Biological Activities of Gel Containing *Kaempferia marginata* Extract

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ ฤทธิ์ทางชีวภาพของเจลที่มีส่วนผสมของสารสกัดเปราะป่า

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บทคัดย่อ

เปราะป่ามีชื่อทางวิทยาศาสตร์ว่า *Kaempferia marginata* Carey เป็นพืชสมุนไพรที่ใช้ดั้งเดิมในการรักษาการอักเสบ สารสกัดชั้นเอทานอลของเปราะป่า มีคุณสมบัติต้านการอักเสบ งานวิจัยครั้งนี้จึงศึกษาฤทธิ์ทางชีวภาพที่เกี่ยวข้องกับฤทธิ์ต้านการอักเสบ ฤทธิ์สมานแผล และฤทธิ์ต้านอนุมูลอิสระ และนำสารสกัดเปราะป่าพัฒนาเป็นตำรับในรูปแบบเจลที่มีความคงตัวทางกายภาพ ทางเคมี และทางชีวภาพ การทดสอบคุณสมบัติทางชีวภาพของฤทธิ์ต้านการอักเสบเป็นการตรวจสอบการต้านการผลิตไนตริกออกไซด์ที่หลั่งออกมาเนื่องจากการอักเสบ ฤทธิ์การสมานแผลเป็นการตรวจสอบการเพิ่มขึ้นของจำนวนเซลล์ที่มีชีวิตและการเคลื่อนที่ของเซลล์เพื่อปิดบาดแผล และฤทธิ์ต้านอนุมูลอิสระเป็นวิธีการวิเคราะห์ความสามารถในการเป็นสารต้านออกซิเดชันซึ่งใช้รีเอเจนต์คือ 2,2-diphenyl-1-picrylhydrazyl (DPPH) และตรวจสอบการรอดชีวิตของเซลล์จากการเหนี่ยวนำการตายของเซลล์ด้วย hydrogen peroxide (H_2O_2) จากการศึกษาฤทธิ์ต้านการอักเสบพบว่า ตำรับเจลจากสารสกัดเปราะป่า 10% w/w แสดงฤทธิ์ที่ดีที่สุดทั้งก่อนและหลังสภาวะเร่งที่ ค่า IC_{50} เท่ากับ 12.50 (ก่อน) และ 12.83 µg/ml (หลัง) (เจล ไดโคลฟีแนก, ค่า IC_{50} เท่ากับ 64.90 µg/ml) สำหรับฤทธิ์สมานแผลพบว่า ตำรับเจลจากสารสกัดเปราะป่า 5% w/w แสดงฤทธิ์การเพิ่มขึ้นของจำนวนเซลล์ที่มีชีวิตมากที่สุดทั้งก่อนและหลังสภาวะเร่งที่ % การอยู่รอดของเซลล์ เท่ากับ 134.05 (ก่อน) และ 134.65% (หลัง) (เจลว่านหางจระเข้, % การอยู่รอดของเซลล์เท่ากับ 92.64%) และทำให้เกิดการเคลื่อนที่ของเซลล์เพื่อปิดบาดแผลมากที่สุดเช่นกันทั้งก่อนและหลังสภาวะเร่งที่ % การเคลื่อนที่ของเซลล์ เท่ากับ 85.22 (ก่อน) และ 85.71% (หลัง) (เจลว่านหางจระเข้, % การเคลื่อนที่ของเซลล์ เท่ากับ 72.64%) นอกจากนี้ยังแสดงฤทธิ์การต้านอนุมูลอิสระจากการตรวจสอบการรอดชีวิตของเซลล์จากการเหนี่ยวนำเซลล์ตายด้วย H_2O_2 พบว่าตำรับเจลจากสารสกัดเปราะป่า 10% w/w แสดงฤทธิ์ที่ดีที่สุดทั้งก่อนและหลังสภาวะเร่งที่ % การอยู่รอดของเซลล์

เท่ากับ 88.66 (ก่อน) และ 87.94% (หลัง) (วิตามิน C และวิตามิน E, % การอยู่รอดของเซลล์ เท่ากับ 68.45 และ 78.12%) แต่อย่างไรก็ตามตำรับเจลจากสารสกัดเปราะป่าไม่มีความสามารถในการเป็นสารต้าน DDPH ตำรับเจลจากสารสกัดเปราะป่ามีความคงตัวทางเคมีที่ดีอีกด้วย โดยวัดจากการวิเคราะห์ด้วย HPLC พบว่า % desmethoxyyangonin ทั้งก่อนและหลังสภาวะเร่งของ 2.5% w/w เจล เท่ากับ 75.1 และ 78.7% และ 5.0% w/w เจล เท่ากับ 77.1 และ 81.3% ในขณะที่ 10.0% w/w เจล (77.7 และ 84.2 %) มีความแตกต่างกันเล็กน้อย ตำรับเจลจากสารสกัดยังมีความคงตัวทางกายภาพที่ดีเช่นกัน โดยประเมินจากสี กลิ่น และลักษณะภายนอกทั้งก่อนและหลังสภาวะเร่ง ไม่มีความแตกต่างกัน ค่า pH ของ 2.5% เจล (5.63 และ 5.72), 5.0% เจล (5.41 และ 5.91) และ 10.0% เจล (6.42 และ 6.78) และค่าความหนืดของ 2.5% เจล (7.05 และ 7.51 $\times 10^3$cP), 5.0% เจล (6.03 และ 6.43 $\times 10^3$cP) และ 10.0% เจล (6.07 และ 7.06 $\times 10^3$cP) ก็ไม่มีความแตกต่างด้วยเช่นกัน ดังนั้นจากการวิจัยการศึกษาครั้งนี้ตำรับเจลจากสารสกัดเปราะป่ามีคุณสมบัติทางชีวภาพที่ดี และมีความคงตัวทั้งทางกายภาพ ทางเคมี และทางชีวภาพ คุณสมบัติทางชีวภาพของพืชนี้สามารถช่วยสนับสนุนการใช้พื้นบ้านแต่ดั้งเดิมและได้ผลิตภัณฑ์ทางยาใหม่ที่มีความคงตัว

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ABSTRACT

Kaempferia marginata Carey has been traditionally used in the treatment of inflammation. The ethanol extract exhibited the potent anti-inflammatory properties, therefore this study aimed to investigate biological activities on anti-inflammatory, wound healing and anti-oxidant activities. The extract was developed as gel formulations with the physical, chemical and biological stabilities. The anti-inflammatory activity was tested on anti-nitric oxide (NO) production, the wound healing activities were tested on cell proliferation and migration whereas, anti-oxidant activities were tested on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and hydrogen peroxide (H_2O_2)-induced oxidative stress. The anti-inflammatory activity of gel containing *K. marginata* of 10% w/w showed the highest activity with an IC_{50} value of 12.50 (before) and 12.83 µg/ml (after) (Diclofenac gel, IC_{50} value = 64.90 µg/ml). The wound healing activities of 5% w/w gel showed the highest % cell viability at 134.05 (before) and 134.65% (after) (*Aloe vera* gel, % cell viability = 92.64%) and the highest % cell migration was at 85.22 (before) and 85.71% (after) (*Aloe vera* gel, % cell migration = 72.64%). Moreover, the anti-oxidant activity on H_2O_2-induced oxidative stress activity assay of 10% w/w gel showed the highest % cell viability at 88.66 (before) and 87.94% (after) (vitamin C and vitamin E, % cell viability = 68.45 and 78.12%). However, the study on DPPH radical scavenging assay of samples were inactive. *K. marginata* gels showed good chemical stabilities which were evaluated by HPLC analysis. The samples were indicated that % desmethoxyyangonin both before and after accelerating conditions of 2.5% w/w were 75.1 and 78.7% and 5.0% w/w were 77.1 and 81.3%

whereas at 10.0% w/w (77.7% and 84.8%) was slightly different. *K. marginata* gels also exhibited the good physical stabilities. The evaluations (color, odor and texture) both before and after accelerating conditions of samples were not different. The pH values of 2.5% w/w were 5.63 and 5.72, 5.0% w/w were 5.41 and 5.91 and 10.0% w/w were 6.42 and 6.78. The viscosity (10^3cP) values of 2.5% w/w were 7.05 and 7.51 $\times 10^3$cP, 5.0% w/w were 6.03 and 6.43 $\times 10^3$cP and 10.0% w/w were 6.07 and 7.06 $\times 10^3$cP which also were not different.

Gel containing *K. marginata* extracts have good anti-inflammatory, wound healing and anti-oxidant properties *in vitro* and showed physical, chemical and biological stabilities after accelerating conditions. The biological properties from this plant could support its traditional uses and obtain a new pharmaceutical product that has physical, chemical and biological stabilities.

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Most importantly, I would like to thank my parents for their understanding, encouragement, love and support throughout my life. I could not have done any of this without them.

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LIST OF ABBREVIATION AND SYMBOLS

°C	degree celsius
cP	centipoise
cm, nm	centimeter, nanometer
COX	cyclooxygenase
DMEM	Dulbecco's modified eagle medium
FBS	fetal bovine serum
g, mg, kg	gram, milligram, kilogram
h	hour
HDF	human dermal fibroblast cells
IC50	50% inhibitory concentration
LPS	lipopolysaccharide
MIC	minimum inhibitory concentrations
min	minute
ml	milliliter
mM	millimolar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NO	nitric oxide
PBS	phosphate buffered saline
pH	potential of hydrogen (-log hydrogen concentration)
RPMI	Roswell park memorial institute
S.E.M.	standard error of the mean
TNF-α	tumor necrosis factor-alpha
w/w	weight by weight
µg	microgram
µl	microliter
%	percent

CHAPTER 1

INTRODUCTION

1. Background and Rationale

Inflammation is a complex biological response of body tissues to harmful stimuli, such as damaged cells, pathogens and irritants or free radicals. It is a protective response involving molecular mediators, blood vessels and immune cells. Inflammation is classified as acute or chronic. Acute inflammation is achieved by the increased movement of leukocytes and plasma and from the blood into the injured tissues that is initial response of the body to harmful stimuli (Harty et al., 2003). Events of biochemical series mature and proliferate the inflammatory reaction, relate to system of topical vascular and the immune, and diverse cells inside injured cell. Chronic inflammation, bring to a cumulative shift in the type of cells show at the site of inflammation, such as mononuclear cells, and is characterized by simultaneous disruption and healing of the inflammatory cell; inflammatory process is the first step of the wound healing process (Gilroy and De Maeyer, 2015). The inflammatory function is to eradicate the preliminary cause of cell damage; to clear necrotic cells away and tissues hazard from the primitive insult and the process of inflammation; to institute tissue restore or wound healing in the step of cell proliferation and migration; and finally, to promote the remodeling process (Ferrero et al., 2007).

