



DEVELOPMENT OF HERBAL EXTRACT-LOADED PATCHES FOR WOUND
HEALING



By
MISS Pattaranut EAKWAROPAS

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL TECHNOLOGY
Department of PHARMACEUTICAL TECHNOLOGY
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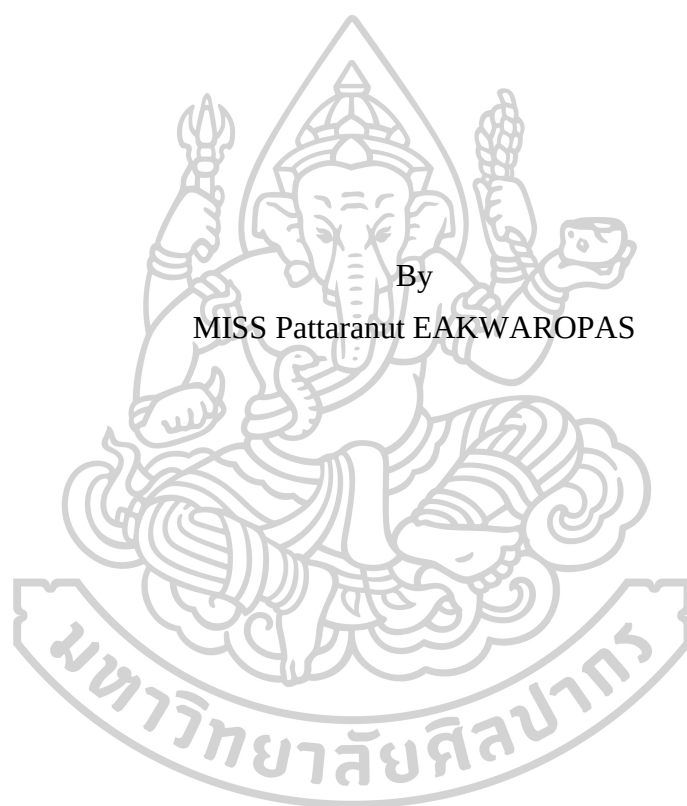
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In this study, *Boesenbergia rotunda* and *Dioscorea bulbifera* were extracted and characterized for wound healing proposes. *B. rotunda* ethanolic extract exhibited antioxidant and antimicrobial activities against *Staphylococcus aureus*. The extract showed no toxicity to fibroblast cells and was able to enhance fibroblast cell growth. However, it did not affect fibroblast migration. *D. bulbifera* extract was also obtained by maceration with ethanol. The extract presented a strong antioxidant activity whereas it provide no antibacterial activity. The *D. bulbifera* extract was able to increase cell proliferation but it did not affect cell migration. *B. rotunda* extract-loaded polyvinyl alcohol (PVA) hydrogel patches were fabricated by a physical crosslinking method. The hydrogel patches were designed and optimized using a Box-Behnken design with 3 factors and 3 levels. The optimized hydrogel patch was composed of 17.07 % w/w of PVA and 1.00 % w/w of *B. rotunda* extract. The optimized crosslinked hydrogel was obtained with 3.86 freeze-thaw cycles. The *B. rotunda* extract-loaded hydrogel patch showed antimicrobial activity and infiltration capacity against bacterial contamination. Moreover, it facilitated fibroblast migration after 72-h incubation. On the other hand, the *D. bulbifera* extract/honey-loaded Gantrez/Xyloglucan (Gan/XG) hydrogel films were prepared by the thermal crosslinking method. A full central composite design was used to create and optimize the hydrogel films with 2 factors including Gan and XG concentrations. The optimized film consisted of Gan and XG at a concentration of 17.78 % and 0.1 % w/w, respectively. After crosslinking at 130 °C for 30 min, the *D. bulbifera* extract and honey were loaded in the optimized film by absorption. The results showed that the *D. bulbifera* extract/honey-loaded Gan/XG presented strong

antioxidants comparable to the *D. bulbifera* extract. In addition, both honey-loaded and the *D. bulbifera* extract/honey-loaded Gan/XG could protect the wound bed from environmental bacteria and improved normal human fibroblast migration. Therefore, *B. rotunda* extract-loaded PVA hydrogel patches, the honey-loaded and the *D. bulbifera* extract/honey-loaded Gan/XG may be potential candidates for the development of functional wound dressing materials.



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For any mistake or inadequate information that may still be

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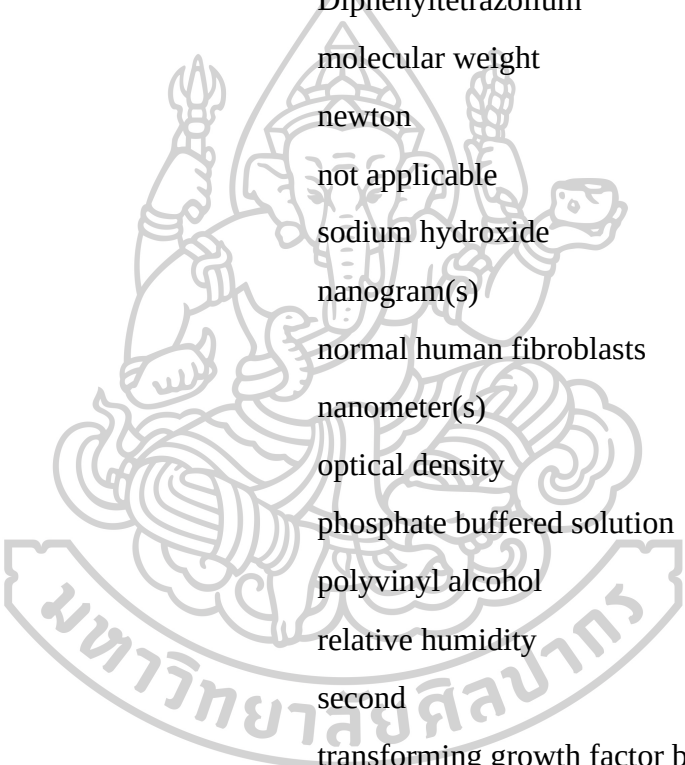
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LIST OF ABBREVIATIONS

Abs.	Absorbance
ANOVA	analysis of variance
ATR	attenuated total reflectance
°C	degree Celsius
CFU	colony forming unit
cm	centimeter(s)
CPD	critical point drying
CO ₂	carbon dioxide
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl radical
DSC	Differential scanning calorimetry
<i>E. coli</i>	<i>Escherichia coli</i>
Eq.	equation
<i>et al.</i>	and others
etc	Et cetera
FBS	fetal bovine serum
FT	freeze-thaw
FT-IR	Fourier-transform infrared spectroscopy
g	gram
Gan	Gantrez TM S-97
h	hour(s)
HPLC	high-performance liquid chromatography
IC ₅₀	The half-maximal inhibitory concentration
i.e.	<i>id est</i> (Latin abbreviation); that is
kg	kilogram(s)
LC	loading capacity
LE	loading efficiency
MBC	Minimum Bactericidal Concentration



mg	milligram(s)
min	minute(s)
MIC	minimum inhibitory concentration
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar
MPa	Mega Pascal
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium
MW	molecular weight
N	newton
N/A	not applicable
NaOH	sodium hydroxide
ng	nanogram(s)
NHFs	normal human fibroblasts
nm	nanometer(s)
O.D.	optical density
PBS	phosphate buffered solution
PVA	polyvinyl alcohol
RH	relative humidity
s	second
TGF-β1	transforming growth factor beta 1
TSA	tryptic soy agar
TSB	tryptic soy broth
UV	ultraviolet
v/v	volume by volume
WVTR	water vapor transmission rate
w/w	weight by weight
XG	xyloglucan
μg	microgram(s)
μL	microliter(s)

