being absorbed, isoflavones are then reconjugated mainly to glucuronic and sulfuric acids (Cassidy, 2003). As dietary phytoestrogen metabolism is mainly determined by the gut microflora; thus, antibiotic use and bowel diseases will modify metabolism.

Level of lignans and isoflavones can be measured in urine, plasma, feces, bile, saliva, semen, and breast milk (Adlercreutz et al., 1995) Concentrations of the different phytoestrogen metabolites are variable between individuals even when a controlled quantity of an isoflavone or lignan supplement is administered.

3. Phytoestrogens Mechanism of Actions

Phytoestrogens have a chemical structure similar to mammalian estrogen, estradiol, and bind to estrogen receptors α and β with a preference for ERβ. After binding with ligand, these receptors move from cytoplasm to the nucleus and bind to the transcription-control center of DNA or small RNAs; therefore, they can affect the expression of specific genes. Phytoestrogens can potentially affect all the processes regulated by estrogens such as induction of sex hormone binding globulin and inhibition of aromatase. Estrogen receptors are presented in various tissues: central nervous system (including hypothalamo–hypophysial axis), gonads, reproductive tract, mammary gland, bones, gastrointestinal tract, and lungs. This suggests that phytoestrogens may have tissue specific hormonal effects. The estrogen receptor-specific effects may occur too. For example, ERα is considered as a promoter of cell proliferation, whilst ERβ promotes mainly cellular apoptosis (Rietjens et al., 2013).

The biological potencies of phytoestrogens vary. Most of these compounds are non-steroidal in structure and much less potent than the synthetic estrogens. They vary between species, routes of administration, and end points expected. The relative potencies as determined by human cell culture are: coumestrol 0.202, genistein 0.084, equol 0.061, daidzein 0.013, and formononetin 0.0006 (Adlercreutz, 1990; Markiewicz et al., 1993).

Phytoestrogens can exhibit both weak estrogenic and anti-estrogenic actions. Genistein has been the phytoestrogen of highest interest and has been shown to exert both proliferative (estrogenic) and anti-proliferative (anti-estrogenic) effects in human cell line. In the human estrogen receptor-positive MCF-7 breast cancer cell line, the effects of genistein are biphasic and concentration dependent, with stimulation of cell growth occurring at low concentrations of genistein, and inhibition at higher concentrations (Wang et al., 1996).

The anti-proliferative effects of genistein occurred in both ER-positive and ER-negative cell lines; thus, they appear not to be related with the ER. It has been suggested that genistein, and perhaps other phytoestrogens, inhibit tumor cell growth by interrupting the tyrosine kinase activity of activated growth factor receptors and cytoplasmic tyrosine kinases, which are crucial for the transduction of mitogenic signals (Wang et al., 1996).

Besides their ability to bind to estrogen receptors, Phytoestrogens also have other biological effects, which are not mediated by these receptors, for example, activation of serotoninergic receptors, IGF-1 receptors, binding of free radicals, inducting DNA methylation, affecting tyrosine kinase, transcription factors NF-kappaB and DNA topoisomerase activities, histone modification, RNA expression and other intracellular regulators of cell cycle and apoptosis. These abilities are responsible for anti-oxidant, anti-proliferative, anti-mutagenic and anti-angiogenic effects of phytoestrogens and their ability to promote human health and longevity (Cassidy, 2003).

4. Phytoestrogens and Skin

Phytoestrogen may exert anti-aging effect on the skin via estrogen receptors, increase in hyaluronic acid production, collagen, extracellular matrix proteins (Gopaul et al., 2012) or via promotion of skin vascularization, cell proliferation, protection against oxidative stress and apoptosis. Skin aging in menopausal women can be significantly delayed by the administration of estrogen, selective estrogen receptor modulators and phytoestrogens (Thornton, 2013).

5. Possible Side Effects of Phytoestrogens

Despite being widely used by postmenopausal women for the treatment of the climacteric syndrome, the risk of adverse effects of this treatment is still unclear. A meta-analysis

which identified 174 randomized controlled trials with 9629 participants reported 92 side effects. The overall incidence of side effects in the phytoestrogen and control group was 36.7% and 38.0%, respectively. Significantly higher rates of gastrointestinal side effects were found among phytoestrogen users comparing with other side effect categories; whilst musculoskeletal, neurological, and unspecific side were not significantly different between groups. Within side effect categories, there was no significant difference in rates of side effects between women using phytoestrogens and control group including the rates of endometrial hyperplasia, endometrial cancer, and breast cancer (Tempfer et al., 2009).

Based on the available evidences, phytoestrogens have a safe side-effect profile with slightly elevated rates of gastrointestinal side effects. Rates of vaginal bleeding, endometrial hyperplasia, endometrial cancer, and breast cancer were not significantly increased among phytoestrogen users (Tempfer et al., 2009).

2.8 Nutraceuticals used in This Study

1. Soy Isoflavones

Soy isoflavone is a type of flavonoids contained in legumes, with the richest source in soybeans (glycine max). There are three types of isoflavone aglycones; genistein, daidzein and glycitein. These also exist as their respective glycosides (genistin, daidzin, glycitin); glycoside acetyl forms and malonyl forms. They are structurally similar to estradiol (Munro et al., 2003).

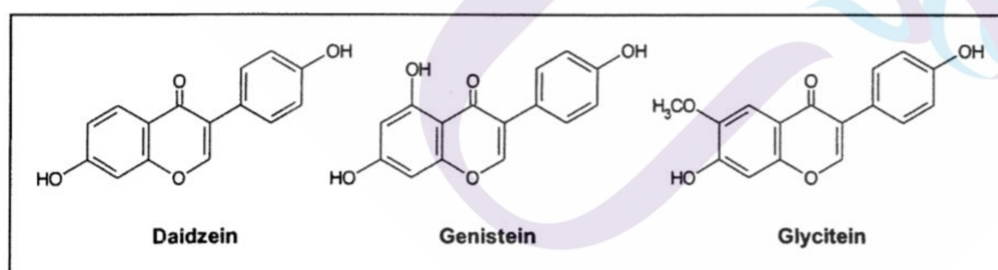


Figure 2.5 Chemical structures of the isoflavones, daidzein, genistein and glycitein in aglycones

Source: Food Safety Commission of Japan, 2006

Soy isoflavone glycosides are hydrolyzed by enzymes in saliva and small intestine, and also by ß-glucosidase in intestinal microflora, whereby the aglycones daidzein and genistein are produced. The aglycones and metabolites are absorbed by enterocyte and then transported to

the liver. The glucuronic acid and sulfate conjugates are then excreted into the bile. The metabolites are deconjugated at the intestine mostly by ß-glucuronidase contained in intestinal microflora and subjected to enterohepatic circulation through the reabsorption. They are then excreted in the urine. The secondary metabolites of soy isoflavone are equol and o-desmethylangolensin (O-DMA), which are formed from daidzein. Furthermore, dihydro-genistein is produced from genistein (Munro et al., 2003).

Isoflavones competitively bind to ERs, causing an increase in transcriptional activity of genes with ER response elements. Genistein shows a stronger affinity for binding activity to the ER than daidzein. While equol, a metabolite of daidzein, has a slightly stronger affinity than genistein. For ER-α, genistein has a binding affinity estimated as 4/100 of E2 and daidzein was 1/1000 in the solid-phase binding assay. For ER-ß, genistein has a binding affinity estimated as 87/100 of E2 and daidzein was 5/1000 in the solid-phase binding assay. Variations between individual in the biological effects of soy isoflavones may come from differences of absorption, metabolism, and equol production. The prevalence of people that produce equol, although it depends on race and sex, ranges between 20-60% and it is thought that certain types of intestinal microflora may affect the production of equol (Food Safety Commission of Japan, 2006).

Soy isoflavone is reported to have the synergistic and antagonistic effects in vitro and in experimental animal tests. It is also reported to induce apoptosis in human cancer cells, inhibit cell proliferation and inhibit carcinogenesis. There are also reports of an anti-carcinogenetic effect in vivo (Food Safety Commission of Japan, 2006).

Apart from their estrogenic effects, Isoflavones also have been linked to decreased risk of cardiovascular disease, osteoporosis, endocrine-responsive, and menopausal symptoms, due to their possible antioxidant activities. Research on the antioxidant effect of isoflavones suggests free radical scavenging ability, ability to reduce LDL and DNA susceptibility to oxidative stress and ability to increase the activity of antioxidant enzymes (Yoon & Park, 2014).

In a randomized, double-blind, placebo-controlled trial, 26 women in their late 30s and early 40s were randomly assigned to receive either an oral intake of 40 mg soy isoflavone aglycones per day or placebo for 12 weeks. It was reported that the isoflavones improved fine wrinkles and malar skin elasticity at the end of the study period (Izumi et al., 2007).

Another study was performed in 30 post-menopausal women to evaluate before and immediately after the end of treatment with 100 mg/day of an isoflavones-rich, concentrated soy extract for six months. A skin punch was done in the gluteal region for sample collection before and immediately after the treatment program. The epidermal thickness, the amount of dermal elastic and collagen fibers, and the amount of blood vessels in the sample was evaluated. A 9.46% increase in the epidermal thickness was observed in 23 patients after isoflavone treatment. The amount of collagen fibers in the dermis was increased in 25 women (86.2%). In 22 women (75.8%), the increase in the number of elastic fibers was observed. The number of dermal blood vessels was also significantly increased in 21 women (Accorsi-Neto et al., 2009).

2. Evening Primrose Oil (EPO)

Lipids are crucial components of the skin and of these the omega-6 fatty acid linoleic acid (LA), recognized to be an essential fatty acid. Linoleic acid is the main fatty acid in ceramides, the largest polar lipid class of the stratum corneum. Insufficient intakes of LA lead to scaling and increased loss of water across the epidermis, which is a sign of a disturbed epidermal skin barrier. Administration of LA has been shown to regenerate a defective skin barrier (Muggli, 2005).

In addition to its structural function, LA has a metabolic role as the parent fatty acid of the long-chain gamma-linolenic acid (GLA), a precursor of prostaglandin E1 and 15-hydroxy-eicosatrienoic acid, which have potent anti-inflammatory effects (Ziboh et al., 2000)

In industrialized societies, the diet is rather oversupplied with LA due to the widespread use of vegetable oils. However, the skin may still be undersupplied with GLA. The delta-6-desaturase, the enzyme that transforms LA into GLA, can be impaired by age, genetics and metabolic diseases such as diabetes. Excessive intakes of trans-fatty acids, saturated fatty acids, insufficient intakes of zinc, magnesium, and vitamin B6 as well as smoking, alcohol drinking and psychological stress may decrease the activity of the enzyme (Horribin, 1990). Therefore, insufficient amounts of GLA may occur in the skin even in the presence of adequate LA (Muggli, 2005).

Evening primrose oil (EPO) is a natural source of LA and GLA. The anti-inflammatory properties of gamma-linolenic acid are useful in supporting menopause and PMS,

to help alleviate symptoms associated with vasomotor function and neurological stress responses. Furthermore, Evening primrose oil has been reported to restore a defective epidermal skin barrier, to normalize transepidermal water loss (TEWL) and to improve the skin smoothness both after topical application to healthy adults (Nissen et al., 1995) and after systemic administration to eczematous adults (Nissen et al., 1988). The skin-improving effects have been ascribed to the effects of GLA. Several studies strongly suggest that skin may have an inherent requirement for GLA for optimal structure and function (Muggli, 2005).

A randomized, double-blind, placebo-controlled study in healthy adults evaluated the effect of Evening primrose oil on skin moisture, transepidermal water loss (TEWL), redness, firmness, elasticity, fatigue resistance and roughness. EPO 3g/day was administered orally for 12 weeks. After the treatment, all measured variables, with the exception of skin redness, were significantly different in the EPO group comparing to the placebo group. Skin moisture, TEWL, elasticity, firmness, fatigue resistance and roughness were significantly improved (Muggli, 2005).