Modern medication used to reduce inflammation called NSAIDs (Non-Steroidal Anti-Inflammatory Drugs). NSAIDs may cause side effects and complications, particularly in the central nervous system and gastrointestinal tract.

This will cause nausea, vomiting, abdominal pain, headache, dizziness and potentially with other systems (Wolfe et al., 1999; Singh et al., 1996; Hoppmann et al., 1991).

K. marginata Carey is a herb of the Zingiberaceae family that has been used as an alternative anti-inflammatory agent. It is a genus with approximately 60 species, widely distributed in Myanmar, India, China, Laos, Thailand and Cambodia. In Thailand, the leaves and root have been used by native people for curries and this plant is used as a medicine. Thailand is the wealthy biodiversity. There are more than 20 species in this area. Bioactive components isolated from various *Kaempferia* species are cinnamate derivatives, cyclohexane oxides, chalcones, monoterpenes, flavonoids and diterpenes (Thongnest et al., 2005). The tuber is locally used as a medicine and for toothache relief. Its rhizomes have been used in the treatment of allergies, swollen leg and fever, and they have been found to relieve inflammation occurring as a result of the insects bites (Chuakul and Boonpleng, 2003; Saensouk and Jenjittikul, 2001). A introductory of biological assay on anti-malarial parasite (*Plasmodium falciparum*) from dichloromethane of *K. marginata* extract showed the IC_{50} value at 26.4 µg/ml (Desjardins et al., 1979). The antimycobacterial activity (anti-TB) on microplate alamar blue assay showed minimum inhibitory concentrations (MIC) at 0.0023 µg/ml which inhibited *Mycobacterium tuberculosis* H37Ra. Quantitative measurement *in vitro* was using by the microculture radioisotope technique and the standard drugs are rifampicin and isoniazid (kanamycin used as positive controls). The antifungal activity on tetrazolium/formazan assays showed IC_{50} value at 0.068-0.092 µg/ml which inhibited *Candida albicans* (ATCC 90028). The ethanol extract of *K. marginata* rhizomes

showed a potent inhibitory effect against lipopolysaccharide (LPS)-induced NO and tumor necrosis factor-alpha (TNF-α) release in RAW264.7 cells (Kaewkroek et al., 2013). NO is an important cellular signaling molecule that is a powerful vasodilator such as immunoregulatory and inflammatory processes. Chemical constituents of *K. marginata* rhizomes are groups of diterpenes, kavalactone and curcuminoids. Bisdemethoxycurcumin has been found to show wound healing activities (Thongnest et al., 2005).

2. Objectives

1. To investigate anti-inflammatory, anti-oxidant and wound healing activities of *K. marginata* extract *in vitro*
2. To determine anti-inflammatory effect of a gel preparation of *K. marginata*
3. To evaluate the physical, chemical and biological stabilities of *K. marginata* gel

3. Literatures review

3.1 Botanical characteristic of *K. marginata*

K. marginata is the herb of Zingiberaceae family (Figure 1) which has the common name as peacock ginger and the local name is proh-paa or tup mup. This is a low-growing, deciduous herbaceous perennial with underground rhizomes. It is closely related to galangal and it is also grown for the use as a spice. Leaves are green to purplish and lie very nearly flat on the ground. The roll at the margin of the leaf gives it more rigidity than the leaves of most *Kaempferia* species. For the flower, only lives less than a day, in the center of flowers are white with a purple spot, but the plant keeps producing orchid-like flowers for weeks during the growing season, in shade it grows well like other *Kaempferia* species (สุดารัตน์ หอมหวล, 2556; กรมส่งเสริมการเกษตร, 2556).

Figure 1. *K. marginata* Carey

The tuber is locally used as a medicine and it relieves toothache. Allergy, fever, and swollen leg were treated by its roots (Chuakul and Boonpleng, 2003; Ferrero et al., 2007). Chemical constiuents that have been recorded including cyclohexane oxides,

chalcones, diterpenes, cinnamates, monoterpenes and flavonoids. The compounds isolated from *K. marginata, K. paviflora* and *K. galanga* are shown in Table 1.

Table 1. The compounds isolated from *K. marginata, K. paviflora and K. galanga*

Plant species	Compounds	References
1. *K. marginata*	pimarane diterpenes (Sandaracopimaradien-7-one, sandaracopimaradien-1α-2α-diol, 2α-acetoxysandaracopimaradien-1α-ol, *ent*-sandaracopimaradien-2-ol, marginatol 1α-acetoxysandaracopimaradien-2α-ol) kavalactone (desmethoxyyangonin) steroids (β-sitosterol, sitosterol-β-D-glucoside) diarylheptanoid (bisdemethoxycurcumin)	Abas et al., 2005 Kaewkroek et al., 2013 Kaewkroek et al., 2017 Thongnest et al., 2005
2. *K. parviflora*	5,7-dimethoxyflavone, α-copaene, dauca-5, 8-diene, camphene, β-pinene, borneol, linalool, limonene, linalool, α-pinene, caryophyllene oxide, longiborneol acetate	Pitakpawasutthi et al., 2018 Rujjanawate et al., 2005 Tuntiyasawasdikul et al., 2014
3. *K. galanga*	Pentadecane, ethyl-trans p-methoxycinnamate, 1,8-cineole, g-carene and borneole, kaempferol, cinnamaldehyde, kaempferide, terpene, methoxycinnamic acid, camphene, ethyl cinnamate, cyclohexane oxide-derivatives ,diterpenes	Munda et al., 2018 Sahoo et al., 2014 Tewtrakul et al., 2008

3.2 The description from flora of China of *K. marginata* is shown in Table 2.

Table 2. Description of *K. marginata* (Chen et al., 1984)

Plant parts	Descriptions
1. Rhizomes	white (non-toxic) or yellow (toxic) inside, tuberous
2. Leaves	spreading flat on ground, ligule triangular; sessile; adaxially green , leaf blade purple-brown, abaxially purple, or rarely green or green variegated purple, suborbicular, 8-11 × 6-9 cm, base rounded or cordate, margin thickened.
3. Flowers	enclosed by 2 leaf sheaths at terminal of inflorescences on pseudostems, lanceolate, ca. 3.8 cm, 15-30-flowered; bracts greenish white. Flowers lightly purple. Lateral staminodes white, suborbicular. Calyx ca. 2.5 cm, apex 2-cleft. Corolla tube ca. 4 cm; lobes linear, ca. 2.5 cm, central one hooked at apex. Filament ca. 1 mm; anther ca. 4 mm; connective appendage oblong, 2-cleft. Labellum white with longitudinal, at center with purple bands, oblate, 1.2-1.8 × 2-2.8 cm, apically 2-cleft to base; lobes suborbicular.

3.3 Inflammation

Inflammation is a part of innate immunity. Inflammation can occur from many causes and one of that is infection, even when an infection causes inflammation. Infection is caused by virus, fungus or a bacterium, inflammation is

like the body's response. The body's attempt at self-protection, there is a biological response; the aim being to remove harmful stimuli, including, irritants or pathogens, damaged cells and begin the healing process (Wichmann et al., 2014). The signs and symptoms of inflammation, specifically acute inflammation, show that the body is trying to heal itself. If the inflammatory process does not occur, the body cannot get rid of the foreign matter. Inflammatory reaction is formed to the existing inflammation which leads to chronic inflammation that destroys the tissue causing the abnormal tissue function (Harty et al., 2003). A series of matures the inflammatory response and biochemical events propagates, involving the local vascular system, various cells within the immune system and the injured tissue. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells, and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Gilroy and De Maeyer, 2015) (Figure 2).

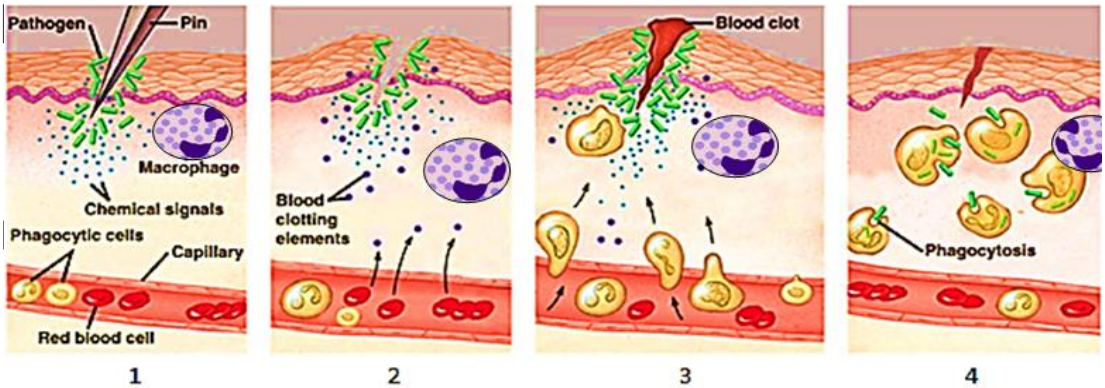

Figure 2. Inflammatory process (Gilroy and De Maeyer, 2015)

3.3.1 The processes of inflammatory events are shown in Table 3.

Table 3. A series of the process of inflammation (Olivera et al., 2012; Reinehr et al., 2006)

The inflammatory processes	Function
1. Vasodilation	brings to more blood flow into the inflammatory area, resulting in heat and redness.
2. Vascular permeability	endothelials become "leaky" from either direct injury of endothelial cell or by chemical mediators.
3. Exudation	white blood cells red blood cells, proteins and fluid evade from the intravascular space due to increased hydrostatic pressure intravascular and increased osmotic pressure extravascular.
4. Vascular stasis	fluid exudation and vasodilation of blood in the bloodstream to let inflammatory cells to maintain and chemical mediators and respond to the stimulus.

3.3.2 Series of processes of inflammatory events are affected by mediators are shown in Table 4.

Table 4. Series of events in the inflammatory process affected by mediators (Rozanski et al., 2011; Fougère et al., 2016; Van Faassen et al., 2009)

Mediators of inflammation	Function
1. Selectins	Endothelium (E-selectin, P-selectin) and molecules on leukocytes (L-selectin) perform as receptors to offer loose binding for rolling.
2. ICAM-1	Intercellular adhesion molecule 1 offer more firm adhesion of the neutrophil, by integrins on neutrophil surfaces, to the endothelium.
3. CD31	The cell to cell adhesion molecule assist in diapedesis.
4. C5a and LTB4	Chemotaxis is assisted by the C5a component from complement activation, together with leukotriene B4, the product from metabolism of the lipo-oxygenase pathway of arachidonic acid.
5. C3b and IgG	Coat foreign objects as bacteria to assist in phagocytosis by binding to leukocyte receptors. opsonins as the C3b component from complement activation, as well as immunoglobulin G.
6. Myeloperoxidase, lysozyme	Generation of toxic oxygen species (superoxide) converted to hydrogen peroxide and then changed to a hypochlorous radical by myeloperoxidase from neutrophil granules after engulfment, killing of bacteria occurs. In the absence of oxidation, lysozyme from neutrophil granules can build holes in microbial membranes.