μm	micrometer(s)
%	percentage(s)
TM	trademark
®	registered trademark
°C	degree Celsius



CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the research problem

Boesenbergia rotunda is a herbal plant in the Zingiberaceae family that is commonly used as a food component and in ethnomedicinal formulations for the treatment of illnesses including rheumatism, muscle pain, fever, gout, gastrointestinal disorders, flatulence, stomach ache, dyspepsia, peptic ulcer, dermatitis, dry cough, tooth and gum diseases. Nowadays, almost a hundred chemical compounds are extracted from *B. rotunda* such as flavonoids, chalcones, esters, terpenes, and terpenoids (1). Numerous studies reported no toxicity of *B. rotunda*, for instance, mutagenic effect, changes in body weight, and hematological, and histopathological effects (1-3). Teethaisong *et al.* (2018) reported the antibacterial activity of *B. rotunda* rhizome ethanolic extract against β -lactam-resistant *staphylococci* including *Staphylococcus aureus* which is an important cause of wound infection. The extract showed inhibition against β -lactam-resistant *S. aureus* with a minimum inhibition concentration (MIC) of 16 $\mu\text{g/mL}$. Moreover, the extract combination with ampicillin, cloxacillin and cefazolin exhibited synergistic activity against bacterial growth (4). For wound healing enhancement, *B. rotunda* rhizome ethanolic extract was evaluated on human keratinocyte cells which are one of the key cells in the healing process. The extract could increase cell proliferation through stimulation of the MAPK and PI3K/Akt signal transduction pathways (5). In addition, the wound-healing activity of *B. rotunda* rhizome ethanolic extract was evaluated using excision and linear incision wound in the rat model. The results showed that topical application of the extract significantly increased the percentage of wound contraction on day 12 compared with the control group. The histological results showed a complete epidermis layer, collagen fibers, and hair follicles in the dermis layer (6). Furthermore, Mahmood *et al.* (2010) exhibited the enhancement of wound healing enclosure of rhizome ethanolic extract in a rat model. Histological results of the healed wound showed smaller scar width, less inflammatory cells, more collagen, and more blood capillaries compared to the vehicle (7).

Dioscorea bulbifera L. is a medicinal plant used as a food ingredient and in traditional medicine. The chemical components isolated from *D. bulbifera* are different depending on the cultivated area, part of the plant, and the extraction method (8). Numerous studies reviewed the pharmacological activities of this plant such as anticancer, antibacterial, anthelmintic, antihyperglycemic, antihyperlipidemic, analgesic, and anti-inflammatory activities (8-12). Analgesic and anti-inflammatory properties of aqueous and methanolic extracts of the bulbils in mice and rats were investigated. The results showed that the bulbils of *D. bulbifera* consisted of potent analgesic and anti-inflammatory activities (13). However, the toxicities of *D. bulbifera* extract have been reported such as hepatotoxicity and nephrotoxicity (8, 10, 14). Two detoxification methods were possible including leaching and combining with other herbs, for instance, *Angelica sinensis*, *Schisandra chinensis* and *Glycyrrhiza uralensis* (8, 10). Nevertheless, infrequent research conveyed the wound-healing activity of *D. bulbifera* extract. Panduraju *et al.* (2010) found that the tuber ethanolic extract was able to enhance wound contraction and decrease the epithelialization process in a rat model compared to standard ointment (15).

Maceration is one of the famous extraction methods because it is a very simple and non-heating method. It is suitable for thermolabile components. Plant material is contained in a well-closed container with an optimal solvent and allowed extraction at room temperature for a minimum of 3 days with frequent stirring. The soluble phytochemicals are released from the soft plant cells. After the maceration, the extract is isolated from crude fiber by filtration. The polarity of the isolated components is different depending on the extraction solvent (16, 17).

Wound healing is a continuous process that is divided into four overlapping phases including hemostasis, inflammation, proliferation, and remodeling. The hemostatic phase first stops bleeding. The endothelial cells secrete molecules that affect platelet aggregation. Moreover, coagulation occurs through the catalytic transformation of fibrinogen into fibrin. Next, the inflammatory phase begins with increased vascular permeability and infiltration of phagocytes (neutrophils and macrophages) to the wound area by the local release of growth factors and cell mediators. Neutrophils are the first inflammatory cells that enter the wound site. The

main role of neutrophils is bacteria and tissue debris phagocytosis. Macrophages, derived from circulating monocytes under the influence of inflammatory mediators, migrate next to the wound. In the early inflammatory phase, macrophages have a part in bacteria phagocytosis, cell debris removal, and cytokine release. At the end of the inflammatory phase, the descent number of neutrophils occurs in a non-infected wound by the apoptosis process influenced by macrophages. Lymphocytes migrate into the wound bed for complete pathogen defense by the influence of cytokines secreted from macrophages. The proliferation phase starts with the migration and proliferation of fibroblasts and endothelial cells to form granulation tissue under the influence of macrophages. In the early proliferation phase, type III collagen synthesis predominance over type I collagen provides suitable tensile strength to the newly formed tissue. The new blood vessel formation (angiogenesis) is essential before granulation tissue creation. Angiogenesis starts during endothelial cells migrate to the temporary matrix of the wound. Granulation tissue is constructed from epithelial cells, fibroblasts, and keratinocytes. While granulation tissue and collagen synthesis are processed deep in the wound bed, epithelialization occurs at the wound surface by migration from the wound margins under the scab and over the underlying viable connective tissue. The last remodeling phase is identified by the transformation of the granulation tissue into the scar tissue to increase wound strength. The fibroblast cells differentiate into myofibroblasts and the total collagen content enlargement; type I collagen becomes more plentiful than type III collagen (18-21).

The infected wounds immediately and temporarily stop the inflammatory phase and consequently fail to conform to the normal healing process. Wound debridement, which consists of impracticable tissue and microorganism removal, is a crucial stage of the healing process. Chronic wounds progress from the combination of poor debridement and microbial intrusion, which postpone proliferation and tissue regeneration processes (19, 21).

Numerous studies reported about the moist environment in wound beds could enhance the healing process (22, 23). Winter (1962) studied the formation of scabs and the epithelization rate of superficial wounds in a pig. The results showed that a moist wound inhibited scab formation and epithelization of the moist wound was

more rapid than dried wound (22). In addition, Dyson *et al.* (1988) concluded that the inflammatory and proliferative phases of full-thickness excision wounds in pigs were faster under moist conditions than in dry conditions (23). Nowadays, wound dressings are classified into numerous groups, for instance, semipermeable film, hydrogel, hydrocolloid, alginate dressing, foam, antimicrobial dressing, and deodorizing dressings (24-27). The goals of dressing application on the wound are fast epidermal migration, angiogenesis, and connective tissue synthesis (26). An ideal dressing could provide an optimal healing environment including maintaining high humidity, displacing excess wound exudate, protecting the wound from further trauma, preserving appropriate temperature, allowing gaseous exchange between the wound bed and environment, minimizing scar formation, inhibiting extraneous bacteria contamination, and easy to remove after healing (24-27).

To minimize infection and maximize the proliferation of the healing process, the present study aims to develop herbal extract-loaded patches for wound healing. Crude extracts from *B. rotunda* and *D. bulbifera* are prepared using the maceration method. The *in vitro* biological activities of the extract, that supported the wound healing processes, such as antimicrobial activity, antioxidant activity, viability, cell proliferation, and cell migration effects are investigated. Thereafter, herbal extract-loaded patches are fabricated by physical crosslinking and thermal crosslinking methods and optimized by experimental design. The physicochemical properties of the optimized herbal extract-loaded patches including, water content, water absorption, erosion, water vapor transmission rate (WVTR), and mechanical properties are characterized. Moreover, the *in vitro* cytotoxicity and the effects on cell proliferation and migration of the optimized patches are evaluated. Finally, the physicochemical stabilities of optimized patches are assessed.