A randomized, double-blinded, controlled clinical trial to evaluate the efficacy of EPO was performed in Korean patients with AD. 50 mild AD patients with an Eczema Area Severity Index (EASI) score of 10 or less were randomly assigned into two groups. The first group received 450 mg of EPO (40 mg of GLA) per day, while placebo capsules were given to the other group for 4 months. At the end of the treatment, the patients of the EPO group showed a significant improvement in the EASI, whilst the patients of the placebo group did not. Although not statistically significant, the TEWL and skin hydration also slightly improved in the EPO patients group (Chung et al., 2018).

3. Black Cohosh

Black Cohosh (*Actaea racemosa L.*, formerly *Cimicifuga racemosa*) is a perennial woodland herb native to North America with large compound leaves and a thick, knotted rhizome system. Its rhizomes contain several different active compounds such as triterpene glycosides and polyphenols. Most triterpene aglycones are cycloartanes, whereas the polyphenolics are diverse, including isoferulic and salicylic acids, tannins, and other compounds (Betz et al., 2010).

Black cohosh extract (BCE) has long been used to alleviate menopausal symptoms. The study of BCE is limited due to the lack of standardization of the extract to one or more active

compositions. It remains unclear which ingredients are essential for menopausal symptom relief because most of the research on efficacy has been focused on the whole extract, not individual components present in the extract. Although the mechanism by which BCE relieves symptoms remain unclear, several hypotheses have been proposed: it acts as a selective estrogen receptor modulator, acts on serotonergic pathways, acts as an antioxidant, or acts through inflammatory pathways (Ruhlen, 2008).

Black cohosh extracts show serotonergic properties which help controlling thermoregulation, mood and the balance of LH and GnRH under estrogen withdrawal. However, the studies to determine whether BCE has estrogenic activity have revealed conflicting results. Human data of estrogenic effects of BCE are not consistent; however, reports of BCE estrogenic effect are consistent with the effects of a selective estrogen receptor modulator (SERM), which acts as an estrogen agonist in some tissues, and as an estrogen antagonist in other tissues. BCE does not induce the proliferation of hormone dependent or independent breast or prostate cancer cell lines, but it inhibits cell proliferation via the induction of apoptosis. Some study revealed that BCE competed with estradiol for binding to ERs in uterine cytosol but did not bind recombinant ERs. The Variation of the study outcomes may be due to inconsistent triterpene concentrations present in the various preparations used in each BCE products (Ruhlen, 2008).

To date, the effects of black cohosh on skin health in post-menopausal women have not yet been identified. However, according to their possible mild estrogenic activity and antioxidant properties, we assume that black cohosh may also exert beneficial effects on skin health.

The safety of black cohosh has been extensively reviewed in many studies. The data from 78 case reports suspected black cohosh hepatotoxicity have been evaluated, all of the cases were poorly documented, and there were many confounding factors including failure to identify the product, use of multiple ingredients in addition to black cohosh, co-medication with other synthetic drugs and herbs, failure to specify the modalities of black cohosh treatment and preexisting liver disease. Due to the insufficient of significant circumstantial evidence, the review suggests that there are few hepatotoxic risks of black cohosh (Soni et al., 2011).

4. Chasteberry

Chasteberry (*Vitex agnus-castus*) is a well-known herb that grows in middle-Asia and Mediterranean countries. It has been used as a reproductive system toner and used to regulate menopause, sexual ability and menstruation. Its precise mechanism of action have not been established yet. Chasteberry contains a wide variety of synergistic active constituents which include flavonoids, diterpenes, iridoid glycosides and essential oils. Some constituents may have anti-inflammatory, sedative, or analgesic properties (Gardiner, 2000). Chasteberry also has dopaminergic properties; this can improve several physical and psychological symptoms of menopause, while also inhibit excessive or fluctuating prolactin production to help regulate estrogen and progesterone level. Flavonoids from chasteberry are agonists for μ- and δ-opioid receptors; thus it helps regulate hormone levels, provide relief from pain and are able to modify other opioid mediated symptoms (Slopien et al., 2015). A recent study investigated effects of the chasteberry crude extract on prostate cancer reported that chasteberry extract significantly inhibit cyclooxygenase-2; therefore, it suggested that chasteberry crude extract has a selective anti-inflammatory effect. Furthermore, the study also showed that chasteberry extract has a crucial role in improving oxidative stress produced in cancer-induced animals as it increased reduced glutathione (GSH) concentration and induced glutathione reductase, glutathione-S-transferase, glutathione peroxidase, and catalase activities (Ibrahim et al., 2017).

Only few clinical studies have investigated the use of chasteberry for the skin health improvement. Therefore, more studies are required to indicate whether it has beneficial effects on the skin or not.

2.9 Safety Information

Table 2.2 Safety Information

Nutraceutical	The dosage used in this study	Max. dosage used in other previous studies	Safety Information
Soy isoflavones	100 mg/day	160 mg/day	A systematic review of total 43 studies evaluating multidimensional effects of phytoestrogen in postmenopausal women using isoflavones maximum dosage of 160 mg/day reported no significant side effects (Perna et al., 2016)
Black cohosh	80 mg/day	160 mg/day	A randomized study conducted in 351 menopausal women using black cohosh 160 mg/day (contain 2.5% triterpene glycosides) for 12 months to treat menopausal symptoms. No significant side effects reported (Newton et al., 2006) The German Commission E Monograph recommends a maximum treatment duration of 6 months, crude drug: 40–80 mg/day (American Botanical Council, 2003).
Evening Primrose Oil	500 mg/day	6000 mg/day	A randomized, placebo controlled trial conducted in 53 AD patients using 2000-6000 mg EPO per day for 5 months reported no adverse effects (Senapati et al., 2008)
Chasteberry	40 mg/day	40 mg/day	A randomized, double-blind, placebo controlled trial conducted in an academic center in Gorgan-Iran evaluating 60 postmenopausal teachers aged 45-60 years old treated with 40 mg of Vitex per day for 8 weeks reported no serious side effects (Abbaspoor, 2011)

Chapter 3

Research Methodology

3.1 Research Design

This experimental, randomized, double-blind, placebo-controlled clinical trial has been approved by the College of Integrative Medicine's Ethical Review Committee for Human Research (006/62EX) and was conducted in accordance with the Declaration of Helsinki on human subjects. Also, the clinical trial registration was approved by Thai Clinical Trials Registry (TCTR20190417001)/ the WHO International Clinical Trials Registry Platform (WHO-ICTRP) dataset.

3.2 Population

Post-menopausal women in their 45-60 years of age whom were recruited at the Department of Nutrition, Faculty of Public Health, Mahidol University, Thailand.

3.3 Sample

110 subjects were selected from candidates according to the inclusion criteria.

The sample size of this clinical study was referred to the previous studies about soy isoflavone and menopause symptoms which recruited at least 58 subjects. (Maturitas 2005;51:127-34., Menopause 2004;11:400-404., Obstet Gynecol 2003;101:1213-1220.)

3.4 Sample Selection

1. Inclusion Criteria

    1.1 Women 45-60 years of age

    1.2. Women whom menstruation have ceased for at least 12 consecutive months

    1.3. Type II-III fine lines and wrinkles (assessed by Glogau wrinkle classification)

    1.4. Willing to attend the project

1.5. Women who have not been taking any herbs, dietary supplements, medicine, or hormonal therapy that contains estrogenic effects, contains antioxidants, affects estrogen level or skin health for 1 month prior to this study (a washout period of 1 month was according to the article: A multicenter, placebo-controlled, double-blind clinical trial assessing the effects of a multicomponent nutritional supplement for treating photoaged skin in healthy women by Casini et al., 2006)

1.6. No botulinum toxin or fillers injected into the facial area in less than 6 months

1.7. No Thermage or Ulthera done on the face in less than 6 months

1.8. No any of the following procedures done on their facial area in less than 1 month prior to study: lasers, IPL, dermabrasion, iontophoresis, PRP injection, chemical peels, or other procedures that can alter skin wrinkling and skin aging

1.9. No underlying diseases or health conditions which may interfere with the treatment or cause higher risk to develop side effects; for example, diabetes mellitus, liver diseases, renal diseases, cardiovascular diseases, immunological disorders, and hypo/hyperthyroid disease

1.10. No history of tumors or cancers

1.11. Not currently smoking

1.12. No history of food allergy

2. Exclusion Criteria

2.1. Volunteers who develop any adverse reactions or request to quit the treatment

2.2. Fail to follow the instructions during the study: compliance <80%

2.3. Receive any other treatments or procedures during the study

2.4. Fail to follow the appointments more than once or fail to show up at the first visit

3.5 Glogau Wrinkle Classification

The Glogau classification was developed by dermatology professor Dr Richard Glogau in 1996 to categorize severity of wrinkles. Referenced by dermatologists and aesthetic practitioners worldwide, it is a practical and easy-to-use clinical scale that provides another useful measurement of the effect of treatment.

Type I: ‘No Wrinkles’

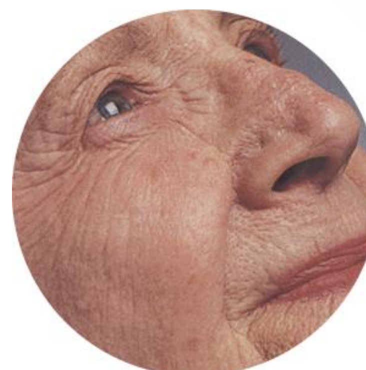
- Early photo-aging
- Mild degree of pigment changes
- Minimal to no wrinkles
- No age spots

Type II: ‘Wrinkles in Motion’

- Typical age: 30s-40s
- Early to moderate photo-aging
- Appearance of lines only when face moves
- More prominent skin pores
- Early alterations in skin texture

Type III: ‘Wrinkles at Rest’

- Typical age: 50s-60s
- advanced photo-aging
- Appearance of wrinkles at rest
- Visible brown age spots
- Prominent, small blood vessels



Type IV: ‘Only Wrinkles’

- Typical age: 60s and older
- Severe photo-aging
- Wrinkles everywhere, at rest or moving
- Yellow-gray skin color
- Prior skin cancers
- Pre-cancerous skin changes (actinic keratosis

Figure 3.1 Glogau Classification

Source: Richard G. Glogau

Table 3.1 Glogau Classification

Group	Classification	Typical Age	Description	Skin Characteristics
I	Mild	28-35	No wrinkles	Early photoageing: mild pigment changes, no keratosis, minimal wrinkles, minimal or no makeup required
II	Moderate	35-50	Wrinkles in motion	Early to moderate photoageing: early brown spots visible, keratosis palpable but not visible, parallel smile lines begin to appear, wears some foundation
III	Advanced	50-65	Wrinkles at rest	Advanced photoageing: discolouration, visible capillaries, visible keratosis, wears heavier foundation
IV	Severe	60 and above	Only wrinkles	Severe photoageing: yellow/grey skin colour, prior skin malignancies, wrinkles throughout, no normal skin, cannot wear makeup because it cracks and cakes

Source: Aesthetics journal, 2017

3.6 Research Instruments

1. Combined nutraceuticals product (Belle Dame; Max Biocare, Australia):

Composition (per tablet)	Content
SoyLife® isoflavones (mainly as daidzin/ein, genistin/ein, glycitin/ein) *from Glycine max (Soya Bean) seed germ ext. dry conc. std. equiv. to fresh*	100 mg *7.5 g*
Actaea racemosa (Black Cohosh) root & rhizome ext. dry conc. *equiv. Triterpene glycosides calc. 27-desoxyactein* *equiv. dry*	80mg *2 mg* *520 mg*
Vitex agnus-castus (Chasteberry) fruit ext. dry conc. *equiv. dry*	40 mg *400 mg*
Evening Primrose Oil *Containing: gamma-Linolenic acid 50mg & Linoleic acid 325 mg*	500 mg

2. Placebo: soybean oil
3. Study record form
4. Consent form
5. Satisfaction self-evaluation form

6. 3D Camera: QuantifiCare 3D LifeViz® Mini

7. Visioscan®

8. Cutometer® (Probes: Cutometer®, Corneometer®, Tewameter®)

9. Mexameter®

10. Skin-Glossymeter®

3.7 Skin Parameters Measurement

The skin parameters in this study were evaluated using the following equipments:

1. Cutometer®

Figure 3.2 Cutometer® dual MPA 580

Source: Courage + Khazaka electronic GmbH

The Cutometer® dual MPA 580 is a standard device to measure viscoelastic properties (elasticity, firmness, tonicity and suppleness) of the skin. The Multiprobe Adaptor function allows to connect additional probes other than the two Cutometer® probes.

