DETERMINATION OF PREVENTIVE EFFECT OF *AQUILARIA CRASSNA* EXTRACT ON 7, 12 DIMETHYLBENZ[A]ANTHRACENE (DMBA)/UVB RADIATION INDUCED SKIN DAMAGE IN MICE MODEL.

SUCHADA CHUMSANG

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Pharmacology and Biomolecular Sciences)
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By SUCHADA CHUMSANG

has been approved by the Graduate School as partial fulfillment of the requirements for the Master of Science in Pharmacology and Biomolecular Sciences of Naresuan University

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ABSTRACT

Repeated ultraviolet (UV) exposure, especially UVB radiation, is a risk factor for skin disorders. UVB directly destroys the cellular structure of the skin including proteins and lipids, and can damage the DNA of the skin which leads to gene mutation. As well, UVB can generate reactive oxygen species (ROS) and free radicals in skin cells. The mechanism for decreasing ROS and free radicals is called the antioxidant activity. The bioactive components of fruits, vegetables and herbal plants are extracted by that same mechanism. Herbal plants have been used for many years in traditional medicines to prevent and treat various illnesses. In Thailand, *Aquilaria crassna* (*A. crassna*), which is also called Krissana has been reported to have antioxidant activity, anti-inflammation activity and decreasing the pro-inflammatory cytokines.

The aims of this study were to determine the effects of *A. crassna* leaf extract (ACE) on DMBA-induced skin damage on 3-week old ICR mice, and also to determine the effects of the Mangiferin standard (MGF) on similarly induced skin damage on the ICR mice. The DMBA was applied once on the dorsal skin with repeated UVB irradiation for 16 weeks. The epidermal thickness, the expression of 4-HNE and COX-2 level were observed. The mice were divided into 5 groups, one of

which was the Normal group comprising non-DMBA/non UVB irradiated mice. A second group was the Control group comprising DMBA/UVB-irradiated mice that received water). The group called Test Group 1 was DMBA/non UVB-irradiated mice receiving water, with Test Group 2 being orally administered ACE at a concentration of 250 mg/kg body weight/day and Test Group 3 was administered MGF standard at a dosage of 19.4 mg/kg body weight/day. The UVB-exposure dose was increased from 54 mJ/cm^2 per exposure at week 1 to 126 mJ/cm^2 at week 16 in varying weekly increments. A significant increase in epidermal skin thickness was observed in all groups, and a higher expression of 4-HNE and COX-2 showed in the Control group when compared with the Normal group, and both Test Group 1 and Test Group 3. In Test Group 2, the results showed that ACE can decrease the level of expression of 4-HNE, while the epidermal thickness and the level of expression of COX-2 were not significantly different in in this group when compared with the Control group. Further, comparing between the Control Group and Test Group 3 showed that MGF decreased the epidermal thickness in Test Group 3, and that the level of expression of 4-HNE and COX-2 in Test Group 3 differed significantly from the Control group. The comparison between the Control group and Test Group 2 showed no difference in epidermal thickness and the level of expression of COX-2. Also, it was found that both Test Group 2 and Test Group 3 showed a decrease in the level of expression of 4 HNE level. Therefore, it can be concluded that the dietary uptake of herbal plants can reduce skin damage from repeated UV exposure by decreasing epidermal thickness with an over-proliferation of keratinocytes, which is the expression of 4-HNE, while the uptake of a pure compound of MGF can decrease skin damage which showed as lower epidermal thickness, and a lower level of expression of both 4-HNE and COX-2.

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SUCHADA CHUMSANG

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CHAPTER I

INTRODUCTION

The rationale of study

The UV radiation from the sun is a risk factor in daily life, which is divided into three types depending on the wavelength: UVA (320-400 nm), UVB (290-320 nm), and UVC (100-290 nm). The energy level of UVA and UVB is high enough to penetrate into our skin, which causes skin disorder leading to skin damage and develop to skin cancer, while UVC can be absorbed by ozone layer (Ichihashi et al., 2003). UVB, a shorter wavelength than UVA, can cause sunburn on the skin surface and directly action with DNA and other biomolecules within skin cells including disruption of the extracellular matrix for the processing of elastic fibers and collagens in skin cells.

On the molecular level, UVB can be absorbed by DNA, while nucleic acids can absorb electromagnetic energy at a wavelength of 260 nm. These occur from pyrimidines bases adjacent in the same DNA strand binding together and lead to abnormal structure of DNA, which mainly affect to cells mutation. Moreover, UVB can generate reactive oxygen species (ROS) or free radicals containing an unpaired electron in the molecule, such as singlet oxygen (O^1), superoxide anion (O^{-2}), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO.) (Durbeej & Eriksson, 2002). These ROS are able to steal electron from other molecules and change them into free radicals, also damage cellular components in cells, including protein, lipid and DNA. Generally, ROS can be generated from metabolism processes in the body including inflammation activities, electron transport chain (ETC) in mitochondrial process and the respiratory burst in phagocytic cells (Moloney & Cotter, 2018). The defensive systems in our body have processes to against ROS and control these molecules to be in balance stage called endogenous antioxidants. This process can prevent or slow down the damage of cells caused by free radicals such as catalase, superoxide dismutase, glutathione and related enzyme via scavenging free radicals. However, repeated UV exposure is mean accumulative of ROS production in skin cells which

causes imbalance between ROS and endogenous antioxidant. The over-production of ROS has been reported as the important role of oxidative stress by oxidizing protein and lipid in skin cells, resulting in aging and skin damage. ROS can also induce lipid peroxidation process via direct reaction with polyunsaturated fatty acid, one of components in membrane, resulting in chain reaction of lipid molecule on membrane and subsequent lipid free radicals. The products from this oxidation are Malondialdehyde (MDA) and 4-Hydroxynonenal (4-HNE). These reactive aldehydes can form adducts with DNAs and proteins, leading to cause various diseases (Rinnerthaler et al. , 2015). In addition, the resulting of protein oxidation can generate carbonyl groups that may introduce into proteins by reactions with aldehydes (MDA and 4-HNE) produced during lipid peroxidation (Stadtman & Berlett, 2001)

On the other hand, the effect of repeated UV exposure shows sunburn which leads to inflammation activities on skin. An important dermal marker of inflammation activities is cyclooxygenase 2 (COX-2) which is the rate-limiting enzyme for the production of prostaglandin E2 (PGE2) from arachidonic acid on membrane. The expression of COX-2 consistently increased in the skin hyperplasia on epidermis layer (An et al., 2002)

The best way to prevent or decrease the accumulation of ROS is to avoid an exposure to UV and collaborate with enhancing antioxidant activities. The alternative for enhancing antioxidants into the body is intake antioxidants supplement such as polyphenolic compound including flavonoids, β-carotene and vitamin from vegetables and fruits. Another way is intake commercial products that contain various vitamins leading to decrease ROS. Nowadays, plants and herbals are popular for using as medicinal prevention and treatment in various illnesses. Phytochemistry in herbal plants is source of bioactive compound that useful to be antioxidant for scavenging the over-production of ROS. The previous study found that oil of Aquilaria family can inhibit pro-inflammatory cytokine (IL-1, IL-6 and TNF-α) in mice with TPA-induced ear inflammation for increasing pro-inflammatory cytokine compared with normal group. Likewise, the mice were induced tumor by using sub-chronic toxicity and then treated with Aquilaria crassna essential oil. The results showed *A. crassna* essential oils can reduce size of tumor when compared with untreated group (Dahham et al., 2016; Yadav et al., 2013).

A. crassna classified in Thymelaeaceae family, is a highly valuable medicinal plant found in the South of Asia such as China, Philippines, Cambodia, Vietnam, Laos and Thailand (Wang et al., 2018). In Thailand, the local name is Krissana. This plant is a medium to large tree reaching 10–30 m. The leaves are green, smooth, and shiny. The pharmacological effect of *A. crassna* have been reported in various researches: anti-inflammation, anti-oxidant activity, anti-diabetic and, anti-tumor (Wongwad et al., 2019).

Bioactive compounds in *A. crassna* leaf extract were studied using LC / MS / MS coupling with DPPH assay and HPLC (Ito et al., 2012). The study revealed bioactive contents in *A. crassna* including phenolic compound, flavonoids group, xanthone, steroids, inter alia. The main compound in *A. crassna* leaves is Mangiferin (MGF), which were approximately 9.5 % in the water extraction (Wisutthathum et al., 2018). MGF has high antioxidant activity equivalent to trolox and showed IC50 of 24.6 µg/ml (Wongwad et al., 2019). The previous study interested in *A. crassna* water extract for treatment of type 2 diabetic in mice induced by streptozotocin (STZ)-nicotinamide and determine IC50. An antioxidant activity of the extract was confirmed using DPPH radical scavenging assay to be 34.6 µg/ml. Moreover, *A. crassna* leaf extract can decrease the blood glucose levels when compared with control group (Manok et al., 2016).

In this study, the preventive effect of *A. crassna* leaf extract on 7, 12 dimethylbenz[a]anthracene (DMBA)/UVB radiation induced skin damage in mice model were determined. The mice will be treated *A. crassna* leaf extract by orally administration at concentration 250mg / kg body weight for 4 months. The protein expression and the level of 4-HNE and COX-2 on skin will be evaluated by comparing with pure MG treatment. The result of in-depth study will lead to further development of food products from the *A. crassna* leaves to treatment and prevent disease in humans such as cancer in the future and to present herbals, plants or animals that were used in prevention and treatment. Biological activity and safety of these extracts on human will be discovered by observation and trial an error, then take the fact to the future generations. Currently, scientific advances can identify and support those facts. To maximize the benefits and safety to be natural developed in various ways to benefit for humans.

Objectives of the study

1. To determine antioxidant activities in *A. crassna* leaf extract.

2. To evaluate preventive effects of *A. crassna* leaf extract on 7, 12 Dimethylbenz [a] anthracene (DMBA)/UVB irradiated induced skin damage in mice.

Expected output of the study

The study can provide scientific knowledge, which promotes Thai herbal plants for medicinal prevention or treatment of the illnesses from repeated UV exposure in daily life.

Scope of research

In this study, the preventative effect of Aquilaria crassna leaf extract on 7, 12 dimethylbenz[a] anthracene (DMBA)/UVB radiation induced skin damage in mice will be evaluated. In addition, the amount of MGF, an interested bioactive compound in *A. crassna* leaf extract will be determined using High Performance Liquid Chromatography (HPLC). Then, DPPH assay were used to evaluate the antioxidant activities of *A. crassna* leaf extract compared with ascorbic acid (Vitamin C). In part of animal model, mice were divided into 5 groups compared among normal group (non UVB-irradiated mice), control group (DMBA/UVB-irradiated) receiving water, Test Group 1 (DMBA/non UVB-irradiated), Test Group 2 (DMBA/UVB-irradiated) receiving *A. crassna* leaf extract and Test Group 3 (DMBA/UVB-irradiated) receiving MGF standard. All of 5 groups were treated for 16 weeks, followed by collected the dorsal skin tissue and determine the end products from oxidation processes, 4-HNE and COX-2.