1.2 Aims and objectives

1. To prepare extracts from herbs, characterize and determine the *in vitro* biological activities
2. To develop herbal extract-loaded patches for wound healing

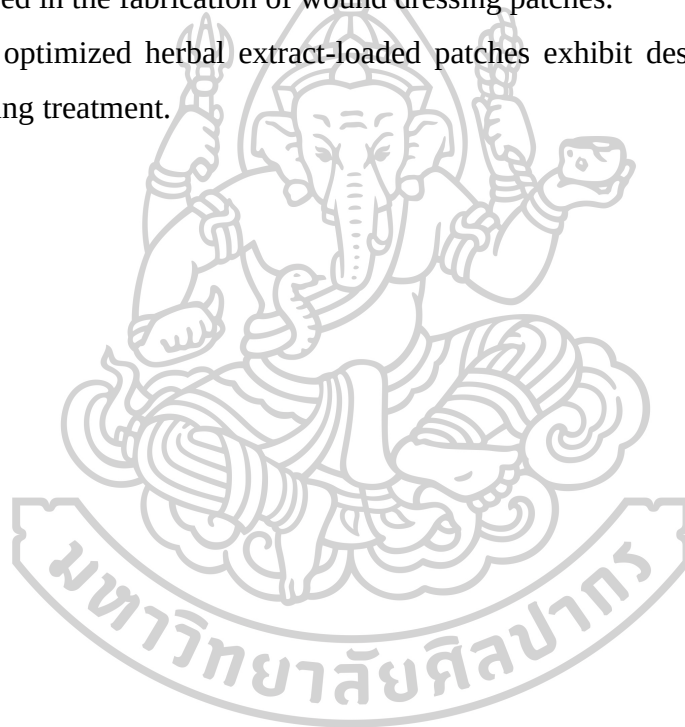
3. To evaluate the physicochemical properties, biological activities, and stability of the optimized herbal extract-loaded patches

1.3 The research hypothesis

1. Crude extracts from *B. rotunda* and *D. bulbifera* prepared by maceration method have numerous biological activities, for example, antimicrobial activity, antioxidant activity, and the ability to increase viability, proliferation, and migration of fibroblast cells and consequently improve the wound healing processes.

2. Crude extracts from *B. rotunda* and *D. bulbifera* are compatible with the polymers used in the fabrication of wound dressing patches.

3. The optimized herbal extract-loaded patches exhibit desirable properties for wound healing treatment.



CHAPTER 2

INTRODUCTION

2.1 Wound

2.1.1 Wound types

Wounds can be classified as acute or chronic wounds. Acute wounds are divided into accidental wounds (for instance, bites, burns, minor cuts, and abrasions) and surgical wounds. Chronic wounds are wounds that could not heal within 6 weeks; for example, pressure ulcers, diabetic foot ulcers, arterial occlusive, venous stasis, lymphedema, calciphylaxis, warfarin-induced skin necrosis and infection. The main cause of chronic wounds is bacterial infection (18, 24, 28). On the other hand, wounds can be separated into four categories based on their appearance: necrotic, sloughy, granulating, and epithelializing (27).

2.1.2 Wound healing process

The wound healing process is divided into 4 overlapping phases including hemostasis, inflammation, proliferation, and remodeling as shown in **Figure 1** (18, 19, 28).

2.1.2.1 Hemostasis

Hemostasis is the normal reaction of a vessel to injury by forming a clot that inhibits hemorrhage. Hemostasis is the beginning step followed by inflammation. The reduced blood flow occurs by arteriolar narrowing in a few minutes which affects tissue hypoxia and acidosis. After injury and vessel leakage, platelets are exposed to subendothelial collagen and become aggregated and coagulated. Moreover, thrombin and fibronectin stimulate the release of cytokines and growth factors. Next, the fibrin clot is formed and then, neutrophils, monocytes, fibroblasts, and endothelial cells migrate into the wound area (18, 28).

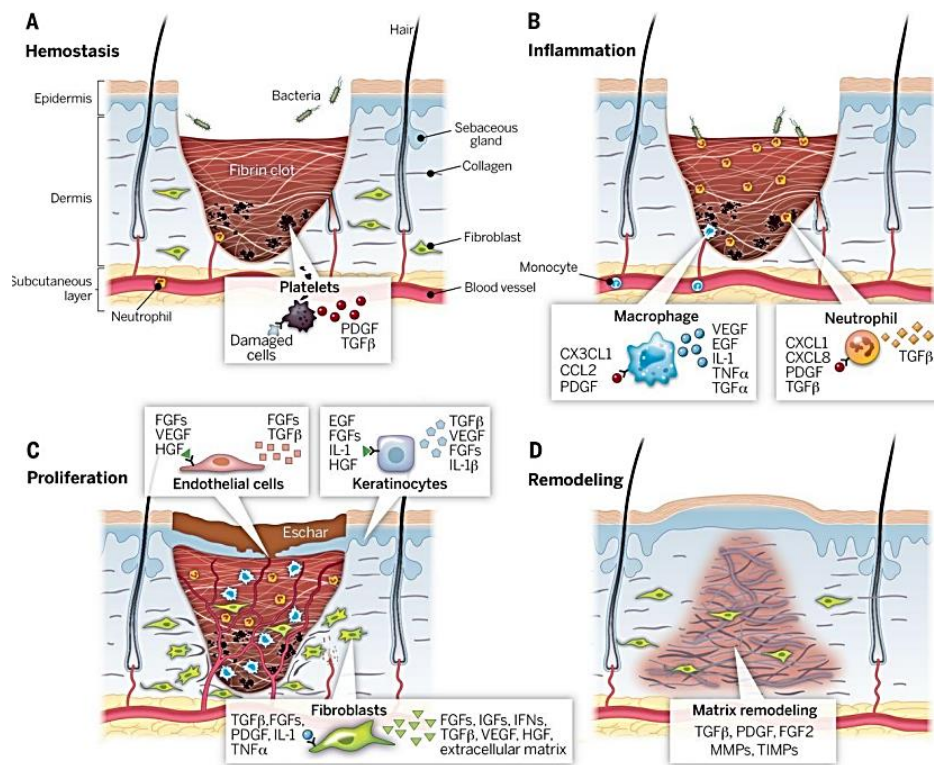


Figure 1 Wound healing phases; (A) hemostasis, (B) inflammation, (C) proliferation, and (D) remodeling (19).

2.1.2.2 Inflammation

The inflammatory phase is identified by increased vascular permeability, the local release of cytokines and growth factors, and migrating of cells into the wound bed. Increased vascular permeability due to inflammation and the prostaglandin release, together with a concentration gradient of chemotactic substances including complement factors, interleukin (IL)-1, tumor necrosis factor (TNF)-α, TGF-β, platelet factor-4, and bacterial products. The circulating immune cells move into the wound within 6 h. Neutrophils are the first leukocytes that migrate to the wound site caused by vascular permeability, and their maximum numbers are within 24–48 h after injury. The main role of neutrophils is the phagocytosis of bacteria and dead tissue. Macrophages, originating from circulating monocytes, enter the wound and maximum at 72 h after damage. Macrophages have a much longer lifetime than neutrophils and remain in the wound until the healing process is complete. Next, lymphocytes occur in significant numbers in wounds around the 5 day after damage. The cooperation of neutrophils, macrophages and lymphocytes in the wound area is

critical for the normal healing process. The release of factors from platelets stimulates macrophage activation. The macrophage activation has numerous effects on healing process, including debridement, matrix synthesis, and angiogenesis. The activated macrophages release cytokines that affect angiogenesis, fibroplasia, and nitric oxide (NO) synthesis, which itself has antimicrobial capacities and encourages collagen synthesis. The inflammatory phase is an essence to the suitable development of the following healing phases. Inflammatory reduction importantly affects the healing process, as clinically observed in patients who have chronic wounds. On the other side, the excess inflammatory phase will be excessive fibrosis (18, 28).