The principle is based on a mechanical deformation of the skin via the suction method. A negative pressure is created in the device and the skin is drawn into the 2 mm aperture of the probe. The resistance of the skin to the negative pressure (firmness) and its ability to return into its original position (elasticity) are displayed as real time measurement curves (penetration depth in mm/time).

The probe has to be applied to the skin at a right angle and has to be hold very steady during the measurement. A spring in the probe head ensures constant pressure on the skin. It must not be pressed too tightly onto the skin, otherwise, the skin is pressed into the probe and could touch or grease the glass prisms. Pressing too tightly also leads to disturbed blood circulation, thus influencing the measurement. It must sit plainly without gap on the skin throughout the complete measurement.

During the first phase of the measurement, the skin is sucked into the probe opening by negative pressure and the sound of the pump can be heard. Then the negative pressure is cut off and no sound is heard while the skin is relaxed in the second phase of the measurement. Leave the probe still and plainly throughout both phases of the measurement.

2. Corneometer®

The Corneometer® CM 825 is an instrument used to determine the hydration level of the skin surface, primarily the stratum corneum. The measurements are performed by the application of a probe to the skin surface. Upon contact, an electric field passes through the stratum corneum and the dielectric constant is obtained. The value of the dielectric constant (in arbitrary units) is directly proportional to the level of skin hydration.

Place the Corneometer®-probe head straight on the skin area to be measured according to the pressure of the spring in the probe. Hold it still. The measurement is triggered by the skin contact and after one second the result is displayed accompanied by an acoustical signal.

Putting the probe on the same skin area can cause accumulation of water under the probe head; thus, measuring values become higher even though the water content in the stratum corneum has not changed. The measurement should be repeated on different spots or adjacent skin area. Taking three measurements at adjacent skin areas and regard the average value is recommended.

Figure 3.3 Corneometer® probe

Source: Courage + Khazaka electronic GmbH

3. Visioscan®

The Visioscan® VC 98 USB is a special UVA-light video camera with high resolution to study the skin surface directly. The images show skin structure and level of dryness of the skin. A variety of interesting parameters can be evaluated: SELS (Surface Evaluation of the Living Skin), analyses the grey level distribution and allows the calculation of four clinical parameters to qualitatively and quantitatively describe the skin surface as an index: Skin smoothness (SEsm), Skin roughness (SEr), Scaliness (SEsc), Wrinkles (SEw).

Figure 3.4 Visioscan® VC 98 USB

Source: Courage + Khazaka electronic GmbH

When the camera is lit and put on the skin, a live image can be seen. The camera turns off automatically when not moved for a few seconds to save energy and prevent overheating.

Pressing the camera button shortly will turn it on again. Press the camera button a little bit longer to freeze the image. The exposure time is set to 60 for skin images. It may be increased or decreased for very fair or dark skin but only the results from images taken with the same exposure time can be compared.

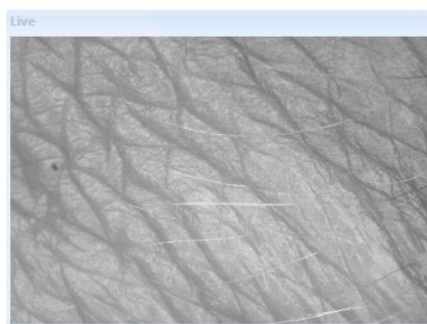


Figure 3.5 Live image from Visioscan®

Source: Courage + Khazaka electronic GmbH

4. Mexameter®

The Mexameter® MX 18 is a device that measures simultaneously two major components of skin pigmentation: melanin and hemoglobin.

The measurements are performed by the application of a probe to the skin surface. Place the probe head straight on the skin area to be measured according to the pressure of the spring in the probe. Hold it still. The measurement is triggered by the skin contact and after one second the results for melanin and erythema are displayed accompanied by an acoustical signal.

The probe has a 5 mm aperture that emits radiations. These radiations are reflected by the skin and captured back by the same probe.

Figure 3.6 Mexameter® probe

Source: Courage + Khazaka electronic GmbH

The results are expressed as index value for each parameter (melanic index and erythematic index) in arbitrary units on a scale from 0 to 999.

5. Tewameter®

The Tewameter® TM 300 is a device for the assessment of the transepidermal water loss (TEWL). This parameter provides information on the skin integrity or the skin protective barrier functions.

Place the short end of the probe head on the skin. To avoid skin entering the probe, it should not be pressed too tightly against the skin. Also the blood circulation can be disturbed thus influencing the measurement. If the probe does not sit tightly enough on the skin, water may evaporate through the gap between probe and skin. The probe should be held absolutely still during the measurement. Under normal room conditions, a stable measurement can be achieved in 20 seconds. Upon contact, the evaporation rate of the water (g/h/m²) is obtained. High values of TEWL reflect a loss of skin barrier function.

Figure 3.7 Tewameter® probe

Source: Courage + Khazaka electronic GmbH

6. Skin-Glossymeter

The Skin-Glossymeter GL 200 is a tool used for measuring the gloss especially on skin. Applications on lips, hair, teeth and other materials are also possible. The measurement is based on reflection. Parallel white light is created by the LEDs in the Glossymeter probe head and sent via a mirror in a 60° angle onto the skin surface. One of the two sensors measures the directly reflected light by a mirror, the other measures the diffuse reflected light vertically above the

surface. So the Skin-Glossymeter GL 200 measures both the portion of directly reflected light, which is related to the gloss, and the scattered portion from the surface.

Press the Skin-Glossymeter-probe on the skin surface lightly according to the pressure of the spring in the probe. It should be placed straight (as close to 90°as possible) and quickly on the skin. Make sure to use only a very little bit of pressure in order to avoid the skin entering the probe head and spoiling the mirrors and the optics inside the probe. After one second the measuring results are displayed accompanied by an acoustical signal.

Figure 3.8 Skin-Glossymeter Probe

Source: Courage + Khazaka electronic GmbH

3.8 Research Method

A total of 110 post-menopausal women were randomly allocated to group A or group B based on a sequence provided by an independent researcher and computer generated using a randomization plan from www.randomization.com. The manufacturer then labeled the combined nutraceuticals and placebo as supplement A or supplement B. Subjects in group A were administered with supplement A, while subjects in group B were administered with supplement B. Neither the subjects nor the researchers knew which group received the combined nutraceuticals and which group received the placebo. The study was conducted for 12 weeks. Assessments were made at week 0 (baseline), week 6, and week 12.

1st Visit**:** week 0

1. The subjects were selected according to the inclusion and exclusion criterias. The details about the objectives, potential benefits, potential side effects and

instruction of the study was informed to the subjects. The consent form was signed

2. 110 subjects were randomly assigned into 2 groups (55 subjects per group).
    2.1. Treatment group
    2.2. Placebo group

    Neither the participants nor the researchers knew which participants belonged to the control group, or the treatment group.
3. General information and past history of the subjects were obtained
4. Height and weight were measured by nurses
5. Blood pressure and pulse rate were measured by nurses
6. The skin in the facial area was cleaned
7. Photographs of the subjects' faces were taken with a 3D Camera
    7.1. Straight angle
    7.2. 45 degree angle of both sides
8. The skin parameters were assessed by a physician. The points of assessment were
    8.1. Point A: 1.5 cm lateral to lateral canthus of right side
    8.2. Point B: 1.5 cm lateral to lateral canthus of left side
9. The following parameters were assessed:
    9.1. Skin smoothness (SEsm), Skin roughness (SEr), Scaliness (SEsc), Wrinkles (SEw) were measured by Visioscan®
    9.2. Skin elasticity was measured by Cutometer®
    9.3. Skin hydration was measured by Corneometer®
    9.4. Transepidermal Water Loss (TEWL) was measured by Tewameter®
    9.5. Melanin index was measured by Mexameter®
    9.6. Skin gloss was measured by Skin-Glossymeter

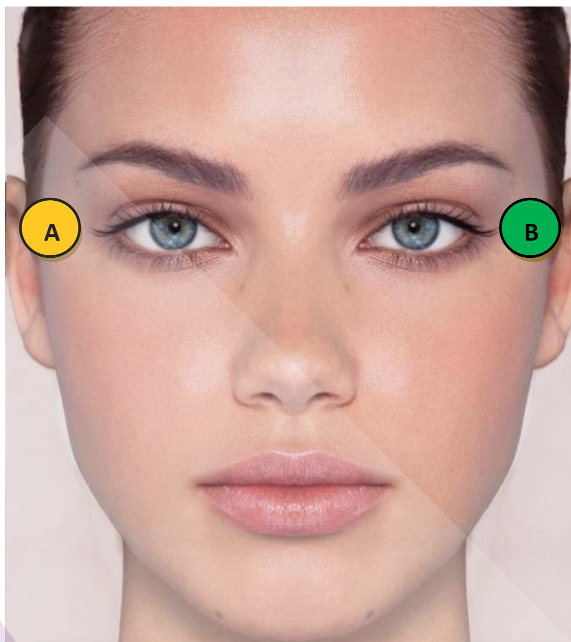


Figure 3.9 The points of assessment of skin parameters

10. Diet consumption information was assessed by nutritionists using 24-hour diet recall
11. Fasting blood sample was collected by nurses (blood sample volume: 15 ml). All biochemical analyses were carried out at N Health Asia Lab, Bangkok, Thailand, which is a medical laboratory with ISO15189:2007 certification.
    11.1. Malondialdehyde (MDA) was measured by the thiobarbituric acid reactive substances (TBARS) assay
    11.2. Reduced glutathione (GSH) was measured by the reduction of 5,50-dithiobis-(2-nitrobenzoic acid) method
    11.3. Aspartate transaminase (AST), Alanine transaminase (ALT)
    11.4. Blood urea nitrogen (BUN), Creatinine (Cr)
12. Satisfaction self-evaluation form was assessed by subjects
13. Subjects were administered with combined nutraceuticals or placebo, 1 capsule per day, after breakfast, for 12 weeks

2nd Visit: week 6

1. Height and weight were measured by nurses
2. Blood pressure and pulse rate were measured by nurses

3. The skin in the facial area was cleaned
4. Photographs of the subjects' faces were taken with the same camera, at the same spot, with the same settings and lights
    4.1. Straight angle
    4.2. 45 degree angle of both sides
5. The skin parameters were assessed by the same physician, at the same points. The following parameters were assessed:
    5.1. Skin smoothness (SEsm), Skin roughness (SEr), Scaliness (SEsc), Wrinkles (SEw) were measured by Visioscan®
    5.2. Skin elasticity was measured by Cutometer®
    5.3. Skin hydration was measured by Corneometer®
    5.4. Transepidermal Water Loss (TEWL) was measured by Tewameter®
    5.5. Melanin index was measured by Mexameter®
    5.6. Skin gloss was measured by Skin-Glossymeter
6. Diet consumption information was assessed by nutritionists using 24-hour diet recall
7. Satisfaction self-evaluation form was assessed by subjects

3rd Visit: week 12

1. Height and weight were measured by nurses
2. Blood pressure and pulse rate were measured by nurses
3. The skin in the facial area was cleaned
4. Photographs of the subjects' faces were taken with the same camera, at the same spot, with the same settings and lights
    4.1. Straight angle
    4.2. 45 degree angle of both sides
5. The skin parameters were assessed by the same physician, at the same points. The following parameters were assessed:
    5.1. Skin smoothness (SEsm), Skin roughness (SEr), Scaliness (SEsc), Wrinkles (SEw) were measured by Visioscan®

5.2. Skin elasticity was measured by Cutometer®

5.3. Skin hydration was measured by Corneometer®

5.4. Transepidermal Water Loss (TEWL) was measured by Tewameter®

5.5. Melanin index was measured by Mexameter®

5.6. Skin gloss was measured by Skin-Glossymeter

6. Diet consumption information was assessed by nutritionists using 24-hour diet recall
7. Fasting blood sample was collected by nurses (blood sample volume: 15 ml)

   Blood biochemistry test:

   7.1. MDA, GSH

   7.2. AST, ALT

   7.3. BUN, Cr
8. Satisfaction self-evaluation form was assessed by subjects
9. Compliance was assessed at the last visit. We calculated the amount of tablets the subjects should have taken in order to be fully compliant. The "Compliance Index" was calculated as the number of taken tablets/the number of tablets that should have taken if compliance is 100%.