CHAPTER II

LITERATURE REVIEW

Skin structure

Skin is an organ for prevention of the body from external environment including microorganism, pollution, and UV radiation. Moreover, it prevents the biomolecules including protein lipid and DNA in skin cells from being destroyed. The skin is composed of three layers: epidermis, dermis, and subcutaneous layer. Epidermis is the outer layer that serves as a physical and chemical barrier between the interior body and exterior environment. The main cells of the epidermis are keratinocytes, which produce keratin.

The next layer is dermis, which lies between the epidermis and the subcutaneous fat. It carries the epidermis structurally and nutritionally. Generally, the dermis layer is thicker than the epidermis layer. Collagens and elastin fibers are the main components of the dermis (Igarashi, Nishino, & Nayar, 2007). The last layer is subcutaneous layer that lies below the dermis and is mainly composed of fat where the blood vessels and nerves are located (McGrath, Eady, & Pope, 2004).

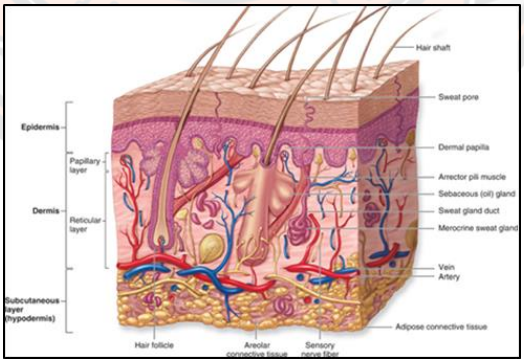

Figure 1 Structure of the skin

Source: http://cosbiology.pbworks.com/

The epidermis

The epidermis is the outer layer of skin. It serves as the physical and chemical barrier between the interior body and exterior environment. The main cells of the epidermis are keratinocytes, which synthesize keratin. The epidermis also secretes a variety of chemokine and growth factors. There are 5 different layers in the epidermis: stratum basal, stratum spinosum, stratum granulosum, stratum lucidum, and stratum corneum or horny layer (McGrath et al., 2004).

The dermis

The dermis lies between the epidermis and the subcutaneous fat. It carries the epidermis structurally and nutritionally. Normally, the dermis layer is much thicker than the epidermis. Collagens and elastin fibers are the main components of the dermis. These cellular matrixes are produced by fibroblasts, the main cells of the dermis layer. The collagen fibers make up 70% of the dermis. It provides strength and firmness to the skin. Elasticity and flexibility are provided by the elastin component of the dermis. The dermis contains nerve endings, sweat glands and oil (sebaceous) glands, hair follicles, and blood vessels.

The Subcutaneous

This layer comprises connective tissue and fat.

Ultraviolet (UV) radiation

The sun is the most important natural source of ultraviolet rays which have radiation wavelengths are between 200 and 400 nm. It is divided into three components: UVA, UVB, and UVC. UVC, with wavelength of 200-280 nm, are almost completely absorbed by the ozone layer. The UVA and UVB rays, which are invisible to the human eye, reach the Earth. Depending on its wavelength and intensity, UV radiation can induce acute and chronic reactions in human skin leading to skin damage and develop to skin cancer. (Biniek, Levi, & Dauskardt, 2012)

Ultraviolet B radiation (UVB)

UVB (280-320 nm) controls the gene expression through intracellular signal transduction pathways. UVB produces extensive dermal connective tissue damage (Grimbaldeston, Simpson, Finlay-Jones, & Hart, 2003), and generates reactive oxygen species (ROS) or free radicals in skin cells which directly destroys DNA (Schuch et al., 2017).

Ultraviolet A radiation (UVA) radiation

UVA (320-400 nm) is the largest spectrum of solar UV radiation (90-95%). UVA rays are weaker than UVB but are 1,000 times more common. Natural UVA rays do not generally cause sunburn but can penetrate more deeply into the lower skin layers where they can produce, consequently, the deep dark tan, premature aging and wrinkles (Rünger, 2016).

Effect of UVB on skin and related disease

The human skin is regularly exposed to environmental conditions which results in skin damage. Daily exposure to air pollution, cigarette smoke, and chemicals, and especially UVB irradiation, generate free radicals as well as reactive oxygen species (ROS) of our own metabolism. Free radicals, as a chemical species, possess an unpaired electron which is a common property in most radicals (Reelfs, Tyrrell, & Pourzand, 2004). The free radicals and ROS generated by UVB irradiation including singlet oxygen ($^1O_2$), hydroxyl radical (•OH), and lipid radicals which are produced from lipid oxidation processes. The free radicals can damage most cellular components, including lipids, proteins, and nucleic acid. These ROS have been reported to create cross-linking and cause mutation of DNA and proteins, as well as the oxidization of the functional groups of these important biological molecules in the cells.

The effect of UVB on the development of skin damage

Cellular damage and alterations in immunological functions are caused by UVB which initiates production of DNA photoproducts, gene mutations, oxidative stress, and inflammatory responses, which subsequently lead to skin cancer (Neagu et al., 2016). Chronic UVB irradiation causes over production of reactive oxygen species (ROS), DNA damage and DNA mutations leading to oxidative stress that can oxidize biomolecules in skin cells, causing skin damage (Moloney & Cotter, 2018). On the cellular level, UVB can directly damage DNA because nucleic acids are the primary chromophores for this electromagnetic energy. DNA and RNA can absorb UV radiation with a wavelength of about 260 nm, creating the occurrence of the pyrimidine dimer incidence the major effect of which is to cross-link adjacent pyrimidine base along a DNA, which caused abnormal DNA replication process leading to the occurrence of mutation genes in cells that subsequently develop into skin diseases (Atillasoy et al., 1998).

Antioxidant

Antioxidants are molecules that inhibit or scavenge free radical reactions and decrease or inhibit cellular damage (Young & Woodside, 2001). Although the antioxidant defenses differ from species to species, the incidence of the antioxidant defense occurs regardless of species. Antioxidants exist in both enzymatic and non-enzymatic proteins which can be derived from both the body's internal environment and the external environment: from the consumption of food, for example.

The enzymatic systems include superoxide dismutase, glutathione peroxidase, glutathione reductase and catalases. The non-enzymatic systems include vitamin C, vitamin E and glutathione. They are known as endogenous antioxidants which help to protect against free radical produced in cells by the metabolism processes in the cells. Antioxidants have functions to maintain ROS to the equilibrium stage. As antioxidant substances that include active compounds such as phenolic compounds and flavonoids increase antioxidant activity in the body (Koruk et al., 2004), researchers have recommended dietary intake high in these substances.

The mechanism of antioxidant molecules transfers their electrons to unpaired free radicals, creating stable molecules (Santos Sánchez et al., 2019).

Lipid peroxidation

Skin damage results from lipid peroxidation, the production of which leads to the spread of free radical reactions. The target substrates for lipid peroxidation are the polyunsaturated fatty acids because of the presence of active bis-allylic methylene groups. Unsaturated fatty acids; phospholipids, glycolipids and other lipid compounds, in the skin, can generate lipid hydrogen peroxides which are the non-radical intermediates (Catala, 2008). These ROS include hydroxyl radicals that effect tissue damage. The breakdown of lipids and the formation of primary oxidation result from lipid peroxidation. MDA and 4-HNE (Figure 2) are generated as lipid peroxidation end products. MDA and 4-HNE adduction with other cellular components have been reported as toxicant to tissue. The lipid peroxidation affects the formation of reactive compounds leading to changes in the permeability of the membrane lipid bilayer and can dramatically alter cell integrity (Barrera, 2012).

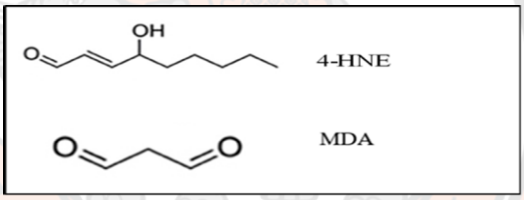

Figure 2 Structures of end products of lipid peroxidation

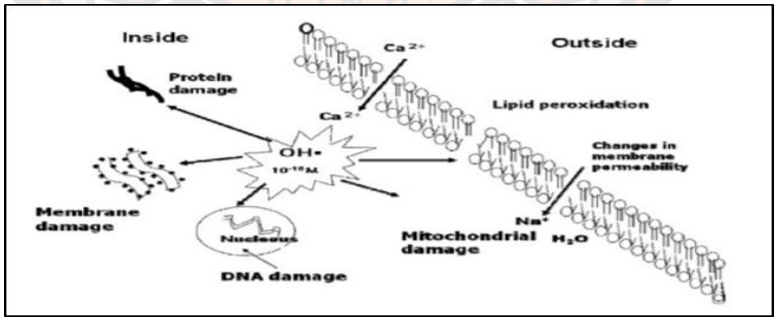

Figure 3 Lipid, DNA and protein oxidative damage from reactive hydroxyl radical

Source: Repetto et al., 2012

Cyclooxygenase-2 (COX-2)

Repeated UVB exposure will promote prostaglandins via a conversion of arachidonic acids in the phospholipid membrane by cyclooxygenase (COX) mediation. Two types of cyclooxygenase enzymes have been identified in humans: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) . The COX-1 type enzyme, which has been called the housekeeping gene, regulates physiological processes in the body such as the regulation of vascular homeostasis, gastroprotection in the stomach, and regulation of renal activities. COX-2 is an inducible enzyme expressed at the site of any inflammation and stimulates the proinflammatory cytokines such as IL-1β, IL-6, and TNFα which cause chronic inflammation (An et al. , 2002) . An overexpression of COX-2 can indicate the longevity of chronic inflammation, which may be indicative of the development of cancer (Kirkby et al. , 2016).

Botanical Classification of *Aquilaria crassna*

Aquilaria crassna (*A. crassna*) is classified in the Thymelaeaceae family, and has been widely used in traditional medicine. It is found in in China and India and in South East Asia: the Philippines, Cambodia, Vietnam, Laos, Thailand, and Malaysia (Tay et al. , 2014) . Krissana is the local name in Thailand. Its resin wood has been used in Ayurveda and other traditional medicines for the treatment of various illnesses such as joint pain, inflammation-related disorders, and diarrhea (Sattayasai et al. , 2012) . There are many biological activities found in the leaves, including anti-oxidant, antipyretic, anti-diabetic, anti-microbial and, hepatoprotective activities. Phenolic compounds, phytosterols/steroids, fatty acids, benzophenones, xanthonoids, flavonoids, terpenoids and alkaloids are listed as bioactive compounds found in this plant (Hashim et al., 2016).