2.1.2.3 Proliferation

The proliferative phase happens for 4 to 21 days, and consists of angiogenesis, extracellular matrix (ECM) formation and epithelization. Revascularization of the wound appears in collateral with fibroplasia. Angiogenesis occurs through a combination of both proliferation and migration. Capillary buds grow from blood vessels near the wound and expand into the wound area. Endothelial cells from the venue area closest to the wound start to move in response to angiogenic stimuli. This capillary grows ultimately branch at their tips and combine to form capillary loops, through which blood starts to flow. Then, new buds spread from these loops to form a capillary plexus. Fibroblasts first migrate in significant numbers on the 3 days after trauma and reach maximum numbers around the 7 days. Growth factors stimulate fibroblast proliferation. The rapid enlargement of the fibroblast population at the wound area caused by the combination of proliferation and migration. Fibroblasts derived from local mesenchymal cells migrate to the wound and are stimulated to proliferate by cytokines released from platelets, macrophages and lymphocytes, respectively. Fibroblasts are the primary synthetic component that produces the main structural proteins necessary for tissue reorganization including collagen. Granulation tissue and collagen synthesis are progressing deep in the wound, while epithelial repair at the superficial surface. Epithelialization of the wound occurs by epithelial cells moving from the wound borders and residual dermal epithelial appendages within the wound area start to migrate below the scab and above the underlying viable connective tissue. Fixed basal cells in an area near the trauma edge rapidly mitotic

divide and migrate by moving over one another until the wound area is fully covered and the superficial layer is finally keratinized. Neutrophils exhibit apoptosis and are ingested by macrophages. Macrophages also undergo apoptosis and in the process release arginase, which affects wound metabolism (18, 28).

2.1.2.4 Remodeling

Remodeling is the last phase of healing, starting two to three weeks after injury and can continue for one year or more. Collagen is the main scar protein. Fibroblasts are also responsible to produce other matrix constituents, such as fibronectin and glycosaminoglycans. Net collagen synthesis is higher from the higher number of fibroblasts in the wound area and a collagen building augmentation by each cell. Wound contraction occurs from the wound edges together. Numerous theories have been reported on the mechanisms of wound contraction. It has been hypothesized that a special cell, the myofibroblast, is accountable for contraction, whereas another theory suggested that the movement of all fibroblasts affect contraction (18, 28).

Closure of acute wounds follows the normal process and completely heals within 8–12 weeks. However, chronic wounds have a longer inflammatory phase and may have excess exudate, heavy infections, pain, or tissue debris. Thus, chronic wounds may take several months or years to fully heal (19). The normal and chronic wound (such as in diabetes) healing processes are shown in **Figure 2**.

2.1.3 Wound dressings

The ideal wound dressings must preserve moist conditions, remove excess exudate, permit thermal insulation, allow vapor exchange, conform to wound surface, reduce scar formation, protect from outside bacteria, and be safe and non-adhere (24-27). Wound dressings are classified into many types such as gauze, film, foam, alginate, hydrocolloid, nanofiber, antimicrobial dressing, deodorizing dressing and hydrogel. The selection of appropriate wound dressing is presented in **Figure 3**.

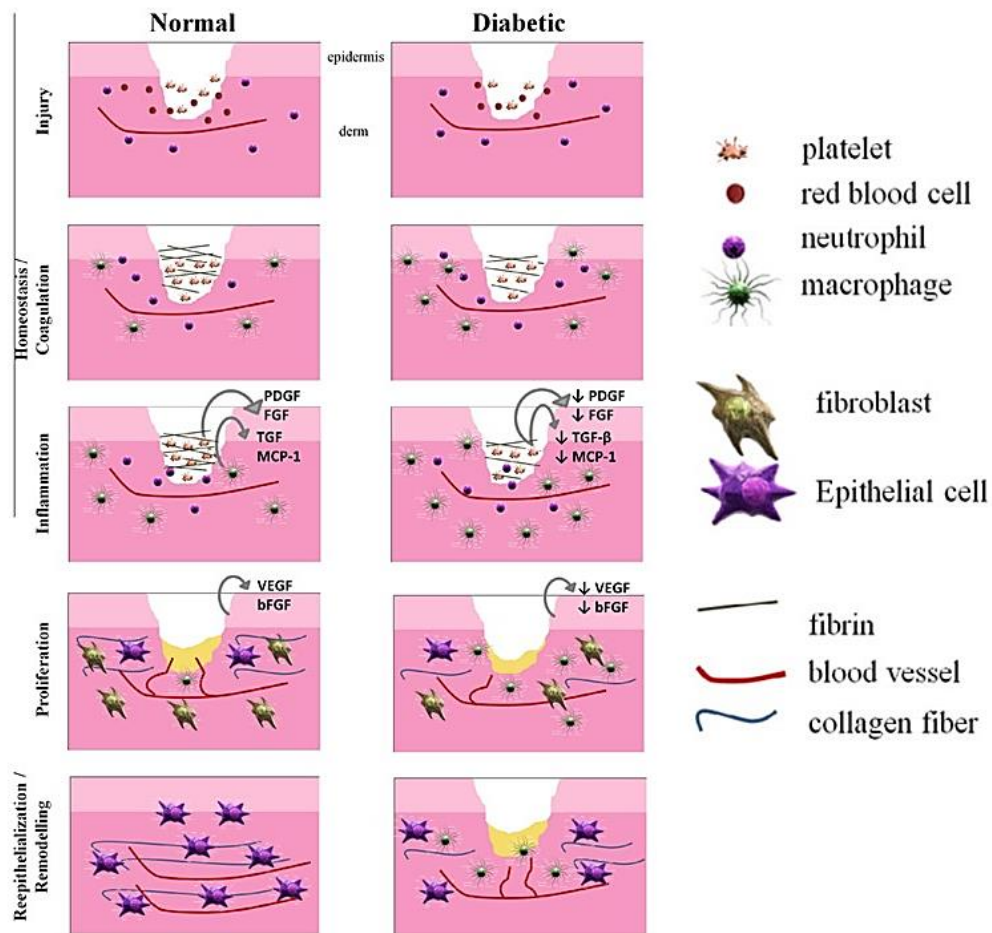


Figure 2 Differences between the normal and diabetic wound healing phases (29).

2.1.3.1 Gauzes

Gauze is a primitive dressing used in the Egyptian period. It is separated into 2 subtypes: woven and non-woven gauzes depending on the fabrication and composition. Non-woven gauze dressings are fabricated from rayon or synthetic fibers. Woven gauzes, acquainted as absorbent gauze, are fabricated from pure natural cotton yarns. In antiquity, gauzes that quickly dried have been used to only cover the wound area. The advantages of gauze are cheap, compatible, readily usable, and often used in surgical and clinical practices. Nowadays, the developed gauzes are altered with polymers, nanoparticles, and other substances for desirable use in several wound managements such as Vaseline[®] petrolatum gauze and Irradiated atrauman[®] Ag gauze, etc. (30).