Safety parameters including liver and kidney function were assessed before and after the intervention. The surveillance for adverse reactions including anaphylaxis (rash, pruritus, and dyspnea), headache, dizziness, nausea, emesis, abdominal discomfort, weight increase, breast distending pain, and vaginal bleeding or spotting of unknown etiology was done during the study.

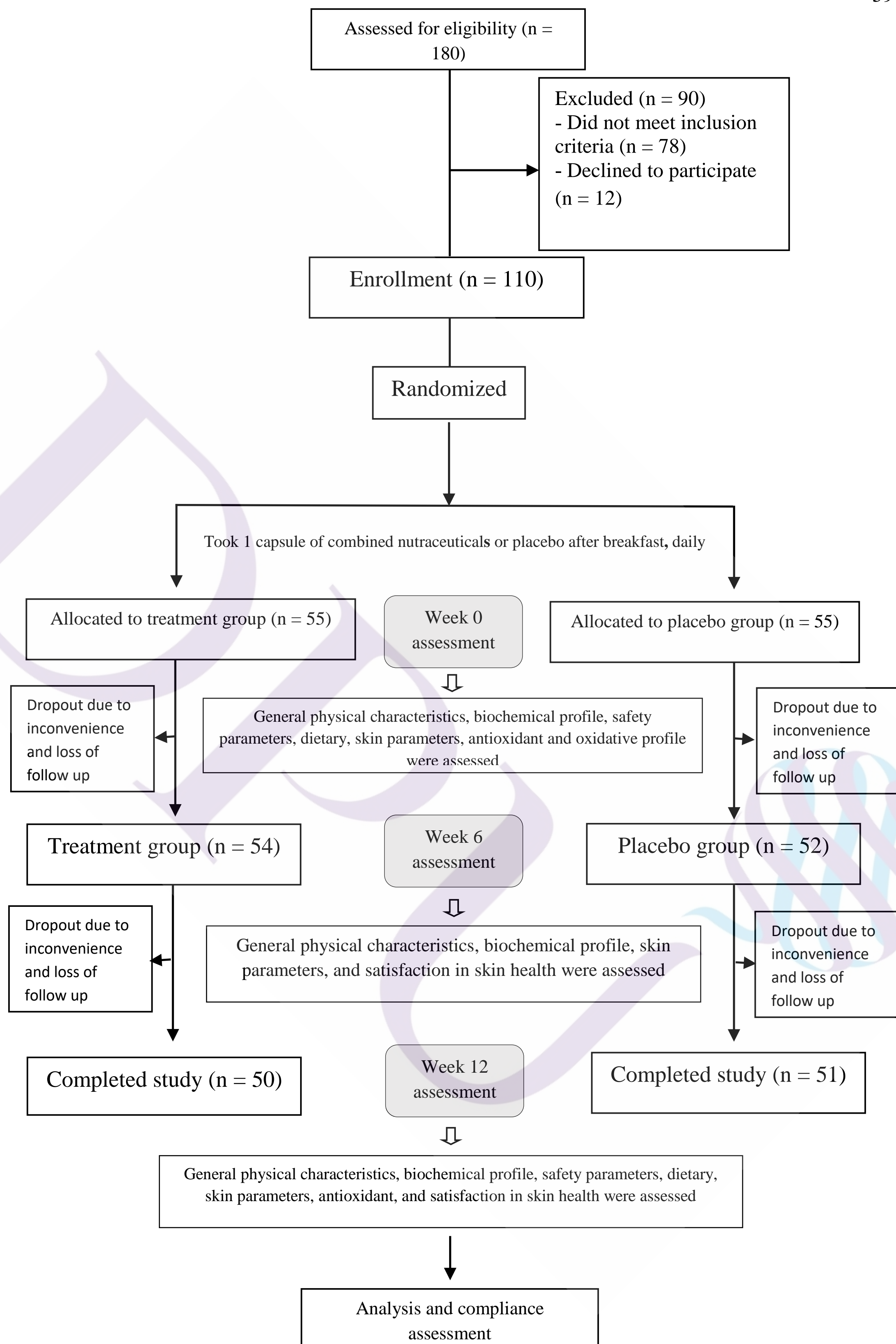
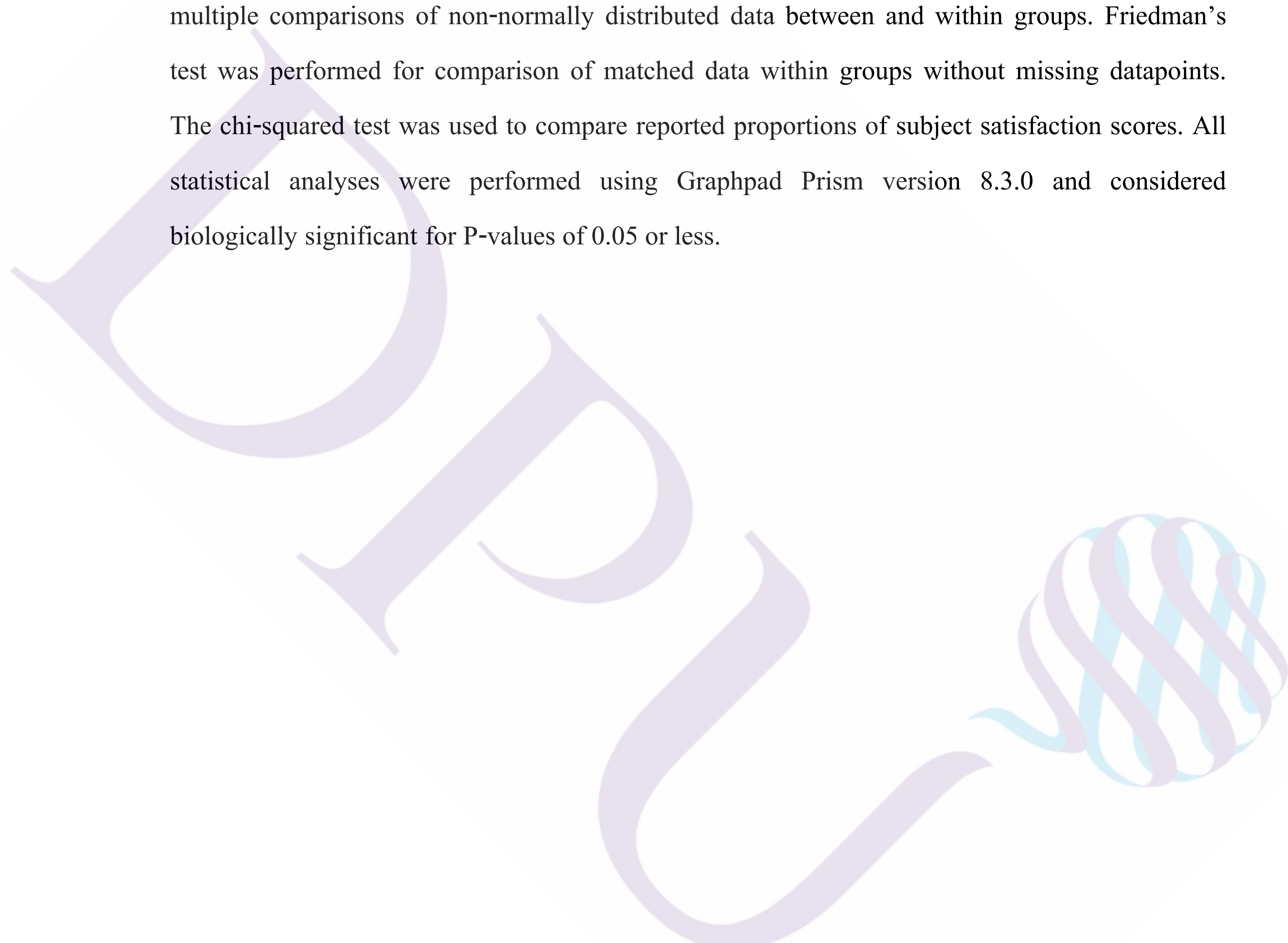


Figure 3.10 Study design and flow diagram

3.9 Statistics

Data obtained at baseline, 6 and 12 weeks, for treatment and placebo groups. Endpoint measures of dietary intakes and dermatological parameters were assessed for normality using the Shapiro-Wilk test. The ROUT test was used to identify outliers, which were omitted at Q=0.1%. For comparison of normally distributed groups, repeated measures ANOVA with Bonferroni correction was performed. The Kruskal-Wallis rank sum test was performed for multiple comparisons of non-normally distributed data between and within groups. Friedman's test was performed for comparison of matched data within groups without missing datapoints. The chi-squared test was used to compare reported proportions of subject satisfaction scores. All statistical analyses were performed using Graphpad Prism version 8.3.0 and considered biologically significant for P-values of 0.05 or less.



Chapter 4

Results

A total of 110 subjects were enrolled according to the inclusion and exclusion criteria and randomized into either the treatment group or the placebo group. During the study, 5 subjects from the treatment group and 4 subjects from the placebo group dropped out due to loss of follow up. Finally, a total of 101 post-menopausal women ranging in age from 47 to 58 years completed the clinical study.

In this chapter, the following information will be reported:

4.1. Characteristics of the subjects

4.2. Dietary assessment

4.3. Evaluation of clinical improvement in skin parameters

4.4. Antioxidant and oxidative stress profile

4.5. Satisfaction assessment

4.1 Characteristics of Subjects

The mean age of the subjects was 53.3 years in the treatment group, and 52.6 years in the placebo group; the difference in the mean ages was not significant. Table 4.1 shows the general physical characteristics including age, weight, BMI, body fat, blood pressure and pulse rate at baseline and week 12. No significant difference was observed in general physical characteristics of the subjects between the treatment group and the placebo group at baseline and at the end of the study.

BUN, Cr, AST and ALT were evaluated as safety parameters to monitor renal and liver function throughout the study. No significant difference was found between both groups and no significant change in these safety parameters was found within each group.

Table 4.1 General Characteristics and Blood Chemistry of Subjects

Characteristics and Blood Chemistry	Treatment		Placebo		P1	P2
	Baseline	**Week 12**	**Baseline**	**Week 12**		
Age (year)	53.3 (47-57)	-	52.6 (45-58)	-	0.055	-
Weight (kg)	59.79 ± 10.18	59.20 ± 10.17	62.13 ± 11.19	60.45 ± 13.67	>0.999	>0.999
BMI (kg/m2)	23.94 ± 4.03	23.08 ± 5.14	24.72 ± 4.28	24.47 ± 4.33	>0.999	>0.999
Body fat (%)	34.42 ± 6.27	33.86 ± 6.52	36.21 ± 6.49	36.00 ± 6.71	>0.999	>0.999
Blood pressure (mmHg)						
- Systolic	123.1 ± 19.32	120.8 ± 16.30	126.5 ± 13.82	124.7 ± 15.54	>0.999	>0.999
- Diastolic	78.92 ± 11.27	77.08 ± 12.15	82.36 ± 9.209	78.62 ± 10.55	0.662	>0.999
Pulse rate (bpm)	72.60 ± 9.52	72.58 ± 9.47	73.96 ± 11.00	75.94 ± 10.75	>0.999	>0.999
BUN (mg/dL)	12.58 ± 2.41	12.94 ± 2.46	12.58 ± 1.73	13.73 ± 2.59	>0.999	>0.999
Cr (mg/dL)	0.71 ± 0.16	0.66 ± 0.17	0.72 ± 0.22	0.67 ± 0.17	>0.999	>0.999
AST (U/L)	21.89 ± 4.80	21.76 ± 4.55	23.29 ± 5.44	23.55 ± 6.37	>0.999	>0.999
ALT (U/L)	20.59 ± 8.61	21.80 ± 7.64	18.33 ± 5.37	21.92 ± 9.59	>0.999	>0.999

Values are means ± SD. Means in a row with superscript letters without a common letter differ within group; Significant differences at $p < 0.05$. P1 = Comparison of mean between the two groups at baseline; P2 = Comparison of mean between the two groups at week 12; Significant differences at $p < 0.05$. BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

4.2 Dietary Assessment

Table 4.2 Total Energy and Nutrients Intake of the Subjects

Dietary Assessment	Treatment		Placebo		P1	P2
	Baseline	Week 12	Baseline	Week 12		
Energy (kcal/d)	1583 ± 448.10	1569 ± 477.40	1508 ± 455.90	1536 ± 409.10	>0.999	>0.999
Carbohydrate (% of energy)	57.17 ± 9.89	53.89 ± 8.14	57.90 ± 10.36	55.94 ± 12.47	>0.999	0.452
Protein (% of energy)	15.09 ± 4.31	17.79 ± 6.63	15.10 ± 4.76	15.67 ± 4.00	>0.999	>0.999
Fat (% of energy)	27.74 ± 8.56	28.32 ± 7.27	27.39 ± 8.83	28.39 ±10.31	>0.999	>0.999
Cholesterol (mg/d)	295.30 ± 95.92	289.70 ± 83.81	292.4 ± 90.29	282.5 ± 62.09	>0.999	>0.999
Fiber (g/d)	11.21 ± 4.87	11.56 ± 5.85	12.26 ± 7.03	12.29 ± 6.26	>0.999	>0.999

Values are means ± SD. P1 = Comparison of mean between the two groups at baseline; P2 = Comparison of mean between the two groups at 12 wk; Significant differences at $p< 0.05$.