Figure 4 The herbarium of A. crassna were collected in Phitsanulok Province

Source: Wisutthathum et al., 2018

Table 1 Summary of various bioactive compounds presented in *A. crassna* leave

Class	Bioactive compounds
Phenolic acids	4-hydroxybenzoic acid, isovanillic acid, methylparaben, protocatechuic acid, syringic acid and vanillic acid
Phytosterols/Steroids	Stigmasterola, stigmasta-4,22-dien-3-one β-sitostenone and β-sitosterol)
Fatty acids	triacontenoic acid, n-hexadecanoic acid, hexacosanoic acid and 1,2,3-propanetriol
Benzophenones	iriflophenone 3,5-C-β-D-diglucopyranoside, iriflophenone 3-C-β-glucoside
Xanthonoids	aquilarixanthone, homomangiferin, isomangiferin and Mangiferin (Monoglucoside group)
Flavonoids	epicatechin gallate, epigallocatechin gallate and genkwanin (flavonols group)
Terpenoids	2-α-hydroxyursane, 2-α-hydroxyursolic acid 3-friedelanola, epifriedelanol and squalene
Alkaloids	isocorydine

To achieve the objectives of our study, we determined the bioactive compounds of *A. crassna* leaves and developed a procedure to purify the compounds for using in pharmacological research.

Table 2 Summary of pharmacological activities of some compounds from *A. crassna* leaves

Compounds	Pharmacological activities
Mangiferin	Anti-oxidant, Anti-diabetic, Anti-inflammatory and Laxative.
Genkwanin	Anti-oxidant, Anti-inflammatory
Iriflophenone 3-C-β-glucoside	Anti-hyperglycemic(Pranakhon, Aromdee, & Pannangpetch, 2015)

Mangiferin (MGF)

Mangiferin, MGF, 1,3,6,7-terahydroxyxanthone-C2-b-D-glucoside, is a polyphenol that is found in several plants. The structure of MGF is C-glucosyl xanthone contains aromatic ring attached to the C-C bond of a glucose (Figure 5).

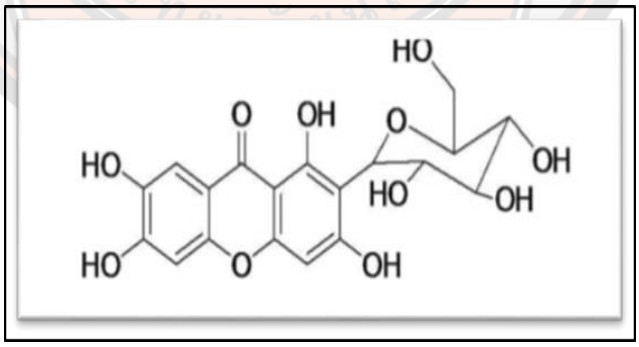

Figure 5 Structure of MGF

Source: Imran et al., 2017

MGF has various pharmacological effects including being an antioxidant, anti-inflammatory, anti-viral, analgesic, immunomodulatory, antidiabetic, and anti-cancer. The chemical properties of MGF have been studied and reported in Table 1.

Table 3 The chemical properties of MGF

Chemical properties	
Formula	$C_{19}H_{18}O_{11}$
Molecular weight	422.342 g/mol
Water solubility	5.02 g/L
Melting point	271°C
Log P	2.73

Pharmacokinetic of MGF)

The metabolism process of MGF contains three pathways: methylation, glucuronidation, and deglycosylation. The metabolite from these pathways are monomethyl mangferin, mangiferinglucuronide, and noratylrioll, respectively (Figure 6).

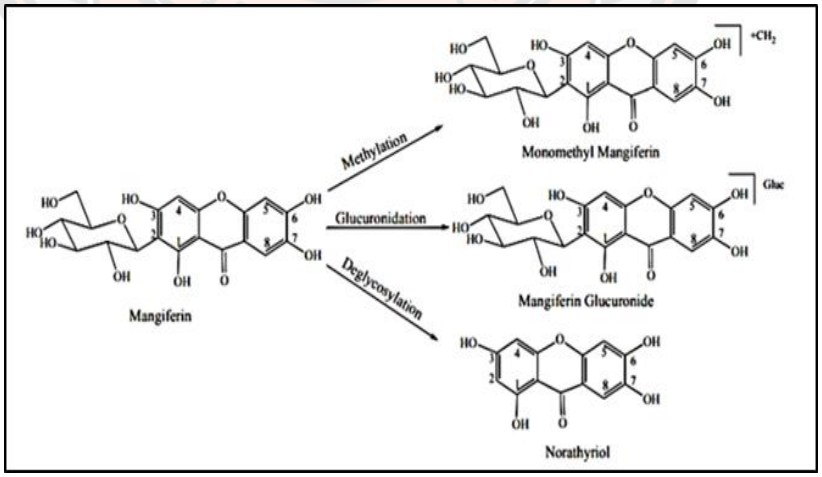

Figure 6 Metabolism of MGF

Source: Benard & Chi, 2015

The pharmacokinetics of MGF with oral administration have been studied in vitro and measured in plasma, urine and tissues. The results showed that MGF and its metabolites have been directly absorbed and can be detected in many organs such as heart, small intestine, stomach, liver, lung, and spleen. A previous study (Tian et al., 2016), showed pharmacokinetic parameters of MGF including maximum plasma concentration (Cmax), area under the curve (AUC), half-life (t1/2), and time of the maximum plasma concentration (Tmax) (Table 4). The bioavailability of MGF in humans is very low (1.2%) due to its low solubility in water and organic solvents.

Table 4 Pharmacokinetic parameters of MGF with oral administrations in *in vivo*

Administration Dose	**Oral (mg/kg)**	
	25	**30**
Cmax (μg/mL)	0.181 - 0.301	15.23
AUC0-t (μg h/mL)	1.040 - 1.855	155.03
t1/2 (h)	3.2 - 4.49	1.06
Tmax (h)	2.5 - 3.0	0.67

CHAPTER III

RESEARCH METHODOLOGY

Our study was divided into two parts. For our first objective, to determine antioxidant activities in *A. crassna* leaves, undertook a full chemical assay of the extract from *A. crassna* leaves, and then evaluated the preventive effects of *A. crassna* leaf extract on DMBA/UVB induced skin damage with an animal model to achieve the second objective.

For the chemical assay, the MGF content in *A. crassna* leaf extract was quantified using HPLC and its antioxidant activity was investigated using DPPH assay. For animal model part, an expression of 4-HNE and COX-2 and collagen content in dorsal skin tissue of mice model were determined using immunofluorescence and colorimetric, respectively (Figure 7).

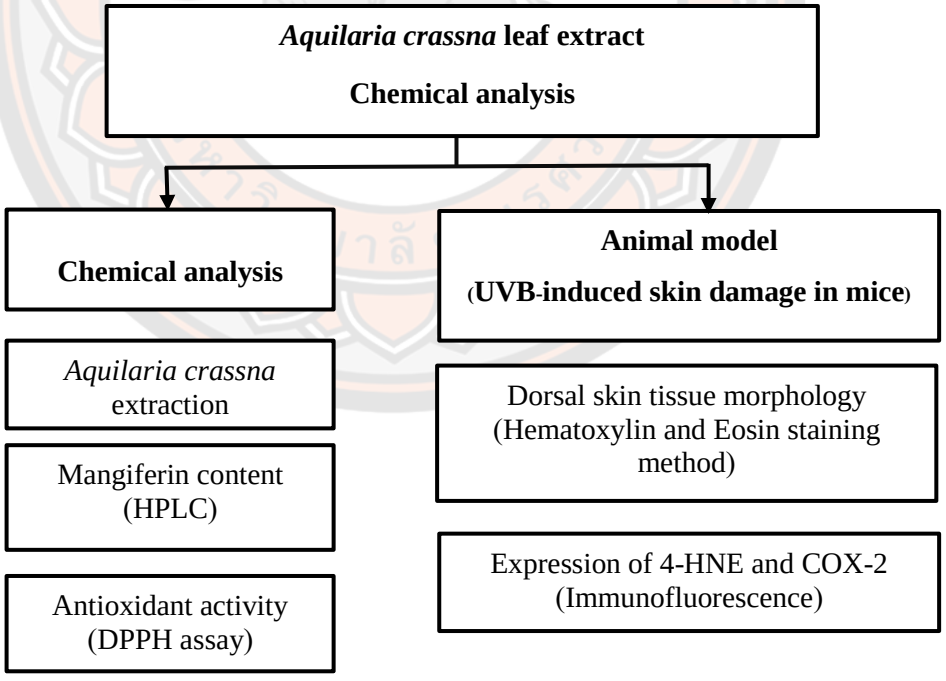

Figure 7 Experimental design in this study

Animal

1. Male ICR mice (3 weeks old, Nomura Siam International Co. , Ltd. Thailand.)

Chemicals and reagents

1. Acetone AR grade reagent (RCI LabScan, Thailand)
2. Anti-Cox2/Cyclooxygenase 2 antibody ab15191 (Cambridge, UK)
3. Anti-4 Hydroxynonenal antibody ab46545 (Cambridge, UK)
4. Bovine serum albumin (Lot. 051M1845V Sigma-Aldrich, Co., St. Louis, USA)
5. Deionized water (Batch No. 15128066, LabScan Asia Co. LTD. , Bangkok, Thailand)
6. DMBA (7,12-Dimethylbenz[a] anthracene. 95% Lot# D3254, Sigma-Aldrich, Co., St. Louis, USA)
7. Ethanol (Lot. K31369783 245, Merck, Darmstadt, Germany)
8. Frozen section compound (Lot#010515, Leica, USA)
9. Goat anti-rabbit IgG H&L, Alexa Fluor®488 (abcam150077, ABCAM, Cambridge, UK)
10. Goat anti-rabbit IgG H&L antibody (ab 6564, Cambridge, UK)
11. Histological staining for PATH. 1 Counter staining Hematoxylin and Eosin staining (Lot. SEP. 2015, C.V. Laboratories CO., LTD., Bangkok Thailand)
12. Histological staining for nuclear staining Hematoxylin and Eosin staining (Lot. SEP. 2015, C.V. Laboratories CO. LTD., Bangkok Thailand)
13. Histological mounting medium Permount) (Lot. 1813446, Fisher Scientific, Massachusetts, USA)
14. Injection water (A.N.B. LABORATORIES co., Ltd, Thailand)
15. L-ascorbic acid (Lot. 529150113, Poch, Sowiñskiego, Poland)
16. Mangiferin Mangifera indica (Lot#SLBV4993, Sigma-Aldrich, Co., St. Louis, USA)
17. Poly-L-lysine solution 0.1% (w/v) in H2O (Lot#SLBN6266V, Sigma, USA)
18. Sodium sulfide X hydrate (Solo, Ohio, USA)

19. Sodium chloride (B/NO. 1306251725, Univar, Australia)

20. Triton X-100 (Lot#MKBQ0896V, Sigma, USA)

21. Tween 20 (Srichand United Dispensary CO., LTD., Bangkok Thailand)

Anesthetic

1. Ketamine-Hameln (Hameln pharmaceuticals, Batch no. 408069, Germany)

2. Xylazine (L.B.S. laboratory LTD., PART, Lot No. 1790038, Thailand)

3. Thiopental (Nembutal)

Equipment

1. Carl Zeiss-axiom observer z1 fluorescent microscope (Carl Zeiss Microscopy Ltd., Cambridge, England)

2. Cryostat (Leica Microsystem Nussloch GmbH, Nussloch, Germany)

3. Centrifuge (Universal 320, Hettich laboratory, Tuttlingen, Germany)

4. Freeze drying apparatus (Heto Model Lyolab 3000, Thermo Electron Corporation, Mukarov, Czech)

5. Insulin syringe 10 units (BD Ultra-Fine, Lot. 4121921 E, USA)

6. High profile microtome Blades (Leica 818, Nussloch, Germany)

7. Microplate Reader (BioTek Instruments, BioStack Ready, USA)

8. Manual HPLC syringe (Hamilton, Nevada, USA)

9. Magnetic stirrer (HL instrument, MS 115, Harikul Science, Tokyo, Japan)

10. NYLON Membrane Filters, 47 mm, 0.45 µm (Lot. 2332, Vertical, Thailand)

11. pH meter (METTER TOLEDO, S20-k, Metter-Toledo International Inc., Ohio, USA)

12. Ultracentrifuge (Beckman Model J2-MC, Beckman Instruments, Inc., USA)

13. VertiClean nylon syringe filter 0.45 µm (Vertical chromatography Co., Ltd. Thailand)

14. Water bath (model LWB-211A, Daihom Lab Tech CO., LTD., Korea)

Methodology

Preparation of *A. crassna* leaf extract.