Table 4. continued

Mediators of inflammation	Function
7. Histamine	Histamine increases the permeability of the capillaries to some proteins and white blood cells, to allow them to engage pathogens in the infected tissues.
8. Serotonin	Serotonin is stored by blood platelets which release the chemical when they bind to a clot, in order to promote blood clotting.
9. Leukotrienes	Leukotrienes can stimulate proinflammatory activities such as endothelial cell adherence and chemokine production by mast cells. As well as mediating inflammation, they induce asthma and other inflammatory disorders, thereby reducing the airflow to the alveoli.
10. Platelet activating factor	PAF is a lipid mediator that is well-known for its ability to cause allergic response, platelet aggregation, inflammation. This potent signaling compound is secreted from tumors, as well as from cells derived from carcinomas.
11. Cytokines	Cytokines are a large group of peptides or glycoproteins, proteins that are secreted by specific cells of immune system. Cytokines are a category of signaling molecules that mediate and regulate immunity, hematopoiesis and inflammation.
12. Nitric oxide	Nitric oxide dilates blood vessels, lowering blood pressure, establishing blood supply. Conversely, it protect tissues from damage by reason of low blood supply. Also a neurotransmitter, nitric oxide acts in the nitrergic neurons active on smooth muscle, abundant in erectile tissue and the gastrointestinal tract, therefore resulting increasing blood flow and in vasodilation.

3.3.3 Acute inflammation

A few seconds or minutes after tissue is injury can cause acute inflammation. The derogation could be caused by an immune response or could be a physical one. Three major processes occur during and before acute inflammation: Arterioles, small branches of arteries that bring to capillaries that supply blood to the damaged region dilate, resulting in enhanced blood flow (Gharibi et al., 2015; Shimizu et al., 2014). The capillaries become more permeable, so blood and fluid proteins move into interstitial spaces. Some macrophages and neutrophils migrate out of the venules and capillaries (small veins that go from a capillary to a vein) and flow into interstitial spaces (Vaccarino et al., 2009; Kritchevsky et al., 2005). A neutrophil is a type of granulocyte (white blood cell), it is filled with tiny sacs which contain enzymes that digest microorganisms (Zhang et al., 2016). Macrophages are also a type of white blood cells that ingests foreign material. In nature that the human body's defense of first line is neutrophils; they are the major cells that against infections from bacteria (Vasović et al., 2010; Mantovani et al., 2008). When scratch the skin, the area around that scratch becomes red, this is cause the arterioles have dilated and become more permeable and the capillaries have filled up with blood, taking fluid and blood proteins flow into the space between tissues (Garza-Garcia et al., 2010; Singh et al., 1996). Acute inflammation have five cardinal signs include pain which is the injured area, especially when touching by chemicals that trigger nerve endings are released, the area is more sensitive (Stout et al., 2005), redness is caused by filling up with more blood in the capillaries than normal, immobility is no working of function, swelling is the effect for the redness caused by an accumulation

of heat and fluid, in the affected area has more blood which makes it is hot to touch and the loss of function is maybe due to a neurological reflex in response to ache (Rather, 1971; Morrison, 1990; Tracy, 2003) (Table 5).

3.3.4 Chronic inflammation

The chronic inflammation will occur when harmful agent could not be eliminated on acute inflammatory process after several days, months or even years. The chronic inflammation is characterised by the accumulation of macrophages in the injured area. In the body, these cells are normally protective agents with high quantity and could damage the tissues as harmful agents. Therefore, chronic inflammation is almost always get along with tissue destruction (Serhan and Savill, 2005; Lang et al., 2010; Suzuki et al., 2003) (Table 5).

Table 5. Comparison between acute and chronic inflammation

Characteristics	Acute inflammation	Chronic inflammation
Causative agent	Bacterial pathogens, injured tissues	Persistent acute inflammation is responsible to non-degradable pathogens, autoimmune reactions or persistent foreign bodies, viral infection,

Table 5. continued

Characteristics	Acute inflammation	Chronic inflammation
Major cells involved	Neutrophils (primarily), eosinophils and basophils (inflammatory response)	Mononuclear cells (lymphocytes , monocytes, macrophages and plasma cells), fibroblasts
Minor cells involved	Response to parasites, mononuclear cells (macrophages and monocytes) and helminth worms	-
Duration	Few days	Up to many months, or years
Onset	Immediate	Delayed
Outcomes	Resolution, chronic, abscess formation	Necrosis, destruction of tissues

3.3.5 Causes of inflammation (Piira et al ., 2013)

3.3.5.1 Physical: burns, foreign bodies, physical injury, dirt, frostbite, splinters, ionizing radiation and debris, trauma

3.3.5.2 Biological: immune reactions (stress and hypersensitivity) infection by pathogens

3.3.5.3 Chemical: alcohol, chemical irritants and toxins

3.3.5.4 Psychological: excitement and embarrassment

3.4 Wound healing

The proliferative phase is to decrease the wound tissue area by fibroplasia and gathering, designation a viable epithelial barrier to stimulate keratinocytes. This phase is amenable for the enclosing of the wound itself, which includes fibroplasia, reepithelialization and angiogenesis (Guo et al., 2010). In the first 48 hours, the processes start in the microenvironment of the wound and after the incursion can unfold up to the 14^{th} day of the wound. Blood flow is changed by stimulation of vascular remodeling (Horohov et al., 2010; Martin, 1997). Angiogenesis is a coordinated processes, involving rupture, endothelial cellular proliferation and rearrangement of the basal membrane, the recruitment of perivascular cells and association and migration in tubular structures (Jenny et al., 2012; Kim et al., 2010). Angiogenesis is described as necessary for various pathological conditions and physiological, as tumor growth, metastasis and embryogenesis (Ballard-Barbash et al., 2012). The production of collateral veins through two mechanisms of subsequent evolution of the blood vessels: cell division and germination. The resulting vascular plexus is reformed to be distinguished in small and large blood vessels (Meissner et al., 2010). The endothelium is filled with both smooth muscle cells and related cells. The newly formed microvasulature makes it possible to carry oxygen, immune-competent, cells nutrients and fluid to the stroma. Moreover, the active participation of lymphocyte and endothelial cells are involvcd (Brockmann et al., 2017; Mercado et

al., 2012). Remodeling phase, the third healing phase comprises remodeling, that starts two to three weeks after the incursion of that wound and last for one year or more. The main purpose of the remodeling phase is degradation, to reach the maximum tensile strength through rearrangement and resynthesis of the extracellular matrix (Singer et al., 1999). Wound healing in the final phase, the granulation tissue is gradually reformed, a seek to restore the normal tissue structure emerges and forming scar tissue that is less cellular and vascular and that displays a progressive gain in its concentration of collagen fibers. A monolayer of keratinocytes cover the wound, its new stratified epidermis with a subjacent basal lamina is reestablished from the borders of the wound to its inner portion and epidermal migration ceases, this phase is marked by the maturing of the elements with changes in the resolution of the initial inflammation and the extracellular matrix. At this step, subsequent change in its complement and there is a deposition of the matrix. In type III collagen go through synthesis of type I collagen increases and degradation for the closure of the wound. Throughout the remodeling, there is a reduction in fibronectic acid and the hyaluronic, which are released by plasmatic metalloproteinase and cells, and the growing type I collagen expression indicated above is concomitantly processed (Figure 3). Categories of wound healing (Cheon et al., 2002; Clark et al., 1998; Gillitzer et al., 2001) are shown in Table 6.

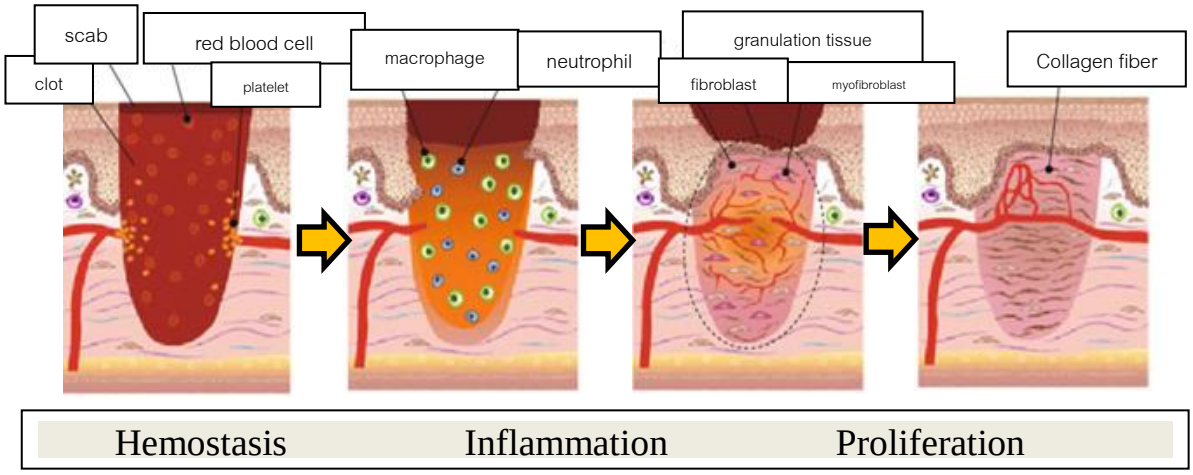

Figure 3. Wound healing processes (Brockmann et al., 2017)

Table 6. Categories of wound healing processes

Sequence of categories	The processes of wound healing
Category 1	Healing or primary wound healing is in hours of repairing a full-thickness surgical wound by first purpose take place. This surgical wound results in the death of cellular constituents that is a minimal number.
Category 2	The insult margins are delayed primary wound healing transpires and not reapproximated immediately, This step of healing could be desired in the case of injured wounds. On day 4, the processes of collagen deposition, maturation and epithelization are taken place and phagocytosis of injured tissues is well underway. Macrophages get rid of foreign matters that may metamorphose into epithelioids
Category 3	Secondary healing is a third type of healing. This step of healing, a full-thickness insult is become to heal and close. Inflammatory reaction in secondary healing is more intense than primary healing.

Table 6. continued

Sequence of categories	The processes of wound healing
Category 3	Moreover, a larger amount of granulomatous tissue is transformed because it has to close the wound. The resemble contractile smooth muscle of fibroblastic differentiation into myofibroblasts is believed to contribute to wound contraction. Secondary healing makes pronounced contraction of wounds. These myofibroblasts are shown in the wound from day 10 to day 21.
Category 4	The step of epithelialization is epithelial cells replicate and migrate by traverse and mitosis the wound. This past of healing which are discussed in sequence of events in the wound healing. In the wounds that are partial thickness, involving only superficial dermis and the epidermis, epithelization is the outstanding method when healing occurs. Contracture of wound is not a normal component of this process if only the superficial dermis and epidermis are involved.