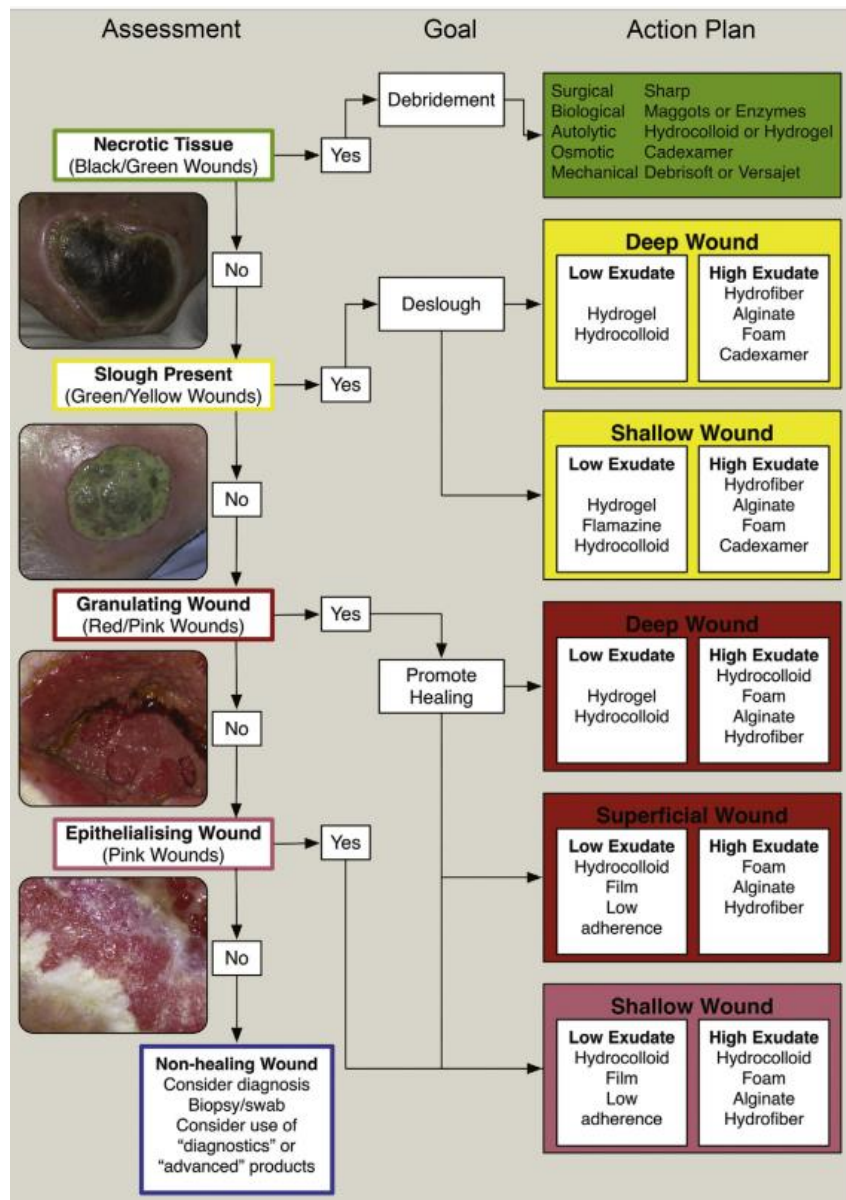


Figure 3 The selection of appropriate wound dressing (24).

2.1.3.2 Films

Films are normally transparent, easy to apply, non-expensive, semi-permeable to oxygen and water vapor, impermeable to liquid and preventive of bacterial contamination. Film dressings are polymer sheets of various thicknesses that had adhesive properties on one side. The polyurethane film is water vapor permeable, decreasing maceration conditions by reducing moisture occlusion in the wound bed. Its transparency consents to observe the wound area without removing it. Films could be used as a primary or secondary wound dressing to treat partial-thickness wounds

with less or no exudate, necrosis, and infection. Films are commercial in many sizes, which are light, flexible, and immediately adhere to the wound area. Film dressings have the potential for use on many wound types, for example, post-operative wounds, superficial burns, and skin grafts, because of the permit in time for wound monitoring. Much research proves that films also utilize for incorporating various biomolecules, drugs, and growth factors. The advantages of film dressings are easily observed by physicians, lowering the infection opportunity, trauma, and pain during dressing changes. The limited point is films are less acceptant by patients who dislike seeing wounds. Film dressings are improper for high exudate wounds or hemostatic use. After removal, the films can cause pain and cell damage. The main disadvantage of films is non-suitable for high exudate wounds (24, 29, 30).

2.1.3.3 Foams

Foams are semipermeable dressings composed of either hydrophilic or hydrophobic with a bacterial barrier. Foams are polyurethane or silicone cores surrounded by semi-obstructive outer layers. The hydrophobic external part repulses fluids and microorganisms. Foam dressings were suitable for applications in moderate to high exudate wounds. Their exudate absorbent capacity depends on the specific fabricated polymer and its thickness. Because of high exudate absorption, foam dressings don't have to be frequently changed. Moreover, foam dressing is non-adhesive, so it could be able to reduce pain and dermal cell damage. They are available in several thicknesses. Furthermore, they equip thermal insulation to the wound and create a moist condition. Foam dressings may be fabricated with an adhesive border and available with active substances such as antimicrobials, and anti-inflammatory analgesics (24, 29, 30)

2.1.3.4 Alginate dressings

Alginate dressings are a non-woven and fibrous patch developed from brown seaweed. The alginate could be able to form a gel when it encounters wound fluid. These dressings could absorb up to 20 times their weight. Absorption capability is achieved by strong hydrophilic gel formation, which limits wound exudates and decreases bacterial contamination. They could be used in both infected and non-infected wounds. Because alginate dressings are highly absorbent ability, they are

suitable for moderate to heavy exudate wounds and they should not be applied to dry or wounds with low exudate. Alginates always need a secondary dressing; foams or hydrocolloids that preserve the alginate from drying out. In the case of the infected wound, the secondary dressing must be non-occlusive. The calcium component of the alginates could be useful in bleeding management (24, 26).

2.1.3.5 Hydrocolloids

Hydrocolloid dressings are composed of absorptive ingredients (regularly carboxymethylcellulose, pectin or gelatin). Hydrocolloids could absorb minimal to moderate amounts of exudate. They can absorb wound exudate and create a moist environment. They appropriately apply in partial- or full-thickness, both acute and chronic wounds. Because of their occlusion, hydrocolloid dressings do not allow vapor or bacteria into the wound. Hydrocolloids also cause the acidic pH of the wound surface which could inhibit bacterial growth. The hydrocolloids can support the wound healing process including granulation, epithelialization, and autolytic debridement in necrotic or sloughy wounds. Nevertheless, the occlusive property of hydrocolloids is not suitable for use in infected wounds and is not recommended for diabetic foot ulceration. In some cases, hydrocolloids may produce unfavorable odor due to the product breakdown (24, 29).

2.1.3.6 Nanofibers/Nanocomposite

Nanofibers are dressings with varying fiber sizes in nanometer ranges that are fabricated through several techniques including template synthesis, phase separation, drawing, self-assembly, and electrospinning. Electrospinning is the most interesting because of its ease of production and scale-up. Because of their high surface-area-to-volume ratio, porous fiber, and plasticity, they are developed in medical applications, including scaffolds for regenerative medicine and therapeutic agent delivery. Electrospinning wound dressings with nanofibers have numerous advantages, for instance, the structural and physiological properties are comparable to the natural ECM. Moreover, the porous structure of the nanofiber sheet allows the effective loading of several physiologically active components, including antimicrobial agents, nanostructured materials, vitamins, growth hormones, and herbal extracts. The release

of biological molecules or therapeutic substances can be controlled by the structure and size of the fiber adjusting (30).