Leaves of *A. crassna* were collected in Phitsanulok Province, Thailand. The leaves were dried in a hot air oven at 70°C for 24 hr. and the dried leaves were then blended into fine powder. A sample of 150g of the leaf powder was boiled in 1500 ml of water at 95°C for 30 minutes and then filtrated through Whatman No. 1 filter paper. The filtrated extract was centrifuged at 15000 rpm for 5 minutes, and then lyophilized in a freeze dryer for 72 hours, and then stored at -20°C until required.

Determination of free radical scavenging activity of the extract.

A DPPH assay was used to screen for free radical scavenging activity of the *A. crassna* leaf extract by comparing the extract with L-ascorbic acid as the positive control. The DPPH assay (2,2—Diphenyl-1-picrylhydrazyl free radical scavenging activity) is one of the most popular antioxidant activity assays used to determine the capability of an extract to scavenge free radical or oxidation molecules. The results of this assay were reported as a percentage of the inhibitory concentration (IC50) of the extract and the L-ascorbic acid. The DPPH assay was modified and evaluated from the previous study (Kalaivani & Mathew, 2010). The *A. crassna* leaf extract and L-ascorbic acid were dissolved in deionizing water at various concentrations. 75µl of the dissolved sample were then added into a 96-well plate, with 150 µl of DPPH solution (78.8 µg/mL) then added with the total solution then kept in dark conditions for 30 minutes. The absorbance was measured using a microplate reader at 515 nm and a sigmoidal curve was plotted in terms of IC50 using the GraphPad Prism 8 Program (Leerach et al., 2017; Viyoch et al., 2012)

The ability of the antioxidant was calculated using the following equation:

$$\text{DPPH radical-scavenging rate (\%)} = (1 - (A_a - A_b)/A_c) \times 100\%$$

Where: Aa is the absorbance of the DPPH with sample.

Ab is the absorbance of the sample.

Ac is the absorbance of the DPPH without a sample.

The percentages of free radical scavenging were plotted and then calculated for IC50 and the results were compared with the L-ascorbic acid as the positive control. All samples were run in triplicate and represented as mean ± SD.

Quantification of Mangiferin content (MGF) in the extract using HPLC.

The amount of MFG in the *A. crassna* leaf extract was quantified using a gradient high-performance liquid chromatograph (HPLC, UV detector). Phenomenex Luna C18 column (150 mm × 4.6 mm, 5 mm particle size) and guard column (5 mm Phenomenex C18, 4 mm × 3 mm) were used. The mobile phase consisted of a mixture of acetate buffer (pH 3.7 at a concentration of 200 μg/mL) (A) and acetonitrile (B). The *A. crassna* leaf extract was dissolved in the mobile phase and filtered through a 0.45 μm nylon filter with 20 μL of sample then injected into the system. A linear gradient program was set as follows: 0 to 5 min, 15% B; 5 to 10 min, 40% B and then held with 15% B for 5 min, with a column temperature set at 30°C, at a flow rate of 1.0 mL/min, and monitoring at 310 nm (Wisutthathum et al., 2018). The amount of MGF was calculated from the integrated peak area using a calibration curve generated from standard MGF.

Study of the preventive effects of *A. crassna* leaf extract on UVB-induced mice skin

Animals and treatments

Male ICR mice (3 weeks old) were purchased from the Nomura Siam International Co., Ltd. Thailand. They were housed with free access to food and water for 1 week in a temperature–controlled room at 22±1°C and 55±10% relative humidity. The animal protocol was previously approved by Naresuan University Animals Ethics Committee, approval code: NUAE-600707.

All mice were divided into 5 groups (5 for each group):

- Normal group: non-DMBA/UVB-induced, receiving water.
- Control group: DMBA/UVB-induced, receiving water
- Test Group1: DMBA/ non UVB-induced, receiving water.
- Test Group 2: DMBA/UVB-induced, receiving 250 mg/kg of body weight/day of ACE
- Test Group 3: DMBA/UVB-induced, receiving MGF standard equivalent MGF content in *A. crassna* leaf extract (19.4 mg/kg per day)

Mice were fed the *A. crassna* leaf extract and MGF standard once a day for 2 weeks. The molecular mechanism underlying the preventive effects of the extract on

UVB-induced skin damage were studied. To start, the dorsal areas of the mice were shaved by applying 8% sodium sulphide x-hydrate on 3x3 cm2 area, and left for 1-2 minutes and then wet cotton swabs were used to clean off the sodium sulphide x-hydrate solution from the shaved area without damaging the skin; this was done twice. Before starting the experiment, the mice of the control group, Test Group1, Test Group 2 and Test Group 3 had a single application of the initiator, 200nM of 7, 12-dimethylbenz (a) anthracene (DMBA), on the dorsal skin and left for 7 days (Sharmila & Shanmugam, 2012). During the ensuring 16 weeks, all of the mice groups were treated with the samples that were mentioned above for each group and the mice were weighed daily before feeding.

UVB radiation on mice model

Mice were anesthetized with Ketamine (40mg/kg) and Xylazine (2mg/kg) before UVB irradiation. Their reflexes were checked to ensure that each animal was anesthetized deeply. In this study, the modified UVB-box with wooden material was used for UVB-irradiated protocol that can prevent UVB spreading. The inside area of the box was W×L×H; 20×72×22 (cm3). It consisted of 4 UVB lamps (alpha type, 18W) which has wavelength of 280-320 nm. The frequency of irradiation was set at 3 times per week; Monday, Wednesday and Friday. The animals were irradiated with UVB once a day (between 08.30am and 11.00am) after the oral administration of a solution of deionized water, *A. crassna* leaf extract and MGF standard. The UVB irradiation period was performed as indicated in Table 5 (Viyoch et al., 2012). Measurement of UVB intensity was performed by UV meter to calculate the exposure time according to the following equation:

$$\text{UVB Dose, mJ/cm}^2 = (\text{Intensity, mW/cm}^2) \times (\text{Time, s})$$

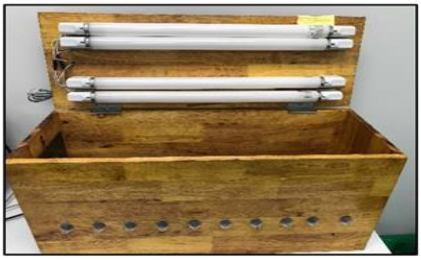
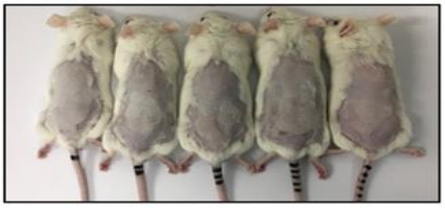

Figure 8 Modified UVB- box and the mice after receiving the sample and before UVB irradiation

Table 5 shows the dose of UVB in each week from week1 to week 16

Table 5 The dose of UVB in each week

Week	Intensity of UVB (mJ/cm2)	Duration time (min)
1-4	54	6.43
5-8	72	8.57
9-12	108	12.86
13-16	126	15.00

Termination and skin collection

After 16 weeks, the mice were sacrificed by thiopental overdose (100 mg/kg, IP). A 4×4 cm^2 section of dorsal skin was collected immediately and frozen using frozen section compound followed by freezing in liquid nitrogen. For the histology study part, the dorsal skin tissue was stored at -80°C until further use.

Measurement of the dorsal skin thickness and histopathology.

Preparation of frozen tissue sections

The frozen dorsal skin was cut in thickness of 8 µm using cryostat. The tissue sections were put on glass slides coated with Poly-L-lysine solution. The tissue section slides were dried at room temperature for 10 minutes and stored in a slide box at -80ºC (Leerach et al., 2017).

Hematoxylin and Eosin (H&E) staining method

H&E staining is a technique used to investigate tissue morphology and pathology. Hematoxylin is a basic dye that stained basophilic tissue such as nuclei. Eosin is an acid dye using a counterstain for hematoxylin used to stain cytoplasm and extracellular matrix that appeared in pink color. In sectioning part, the H&E staining process were done as in Table 6.

Table 6 Steps of Hematoxylin and Eosin staining method

Step	Solution	Time (minute)
1	Acetone at -20°C	15
2	Hematoxylin	2.30
3	Tap water	3
4	Eosin	2
5	95% ethanol	1
6	95% ethanol	1
7	Absolute ethanol	1
8	Absolute ethanol	1

Firstly, the dorsal skin section was rehydrated by soaking in acetone Then, the sections were immersed in a Mayer's hematoxylin chamber for 2 min and rinsed by flowing tap water for 3 min, and then left at room temperature for 5 min. The sections were then stained with eosin and then rehydrated by soaking in 90% ethanol, and then absolute ethanol. Finally, the sections were mounted and covered with a cover slip.

The epidermal thicknesses were measured using the AXIO software (Carl Zeiss Microscopy Ltd. , Cambridge, UK) . The stained slides were subsequently permeated with mounting medium and the permanent slides stored at room temperature. The skin morphology was observed under light microscopy.