3.5 Free radical and anti-oxidant

Free radicals are groups of atoms or atoms with an odd (unpaired) number of electrons and build when occur interaction of oxygen with certain molecules. High reactive radicals will begin a chain reaction form, like dominoes. Their chief danger comes from the harm when they react with important cellular components such as the cell membrane or DNA (Yu et al., 2005). Preventing the free radical damage is antioxidant that is the body defense system (Cheeseman and Slater,

1993). Antioxidants are molecules with free radicals which can stop the damage of molecules. There are various enzyme systems that destroy free radicals within the body, the principle micronutrient (vitamin) antioxidants are β-carotene, vitamin C and vitamin E. Micronutrients cannot be manufactured by the body, therefore they have to supply in the diet. Vitamin E : d-α tocopherol. A fat soluble vitamin can find in seeds, fish oils, nuts, vegetable, apricots etc. It can have side effects if intake above 2,000 mg. The RDA is 60 mg per day. β-Carotene is a precursor of retinol (vitamin A) and is present in egg yolk, liver, milk, spinach, butter, carrots, squash, yams, tomato, broccoli, peaches, cantaloupe, and grains (Figure 4).

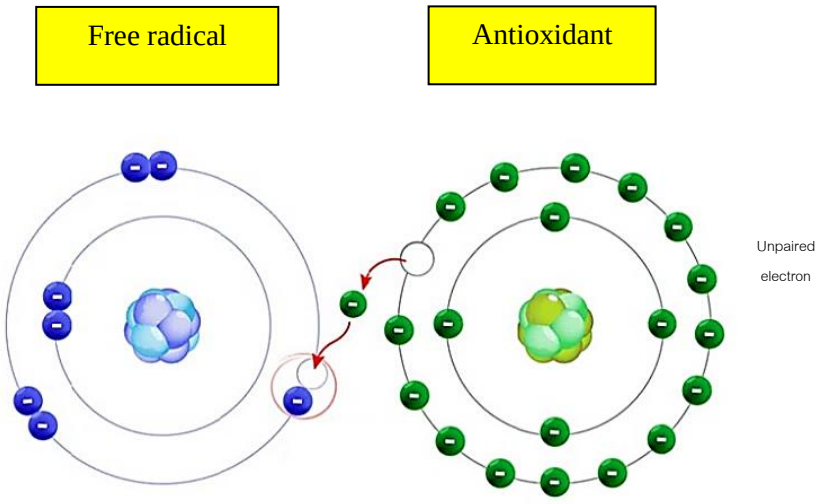

Figure 4. Free radical and antioxidant (Cheeseman and Slater, 1993)

Hydrogen peroxide (H_2O_2) is a pale blue chemical compound, obvious liquid, lightly more condensed than water. It is used like bleaching agent, an oxidizer, and antiseptic with an oxygen-oxygen single bond. High-test peroxide is a reactive oxygen species. H_2O_2 is unstable cause of its instability, H_2O_2 is typically stored in a weakly acidic solution with a stabilizer (Kiecolt-Glaser et al., 1995). It can find in biological systems (the human body). Enzymes that decompose or use H_2O_2 are classified as peroxidases. H_2O_2 is a short-lived product in biochemical processes that formed in human and animals and toxic to cells. The toxicity is due to oxidation of lipids, proteins, DNA and membrane. H_2O_2 is both reducing and an oxidizing agents. The oxidation by sodium hypochlorite yields singlet oxygen. The net reaction of a ferric ion with H_2O_2 is an oxygen and a ferrous ion (Cauley et al., 2007). This proceeds occur hydroxyl radicals and single electron oxidation (Figure 5).

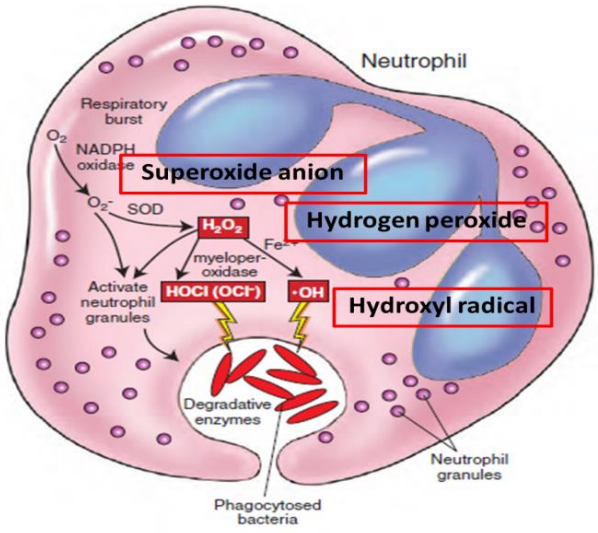

Figure 5. Hydrogen peroxide production (Akhtar et al., 2017)

3.6 Pharmaceutical and natural products containing herbal extracts

3.6.1 Ginger Root

Ginger is used as warming agent and a circulatory tonic. This certified organic formulation supports a healthy inflammatory response in the body and help relief for occasional nausea.

3.6.2 Mushrooms + Herbs Reishi+Turmeric

Reishi and turmeric combines the best-known immune mushroom, Reishi, with turmeric, for a blend of extracts that supports both inflammatory responses and the immune. Reishi and turmeric also contains Shiitake, another immune-supporting mushroom, with fermented ginger root as a warming herb that active as a bioenhancer. Traditionally, Reishi is a mushroom that is used in a several of ways such as heart tonic, body tonic, calming and adaptogen.

3.6.3 Turmeric Supreme Allergy

This herbal formula by turmeric with other herbal extracts to support a healthy response to occasional environmental irritants. Quercetin, a flavonoid was found in many plants, has also been studied for its support of occasional normal histamine response. Black pepper extract is included for bioavailability and increased absorption of curcumins.

3.6.4 Fresh Breath, made with organic peppermint

Fresh Breath is a unique blend of herbs formulated not only to freshen breath, but to support mouth health as well. The pleasant “ginger-mint” taste comes from ginger root extracts, certified organic peppermint leaf. Thyme is included to promote a healthy balance of microbes in the mouth. Prickly Ash bark extract helps support saliva production for long-lasting fresh breath and helps moisten the mouth and adds a tongue “tingle”.

3.6.5 *Aloe vera* gel

Aloe vera has been reported to show antiviral, antifungal and antibacterial activities. It is also rich in compounds which improve energy, improve joint lubrication and mobility. The extracted gel and juice does not contain these and instead have anti-inflammatory compounds which are reduce swelling and pain-killing. One of the most interesting aspects of Aloe are its muco-polysaccharides or MPS (long chain sugars) which are also found in all human body cells. These long-chain sugars help boost the immune system by stimulating macrophage production and producing interleukin and interferon which stops viruses from multiplying and replicating. Giving relief and cooling to hot inflamed skin conditions eg. heal wounds and burns, prevent scarring, sunburn and fire burns. It contains skin regenerative bio-chemistry and cell proliferants which forms new tissue. It equally brings its moisture binding properties to protect the skin from moisture down through all skin layers and moisture loss as well as carrying nutrients, it is naturally non-allergic to all skin types. The whole leaf is also used as a powder to use topically and sometimes internally for

some immune situations. Active components with its properties: *Aloe vera* contains vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acids and amino acids. (American School of Natural Health, 2018).

Aloe vera gel contains glucomannan and a growth hormone (gibberellin) which can react with the fibroblast receptors. The wound healing properties are active on stimulating cell proliferation by increasing collagen synthesis, change collagen composition and increase the collagen cross linked degree. Due to this, it accelerated wound contraction and increased the breaking strength of resulting scar tissue. An increased synthesis of hyaluronic acid and dermatan sulfate in the granulation tissue of a healing wound following oral or topical treatment has been reported. Anti-inflammatory action: *Aloe vera* inhibits the cyclooxygenase pathway and reduces prostaglandin E2 production from arachidonic acid. Recently, the novel anti-inflammatory compound called C-glucosyl chromone was isolated from gel extracts (Surjushe et al., 2008).

3.7 Preformulation and formulation development

Transdermal drug delivery reviewed preformulation aspects for topical and transdermal formulations. This way of delivery show many potential profits compared with the oral path, for example avoidance of fluctuating blood levels, no degradation due to stomach acid and no first-pass metabolism and (Mitragotri and Pankaj, 1997). The transdermal patch has limitation for the barrier function of the skin because polar molecules can not penetrate the stratum corneum. Physicochemical properties of candidate drugs that are necessary in transdermal drug delivery include aqueous solubility, melting point and log P, molecular weight and volume. Furthermore, many substances are weak bases or weak acids, pH will affect their permeation. One way that the carry of zwitterionic drugs through skin is increased by salt formation. Mazzenga who indicated that the rank order of epidermal flux of the salts of phenylalanine across the epidermis was hydrochloride (HCl^-), hydrobromide (HB), phenylalanine (phe) and hydrofluoride (HF^-). Therefore, other delivery paths are worth considering salt choosing issues at the preformulation step to optimize the compound delivery through the skin. The formulations are various forms and they are important factors for selection to transdermal drug delivery including, lotions, ointments, creams and gels. The compound solubility in the vehicle have to determine. Chemical and physical stabilities also have to analyze. For example, it was shown that dithranol displayed a apparent instability in the paraffin base due to light, but was consistent when prevented from light. The degradation in ethanol-water solutions and in a topical cream were very similar in the pH range 2-6. The degradation of compound initiated in compartment phase or an

aqueous phase that was undisturbed by the oily cream excipients. The compound distinguish from oxidation, then an antioxidant may be incorporated (Mitragotri and Pankaj, 2009).

Topical gel formulations are of increasing interest in the dermatology industry. Gel formulations are translucent or typically transparent, water-based semisolids with pleasing aesthetic characteristics and good spreading properties. Gels derive their rheological properties and consistency from polymers that can swell in water and can increase viscosity and thicken the water. Polymers may interact physically, by ionic or chain entanglement, or hydrophilic/hydrophobic interactions. In this case, the polymers form a matrix that can increases the water viscosity and allows for (1) physical stability and protection crystal suspending, (2) preservation the homogenous formulation, (3) well transfer to the skin surface and (4) easy diffusion (Lipp and Müller-Fahrnow, 1999; Shinde et al., 2012).

CHAPTER 2

RESEARCH METHODOLOGY

1. Reagents and Chemicals

Reagents used for anti-inflammatory and wound healing assays were Roswell Park Memorial Institute (RPMI), phosphate buffered saline (PBS), trypsin-EDTA, trypan blue, griess reagent and isopropanol, dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and glutaraldehyde, ethanol, sulfanilamide, phosphoric acid, LPS, penicillin-streptomycin, indomethacin, N-(1-naphthyl) ethylenediamine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Chemicals for gel preparation were paraben concentrate, glycerin, carbopol 940, disodium EDTA, triethanolamine and propylene glycol which purchased from Gibco (Life Technologies, Paisley, Scotland).