2.1.3.7 Antimicrobial dressings

Antimicrobial dressings are used to facilitate the optimum conditions to support fast healing. The antimicrobial agents chosen to decrease or eliminate microorganisms are influenced by their specificity, efficacy, cytotoxicity and allergenicity. Examples of topical antimicrobial agents used such as iodine (cadexomer iodine and povidone-iodine), silver (silver sulfadiazine and ionic silver-impregnated dressings) and antiseptic agents. Honey also acts as an antimicrobial, and debriding agent and could control wound odor. Antimicrobial dressings should only be applied in a short period (recommendations suggest not more than 2 weeks) and if the wounds are not better, other alternative treatments are instead (24, 26, 27).

2.1.3.8 Deodorizing dressings

Wound odor could be a significant problem, especially in non-healing wounds. Charcoal-based dressings can be effective decrease wound odor. Other loaded dressings containing activated charcoal and silver, could be applied as an absorbent wound contact layer that controlled both odor and exudate (24, 27).

2.1.3.9 Hydrogel dressings

Hydrogels are polymeric, moist, non-adherent, and nontoxic dressings. Hydrogels maintain highly moist environments and are composed of single or mixed hydrated polymers. They might be crosslinked by covalent or non-covalently bonds to control their absorption capacities and maintain their conformational structures. An excellent advantage of hydrogels is the dressing change without interfering with wound healing. Because they are almost transparent, the wound can be observed without removing the dressing. Like hydrocolloid dressings, hydrogels could support the autolytic debridement of necrotic tissues and are usually more efficient for wounds with minimal to moderate exudate. A few drawbacks of hydrogels are low mechanical strength and skin maceration. Their application in wounds with heavy exudate can cause wound maceration and lead to a healing crisis (24, 29, 30).

2.2 Hydrogels

Hydrogels are a three-dimensional network of polymers made of natural or synthetic substances that are flexible due to their large water content. Their networks are built of hydrophilic polymer bonds formed by mechanical or covalent crosslinking. The 4-hydrogel fabricating method is composed of ionic interactions, crystallization, complementary group chemical reactions, and radical polymerization (30, 31). Numerous commercial hydrogel dressings are created to preserve a moist environment at the wound bed. Normally, hydrogels support a moist environment for wound healing because they consist of water more than 90% of its weight (19). A moist environment could promote the wound healing process more than dry conditions. Winter (1962) reported the faster epithelialization of moist wounds than dry wounds in the pig model (22). Later, several studies supported the better of a moist environment for wound healing, for example, the dry and moist conditions of 25 x 25 mm² full-thickness wounds were studied in a pig model. The result presented that the inflammatory and proliferation phases under moist conditions were quick than in dry conditions (23).

2.3 Polymers for hydrogel development

2.3.1 Polyvinyl alcohol

Polyvinyl alcohol (PVA) is widely used in numerous medical applications, especially to develop hydrogel because of its safety and biocompatibility (32). PVA is a hydrophilic, non-toxic, and non-carcinogenic agent polymerized from vinyl acetate. In addition, it exhibits good chemical/enzymatic resistance, as well as good mucoadhesive and oxygen permeability properties. Nevertheless, PVA exhibits some undesirable mechanical properties such as strength and elasticity, as well as thermal non-stability. The combination of PVA with other polymers (including naturally based polymers such as chitosan, gelatin, dextran, and hyaluronic acid) by the sufficient crosslinking method can be improved its properties. Chemical crosslinking can be prepared by using glutaraldehyde, genipin, succinyl chloride, adipoyl chloride and sebacoyl chloride, while physical crosslinking can be obtained by repeated freeze-thawing cycles (33).

2.3.2 Gantrez[®]

Gantrez[®] (Gan), a synthetic polymer consisting of methyl vinyl ether and maleic anhydride, has been used to develop pharmaceutical products for several purposes; such as Gan/hyaluronic acid microneedles for transdermal delivery (34), Gan microneedles combined with lyophilized wafer for transdermal delivery (35), Gan/hyaluronic acid hydrogel crosslinked by a solvent-free process for biomedical applications (36), Gan/hyaluronic acid microneedles for ocular delivery (37), thermally-crosslinked mucoadhesive Gan/PVA/hyaluronic acid/catechol nanofibers for transmucosal drug delivery (38), and a thermal crosslinked hydrogel-forming microneedle array using 30% w/v of Gan as the microneedle forming polymer (39).

2.4 Natural materials for hydrogel development

2.4.1 *Boesenbergia rotunda*

Boesenbergia rotunda is a medicinal plant in ginger species that belongs to the family of Zingiberaceae. It has many botanical names such as *Boesenbergia pandurata* (Roxb.) Schltr., *Curcuma rotunda* L., *Gastrochilus panduratus* (Roxb.) Ridl., *Gastrochilus rotundus* (L.) Alston, *Kaempferia cochinchinensis* Gagnep., *Kaempferia ovate* Roscoe, and *Kaempferia pandurata* Roxb. (1). The morphology of *B. rotunda* including aerial part, flower and rhizome are shown in **Figure 4**.

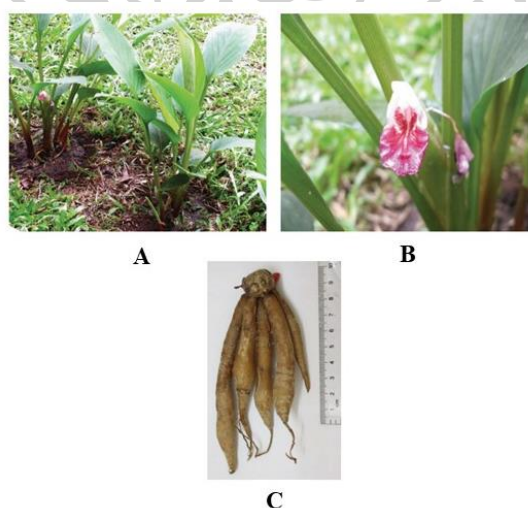


Figure 4 The morphology of *B. rotunda*; (A) aerial part, (B) flower, and (C) rhizomes (1).

Numerous studies reported no toxicity of *B. rotunda* (1). The toxicity of *B. rotunda* extracted with 50 % ethanol with the concentration of 60, 120 and 240 mg/kg/day was investigated in male rats. The toxicity of oral administration for 60 days was not observed. The body weight, hematological parameters, kidney function and liver function were normal (3). In addition, Charoensin *et al.* found that pinocembrin and pinostrobin, the active substances, with a concentration of 500 mg/kg were nontoxic and non-mutagenic in Wistar rats (2). Furthermore, the high dose of ethanolic extract (2 and 5 g/kg) was safe and that no extract-related toxicity (7).

2.4.1.1 Pharmacological activities

The biological activities of *B. rotunda* have been reported such as antiparasitic activity, anti-periodontitis activity, anti-obesity, anti-mutagenicity, antitumor, anticancer, antifungal activity, antiviral activity, anti-inflammatory, antioxidant activity, and antibacterial activity (1, 6). Jitvaropas *et al.* informed that the *B. rotunda* extracted with 95 % ethanol exhibited moderate antioxidant activity. Moreover, it was able to inhibit pathogens including *S. aureus*, *S. epidermidis*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *C. albican*, and *C. cerevisiae* (4, 6, 40). Atun *et al.* also reported the antioxidant activity of *B. rotunda* ethanolic extract. The ethanolic extract had an antioxidant with IC₅₀ at 92.64 mg/mL (40).