Total nutrients consumption was calculated from the dietary record of individual subjects who participated in the study. Based on the data, the difference in the mean energy intake and the consumption of carbohydrate, protein, fat, cholesterol, and fiber at the baseline and at the end of the study between the treatment and the placebo group were not statistically significant.

4.3 Evaluation of Clinical Improvement of Skin Parameters

Table 4.3 illustrates the mean values of the skin parameters between the treatment group and the placebo group at baseline, week 6 and week 12.

Table 4.3 Skin parameters assessment

Skin Parameters	Treatment			Placebo			P1	P2	P3*
	Baseline	Week 6	Week 12	Baseline	Week 6	Week 12			
R2 ratio (Cutometer®)	0.57 ± 0.10	0.66 ± 0.09	0.72 ±0.10*	0.61 ± 0.09	0.61 ± 0.12	0.61 ± 0.09	0.5770	0.2592	<0.0001
Melanin index (Mexameter MX18®)	237.10 ± 62.60	236.30 ± 62.99	233.10 ± 57.13	231.50 ± 57.66	232.00 ± 56.02	230.00 ± 58.74	>0.999	>0.999	>0.999
Gloss DSC Value (Glossymeter GL200®)	5.29 ± 1.23	5.43 ± 1.59	5.28 ± 1.19	5.49 ± 1.77	5.49 ± 1.57	5.41 ± 1.73	>0.999	>0.999	>0.999
Skin hydration (Corneometer CM825®)	72.35 ± 7.06	76.02 ± 8.38	73.21 ± 7.64	73.62 ± 8.85	78.92 ± 7.15	77.77 ± 8.42	>0.999	>0.999	0.0412
TEWL (g/h/ m^2) (Tewameter TM300®)	11.70 ± 3.66	8.87 ± 3.24	10.52 ± 1.73	12.45 ± 3.50	9.74 ± 3.32	10.86 ± 2.02	>0.999	>0.999	>0.999
SELS parameter									
SEsm	212.70 ± 43.23	183.40 ± 33.27	170.70 ± 30.70*	202.20 ± 42.02	199.60 ± 36.99	206.10 ± 42.73	>0.999	0.3386	<0.0001
SEr	2.62 ± 1.01	3.09 ± 1.01	3.48 ± 1.10*	2.71 ± 0.77	2.49 ± 0.56	2.53 ± 0.69	0.206	0.018	0.0001
SEsc	0.62 ± 0.19	0.60 ± 0.11	0.57 ± 0.11*	0.63 ± 0.10	0.64 ± 0.09	0.65 ± 0.10	>0.999	>0.999	0.0052
SEw	76.21 ± 17.19	70.12 ± 13.90	65.12 ± 10.59*	72.35 ± 15.42	72.78 ± 14.36	74.48 ± 14.50	>0.999	>0.999	0.0098

Values are means ± SD. R2 ratio = skin elasticity; TEWL = transepidermal water loss (g/h/m2); SEsm = skin smoothness; SEr = skin roughness; SEsc = skin scaliness; Sew = wrinkles; P1 = Comparison of mean between the two groups at baseline; P2 = Comparison of mean between the two groups at week 6; P3 = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

At baseline, all of the values of the skin parameters did not differ significantly from each other.

At week 6, only the skin roughness (SEr) was found to be significantly improved in the treatment group compared with the placebo group (treatment group vs. placebo group, 3.09 ± 1.01 vs. 2.49 ± 0.56; $p = 0.018$).

At week 12, the skin elasticity (R2 ratio; treatment group vs. placebo group, 0.72 ±0.10 vs. 0.61 ± 0.09; $p = <0.0001$), the skin smoothness (SEsm; treatment group vs. placebo group, 170.70 ± 30.70 vs. 206.10 ± 42.73; $p = <0.0001$), the skin roughness (SEr; treatment group vs. placebo group, 3.48 ± 1.10 vs. 2.53 ± 0.69; $p = 0.0001$), the skin scaliness (SEsc; treatment group vs. placebo group, 0.57 ± 0.11 vs. 0.65 ± 0.10; $p = 0.0052$) and the skin wrinkles (SEw; treatment group vs. placebo group, 65.12 ± 10.59 vs. 74.48 ± 14.50; $p = 0.0098$) in the treatment group improved significantly compared with the placebo group.

The difference in the mean values of the skin hydration between the treatment group and the placebo group at week 12 was found to be statistically significant ($p = 0.041$). However, no significant intragroup improvement was observed in both treatment group ($p = >0.9999$) and placebo group ($p = 0.1100$).

The mean values of the skin elasticity in the treatment group gradually increased from 0.57 ± 0.10, 0.66 ± 0.09, and 0.72 ±0.10 at baseline, week 6, and week 12, respectively. The improvement in the skin elasticity within the treatment group from baseline to week 6 ($p < 0.0001$) and from week 6 to week 12 ($p < 0.0001$) was statistically significant.

The mean values of the SELS parameters including the skin smoothness (SEsm; baseline vs. week 12, 212.70 ± 43.23 vs. 170.70 ± 30.70; $p = <0.0039$), the skin roughness (SEr; baseline vs. week 12, 2.62 ± 1.01 vs. 3.48 ± 1.10; $p = 0.0003$), the skin scaliness (SEsc; baseline vs. week 12, 0.62 ± 0.19 vs. 0.57 ± 0.11; $p = 0.0003$) and the skin wrinkles (SEw; baseline vs. week 12, 76.21 ± 17.19 vs. 65.12 ± 10.59; $p = 0.0032$) in the treatment group also improved significantly at the end of the study compared with the data at baseline.

No significant improvement was found in the skin melanin index (treatment group vs. placebo group, 233.10 ± 57.13 vs. 230.00 ± 58.74; $p = >0.999$), the skin gloss DSC value (treatment group vs. placebo group, 5.28 ± 1.19 vs. 5.41 ± 1.73; $p = >0.999$), and the TEWL

(treatment group vs. placebo group, 10.52 ± 1.73 vs. 10.86 ± 2.02; p = >0.999) of both groups at the end of the study.

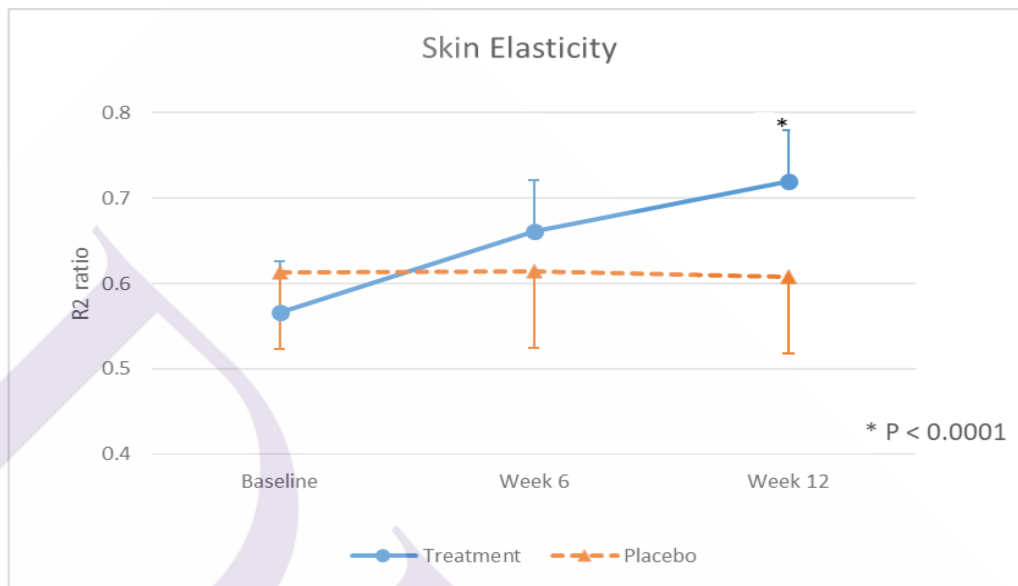


Figure 4.1 Mean R2 ratio at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

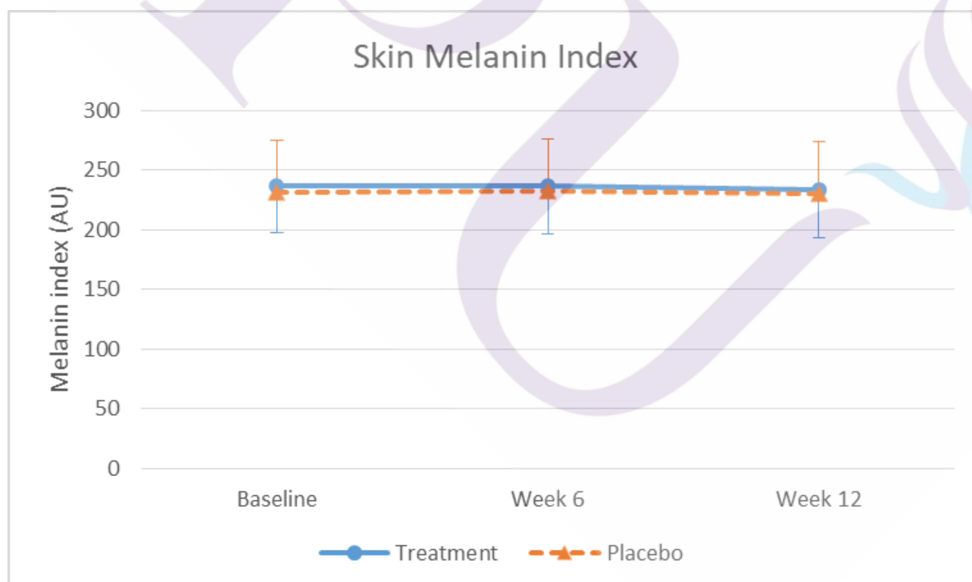


Figure 4.2 Mean melanin index at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

Figure 4.3 Mean gloss DSC value at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