2. Plant material and preparation of extract

The rhizomes of *K. marginata* were bought from a local market in Bangkok in May 2010. A voucher specimen was identified by Mrs Pranee Rattanasuwan (SKP 206111301). Three kg dried weight of *K. marginata* were ground and macerated with ethanol at room temperature (4 x 6L) to obtain the EtOH extract (971.5 g, 32.4% w/w).

3. Anti- inflammatory activity assay (Anti-NO production)

Effect of inhibition on NO production by using the murine macrophage-like RAW264.7 cells was estimated by a modified method from

preliminary reporting (Tewtrakul and Subhadhirasakul, 2008). RAW264.7 cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplemented with 0.1% sodium bicarbonate, 10% fetal bovine serum (FBS), streptomycin (100 µg/ml) and 1% penicillin (100 unit/ml). The cells were diluted to a suspension in a medium and harvested with trypsin-EDTA. Seeding the RAW264.7 cells was 1×10^5 cells/well in a 96 well plate and kept at 37°C in a humidified atmosphere containing 5% CO_2 for 1 h. After that the medium was took the place of a medium containing 1 µg/ml of LPS from *Escherichia coli* together with the tested samples at variety of concentrations (3-100 µg/ml) for the tested group and incubated for 24 h. NO production of RAW264.7 cells was evaluated by measuring the amount of nitrite in the culture supernatant using the griess reagent containing equal volumes of 1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphtyl) ethylenediamine solution. The optical density was measured with a microplate reader at 570 nm. COX inhibitor (Diclofenac gel) was used as a positive control.

4. Cell viability test (MTT assay)

Viability or proliferating cells was detected by using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method. This method was reported by Mosmann in 1983. MTT assay desired mitochondrial reductase enzymes from living cells to reduce MTT, a pale yellow substrate to yield a dark blue crystals of formazan product which was largely impermeable to cell membranes. Thus, the quantitative measure cell respiration and the amount of

formazan produced were proportional to the number of living cells present in the culture.

After 24 h incubation with the tested samples, MTT solution (10 µl, 5 mg/ml in phosphate buffer saline; PBS) (Denizot and Lang, 1986) was filled to the wells. Then after 2 h incubation at 37°C in a 5% CO_2 atmosphere, 100 µl of DMSO was added into each well, in the cells crumble the formazan production. The absorbance of this purple solution was detected with a microplate reader at 570 nm. When the optical density of the formazan production of treated cells was compared with untreated control cells, indicated that the tested samples were cytotoxic. Deductions of non-cytotoxic agents were considered at fewer than 80% of that in the untreated control group. % Cell viability = (Absorbance of sample / Absorbance of control) ×100 (Sudsai et al., 2013).

5. Wound healing activity assays

Cell proliferation and cell viability using human dermal fibroblasts (HDF). Seeding the HDF cells was $2x10^4$cells/well in a 96 well plate in DMEM containing 10% FBS. After 48 h incubated at 37°C in a 5% CO2 atmosphere, cells were taken with various concentrations (1-100 µg/ml) of tested samples and were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 48 h. In each well, MTT solution (10 µl, 5 mg/ml) was mixed directly to the medium, and the plate was incubated for 4 h at 37°C. All medium was aspirated and added with isopropanol containing 0.04 N HCl, and the optical density was detected at 570 nm. The percentage of proliferated cells was calculated and compared with the negative control (Sudsai et al., 2013).

Cell migration of HDF cells was examined using a wound healing method. HDF cells ($5x10^4$ cells/ml) in DMEM containing 10% FBS were seeded into each well of 24 well plates and incubated at 37°C with 5% CO_2. After the confluent monolayer of HDF cells was formed, two horizontal scratches (left and right) were generated in each well by using a sterile pipette tip. Cellular debris was defeated by washing with PBS and substituted for 1 ml of medium in the presence of a tested sample. Photographs were taken on day 0, then plates were incubated at 37°C with 5% CO_2 and photographs were taken at day 1 and 2 and 3. To determine the migration of HDF cells, the images were analyzed using image J computing software. Percentage of the closed area was measured and compared with the value obtained before treatment (day 0). An increase of the percentage of closed area indicates the migration of cells (Sudsai et al., 2013).

6. Anti-oxidant activity assays

6.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Plant is analyzed for the amount of free radicals by DPPH assay. Solution of DPPH in ethanol is prepared at concentrations 6×10^{-5} M and prepared stock solution (10 mg/ml) of the sample in DMSO. The concentrations 10, 30 and 100 µg/ml were diluted with ethanol. The reaction mixture contained 100 µl of samples at various concentrations and 100 µl of DPPH in ethanol. The mixture is shaken vigorously and allow to stand at room temperature for 30 min. Then, absorbance is measured at 517 nm by using spectrophotometer (UV-VIS Shimadzu). The commercial known antioxidant, butylated hydroxytoluene (BHT) is used as a positive control. The DPPH solution in the absence of sample is used as a control and

the absolute ethanol is used as a blank. The IC_{50} value of the sample, the DPPH free radical was inhibited 50% by the concentration of sample. Lower absorbance of the reaction mixture show higher free radical activity. The percent DPPH scavenging effect is calculated by using following equation: DPPH scavenging effect (%) or Percent inhibition = A_0 - A_1 / A_0 × 100. Where A_0 is the absorbance of control reaction which is the absorbance of control minus the absorbance of control blank and A_1 is the absorbance in presence of test or standard sample which is absorbance of sample minus the absorbance of sample blank (Li et al., 2013).

6.2 Hydrogen peroxide (H_2O_2) -induced oxidative stress activity assay

H_2O_2 induced oxidative stress was determined in preliminary description (Jitsanong et al., 2011) with a slight modification. Seeding the HDF cells was $2x10^4$cells/well in a 96 well plate in DMEM medium containing 10% FBS and after 24 h, cells were treated with various concentrations (1-30 µg/ml) of test samples. After that treatment with various concentrations for 1 h incubation with 5% CO_2 at 37°C, the HDF cells were co-incubated with 0.9 mM of H_2O_2 for 24 h. and finally, cell viability was detected by the MTT assay.

7. Formulations of *K. marginata* (KM) gels

KM gel (100 g) was prepared using carbopol 940, propylene glycol, glycerin, disodium EDTA, triethanolamine, ethanol, paraben concentrate, KM extract and purified water, respectively (Table 7).

Table 7. The components of *K. marginata* (KM) gel formulation

Chemicals	Gel base (g)	Gel containing KM extract (g)		
		2.5% w/w	5.0% w/w	10.0% w/w
1. Carbopol 940	1.5	1.5	1.5	1.5
2. Propylene glycol	20.0	20.0	20.0	20.0
3. Glycerin	10.0	10.0	10.0	10.0
4. Disodium EDTA	0.5	0.5	0.5	0.5
5. Triethanolamine	2.5	2.5	2.5	2.5
6. Ethanol	0.4	0.4	0.4	0.4
7. Paraben concentrate	0.9	0.9	0.9	0.9
8. KM extract	-	2.5	5.0	10.0
9. Purified water qs. to	100.0	100.0	100.0	100.0

8. Physical, chemical and biological stabilities of gels after formulations and accelerating conditions (Hadgraft et al., 2005)

Macroscopic organoleptic characteristics (color, odor, appearance and texture) were evaluated. The pH was controlled using a potentiometric method. Rheological profile and the apparent viscosity were determined using a Brookfield rotational viscometer. High performance liquid chromatography (HPLC) with UV detection was used for evaluation the chemical stability. The analysis of the active ingredients and biological effects of the gel were also determined.

8.1 Macroscopic analysis of formulation (Al-Suwayeh et al., 2014). The prepared formulation was examined visually for its homogeneity, color, spreadability and consistency. The clarity was determined by using the natural light and all the macroscopic analyses.

8.2 Heating-cooling test (Hadgraft et al., 2005). The gel sample was subjected to a heating-cooling cycle; the test was operated with six cycles for 24 days. Each cycle, the substance remained in a particular temperature for 48 h. The temperature was 4°C (48 h) and 45°C (48 h) in the incubator. For analysis of the resistance to the heating-cooling cycle, each test was done with samples of 30 g each in triplicate.

8.3 Viscosity study (Islam, et al., 2004). The viscosity measurement was operated with a Viscometer. The gel was circulated at 10, 20, 30, 40, 50, and 60 per minute. At each speed, the consistent dial reading was noted.

8.4 HPLC analysis of a marker compound (desmethoxyyangonin). Determination of chemical properties of *K. marginata* gel before and after heating-cooling test by HPLC.

HPLC chromatogram of *K. marginata* extract and gel formulation from *K. marginata* (1-10%) were tested for the presence of a marker compound (desmethoxyyangonin) in the formula. Desmethoxyyangonin was separated using Shimadzu UFLC series (Kyoto, Japan) on a GL Sciences Inc. inertSustain C(18) analytical column (4.6x250 mm, 5 micron) using H_2O : MeOH (40:60) as a mobile phase at a flow rate of 1.0 ml/min (60 min) with detection at 254 nm.

CHAPTER 3

RESULTS AND DISCUSSIONS

1. Biological activity of *K. marginata* gel before and after heating-cooling test

1.1 Anti-inflammatory assay

NO is synthesized and released into the endothelial cells by the help of NOSs that convert arginine into citrulline producing NO in the process. Oxygen and NADPH are necessary co-factors in such conversion. NO is believed to induce vasodilatation in cardiovascular system and furthermore, it involves in immune responses by cytokine-activated macrophages, which release NO in high concentrations and when there is more inflammation, NO will damage cell or surrounding tissue. Anti-inflammatory agents are important to inhibit cell toxicity. For the study, anti-inflammatory activity assay found that before and after accelerating conditions the IC_{50} value of 10% w/w gel formulation showed the highest activity at 12.50 µg/ml. However, at 5.0% and 2.5% w/w showed moderate activity with the IC_{50} values of 27.05 and 35.38 µg/ml, respectively. The IC_{50} value of the gel base showed mild activity at 88.89 µg/ml. The IC_{50} value of Diclofenac gel that is a positive drug was at 64.90 µg/ml. *K. marginata* gel showed IC_{50} values higher than that of the positive control (Table 8). Therefore, gel formulation at high concentrations could inhibit NO production than low concentrations and every gel formulations had effectiveness than a positive drug.