2.4.1.2 Wound healing activity

Several articles have been studied about the wound-healing activity of *B. rotunda* extract. The effect of *B. rotunda* rhizome extracted with 95 % ethanol on viability, proliferation and migration of human keratinocyte cells that are one of the important cells in the healing process was determined. The extract was able to increase cell proliferation through stimulation of the MAPK and PI3K/Akt signal transduction pathways, while it didn't affect cell migration (5). The *B. rotunda* extracted with 95 % ethanol was evaluated using excision and linear incision wound models. The results showed that 10 and 20 % of extract in petroleum were not different compared with the control (pure petroleum). For incision wound, the contraction of 10 and 20 % of treated groups were higher than control and the

difference between groups were not different (6). Furthermore, Mahmood *et al.* (2010) also investigated the *B. rotunda* extracted with 95 % ethanol in an excision wound. The results exhibited significantly lower healing compared with the control in a rat model. In addition, the differences between extract-treated groups and Intracite were not significant. Histological data of the treated wound showed smaller scar width, fewer inflammatory cells, more collagen, and more blood capillaries compared with the control (7).

2.4.2 *Dioscorea bulbifera*

Dioscorea bulbifera L. is a herbal plant that is used as a food ingredient and in traditional medicine. The phytochemical substances consisted in *D. bulbifera* are different depending on the growth area, part of the plant, and the extraction solvent (8). A lot of chemical compounds were isolated from *D. bulbifera* such as Diosbulbin A–P, Dioscoreanoside A–K, Diosbulbiside A–C and Bafoudiosbulbin A–C, etc. (8, 10). The toxicities of *D. bulbifera* extract have been found including hepatotoxicity and nephrotoxicity (8, 10). The leaves and bulbils of *D. bulbifera* are shown in **Figure 5**.



Figure 5 The morphology of *D. bulbifera*; (A) leaves, (B) and (C) bulbils (41).

2.4.2.1 Pharmacological activities

Many research studies reported the pharmacological activities of this medicinal plant including anticancer, antibacterial, anthelmintic, antihyperglycemic, antihyperlipidemic, antioxidant, analgesic, and anti-inflammatory activities. Moreover, it was used for the treatment of goiter, respiratory disorders, eye disorders, diabetes, and digestive problems(8, 10-12). Marandi *et al.* (2016) studied the antimicrobial activity of tuber extract. The result exhibited that the tuber extracted

with ethanol, methanol and distilled water inhibited *Bacillus cereus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Vibrio cholera* (42).

2.4.2.2 Wound healing activity

Nevertheless, infrequent research investigated the wound-healing activity of *D. bulbifera* extract. Panduraju *et al.* (2010) reported that the tuber ethanolic extract could be able to improve excision wound contraction and reduce the epithelialization process in the rat model compared with 0.2 % nitrofurazone ointment (15). Recently, the effect of *D. bulbifera* bulbil extract, its fractions and compounds isolated from extract on NHF were investigated. The data showed that the ethanolic and water extracts didn't enhance NHF proliferation and migration whereas compounds especially (+)-catechin had strong activities (43). Furthermore, the adverse drug reaction and efficacy of *D. bulbifera* gel from U-thong hospital were present. Hyperpigmentation and itching appeared at 0.2 % which denoted an unserious effect. After 4-week treatment, all 5 chronic ulcers were better (41).

2.4.3 Honey

Honey is extensively used as a food and traditional medicine component. Honey consists of phenolic compounds, flavonoids, sugars, amino acids, proteins, lipids, vitamins, minerals, and organic acids (44, 45).

2.4.3.1 Pharmacological activities

The biological properties of honey were assessed. Honey exhibited antibacterial, antioxidant, and anticancer activity against the MCF-7 cell line and extracellular anti-melanogenic activity (46). Furthermore, stingless bee honey was also described for its antioxidant, antimicrobial, and anti-inflammatory activities (47).

2.4.3.2 Wound healing activity

Honey has desirable properties for the wound healing process, including antibacterial activity and healing enhancement. Its antimicrobial, anti-inflammatory and antioxidant activities, promoting effect on the immune system, debridement

action, and stimulating effect on wound regeneration and wound healing processes were reported (**Figure 6**) (48).

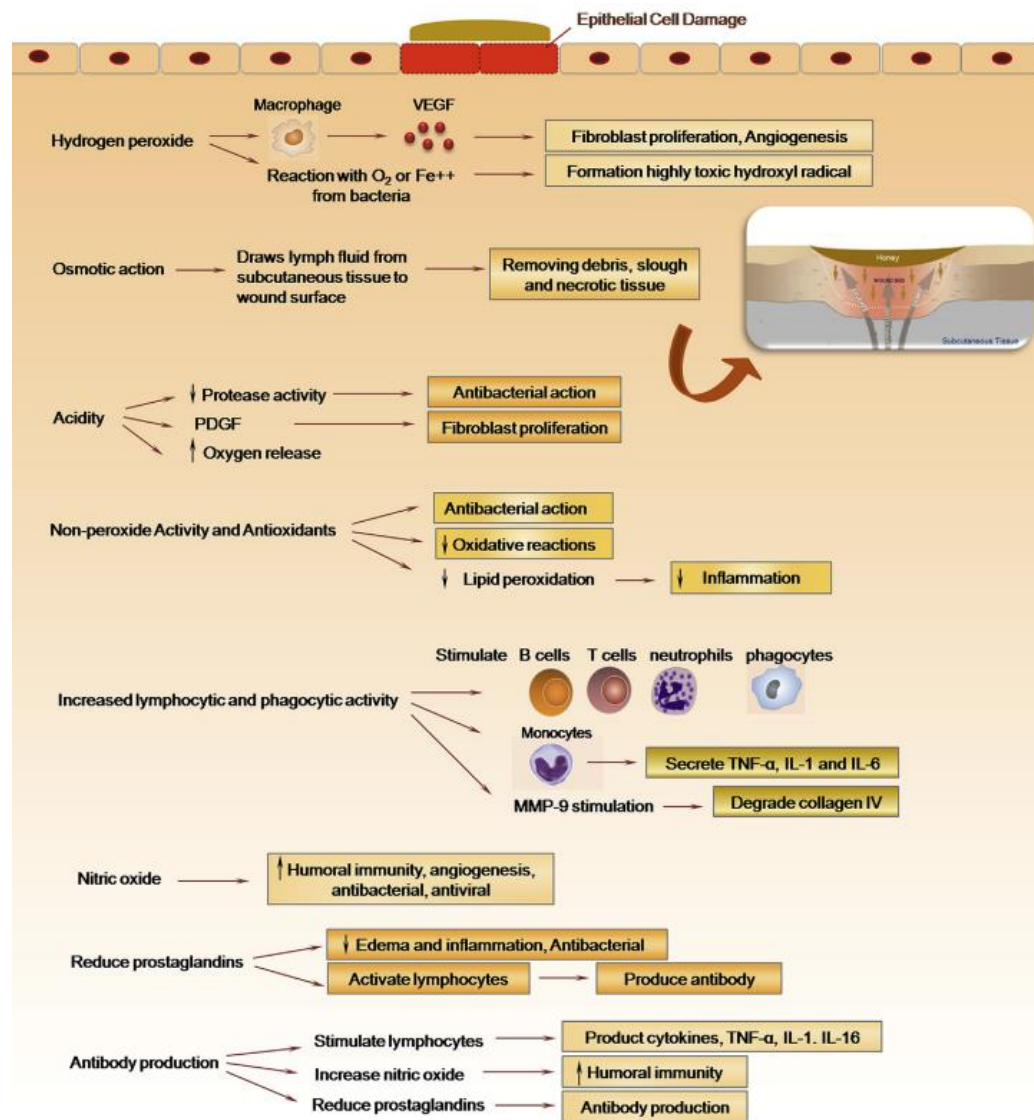


Figure 6 Mechanisms of honey for wound healing (48).