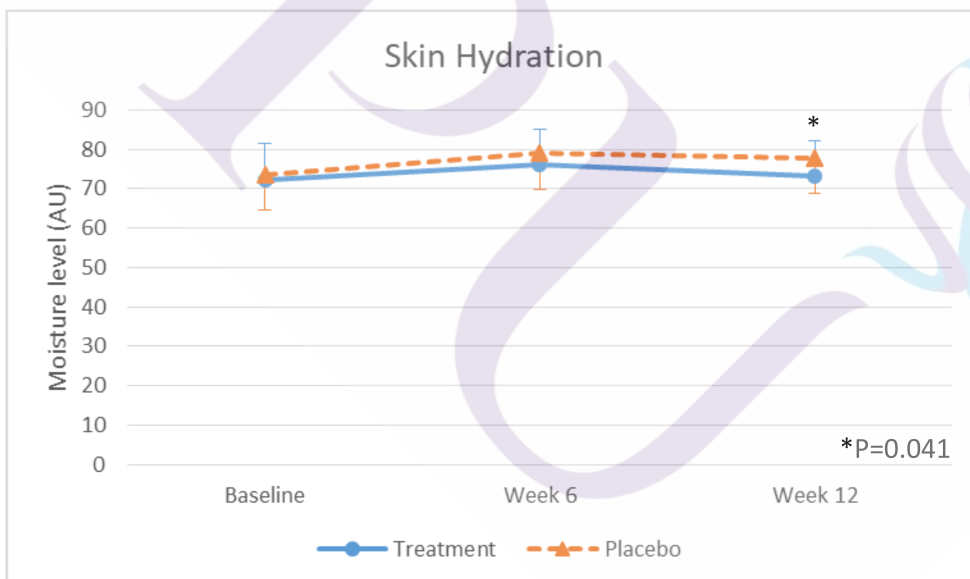


Figure 4.4 Mean moisture level at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

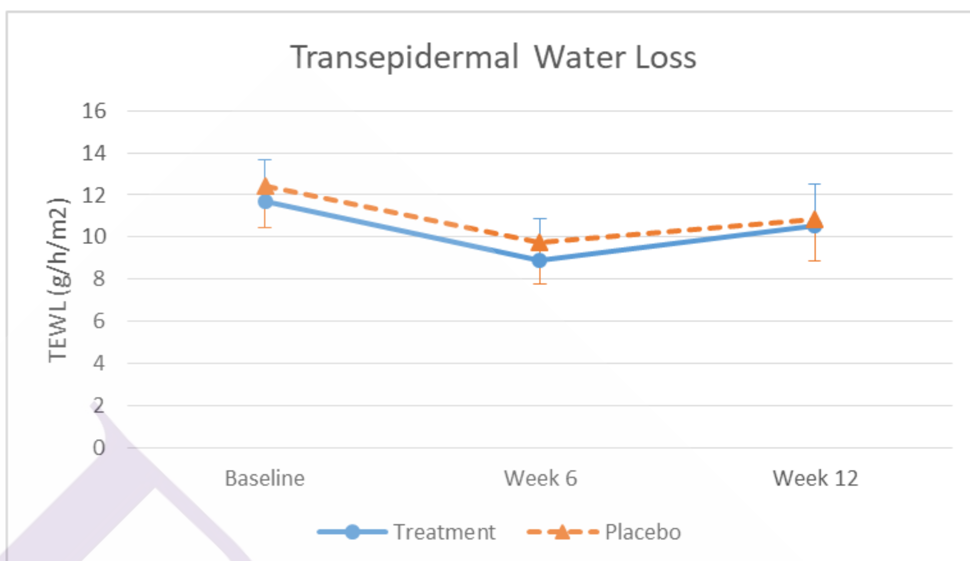


Figure 4.5 Mean TEWL at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

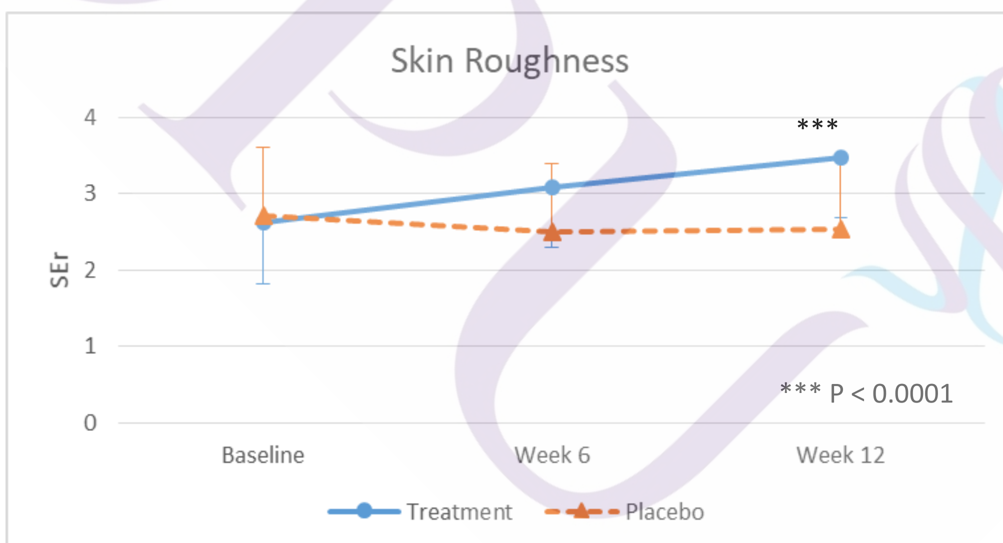


Figure 4.6 Mean SEr at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value

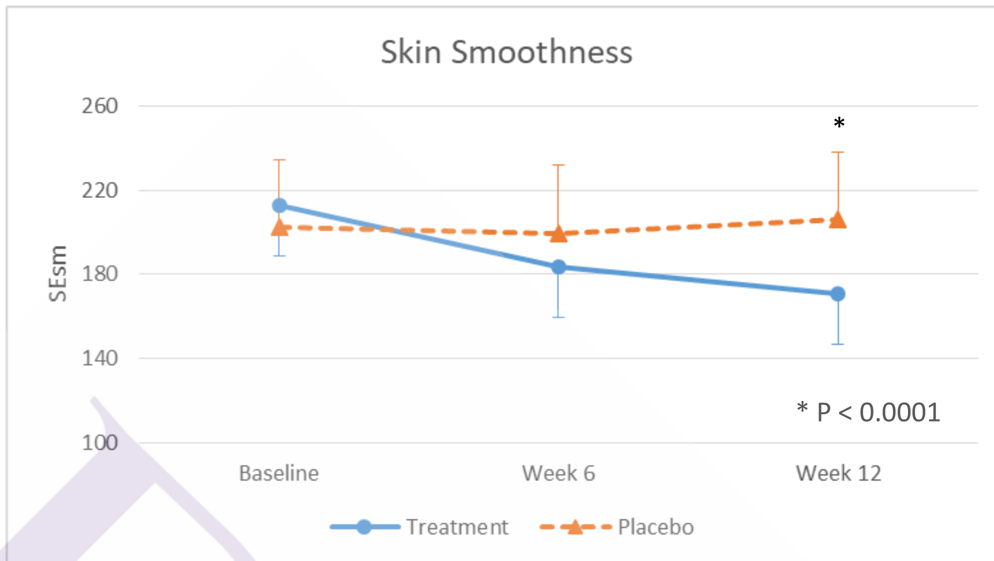


Figure 4.7 Mean SEsm at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value.

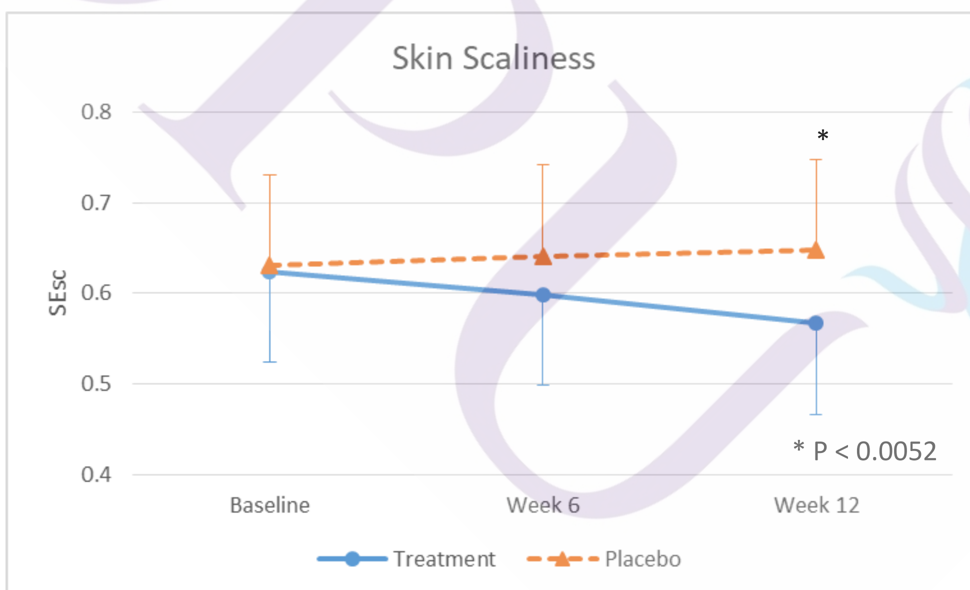


Figure 4.8 Mean SEsc at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value.

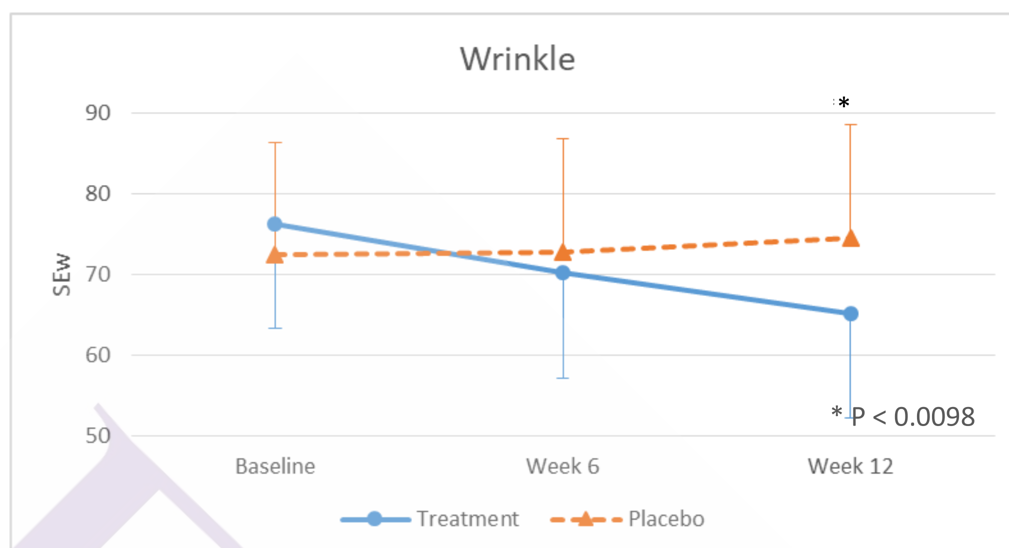


Figure 4.9 Mean SEw at baseline, week 6, and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value.

4.4 Antioxidant and Oxidative Stress Profile

Table 4.4 Anti-oxidant and Oxidative Stress Profile

Anti-oxidant and oxidative stress profile	**Treatment**		**Placebo**		**P1**	**P2***
	Baseline	**Week 12**	**Baseline**	**Week 12**		
GSH (μmol/l)	481.50± 127.40	528.80± 117.80*	460.70± 123.20	458.00± 123.10	>0.999	0.0242
MDA (μmol/l)	4.31 ± 0.88	3.50 ± 0.93*	4.45 ± 0.87	4.75 ± 0.88	>0.999	<0.0001

Values are means ± SD. GSH = reduced glutathione; MDA = Malondialdehyde; P1 = Comparison of mean between the two groups at baseline; P2 = Comparison of mean between the two groups at week 12; Significant differences at $p < 0.05$.

Table 4.4 shows the mean values of GSH and MDA between the treatment group and the placebo group at baseline and week 12. At baseline, the mean values of GSH and MDA between the treatment group and the placebo group did not differ significantly from each other. At week 12, however, the mean values of GSH in the treatment group increased significantly compared with the placebo group (treatment group vs. placebo group, 528.80 ± 117.80 vs. 458.00 ± 123.10; p = 0.0242). The decrease in the mean values of MDA in the treatment group was also statistically significant compared with the placebo group (treatment group vs. placebo group, 3.50 ± 0.93 vs. 4.75 ± 0.88; p <0.0001).