Determination of 4-HNE and COX-2 using immunofluorescence

The amount of 4-HNE and COX-2 expression were detected by immunofluorescence staining. The immunofluorescence staining technique is a technique widely used to investigate the location of antibodies and their antigens (Betterle & Zanchetta, 2012). The dorsal skin sections were fixed in cooled acetone at -20 ºC, then washed with 1X PBS-Tween20 at room temperature for 5 minutes then blocked with 10% BSA in 1X PBS and incubated at 4°C for 30 minutes. After blocking, the tissues were incubated at 4ºC overnight in a humidified chamber with rabbit-anti-4-HNE polyclonal primary antibody (1:200dilution) (ab46545, Cambridge, UK) diluted with 10% BSA in 1X PBS. The tissue was then washed twice with PBS-Tween20 and incubated with goat-anti-rabbit IgG H&L, Alexa Fluor®488secondary antibody (Cambridge, UK) (1:500 dilution) for 1 hour. Fluorescence intensity was analyzed by the Image J program free program (The National Institutes of Health (NIH), (Leerach et al., 2017). Determination of COX-2 expression was the same as for the 4-HNE expression. The primary antibody was changed to Anti-COX-2/ Cyclooxygenase 2 antibody (ab15191, Cambridge, UK) in ratio of 1:200 and also the secondary antibody was changed to Goat anti-rabbit IgG H&L antibody (ab 6564, Cambridge, UK).

CHAPTER IV

RESULTS AND DISCUSSION

Freeze-drying of *A. crassna* leaf extract

The water content in *A. crassna* leaf extract was removed by freeze-drying over 72 hours. The lyophilized of *A. crassna* leaf extract was light brown as shown in Figure 9. The yield of freeze-dried *A. crassna* leaf extract was 30.35g being 20.23% of the 150g weight of the *A. crassna* dried leaves.

Figure 9 The physical characteristics of *A. crassna* leaf extract after freeze-drying

Table 7 The yield of lyophilized *A. crassna* leaf extract

Weight of *A. crassna* leaf extract	Weight of lyophilized of *A. crassna* leaf extract	% Yields
150g	~30-31g	20.23

Measurement of an antioxidant ability of freeze-dried *A. crassna* extracts by DPPH assay

The effectiveness of the *A. crassna* leaf extract and L-ascorbic acid as an antioxidant and for free radical scavenging were compared. In Table 7, the freeze-dried *A. crassna* leaf extract produced IC50 values of 0.030 ± 0.46 mg/ml which were

calculated from Figure 10, while L-ascorbic acid produced IC50 values of 0.007 ± 0.10 mg/ml which were calculated from Figure 11.

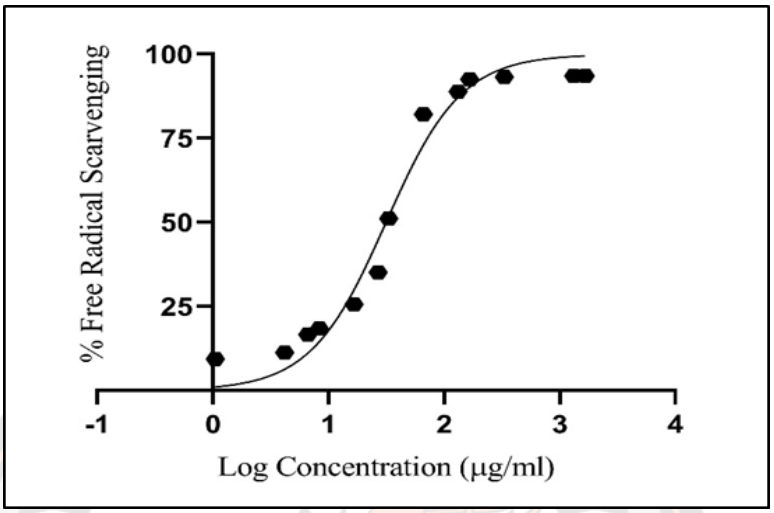

Figure 10 The ability of the antioxidant of *A. crassna* leaf extract

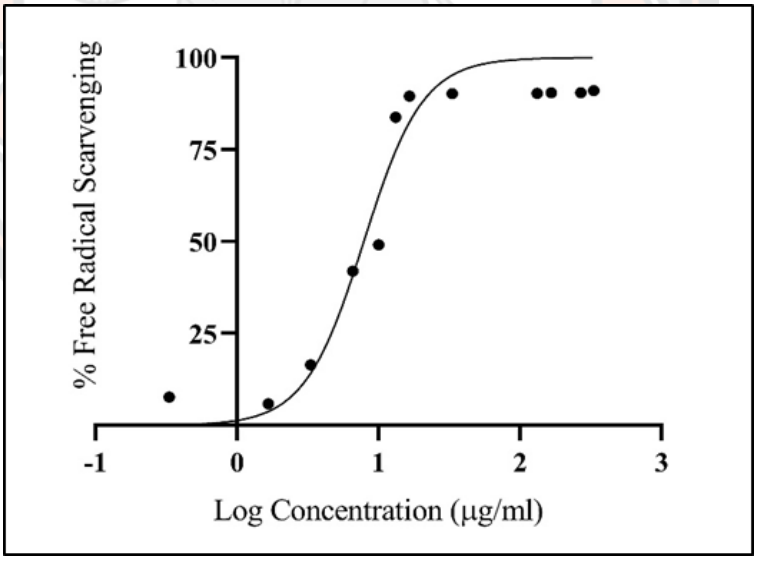

Figure 11 The ability of the antioxidant of L-Ascorbic acid

Quantification of Mangiferin content (MGF) in the extract using HPLC

The result of determining the MGF content in *A. crassna* leaf extract powder was that the *A. crassna* leaf extract at the concentration of 1 mg/mL contained about 21.97% iriflophenone 3, 5-C-β-D-glucoside, 14.02% iriflophenone 3-C-β-D-glucoside, and 7.76% Mangiferin.

Table 8 IC50 value of freeze-dried *A. crassna* leaf extract (mean±SD, n=3)

Samples	% Maximum inhibition	IC50 ± SD (mg/ml)
A. crassna leaf extract (0.5 mg/ml)	92.04	0.030 ± 0.46
L-Ascorbic acid (0.1 mg/ml)	90.22	0.007 ± 0.10

Table 9 MGF content in *A. crassna* leaf extract powder by HPLC method

Standard compounds	Content of bioactive compounds *(%w/w)*	
	Lyophilized ACE	Dried leaves
Flophenone 3, 5-C-β-D-glucoside	21.97±0.77	4.44±0.16
Flophenone 3-C-β-D-glucoside	14.02±0.38	2.84±0.08
MGF	7.76±0.25	1.57±0.05

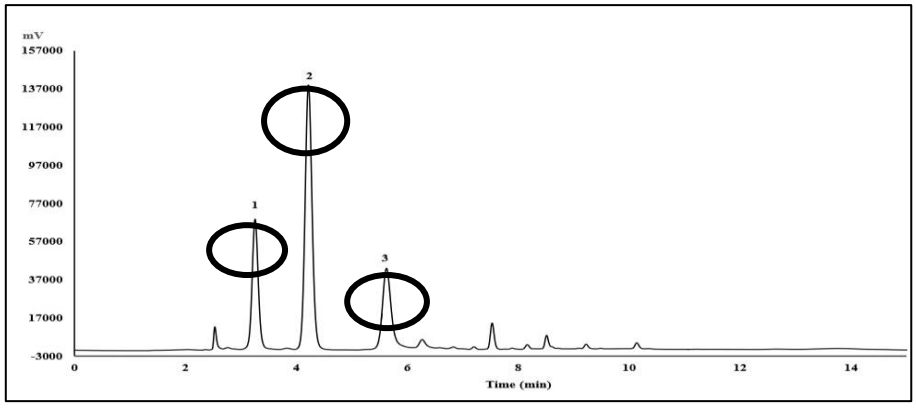

Figure 12 MGF content in A. crassna leaf extract powder by HPLC method (From Table 9)

Effect of *A. crassna* leaf extract administration on mice body weight and DMBA/UVB-induced skin damage

The mice were weighed daily before being orally administered *A. crassna* leaf extract, MGF standard, and water. Administration of the *A. crassna* extract, at a concentration of 250 mg/ kg/day, and MGF standard, did not reduce the body weight of the DMBA/UVB-induced skin damaged mice groups (Figure 13). This indicated that the dosage used in the experiments was appropriate.

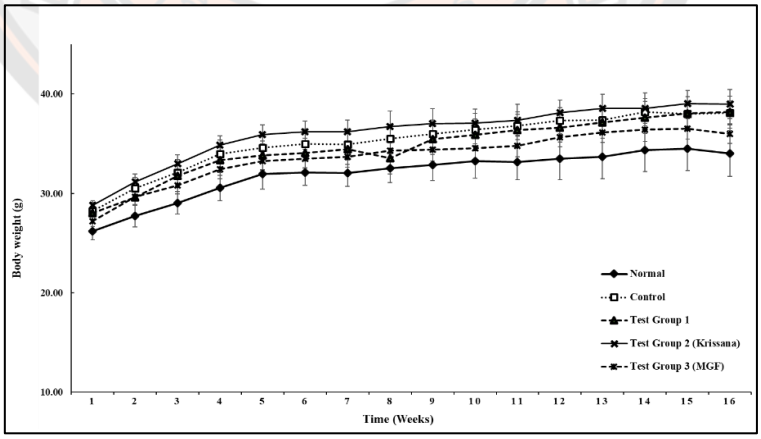

Figure 13 Body weight of mice between week 1 and week 16 of the experiment

After 16 weeks, the appearance of the skin morphology was visualized and compared to a non-DMBA/ UVB-induced group; the Normal Group. The Normal Group's skin morphology was smooth with wrinkles (Figure 14 (A)). The Control

Group - DMBA/ UVB-induced receiving deionized water - had dorsal skin morphology that was wrinkled and rough (Figure 14 (B)), while the DMBA/ non UVB-induced receiving deionized water, Test Group 1, had dorsal skin morphology that was smooth and with no wrinkles (Figure 14 (C), Test Group 2, the DMBA/UVB induced receiving *A. crassna* leaf extract group, and Test Group 3 receiving MGF standard only, both showed roughness and wrinkled skin but less than those in the Control Group (Figure 14 (D) and (E)). These results might suggest that *A. crassna* leaf extract and MGF standard have only a little ability to prevent skin wrinkles from repeated UVB exposure.

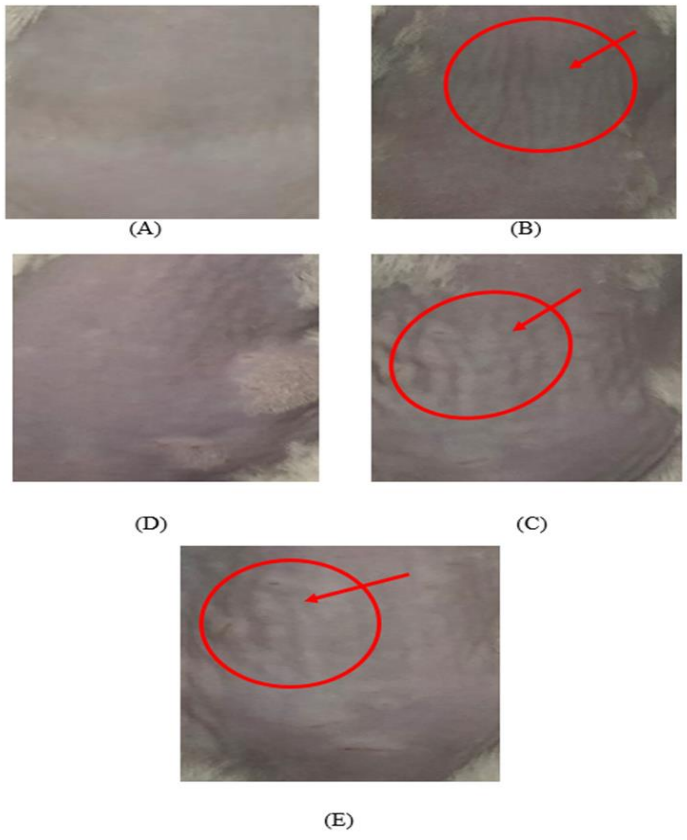

Figure 14 Skin Morphology of each experimental group.