Table 8. Anti-inflammatory activity of *K. marginata* gel before and after heating-cooling test

Samples	IC_{50} (μg/ml)	
	Before	After
Base	88.89±2.30*	88.67±3.40*
KM 2.5%	35.38±0.82*	35.15±2.89*
KM 5.0%	27.05±2.12*	26.77±2.01*
KM 10.0%	12.50±2.91*	12.83±1.09*
KM extract	7.02 ± 1.25*	
Diclofenac gel	64.90 ± 0.63	

*Significant difference from the positive control $p<0.05$
(Mean ± S.E.M. of three determinations)

1.2 Wound healing assay

Wound healing activities were tested on cell proliferation and cell migration of the ethanol extract of *K. marginata* rhizomes in gel form by using HDF cells and comparison the stabilities between before and after accelerating conditions. Cell proliferation was measured amount of cell viability that proliferated after testing by MTT reagent. Cell migration was measured the space of the wound after the cells migrated when treatment with samples and compared treatment of samples between before and after accelerating conditions for evaluation the stability. Cell proliferation is an increase in the number of cells resulting from the normal and healthy cells process by cells growing and dividing. In this regard, cell proliferation can be a good indicator of general cell health. This study was found that % cell viability (mean ±

S.E.M) at 2.5% w/w of before were 70.5-118.2% and after were in the range of 70.8-118.0%. 5% w/w were found to be 86.5-134.0% (before), 86.2-134.6% (after) whereas 10% w/w were in the range of 84.5-131.0% (before) and 86.5-131.0% (after) (Table 9). For a positive drug (99.5% *Aloe vera* gel), the highest % viability was 92.64%. The study of cell proliferation had higher cell viability at low concentrations, especially at 3 µg/ml in 2.5, 5 and 10% w/w and the most at 5% w/w. At high concentrations, cells could not proliferate or cell viability decreased because they were toxic to the cells. Therefore, every gel formulations of the concentrations at 1 and 3 µg/ml had effectiveness than a positive drug that could increase cell viability.

Table 9. Wound healing activity on cell proliferation, % cell viability of *K. marginata* gel before and after heating-cooling test

Samples (µg/ml)	% cell viability							
	Before				After			
	Base	2.5%	5.0%	10.0%	Base	2.5%	5.0%	10.0%
Control	100.00±0.38	100.00±0.88	100.00±0.48	100.00±0.25	100.00±0.40	100.00±0.82	100.00±0.38	100.00±0.31
1	105.10±0.02*	107.02±0.02*	128.05±0.02*	124.05±0.02*	105.63±0.69*	107.05±0.02*	128.34±0.18*	124.72±0.06*
3	112.05±0.22*	118.20±0.22*	134.05±0.32*	131.05±0.12*	112.77±0.01*	118.05±1.25*	134.65±0.34*	131.85±0.22*
10	88.50±0.13*	89.50±0.78*	95.50±0.45	92.50±0.23*	88.53±0.09*	89.76±0.87*	95.34±0.56*	95.50±0.12*
30	82.50±0.43*	72.50±0.13*	90.50±0.53*	87.50±0.17*	82.53±0.09*	72.45±0.22*	90.65±0.84*	88.50±0.13*
100	84.50±0.13*	70.50±0.15*	86.50±0.42*	84.50±0.13*	84.53±0.23*	70.88±0.54*	86.23±0.12*	86.50±0.16*

*Significant difference from the control $p<0.05$

(Mean ± S.E.M. of three determinations)

% Viability (mean ± S.E.M.) of positive control (99.5% *Aloe vera* gel) found at 1-100 µg/ml on Table 10.

Table 10. Wound healing activity on cell proliferation and % viability of 99.5% *Aloe vera* gel

Samples (µg/ml)	% Viability
	99.5% *Aloe vera* gel
Control	100.00±0.52
1	88.58±0.14*
3	89.08±0.36*
10	92.64±0.17*
30	89.79±0.22*
100	89.44±0.11*

*Significant difference from the control $p<0.05$
(Mean ± S.E.M. of three determinations)

For cell migration is a central process in the development and maintenance of multicellular organisms. Tissue formation during embryonic development, wound healing and immune responses all require the orchestrated movement of cells in particular directions to specific locations. Cells often migrate in response to specific external signals, including chemical signals and mechanical signals. Errors during this process have serious consequences, including intellectual disability, vascular disease, tumor formation and metastasis. The study showed that the front of the migration is the site at which the membrane is returned to the cell

surface from internal membrane accumulate at the end of the endocytic cycle. It has led to the hypothesis that extension of the leading edge take place by addition of membrane at the cell front. Thus, the actin filaments that form at the front may stabilize the added membrane in which a structured extension, or lamella, is formed rather than a bubble-like structure (or bleb) at its front. For a cell to move, it is necessary to bring a fresh supply of feet (proteins called integrins, which attach a cell to the surface on which it is crawling) to the front. The study of cell migration was measured the space of cell's moving to close together. It was found that at day 3, % migration (mean ± S.E.M.) of both before and after at 2.5% w/w were 78.5±1.8% and 78.3±1.5%, 5% w/w were 85.2±5.2% and 85.7±5.7%, and 10% w/w were 81.5±2.2% and 83.7 ±2.0%. % Migration (mean ± S.E.M.) of the control both before and after were found to be 50.1 ±1.3% and 50.5 ±1.6%, respectively. For a positive drug (99.5% *Aloe vera* gel), % cell migration ± S.E.M was lower than KM gel 2.5%, 5.0% and 10.0% in all of day 1, 2 and 3; 72.64±2.87, 44.47±1.56 and 29.16±2.15%, respectively (Table 11, Figure 6 and Figure 7). Every concentrations of gel containing *K. marginata* extract showed wound healing activities higher than that of the control group that cells could migrate to close the space or heal the wounds. The highest cell migration was at 5% w/w and when the concentration was higher, it decreased cell movement or cell migration. At low concentrations at 2.5% w/w, cell migration also decreased.

Table 11. Wound healing activity on cell migration (mean of % cell migration and lengths (µm) between the scratch) of *K. marginata* gel before and after heating-cooling test compared with *Aloe vera* gel (commercial product)

Conc.3 µg/ml	Lengths (µm) between the scratch				% Cell migration		
	Day 0	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Before							
Control	1,212.40±4.41	822.00±2.33	661.92±3.16	604.94±2.22	32.21±2.15	45.43±3.27	50.14±1.32
Base	1,295.51±3.36	860.24±4.13	681.45±2.25	619.21±1.13	33.65±3.22	47.42±1.25	52.23±4.47
2.5%	1,256.33±2.78	782.64±2.55	497.46±4.16	270.17±3.25	37.70±2.47*	60.42±2.20*	78.51±1.82*
5.0%	1,228.25±4.15	706.23±3.29	291.02±1.78	181.75±2.55	42.56±3.56*	76.34±2.37*	85.22±5.27*
10.0%	1,284.52±2.14	782.24±2.67	354.52±3.36	237.68±2.67	39.18±1.14*	72.45±3.12*	81.51±2.27*
After							
Control	1,238.63±3.56	836.02±2.74	678.76±1.56	616.83±2.65	32.55±2.24	45.20±3.13	50.28±1.61
Base	1,256.78±3.55	836.94±3.17	658.52±2.99	599.40±2.65	33.42±3.01	47.67±1.55	52.39±4.24
2.5%	1,292.71±2.54	811.89±2.67	515.73±1.57	280.56±1.90	37.28±2.21*	60.14±2.03*	78.30±1.54*
5.0%	1,278.12±4.05	740.07±2.32	300.35±3.65	182.71±2.85	42.10±3.83*	76.52±2.59*	85.71±5.76*
10.0%	1,234.00±3.56	745.38±2.94	307.24±1.56	201.13±5.56	39.63±1.64	75.14±3.52*	83.72±2.01*
Aloe vera gel	1,288.22±1.35	912.55±3.00	715.32±1.80	352.41±2.19	29.16±2.15	44.47±1.56	72.64±2.87

*Significant difference from the control $p<0.05$
(Mean ± S.E.M. of three determinations)

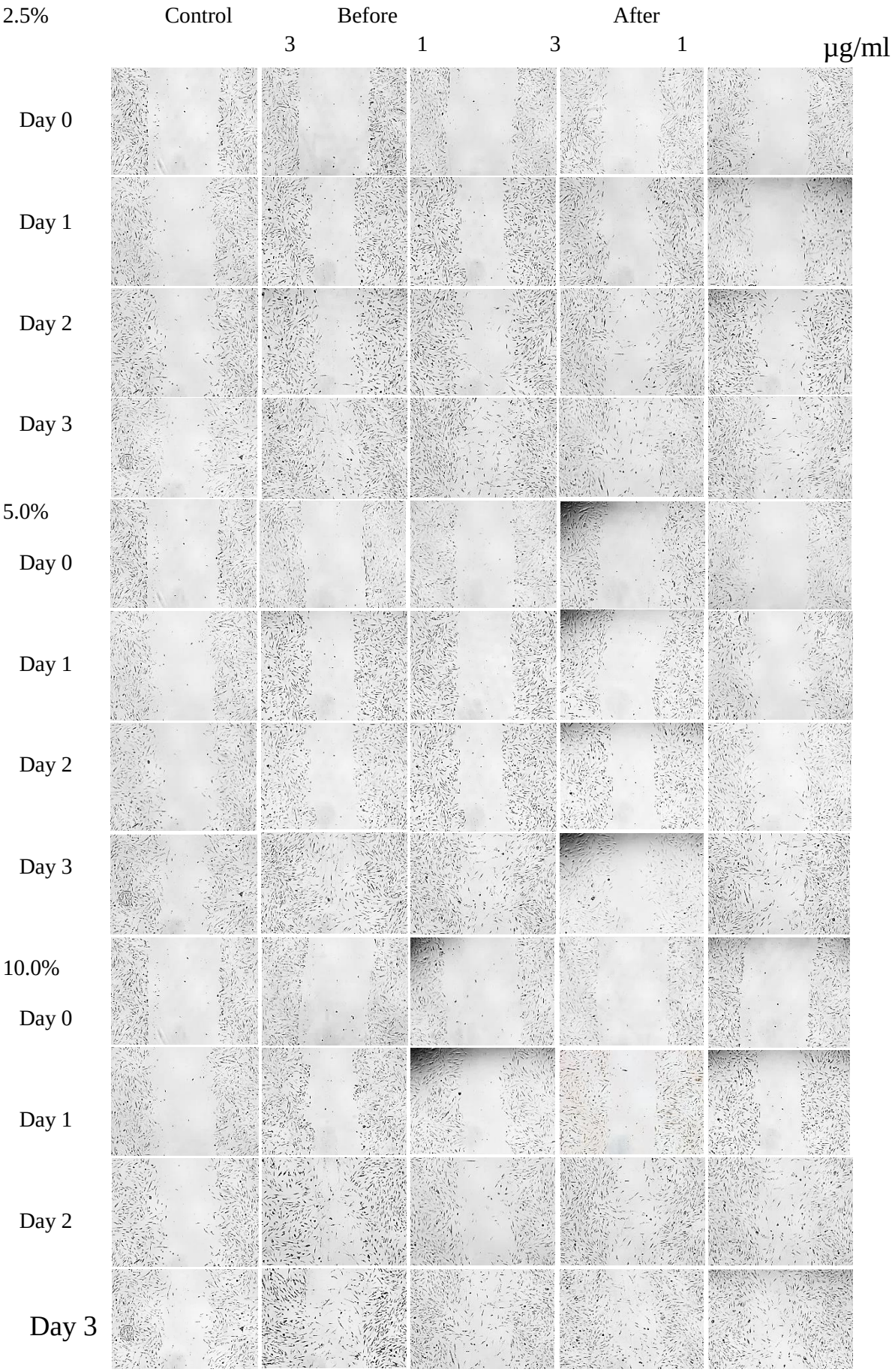

Figure 6. Effect from *K. marginata* gel on HDF cells migration. Images were captured at day 0, 1, 2 and 3 by treating with 2.5%,5.0% and 10% w/w at 3 and 1 µg/ml of samples