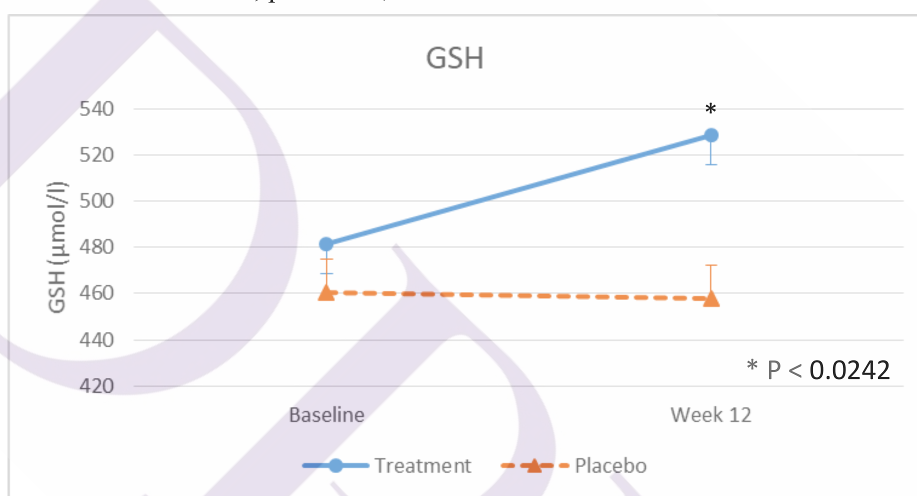


Figure 4.10 Mean GSH at baseline and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value.

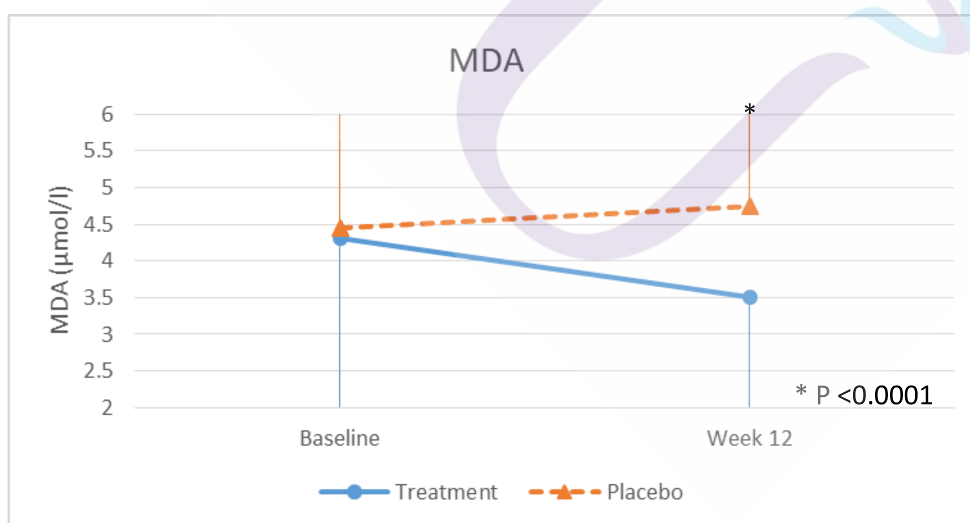


Figure 4.11 Mean MDA at baseline and week 12. P = Comparison of mean between the two groups at week 12. P-value < 0.05, determined as significant value.

4.5 Satisfaction assessment

Table 4.5 Satisfaction assessment: perceived improvement in skin health

Topic	Number of subjects satisfied (percentage)				P*
	Treatment		Placebo		
	Week 6	Week 12	Week 6	Week 12	
Smoothness	34 (65.4%)	42* (80.8%)	12 (23%)	15 (28.8%)	<0.0001
Moisture	28 (53.8%)	34* (65.4%)	16 (30.8%)	19 (36.5%)	0.0012
Elasticity	41 (78.8%)	46* (88.5%)	10 (19.2%)	8 (15.4%)	<0.0001
Dark spots	11 (21.1%)	15 (28.8%)	8 (15.4%)	10 (19.2%)	0.3920
Wrinkles	32 (61.5%)	40* (76.9%)	17 (32.7%)	21 (40.4%)	<0.0001

Values are numbers (percentages). P = Comparison of the value between the two groups at week 12; Significant differences at $p < 0.05$.

According to the subjects' satisfaction evaluation, the satisfaction score was given on a scale of 1 to 5. The subjects who gave a score of 3 to 5 were considered as "satisfied".

Table 4.5 illustrates that subjects in the treatment group were significantly satisfied with almost all aspects of the improvement in skin health at week 6 (percentage of subjects satisfied: smoothness 65.4%, moisture 53.8%, elasticity 78.8%, and wrinkles 61.5%) and even more satisfied at week 12 (percentage of subjects satisfied: smoothness 80.8%, moisture 65.4%, elasticity 88.5%, and wrinkles 76.9%), except for the dark spots which only a few proportion of subjects were satisfied (21.1% and 28.8% of subjects were satisfied at week 6 and week 12, respectively). Meanwhile, in the placebo group, most of the subjects were unsatisfied with the results throughout the study (percentage of subjects satisfied at week 12: smoothness 28.8%, moisture 36.5%, elasticity 15.4%, and wrinkles 40.4%).

The nutraceuticals were well-tolerated and no adverse effect was observed during the period of the study.

Chapter 5

Conclusions, Discussion and Suggestions

5.1 Discussion

In post-menopausal period, a remarkable change in the skin health can be observed in most women as a result of estrogen deficiency and an increase in oxidative stress level. The skin becomes thinner with decreased collagen content, decreased elasticity, decreased skin smoothness, increased skin wrinkling, increased water loss and increased dryness. While the current gold standard for the treatment of menopause symptoms and delaying skin aging process is the hormone replacement therapy, most people are concerned about the possible adverse outcomes which may occur following the HRT treatment. Therefore, this trial was conducted to study the effects of the combined nutraceuticals containing soy isoflavones, evening primrose oil, black cohosh, and chasteberry, on skin health in post-menopausal women as an alternative treatment.

Since soy isoflavones are known to exhibit estrogenic activities and have structural similarities to natural estrogens, several studies have been conducted to identify the effects of soy isoflavone on the skin. Certain authors have reported that an oral intake of 40 mg soy isoflavone aglycones per day for 12 weeks significantly improves fine wrinkles and malar skin elasticity (Izumi et al., 2007). Besides the effects on the skin, an increase in the activity of antioxidant enzyme was also observed in a study of Cai and Wei after taking 30 mg of soy isoflavones for 30 days (Cai & Wei, 1996).

Evening primrose oil contains a large amount of LA and GLA, lipids those are known to play a crucial role in maintaining normal skin physiology. Insufficient amount of LA and GLA in the skin leads to scaling of the epidermis, weakening of skin barrier, accompanied by an increase in water loss across the epidermis causing dryness of the skin. An oral administration of evening primrose oil has been reported to restore a defective epidermal skin barrier and improve the skin smoothness (Nissen et al., 1988). Skin moisture, TEWL, skin elasticity, and skin roughness were also significantly improved following an oral administration of evening primrose oil 3 g per day for 12 weeks in previous study (Muggli, 2005).

Black cohosh extract (BCE) has long been used to alleviate menopausal symptoms. The study of BCE is limited due to the lack of standardization of the extract to one or more active compositions (Ruhlen et al., 2008). However, according to their possible mild estrogenic activity and antioxidant properties, it can be assumed that black cohosh may also exert beneficial effects on skin health and improve oxidative stress level.

Another component of the nutraceuticals is chasteberry. While the study of its effects on the skin is very limited, it has been suggested that chasteberry crude extract exhibits a selective anti-inflammatory effect and may have a crucial role in improving oxidative stress as it increase reduced glutathione (GSH) concentration and induce glutathione reductase, glutathione-S-transferase, glutathione peroxidase, and catalase activities (Ibrahim et al., 2017).

In this trial, the skin at the lateral aspect of both eyes was assessed to evaluate the improvement in the skin parameters. At the end of the study, the results demonstrated that the combined nutraceuticals were effective in improving skin elasticity, skin smoothness, skin scaliness and skin roughness compared with placebo. Significant differences in these parameters were also observed within the treatment group. Presumably, the increased skin elasticity was mediated by the activation of the estrogen receptor by soy isoflavones which resulted in an increase in the amount of collagen and elastic fibers. The improvements in the skin smoothness, skin roughness and the skin scaliness were in line with previous reports suggested that an intake of evening primrose oil helps restoring epidermal barrier structure and function, thus smoothing the surface of the skin and consequently reducing the scaling of epidermis (Muggli, 2005).

Meanwhile, no significant effect of the nutraceuticals was observed on the skin melanin index, skin gloss, skin hydration and TEWL. In previous study, the skin hydration and TEWL significantly improved after administration of evening primrose oil 3 g per day for 12 weeks (Muggli, 2005). This indicated that the concentration of evening primrose oil used in this present study, which is 500 mg/day, might not be enough to significantly regenerate the whole epidermal barrier function. Apart from the skin barrier function, the skin hydration also depends on the vasculature of the skin. Although the activation of estrogen receptor tends to increase the epidermal and vascular-endothelial growth factors, it has been reported that six months of estrogen therapy was not able to restore cutaneous microvasculature (Accorsi-Neto et al., 2009).

With regard to the skin melanin index, evidences related to the effects of isoflavones on melanin pigment are limited and reported controversial findings. It could be suggested that none of the composition of the nutraceuticals had any significant effects on the melanin pigment under the condition in this present study.

In addition, a significant improvement was also observed in GSH and MDA level in the treatment group. This indicated that the nutraceuticals may contain anti-oxidative effects. Accordingly, a previous study also revealed that chasteberry extract increased reduced glutathione (GSH) concentration and induce glutathione reductase, glutathione-S-transferase, glutathione peroxidase, and catalase activities in animal model (Ibrahim et al., 2017). Moreover, in mice fed a basal diet supplemented with either nothing or 1.08 g of isoflavone-rich soy isolate, the level of MDA was measured from the sacrificed liver after 60 days of the study and it was found to be significantly lower in the intervention group compared with the control group (Ibrahim et al., 2008).

The data of baseline general characteristics and blood chemistry of both groups were obtained; no significant difference between the two groups was found. The total energy and nutrients intake between the treatment group and the control group also did not differ significantly; thus the confounding from nutrients intake was unlikely to occur.

Finally, our data allows us to conclude that the intake of these combined nutraceuticals for 12 weeks can provide benefits to not only the skin health, but also the oxidative stress status in post-menopausal women. Additionally, the possibility that an anti-oxidative effect of the nutraceuticals may contribute to the improvement in the skin cannot be ruled out. This nutraceuticals therapy is worthy of further investigations and has a potential to be an alternative treatment for improving skin health in post-menopausal women.

5.2 Suggestions

In this present clinical study, the combined nutraceuticals were observed to effectively improve skin conditions and oxidative stress status in post-menopausal women. The next issue would be to demonstrate the precise mechanisms of action and to evaluate the long-term effects of the treatment. This alternative therapy should be investigated further and should be compared to results obtained from a standard estrogen replacement therapy in post-menopausal women.

5.3 Conclusions

Our study shows that supplementation with combined nutraceuticals containing soy isoflavones, black cohosh, chasteberry, and evening primrose oil in post-menopausal women for 12 weeks improves facial skin health; including elasticity, roughness, smoothness, scaliness, and wrinkle density. This corresponds with increased GSH and lowered MDA levels. Therefore, these findings suggest a potential of the nutraceuticals to be used as an alternative treatment in post-menopausal women with age-related loss of skin structure and integrity.