Note: (A) Non-DMBA/UVB-irradiated (Normal group)

(B) DMBA/UVB-irradiated, receiving water (Control Group)

(C) DMBA/non UVB-irradiated, receiving water (Test Group 1)

(D) DMBA/UVB-irradiated, receiving *A. crassna* leaf extract (Test Group 2)

(E) DMBA/UVB-irradiated, receiving MGF standard (MGF, Test Group 3)

Effect of *A. crassna* leaf extract and Mangiferin standard on epidermal thickness of UVB-irradiated skin

Repeated UVB exposure causes skin damage and dermal connective tissue disarrangement, conditions known as photodamage. After repeated exposure to UVB, free radicals such as ROS are generated. Skin cells, particularly keratinocytes, respond to an occurring ROS by secreting inflammatory mediators such as IL-1, IL-6, and TNF-α. This process can also trigger an expression of several types of keratin, proteins, which are expressed only in epithelia to form intermediate filaments through assembly into bundles leading to the generation of toughness of the epidermis (Feldmeyer et al., 2010; Lichtenberger et al., 2018). Epidermal hyperplasia is marked as a sign of skin disorder. In the present study, epidermal thickness was measured and determined by using hematoxylin and eosin staining. The arrangement of the epidermal layer of the Normal Group showed normally (see Figure 14 (A). In this experiment, skin damage was induced in the dorsal skin of the mice with DMBA before starting UVB-irradiation in appropriate dosage. The results showed that repeated UVB exposure caused the development of highly irregular epidermal hyperplasia in the DMBA/UVB-induced groups. The immune response mechanism was reduced by the keratinocyte cells.

At 16 weeks after the UVB-irradiation, the characteristic epidermal thickness in the DMBA/UVB-induced skin was measured. The observations at that time were that there was a significant increase in the epidermal thickness in the Control Group (35.76 ± 0.96 μm): group of DMBA/UVB-irradiated mice receiving water. For Test Group 2: group of DMBA/UVB-irradiated mice receiving *A. crassna* leaf extract (34.58 ± 1.33 μm) and Test Group 3: group of DMBA/UVB-irradiated mice receiving MGF standard (30.28 ± 0.23). All these groups showed a statistically significant increase in epidermal thickness ($p<0.01$) when compared with the non-UVB irradiated Normal group (16.46±0.61 μm).

In addition, the DMBA/non UVB-irradiated Test Group 1, receiving water (26.49 ± 1.69) was measured and compared with the Normal Group and showed a statistically significant increase in epidermal thickness ($p<0.01$). Test Group 2 (34.58 ± 1.33 μm), the DMBA/UVB-irradiated group receiving *A. crassna* leaf extract, showed no statistically significant difference in epidermal thickness ($p<0.01$)

compared with the Control Group, but showed a statistically significant difference in epidermal thickness when compared with Test Group 1 and Test Group 3 ($p<0.05$). Test Group 3 (30.28 ± 0.23), receiving MGF, showed no statistically significant difference in epidermal thickness ($p<0.01$) as compared with the Test Group 1.

The Control Group, (35.76 ± 0.96 μm) showed a statistically significant increase in epidermal thickness ($p<0.01$) when compared with the Normal Group which showed no increase in epidermal thickness.

In addition, when compared with the mice of Test Group 1 ((26.49 ± 1.69μm) there was no significant difference in epidermal thickness ($p<0.01$) when compared with Test Group 3 (30.28 ± 0.23). Test Group1, however, had not received UVB-irradiation but had received one dose of DMBA before starting UVB radiation method. This implies that applying a sub-carcinogenic dose of DMBA to the dorsal skin penetrated the hair follicles, which are the primary target of the initiation stage. The DMBA is accumulated in the hair follicles causing epidermal thickness. This follows (Abel, Angel, Kiguchi, & DiGiovanni, 2009) who showed that DMBA accumulates at the base of the epidermal proliferative units in the interfollicular epidermis and in the bulge region of the hair follicles.

Our results suggest that the consumption of *A. crassna* leaf extract cannot prevent skin damage from UVB radiation. However, the pure compound MGF is effective in decreasing the skin epidermal thickness arising from repeated UVB exposure. Such beneficial effects might be result from the phenolic groups in MGF, which are natural antioxidants of MGF, which have the ability to scavenge ROS and to also decrease pro-inflammatory cytokine production from the UVB-irradiated skin.

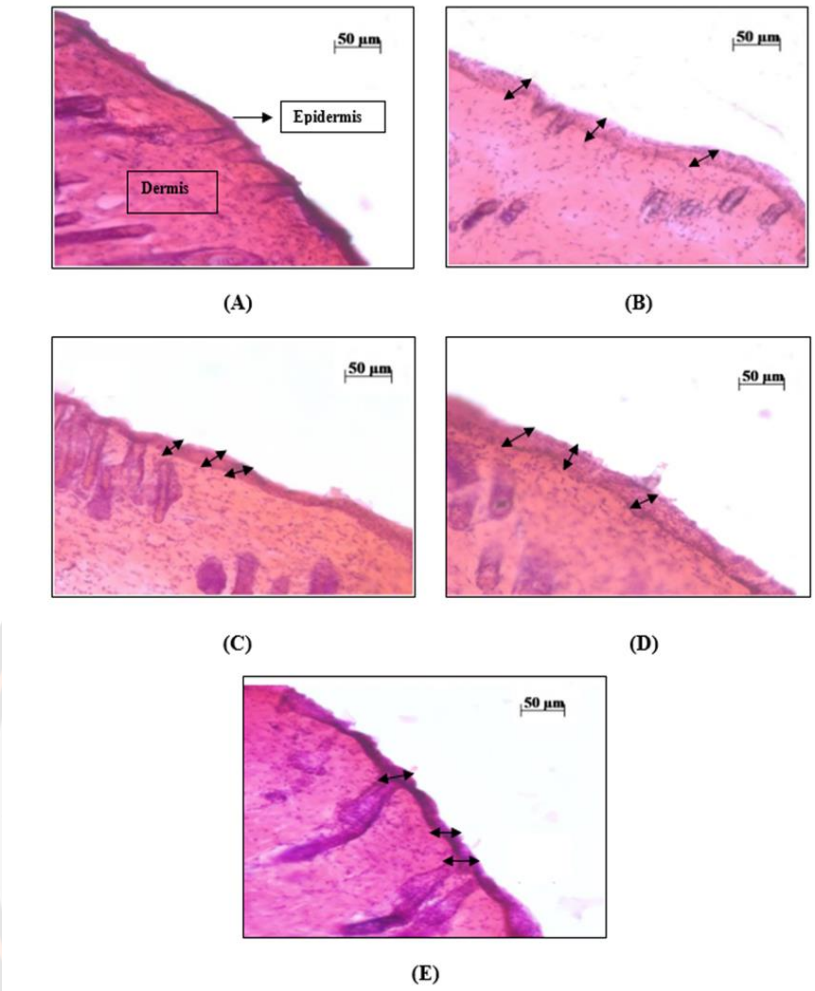

Figure 15 Light micrograph (at magnification, 20x) of tissue stained with hematoxylin and eosin showed the epidermal skin morphology .

Note: (A) Non-DMBA/UVB-irradiated (Normal group)

(B**)** DMBA/UVB-irradiated, receiving water **(**Control group**)**,

(C**)** DMBA/non UVB-irradiated, receiving water **(**Test Group 1**)**

(D**)** DMBA/UVB-irradiated, receiving *A***.** *crassna* leaf extract (Test Group 2**)**

(E**)** DMBA/UVB-irradiated, receiving MGF standard **(**MGF, Test Group 3**)**

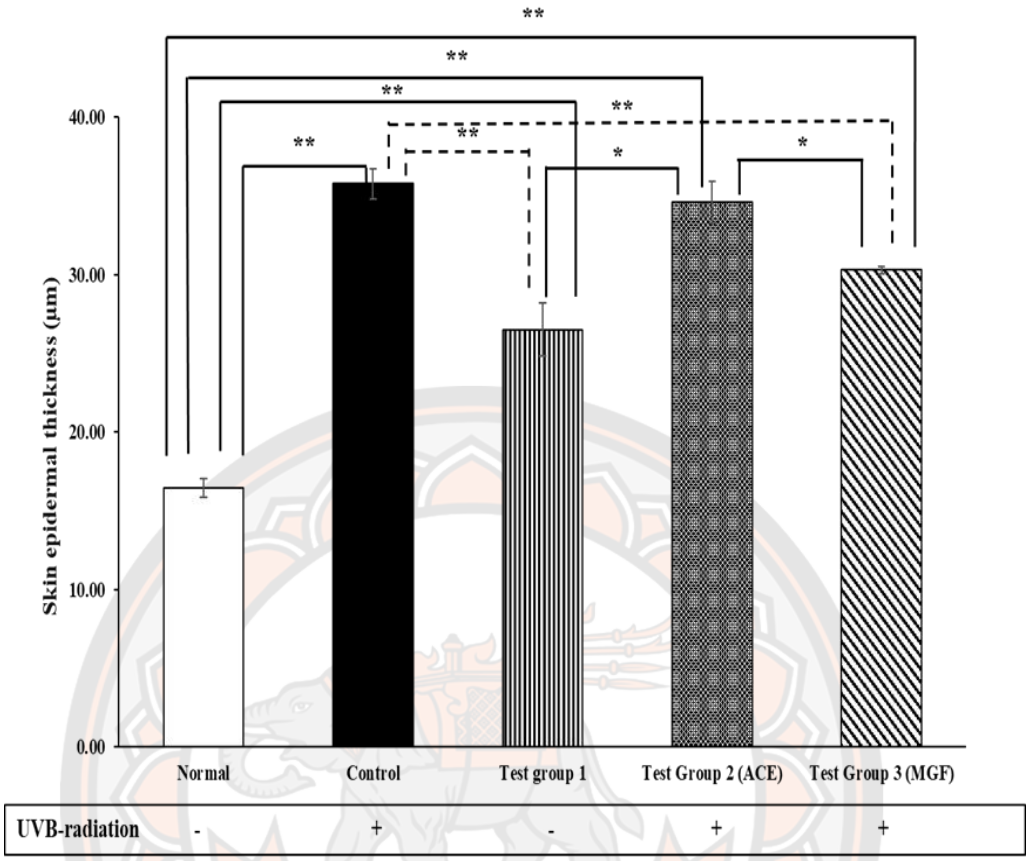

Figure 16 The effect of orally administration of *A. crassna* leaf extract and MGF standard on epidermal thickness in repeated UVB irradiated mice. Each bar represents the mean ± SEM (N=5). The thickness was measured ten points of epidermal of mouse. Student's t-test indicates statistically significant differences between groups. (*p < 0.05) and (p < 0.01)**

Effect of *A. crassna* leaf extract and Mangiferin standard (MGF) administration on 4-HNE and COX-2 expression in DMBA/UVB-induced skin damag

Repeated UVB exposure causes ROS generation and invokes an immune response in skin cells. The expression of 4-HNE and COX-2 in mice dorsal skin was investigated using immunofluorescence staining. 4-HNE is the end product of lipid peroxidation, which plays a role in oxidative damage. The localization of 4-HNE was markedly found in the epidermis layer, which contains a significant quantities of lipids in its elements. In oxidative stress conditions, this affects the cellular components and other molecules that contain lipids. Fluorescence micrograph and fluorescence light intensity tests of the dorsal skin tissue were used to determine the level of 4-HNE expression, as shown in Figures 16 and 17.