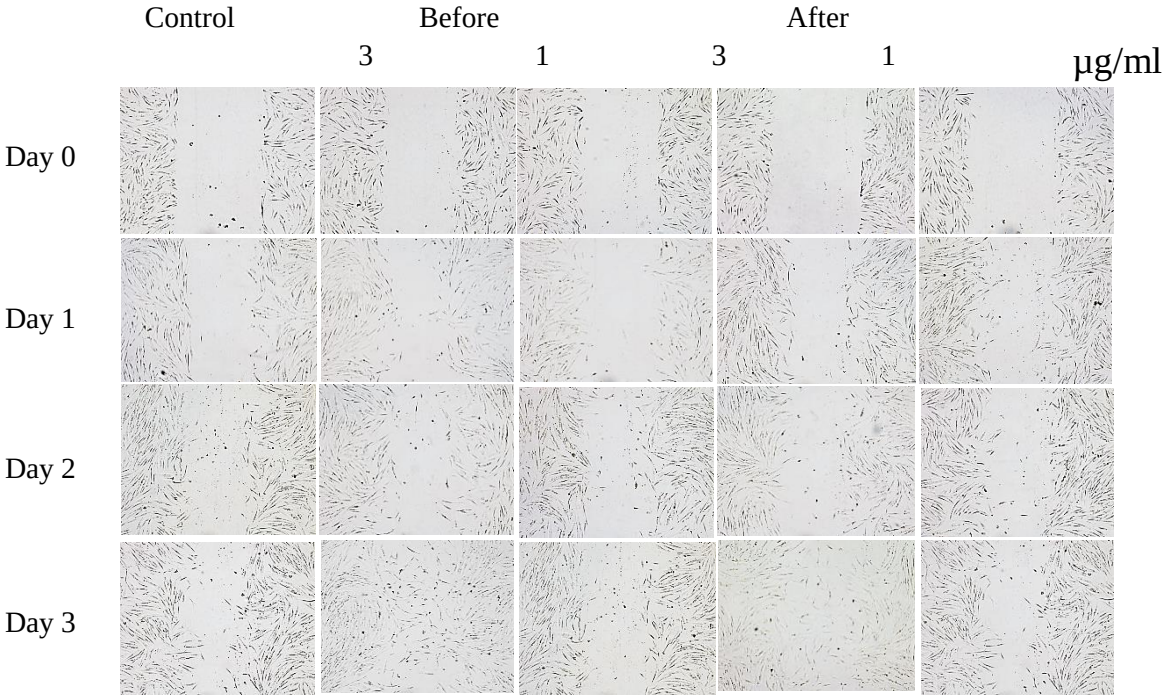

Figure 7. Effect from 99.5% *Aloe vera* gel on HDF cells migration. Images were captured at day 0, 1, 2 and 3 by treating with 2.5%,5.0% and 10% w/w at 3 and 1 µg/ml of samples

1.3 Anti-oxidant activity assays

1.3.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH assay is the method for screening the antioxidant property. The assay is determined on the measurement of the antioxidant effect to scavenge the stable DPPH radical. DPPH is a stable nitrogen-core free radical, it can produces violet color in EtOH solution. DPPH radicals can react with appropriate reducing agent. During the reaction, the electrons become paired off as well as the solution loses color which depending on the number of electrons occupied. In the study, the DPPH radical scavenging activity of KM gel showed IC_{50} values of every concentrations higher than 100 µg/ml both before and after heating-cooling test, positive control (BHT) showed IC_{50} value at 50.91 µg/ml. Whereas KM extract had IC_{50} value at 87.30 µg/ml. Therefore, KM gel in every concentrations has no

inhibitory activity against the DDPH radical both before and after heating-cooling test (Table 12). This may indicate that KM gel could not scavenge DPPH radical which is exogenous oxidant. The antioxidant effect is proportional to the disappearance of DPPH in test samples. DPPH shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by the formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption.

Table 12. An-tioxidant activity of *K. marginata* gel before and after heating-cooling test on DPPH radical scavenging assay

Sample	% inhibition at various concentrations (µg/ml)				IC_{50} (µg/ml)
	0	10	30	100	
Before					
Base	0.00±1.32	-28.52±1.25	-15.22±1.76	-8.13±1.55	>100
2.5%	0.00±1.53	-17.41±0.17	-14.53±2.04	-13.62±0.86	>100
5.0%	0.00±0.71	-28.13±2.76	-15.01±2.12	-5.42±1.70	>100
10.0%	0.00±1.95	-11.50±2.44	-9.87±0.40	5.90±1.65	>100
After					
Base	0.00±2.54	-26.55±1.61	-12.68±2.53	-4.89±1.79	>100
2.5%	0.00±1.63	-14.52±1.45	-14.52±0.22	12.44±0.25	>100
5.0%	0.00±0.16	-29.84±2.48	-16.27±1.51	8.50±2.44	>100
10.0%	0.00±0.89	-10.63±2.00	-7.11±1.22	5.18±1.66	>100
KM extract	0.00±0.32	8.40±0.15	15.61±0.24	58.22±0.36	87.30
BHT (Butylated hydroxytoluene)	0.00±0.56	25.30±1.87	31.91±2.45	66.20±0.89	50.91

(Mean ± S.E.M. of three determinations)

1.3.2 Hydrogen peroxide (H_2O_2)-induced oxidative stress assay

The study was tested on the potent inhibitory effect against H_2O_2-induced oxidative stress of KM gel both before and after accelerating condition to compare the stability. The cells were measured the amount of HDF cell viability after the cells were co-incubated with 0.9 mM of H_2O_2 and detected by MTT assay.

The treatment with 0.9 mM H_2O_2 for 24 h found that it could decrease cell viability from 100% to 28.67±0.78-38.40±0.74% but when treated with samples in every concentrations (1-100 µg/ml) both of before and after heating-cooling test found that they could increase cell viability. Before: for 2.5% KM gel at 1-100 µg/ml increased to 60.42±2.44, 64.30±2.56, 66.42±0.56, 72.57±1.58 and 69.67±1.34%, respectively, for 5.0% KM gel at 1-100 µg/ml increased to 75.13±1.51, 77.33±1.50, 79.21±0.88, 85.65±1.95 and 81.00±1.65%, respectively, for 10.0% KM gel at 1-100 µg/ml increased to 88.66±0.56, 85.44±3.35, 85.52±1.54, 86.71±2.49 and 82.48±1.45%, respectively. After: for 2.5% KM gel at 1-100 µg/ml increased to 60.10±1.22, 65.77±2.75, 64.15±1.55, 71.65±3.44 and 70.08±1.45% respectively, for 5.0% KM gel at 1-100 µg/ml increased to 74.18±2.54, 75.12±2.33, 79.61±1.23, 85.01±2.41 and 81.70±2.24% respectively, for 10.0% KM gel at 1-100 µg/ml increased to 81.45±2.65, 83.32±0.85, 87.94±1.17, 86.26±2.76 and 84.87±1.73%, respectively. The most protective effect of KM gel is 10.0% w/w at 100 µg/ml and has increased cell viability higher than positive controls (vitamin C and vitamin E) in every concentrations. The most protective effect of positive control is vitamin E at 100 µg/ml (78.12±2.44%) (Table 13). This is suggested that KM gel could protect cells from H_2O_2 which is endogenous oxidant.

Table 13. Protective effect of *K. marginata* gel on 0.9 mM H_2O_2-induced HDF cell death, % Viability

Sample	% Viability of HDF cells at various concentrations (µg/ml)						
	Control	H_2O_2	1	3	10	30	100
Before							
Base	100.00±2.26	30.25±0.74	55.71±0.52	56.13±0.50	50.50±0.57	51.21±2.41	50.82±0.61
2.5%	100.00±2.25	31.52±0.77	60.42±2.44	64.30±2.56	66.42±0.56	69.67±1.34	72.57±1.58
5.0%	100.00±2.24	29.88±0.75	75.13±1.51	77.33±1.50	79.21±0.88	81.00±1.65	85.65±1.95
10.0%	100.00±2.25	38.40±0.74	82.48±1.45	85.44±3.35	85.52±1.54	86.71±2.49	88.66±0.56
Vitamin C	100.00±2.26	32.60±0.74	61.33±2.66	65.62±0.56	65.98±1.55	68.45±2.67	20.72±0.90
Vitamin E	100.00±2.20	28.67±0.78	70.42±1.66	72.56±1.64	74.85±2.95	75.83±2.33	78.12±2.44
After							
Base	100.00±2.04	32.55±0.76	56.03±1.43	54.65±1.75	53.60±2.14	53.71±1.12	51.41±2.45
2.5%	100.00±2.06	31.22±0.75	60.10±1.22	65.77±2.75	64.15±1.55	70.08±1.45	71.65±3.44
5.0%	100.00±2.06	29.18±0.71	74.18±2.54	75.12±2.33	79.61±1.23	81.70±2.24	85.01±2.41
10.0%	100.00±2.04	37.21±0.73	81.45±2.65	83.32±0.85	84.87±1.73	86.26±2.76	87.94±1.17
Vitamin C	100.00±2.03	32.10±0.74	61.76±2.57	65.02±2.95	65.18±2.66	68.99±1.22	18.66±2.11
Vitamin E	100.00±2.11	29.44±0.78	70.98±2.45	70.53±2.60	75.90±2.15	76.45±1.60	77.22±0.69

*Significant difference between before and after $p<0.05$
(Mean ± S.E.M. of three determinations)

2. Chemical stability of active ingredient of *K. marginata* gel before and after heating- cooling test by analysis with HPLC (wavelength 254 nm).