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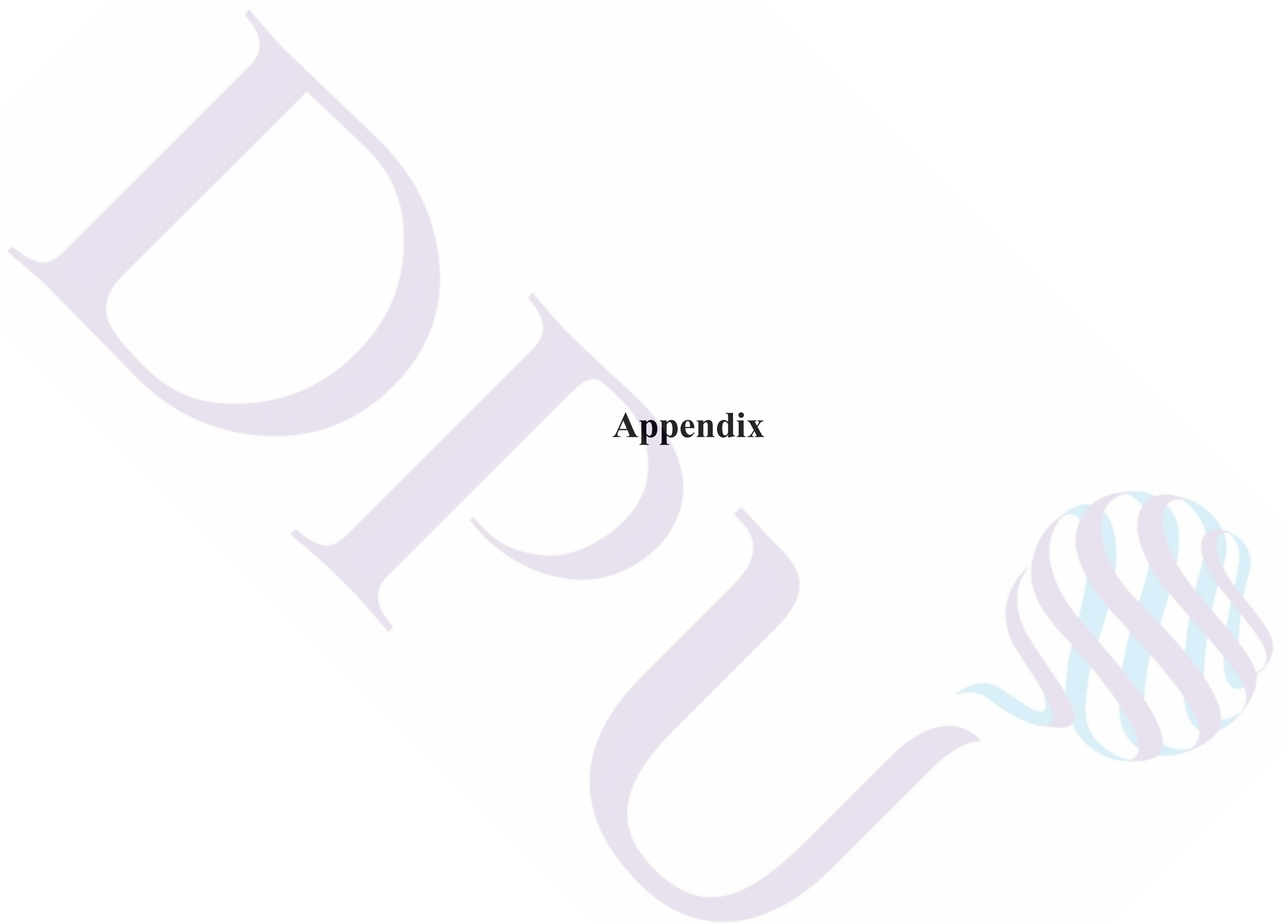

Appendix

Appendix A

Study Record Form

Subject No. ______________

Date ______________

Study Record Form

The Effects of Oral Combined Nutraceuticals on Skin Health

in Post-menopausal Women:

A Randomized, Double-Blind, Placebo-Controlled Trial

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1. General Information

- Date of Birth __________________________ Age _________________

2. Health Record

- Weight _______ kg Height ________ cm BMI ________ kg/m^2
- Blood pressure _______/_______ mmHg Pulse rate ________ bpm
- Last menstrual period ____
- Underlying disease ____
- Current med ____
- History of allergy ____
- History of herbs/supplements/hormone therapy _______________________

____

- History of botulinum toxin or filler injections _________________________
- History of other aesthetic procedures _______________________________

3. Assessment

3.1 Biochemical outcome

	Week 0	Week 12
MDA		
GSH		

3.2 Skin parameters

	Week 0			Week 6			Week 12		
	Point A	Point B	Mean value	Point A	Point B	Mean value	Point A	Point B	Mean value
Assessed by Visioscan® Skin smoothness (SEsm) Skin roughness (Ser) Scaliness (Sesc) Wrinkles (Sew)									
Assessed by Cutometer® Skin elasticity									
Assessed by Corneometer® Skin hydration									
Assessed by Tewameter® Transepidermal Water Loss (TEWL)									
Assessed by Mexameter® Melanin index									
Assessed by Skin-Glossymeter Skin gloss									

3.3 Safety parameters

	Week 0	Week 12
AST (0-48)		
ALT (0-48)		
BUN (12-20)		
Creatinine (0.5-1.1)		

3.4 Adverse reaction events observed

………………………………………………………………………………………………

………………………………………………………………………………………………

Appendix B

Satisfaction Self-Evaluation Form

Subject No. ______________

Date ______________

Satisfaction Self-Evaluation Form

The Effects of Oral Combined Nutraceuticals on Skin Health

in Post-menopausal Women:

A Randomized, Double-Blind, Placebo-Controlled Trial

Evaluation of the satisfaction in taking the nutraceuticals

Score: 5 = Most satisfied 4 = Very satisfied 3 = Moderately satisfied

2 = Minimally satisfied 1 = Least Satisfied

	Score given (1-5 scores)		
Topic	Week 0	Week 6	Week 12
Taste and smell of the supplement			
Skin texture			
Skin hydration			
Skin elasticity			
Skin pigment			
Wrinkles			

Suggestions and opinions

……………………………………………………………………………………………………

……………………………………………………………………………………………………

รหัสอาสาสมัครวิจัย ____________

วันที่ ____________

แบบประเมินความพึงพอใจ

โครงการวิจัย การศึกษาประสิทธิผลของโภชนเภสัชต่อสุขภาพผิว

ในหญิงวัยหมดประจำเดือน

ประเมินความพึงพอใจในการบริโภคโภชนเภสัช

คะแนน: 5 = พอใจมากที่สุด 4 = พอใจมาก 3 = พอใจปานกลาง

2 = พอใจน้อย 1 = พอใจน้อยที่สุด

	ให้คะแนน (1-5 คะแนน)		
หัวข้อ	สัปดาห์ที่ 0	สัปดาห์ที่ 6	สัปดาห์ที่ 12
รสชาติ และกลิ่น			
ความเนียนของผิว			
ความชุ่มชื้นของผิว			
ความยืดหยุ่นของผิว			
สีผิว รอยดำ			
ริ้วรอย			

ความคิดเห็นและข้อเสนอแนะเพิ่มเติม

....

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....

....

Appendix C

Consent Form

หนังสือแสดงเจตนายินยอมเข้าร่วมงานวิจัย

(Consent Form)

โครงการวิจัย การศึกษาประสิทธิผลของโภชนเภสัชต่อสุขภาพผิว ในหญิงวัยหมดประจำเดือน

วันที่ให้คำยินยอม วันที่.................เดือน.....................................พ.ศ.................................

ข้าพเจ้า..อายุ.............ปี อาศัยบ้านเลขที่................

ถนน..หมู่ที่....................แขวง/ตำบล.......................................

เขต/อำเภอ..จังหวัด...

ก่อนที่จะลงนามในใบยินยอมให้ทำการวิจัยนี้ ข้าพเจ้าได้รับการอธิบายจากผู้วิจัยถึงวัตถุประสงค์ของการวิจัย วิธีการวิจัย อันตรายหรืออาการที่อาจเกิดขึ้นจากการวิจัย รวมทั้งประโยชน์ที่อาจเกิดขึ้นจากการวิจัยอย่างละเอียด และมีความเข้าใจดีแล้ว ซึ่งผู้วิจัยได้ตอบคำถามต่าง ๆ ที่ข้าพเจ้าสงสัยด้วยความเต็มใจ ไม่ปิดบังซ่อนเร้น จนข้าพเจ้าพอใจและเข้าร่วมโครงการนี้โดยสมัครใจ

ข้าพเจ้ามีสิทธิที่จะบอกเลิกการเข้าร่วมการวิจัยเมื่อใดก็ได้ ถ้าข้าพเจ้าปรารถนาโดยไม่เสียสิทธิในการรักษาพยาบาลที่จะเกิดขึ้นตามมาในโอกาสต่อไป

ผู้วิจัยรับรองว่าจะเก็บข้อมูลเฉพาะเกี่ยวกับตัวข้าพเจ้าเป็นความลับและจะเปิดเผยได้เฉพาะในรูปแบบที่เป็นสรุปผลงานวิจัย

การเปิดเผยข้อมูลเกี่ยวกับตัวข้าพเจ้าต่อหน่วยงานต่างๆที่เกี่ยวข้องกระทำได้เฉพาะกรณีจำเป็น ด้วยเหตุผลทางวิชาการเท่านั้นและจะต้องได้รับความยินยอมจากข้าพเจ้าเป็นลายลักษณ์อักษร

ผู้วิจัยรับรองว่าหากเกิดภาวะแทรกซ้อนใด ๆ ที่มีสาเหตุจากการวิจัยดังกล่าวข้าพเจ้าจะได้รับ การรักษาพยาบาลโดยไม่คิดค่าใช้จ่าย และหรือจะมีการชดเชยค่าตอบแทน ตลอดจนเงินทดแทนความเจ็บป่วยที่ อาจเกิดขึ้นตามเหมาะสม

ข้าพเจ้ายินยอมให้ผู้กำกับดูแลงานวิจัย ผู้ตรวจสอบ คณะกรรมการจริยธรรมการวิจัยในคนและสามารถเข้าไปตรวจสอบบันทึกข้อมูลทางการแพทย์ของข้าพเจ้าเพื่อเป็นการยืนยันถึงขั้นตอน

โครงการวิจัย ทางคลินิก โดยไม่ล่วงละเมิดเอกสิทธิ์ในการปิดบังข้อมูลของการสมัครตามกรอบที่กฎหมายและกฎระเบียบได้อนุญาตไว้

ข้าพเจ้าได้อ่านข้อความข้างต้นแล้ว และมีความเข้าใจดีทุกประการ จึงได้ลงนามในใบยินยอมนี้ด้วยความเต็มใจ ในกรณีที่ข้าพเจ้าไม่สามารถอ่านหนังสือได้ ผู้วิจัยได้อ่านข้อความในใบยินยอมนี้ให้ข้าพเจ้าฟัง จนเข้าใจดีแล้ว ข้าพเจ้าจึงลงนามในใบยินยอมนี้ด้วยความเต็มใจ ข้าพเจ้าสามารถติดต่อผู้วิจัยได้ที่ 70/170 ซ.รามอินทรา 65 แขวงท่าแร้ง เขตบางเขน จ. กรุงเทพมหานคร 10230 เบอร์โทรศัพท์ 088-010-7110 e-mail: tuangsutti@gmail.com โดยบุคคลที่รับผิดชอบเรื่องนี้ คือ พญ. ภคกมล ตุ้มสุทธิ ในความดูแลของ ผศ.นพ. พันธ์ศักดิ์ ศุกระฤกษ์, ผศ.นพ. มาศ ไม้ประเสริฐ และ ผศ.ดร. เอกราช บำรุงพืชน์

ลงนาม .... ผู้ยินยอม

(...)

วันที่..........เดือน............................พ.ศ.............

ลงนาม .... ผู้วิจัย

(...)

วันที่..........เดือน............................พ.ศ.............

ลงนาม .... พยาน

(...)

วันที่..........เดือน............................พ.ศ.............

ลงนาม .... พยาน

(...)

วันที่..........เดือน............................พ.ศ.............

Appendix D

24-hour Diet Recall Record Form

Subject No. _____________

Date _____________

24-hour Diet Recall Record Form

The Effects of Oral Combined Nutraceuticals on Skin Health

in Post-menopausal Women:

A Randomized, Double-Blind, Placebo-Controlled Trial

Meal	Food Intake	Food Composition	Amount of Intake

Curriculum Vitae

Name	Pakagamon Tumsutti
Background Education	2009-2014 Doctor of medicine, second class honours
	Srinakharinwirot University
Work experiences	2015-2016 Medical internship
	Samut Sakhon Hospital
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