Table 10 The conditions in animal model in each group

Group	DMBA	UVB	Sample
1. Normal group	Not received	Not received	Water
2. Control group	Received	Received	Water
3.Test group 1	Received	Not received	Water
4.Test group 2	Received	Received	ACE
5.Test group 3	Received	Received	MGF

After 16 weeks of DMBA/ UVB irradiation, the following results were observed (Table 10 shows the irradiation status of each group, and the sample of water, *A. crassna* leaf extract and MGF received by each group): The Control Group showed a significantly increased intensity of immunofluorescence staining of level of 4-HNE when compared to the Normal Group. The Control Group also showed significantly increased intensity of immunofluorescence staining of level of 4-HNE when compared to the treated DMBA/ UVB irradiated groups ($p<0.05$) . The comparison between the Normal group and Test Group1 showed that the level of 4-

HNE expression significantly increased in Test Group 1. The Test Group1 was not UVB irradiated but received DMBA applied once on the dorsal skin which causes an accumulation of DMBA in the hair follicles and stimulates lipid peroxidation (Kong & Xu, 2018). Test Group 2, which received *A. crassna* leaf extract, did not show significantly decreased levels of 4-HNE expression when compared with the Control group.

However, the mice in Test Group 3 that received MGF showed significantly decreased levels of 4-HNE expression as compared with the Control group ($p<0.05$).

COX-2, a major mediator of tissue inflammation, can convert arachidonic acid in the phospholipid membrane to prostaglandins at the sites of inflammation (An et al., 2002). In the same way as the overexpression of COX-2, skin repeatedly subjected to UVB exposure can lead to chronic skin inflammation and subsequently may develop into skin cancer. The mechanism of COX-2 is to catalyze the conversion of arachidonic acid from the phospholipids membrane to prostaglandins E2 (PGE2) by using phospholipase A2. Expression of COX-2 plays an important role in the inflammation stage of cells and a high inflammation activity in the skin can stimulate over-proliferation of keratinocyte cells leading to skin damage.

The percentage of fluorescence intensity of the level of COX-2 expression are illustrated in Figures 18-19. The Control Group showed a significantly increased level of COX-2 expression when compared with the Normal Group ($p<0.01$). Test Group 3, one of the DMBA/ UVB irradiation groups, which had received MGF, showed a significantly decreased level of COX-2 expression when compared to the Control Group ($p<0.01$).

However, Test Group 2, also one of the DMBA/ UVB irradiation groups, which had received *A. crassna* left extract, showed no significant difference in COX-2 expression as compared to the Control Group ($p<0.01$). The comparison of the effectiveness of *A. crassna* left extract that Test Group 2 received and MGF, that Test Group 3 received, showed that the level of COX-2 expression was significantly decreased by the MGF, but the *A. crassna* leaf extract had little effect.

An overexpression of COX-2 was found in the mice groups that had DMBA/UVB-irradiated induced skin damage. DMBA is sub-carcinogen, when used as the chemical model for induced skin disorders when applied on the dorsal skin,

leading to oxidative stress in skin cells (Blog & Szabo, 1979). We demonstrated that repeated UVB exposure to the skin also exposed to DMBA promoted inflammation activities and an increase of free radicals and greater stimulation of pro-inflammatory cytokines in the skin.

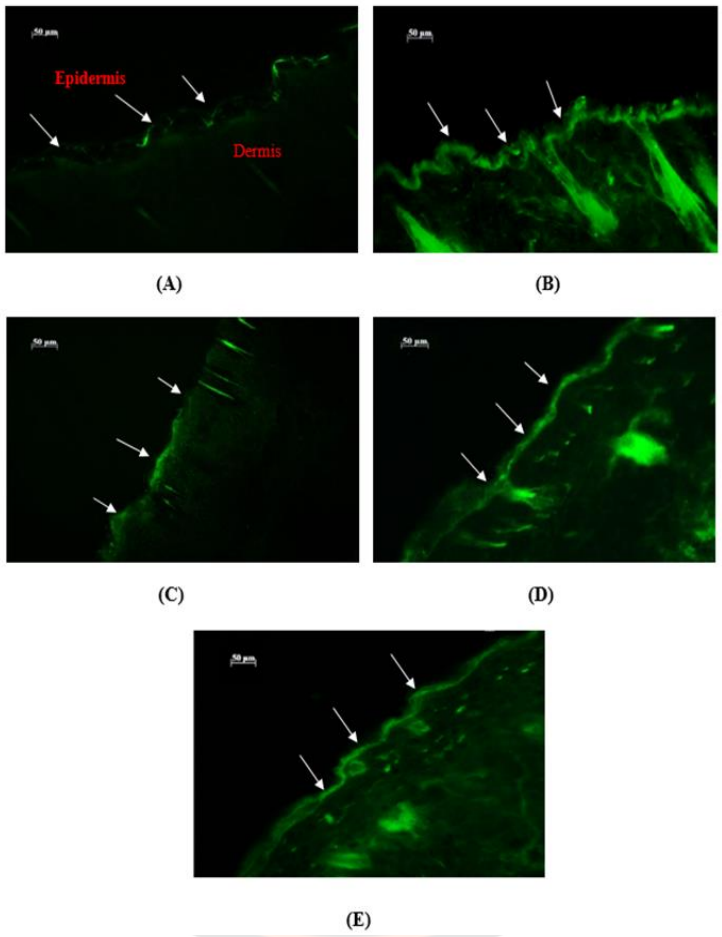

Figure 17 Fluorescence micrograph (at magnification, 20x) of the expression of 4-HNE on dorsal skin sections of mice that stained with immunofluorescence technique

Note: (A) Non-DMBA/UVB-irradiated (Normal group)
(B) DMBA/UVB-irradiated receiving water (Control group),
(C) DMBA/non UVB-irradiated (Test Group 1),
(D) DMBA/UVB-irradiated receiving *A. crassna* leaf extract (Test Group 2)
(E) DMBA/UVB-irradiated receiving MGF (Test Group 3)

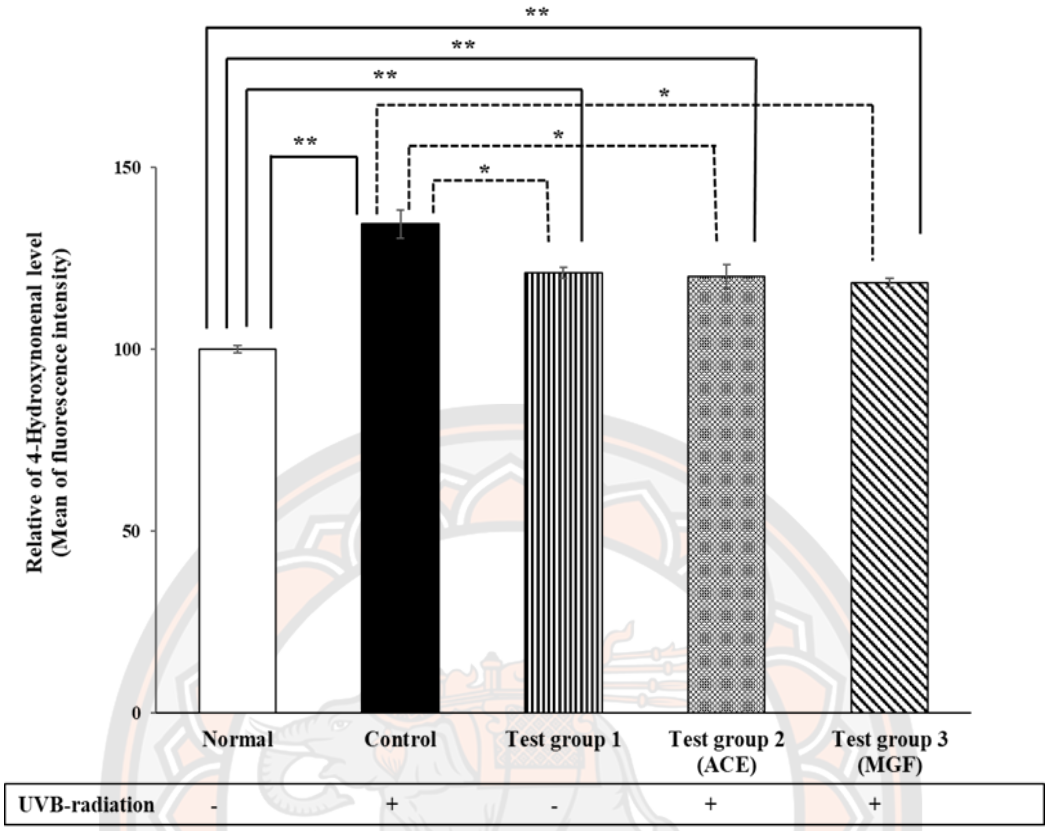

Figure 18 Fluorescence intensity of 4-HNE, immunofluorescence staining of dorsal skin tissue (percentage of control group). The effect of orally administration of *A. crassna* leaf extract and MGF on DMBA/UVB irradiated mouse skin. Each bar represents the mean ± SEM of fluorescence intensity (N=3). Student's t-test indicates statistically significant difference between groups. (*p < 0.05) and (p < 0.01)**