It was found that both before and after accelerating conditions exhibited similar % desmethoxyyangonin in both of 2.5% w/w (75.1 and 78.7%) and 5% w/w (77.1 and 81.3%); whereas at 10% w/w of after accelerating conditions slightly exhibited higher % desmethoxyyagonin (84.8%) than before (77.7%) the heating-cooling test (Table 14 and Figure 8).

Table 14. Chemical stability of active ingredient, % Desmethoxyyangonin of *K. marginata* gel before and after heating-cooling test by analysis with HPLC (wavelength 254 nm)

Samples	% Desmethoxyyangonin before and after heating-cooling test	
	Before	After
Formula		
2.5%	75.17±1.03	78.78±0.25
5.0%	77.14±1.20	81.30±0.23
10.0%	77.71±0.86	84.80±1.84*

*Significant difference between before and after $p<0.05$

(Mean ± S.E.M. of three determinations)

Before **After**

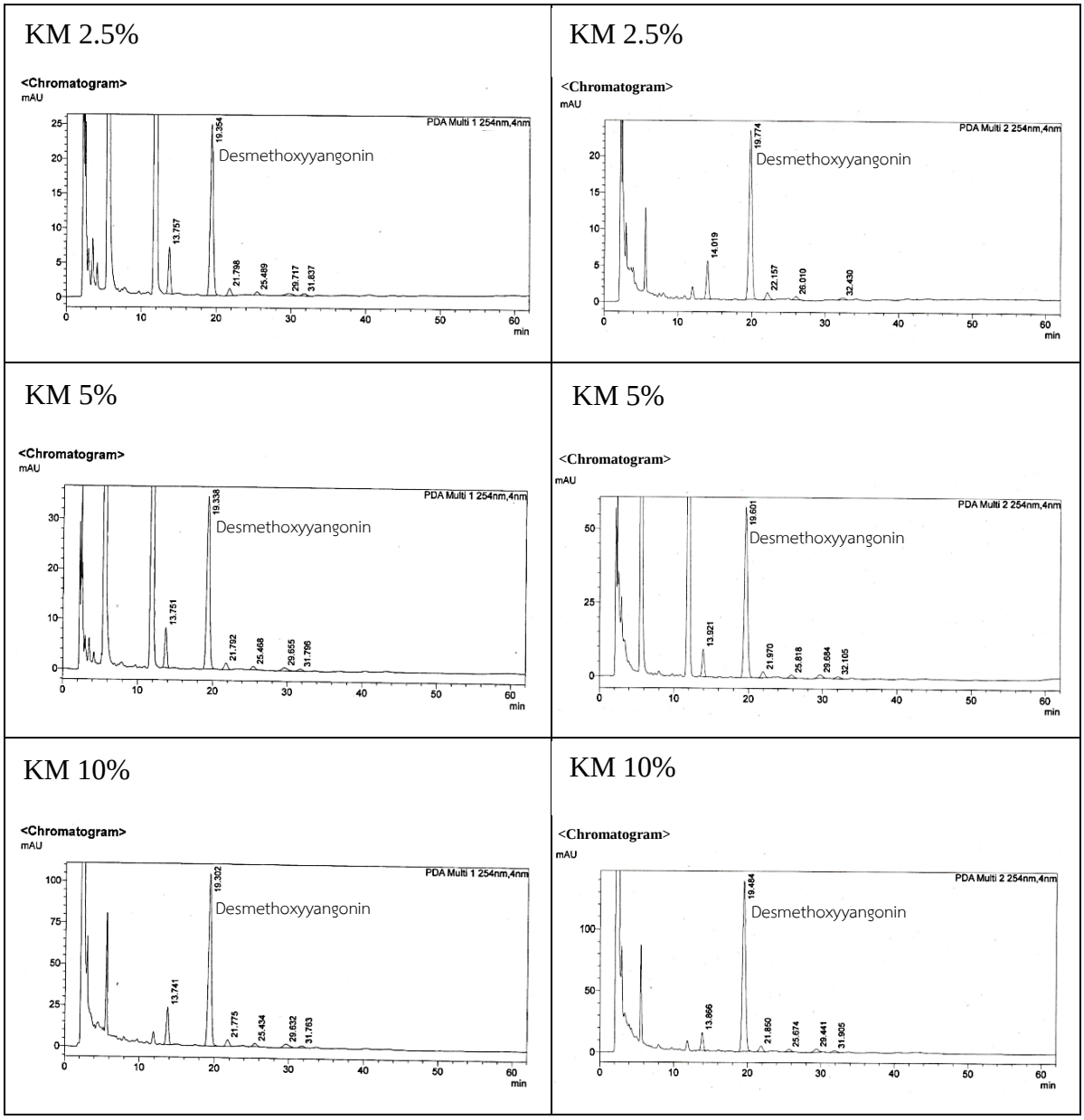

Figure 8. Chemical stability of active ingredient (Desmethoxyyangonin) of *K. marginata* (KM) gel before and after heating-cooling test by analysis with HPLC (wavelength 254 nm).

3. Physical stabilities of *K. marginata* gel before and after heating-cooling test.

The characteristics that were evaluated is color, odor, texture, pH value and viscosity value, both before and after accelerating conditions showed similar color, odor and texture at all concentrations. Viscosity and pH values of all concentrations both before and after heating-cooling test were not significantly different, the pH values of 10.0% gel was 6.42±0.21 (before) and after was at 6.78±0.42; 5.0% gel was 5.41±0.24 (before) and after was at 5.91±0.19; for 2.5% gel was at 5.63±0.22 (before) and after was 5.72±0.32. The viscosity (10^3cP) value of 2.5% was 7.05±0.18 (before) and after was at 7.51±0.34; 5.0% gel was at 6.03±0.23 (before) and after was at 6.43±0.26; 10.0% gel was at 6.07±0.24 (before) and after was at 7.06±0.12 (Table 15).

Table 15. Physical stabilities of *K. marginata* gel before and after heating-cooling test

Samples	Before					After				
	Color	Odor	Texture	pH values	Viscosity (10^3cP)	Color	Odor	Texture	pH values	Viscosity (10^3cP)
Formula Base	White	Tang	Homogeneous	5.58±0.21*	7.03±0.15*	White	Tang	Homogeneous	6.68±0.34*	8.00±0.26*
2.5%	Pale yellow	Tang	Homogeneous	5.63±0.22	7.05±0.18	Pale yellow	Tang	Homogeneous	5.72±0.32	7.51±0.34
5.0%	Dark yellow	Tang	Homogeneous	5.41±0.24	6.03±0.23	Dark yellow	Tang	Homogeneous	5.91±0.19	6.43±0.26
10.0%	Brown	Tang	Homogeneous	6.42±0.21	6.07±0.24*	Brown	Tang	Homogeneous	6.78±0.42	7.06±0.12*

*Significant difference between before and after $p<0.05$
(Mean ± S.E.M. of three determinations)

CHAPTER 4

CONCLUSIONS

The research was studied on objectives that related with biological properties *in vitro* and stabilities of gel preparations. The biological properties of *K. marginata* extract and gel preparations from *K. marginata* rhizomes were studied on 3 three major assays of experiments. The first experiment was to investigate the anti-inflammatory activity assay on the potent inhibitory effect against lipopolysaccharide (LPS)-induced NO by using the murine macrophage-like RAW264.7 cells. The second was to investigate the wound healing activity assays on cell proliferation and cell migration by using human dermal fibroblasts. The third was to investigate the anti-oxidant activity assays on DPPH radical scavenging and H_2O_2-induced oxidative stress activity assay by using human dermal fibroblasts. The gel preparations from *K. marginata* rhizomes extract were evaluated for the physical, chemical and biological stabilities after accelerating conditions.

1. The biological properties of *K. marginata* rhizomes and gel preparations from *K. marginata* rhizomes extract

1.1 Investigation the anti-inflammatory activity assay on the potent inhibitory effect against lipopolysaccharide (LPS)-induced NO production

K. marginata extract and gel preparations from *K. marginata* showed the good activities against NO production (IC_{50}) both before and after accelerating conditions. Before, the extract was found to be 7.02±1.25 µg/ml; 10.0% w/w (12.50±2.91 µg/ml); 5.0% w/w (27.05±2.12 µg/ml) and 2.5% w/w (35.38±0.82

µg/ml). After accelerating conditions, 10.0% w/w was 12.83±1.09 µg/ml; 5.0% w/w (26.77±2.01 µg/ml) and 2.5% w/w (35.15±2.89 µg/ml) and was better than a positive control (Diclofenac gel = 64.90±0.63 µg/ml).

1.2 Investigation the wound healing activity assays on cell proliferation and cell migration

Gel preparations both before and after accelerating conditions of each sample showed the good activity on cell proliferation and cell migration. They were also better than a positive control. The highest activity on cell proliferation was 5.0% w/w gel both before and after accelerating conditions showed % cell viability at 134.05±0.32 and 134.65±0.34, respectively and 99.5% *Aloe vera* gel was at 92.64±0.17. For cell migration, KM gel preparations both before and after accelerating conditions of each sample also showed the good migration and was better than a positive control. The highest effect on cell migration was 5.0% w/w gel both before and after accelerating conditions showed % cell migration at 85.22±5.27 and 85.71±5.76, respectively whereas 99.5% *Aloe vera* gel was at 72.64±2.87.

1.3 Investigation the anti-oxidant activity assays on DPPH radical scavenging and H_2O_2-induced oxidative stress activity assay

The study of DPPH radical scavenging assay did not show potent inhibitory activity of any sample both before and after accelerating conditions. For H_2O_2-induced oxidative stress activity assay showed the good activities in most of samples. They were also higher than positive controls. The highest % cell viability of 10.0% w/w gel both before and after accelerating conditions were at 88.66±0.56

and 87.94±1.17, respectively; vitamin C was at 68.45±2.67 and vitamin E was at 78.12±2.44.

2. The physical, chemical and biological stabilities of gel preparations

Gel preparations both before and after accelerating conditions mostly has no significant difference of each parameter (color, odor, texture, pH value, viscosity value, HPLC analysis and biological activity assays), therefore they have the good physical, chemical and biological stabilities.

The results of all these studies on physical, chemical and biological stabilities had effectiveness enough to support the information for Thai traditional treatment of inflammatory-related diseases and can be developed as a new pharmaceutical product from herbal extract.

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List of Publication and Proceedings

1. Muthachan T, Tewtrakul S. Biological Activities of Gel Containing *Kaempferia marginata* Extract. International conferences of the 5th Current Drug Development 2018 (CDD 2018) and the 3rd Herbal and Traditional Medicine 2018 (HTM2018); 2018 May 23rd-25th; Songkhla, Thailand.

2. อนุสิทธิบัตร เรื่อง สูตรตำรับเจลต้านการอักเสบจากสารสกัดเปราะป่า เลขที่คำขอ: 1803001261 วันที่ยื่นคำขอ 1 มิถุนายน พ.ศ.2561.