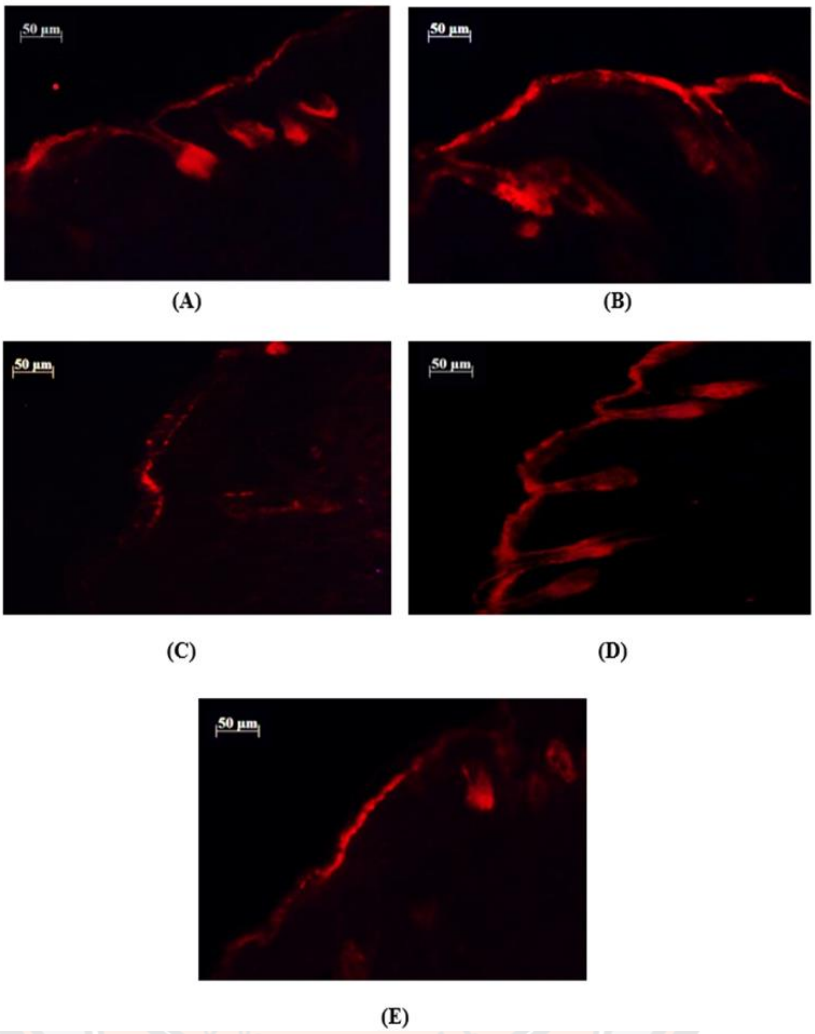

Figure 19 Fluorescence micrograph (at magnification, 20x)of the expression of COX-2 on dorsal skin tissue of mice that stained with immunofluorescence technique.

Note: (A) Non-DMBA/UVB-irradiated (Normal group)

(B) DMBA/UVB-irradiated receiving water (Control group),

(C) DMBA/non UVB-irradiated (Test Group 1),

(D) DMBA/UVB-irradiated receiving *A. crassna* leaf extract (Test Group 2)

(E) DMBA/UVB-irradiated receiving MGF (Test Group 3)

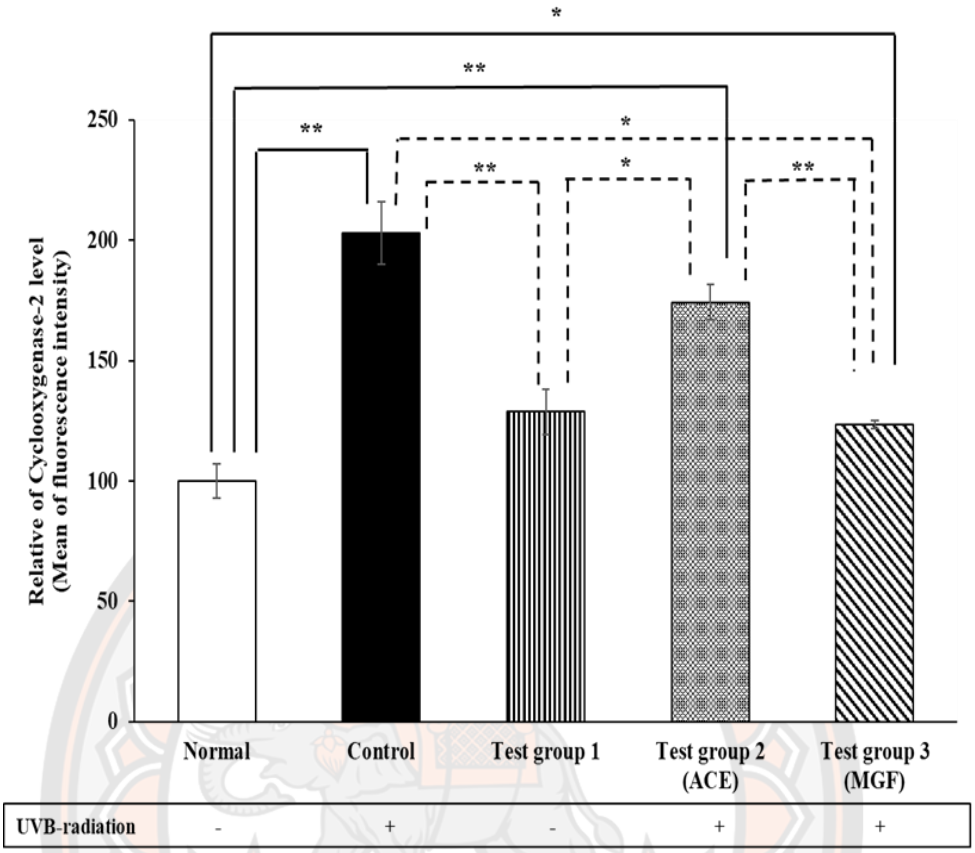

Figure 20 Fluorescence intensity of COX-2, immunofluorescence staining of dorsal skin tissue (percentage of control group). The effect of orally administration of *A. crassna* leaf extract and MGF on DMBA/UVB irradiated mouse skin. Each bar represents the mean ± SEM of fluorescence intensity (N=3). Student's t-test indicates statistically significant difference between groups. (*p < 0.05) and (p < 0.01)**

CHAPTER V

CONCLUSIONS

Repeated ultraviolet radiation (UV) is a risk factor from the external environment that causes sunburn, photoaging, and skin damage that can develop into skin cancer. UV radiation, especially UVB, is harmful to the skin. The radiation energy of UVB can directly destroy DNA and generate reactive oxygen species (ROS) in skin cells. ROS has been reported as playing an important role in oxidizing the cellular components of skin cells including the proteins and lipids leading to skin damage.

Skin damage can be decreased or delayed by dietary intake such as the consumption of certain herbal plants which contain anti-oxidant compounds that can maintain the ability of antioxidants in the body to decrease and delay skin damage that is caused by repeated UVB exposure.

Aquilaria crassna (*A. crassna*) is an important herbal plant in Thailand used in many traditional medicines. Previous studies have reported on the pharmacological effects of this plant such as its anti-oxidant activity, anti-inflammation activity, being an anti-diabetic and anti-pyretic. In this study we researched the possible effects of the antioxidant properties of *A. crassna* leaf extract on DMBA/ UVB induced skin damage. We compared the efficacy of *A. crassna* leaf extract with the pure compound MGF. All groups were subjected to repeated UVB exposure for 16 weeks

For the biological activity tests, the DPPH free radicals scavenging of *A. crassna* leaf extract was compared with L-ascorbic acid. The result showed the IC50 value of *A. crassna* leaf extract was 0.030 ± 0.46 mg/ml and L-ascorbic acid had an IC50 value of 0.007 ± 0.10 mg/ml.

An animal model was applied in an in vivo study to determine the preventive effects of the consumption of *A. crassna* leaf extract and MGF as a comparative study. Repeated UVB exposure to ICR mice was observed to compare the effect of each on epidermal thickness and the level of 4-HNE and COX-2 expression. In the study, repeated UVB exposure caused damage to dermal connective tissue, a situation

known as photodamage which may manifest as Epidermal hyperplasia or epidermal thickness increase. After repeated exposure to UVB, free radicals such as ROS were accumulated. The skin cells, especially the keratinocytes, responded to ROS by secreting inflammatory mediators leading to the over-proliferation of keratinocyte resulting in skin inflammation. The epidermal thickness of all mice in the various groups was measured after 16 weeks. The dorsal skin tissue was first stained with Hematoxylin and Eosin before measurement using the AXIO program. Significant increases were observed in the epidermal thickness in the Control Group, and in Test Group 1, Test Group 2, and Test Group 3. These increases in epidermal thickness in these groups were all statistically significant when compared to the Normal Group. As well, the level of 4-HNE and the level of COX-2 expression were shown to significantly increase in the Control group.

The results of Test Group 2 which received *A. crassna* leaf extract were that the extract was ineffective in preventing skin epidermal thickness increases and was also ineffective in reducing the level of COX-2 expression. It did, however, reduce the level of 4-HNE expression caused by repeated UVB exposure. Furthermore, Test Group 3, which received MGF, showed that MGF can reduce both skin epidermal thickness and the level of 4-HNE expression and COX-2 expression.

The lipid peroxidation end product, 4-HNE, which is a reactive aldehyde that can form an adduct to DNA and proteins, was observed for any increase after exposure to UVB radiation for 16 weeks. It is known that such adducts lead to alterations in the functions of DNA and proteins, such as a mismatching of, or frame shift in, DNA replication which causes various diseases as well as abnormal intracellular signal transduction.

In the comparison between the Control Group and the Normal Group, the fluorescence intensity of staining of 4-HNE products was significantly higher in the Control Group which was treated with DMBA and UVB–irradiated, whereas the Normal Group had not. The Control Group showed increased oxidative stress which was induced by ROS. There was also an increase in lipid peroxidation in the dorsal skin in the Control Group. The consumption of *A. crassna* leaf extract in Test Group 2 and the MGF in Test Group 3 both showed significantly reduced 4-HNE in the DMBA/UVB-irradiated mice skin when compared with the Control Group.

Cyclooxygenase-2 (COX-2) is an enzyme that causes inflammation of the cells by catalyzing arachidonic acid to form prostaglandin E2 (PGE2), which causes the inflammatory response of the cells. This process is promoted by repeated UVB exposure. This was demonstrated in our research when the level of COX-2 expression in the dorsal skin of the mice in the Control Group which had been treated with DMBA/UVB irradiated mice, was significantly higher than in all the other groups.

Our results also showed that the application of *A. crassna* leaf extract cannot reduce the level of COX-2 expression. Test Group 2, which had received, *A. crassna* leaf extract, showed no reduction in the level of COX-2. Test Group 3, however, which had received MGF, had a significantly reduced level of COX-2 expression as compared to the Control Group and Test Group 2, indicating the effectiveness of MGF in reducing COX-2.

It can be concluded, from the results of this study, that *A. crassna* leaf extract is not effective in preventing DMBA/UVB irradiated induced skin damage, but pure compound MGF is effective by significantly decreasing skin epidermal thickness, and reducing the level of 4-HNE and the level of COX-2 expression. The important mechanism of MGF is its antioxidant capacity is its ability to directly scavenge ROS, and also its ability to regulate the antioxidant gene expression of Nrf2/ ARE pathway to increase other antioxidants such as Heam oxygenase, superoxide reductase, and glutathione. Our results are in agreement with previous studies that have reported the ability of MGF to reduce highly reactive radicals

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