Numerous studies described the effect of honey on the wound-healing process in both acute and chronic wounds. The effects of honey on the wound-healing process are shown in **Figure 7**. In the inflammatory phase, honey encourages monocytes to release cytokines that affect collagen synthesis. Moreover, honey could remove necrosis tissues and bacteria by stimulating neutrophils, and macrophages. For the proliferation phase, honey affects angiogenesis by oxygen support because of the acidic property of honey that influences oxygen release from hemoglobin. In addition,

honey could also enhance wound contraction by accelerating fibroblasts, and myofibroblasts. Hydrogen peroxide contained in honey could stimulate the growth of new capillaries. In the re-epithelialization step, honey supports the required glucose as energy for the epithelial cells. Honey also composes of different trace elements which can promote keratinocyte proliferation by modulating integrin expression (48).

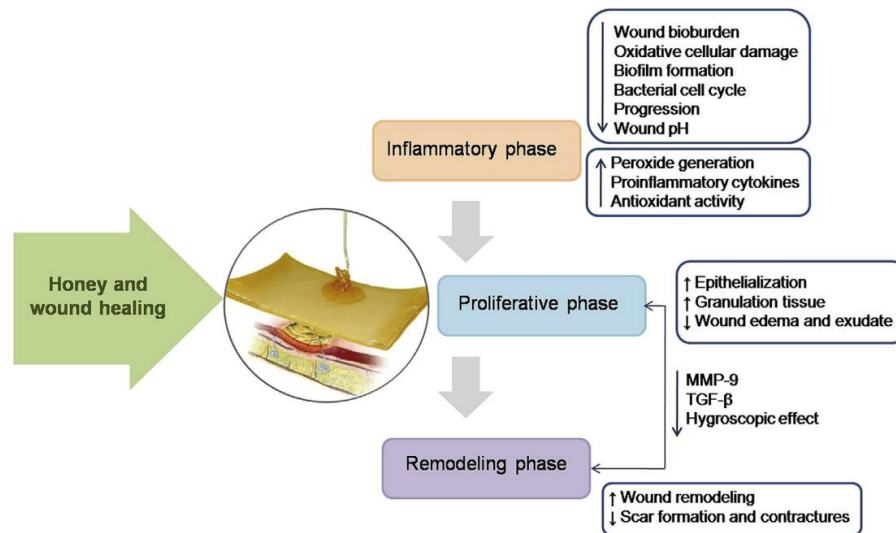


Figure 7 Effects of honey on wound healing process (48).

Nordin *et al.* (2018) investigated the effect of stingless bee honey on NHFs. They found that the morphology of NHFs treated with stingless bee honey for 24 h didn't change. The stingless bee honey was able to improve the proliferation of NHFs without cell cycle change (49). In addition, Eyarefe *et al.* (2017) reported about the multi-flora honey from Nigeria for electroscope incision wound healing in albino rats. They suggested that the honey was able to simplify the healing process (50). The effect of Jamun honey on wound healing in the diabetic mice model was evaluated. The topical application of honey significantly improved wound closure, re-epithelialization, and collagen deposition (I/III) and balanced the myofibroblasts formation (51). A prospective study of open or infected wound treatment in 40 patients reported that honey dressing could decrease bacterial infection, support healing process, and reduce pain (52). Moreover, it was found that honey dressing could decrease the debridement period, and increase the wound healing rate and bacterial clearance rate in diabetic foot ulcers (53). Thus, numerous honey-

incorporated wound dressings were developed, such as a borax-crosslinked honey/PVA film dressing for antibiotic-controlled release (54), a chitosan/honey/gelatin hydrogel sheet dressing for burn wound (55), a honey-incorporated alginate/PVA electrospun dressing (56) and an electrospun honey/silk fibroin fibrous dressing (57), etc.

2.4.4 Tamarind seed xyloglucan

Tamarind seed xyloglucan (XG) is extracted from *Tamarindus indica* L. fine kernel powder by boiling and precipitation in ethanol (58, 59). It consists of many substances such as carbohydrates, protein, oil, and fiber (60); however, the main composition is hexose sugar (58, 59). XG consists of a cellulose-like main chain of (1–4)- β -D-glucan backbone chain which is partially substituted with side chains of α -D-xylose at the O-6 position. Some of these xylose residues are substituted with (1–2)- β -D-galactosyl units at the O-2 position (61, 62). The structure of tamarind seed XG is shown in **Figure 8**.

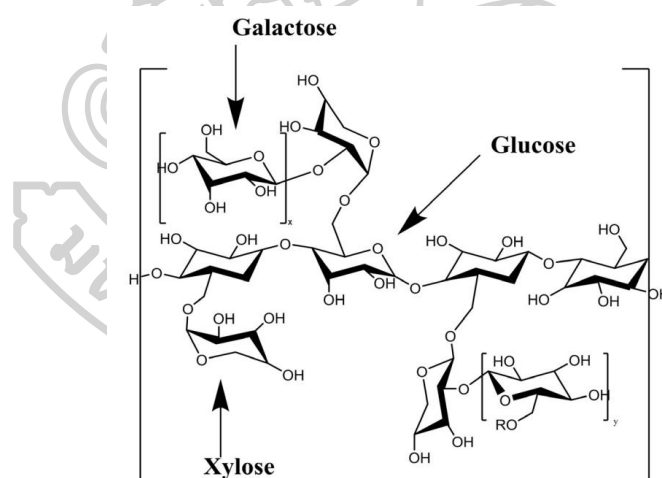


Figure 8 Structure of tamarind seed XG (61).

2.4.4.1 Pharmaceutical applications

Numerous studies proved that XG is safe without carcinogenicity and any adverse effects. Because XG is a non-toxic substance, it is utilized in several pharmaceutical applications including as a suspending agent and emulsifying agent in oral liquids, binder, and as a matrix former in tablets. Furthermore, it has been

developed as drug delivery in various routes, such as buccal, oral, ocular, pulmonary, rectal, periodontal, intraperitoneal, transdermal, and nasal routes (60, 61, 63-65).

2.4.4.2 Wound healing activity

The wound healing capability of XG has been assessed using keratinocytes and fibroblasts. Skin regeneration was reported due to re-epithelialization and remodeling enhancements (66). The effect of tamarind seed extract on excision wounds in ICR mice was reported. The percentages of wound area in all extract-treated groups were lower than in the untreated group. The wound healing rates of treated groups were faster than untreated wounds (67).

Besides, XG was chosen to be combined with other polymers for wound dressing development. The gentamicin-loaded hydroxypropyl methylcellulose/XG film was successfully prepared. The transparency film was desirable for wound observation without removal, moreover, the antimicrobial activity against *S. aureus* and *E. coli* was presented due to the release of gentamicin from the film (68). Ajovalasit *et al.* (2018) fabricated XG/PVA/glycerol film chemically crosslinked using glutaraldehyde. It exhibited non-cytotoxicity and appropriate material for monitoring the healing process (69). In addition, Picone *et al.* (2019) also developed and characterized XG/PVA/glycerol film. The film showed non-cytotoxicity, non-cellular stress-inducing, and hemocompatibility. Moreover, it could protect the wound bed from outside bacteria contamination (70). In addition, the wound-healing activity of the xyloglucan/sucrose sheet was studied using a rat model. The sheet could be able to decrease wound area more than the control (71).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

- 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA)
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium (MTT) (Sigma-Aldrich, St. Louis, MO, USA)
- Acetonitrile (Honeywell, USA)
- Alternative to L-glutamine (GlutamaxTM) (Gibco BRL, Rockville, MD, USA.)
- *B. rotunda* fresh rhizomes (Pathum thani, Thailand)
- *D. bulbifera* fresh bulbils (Uthong Hospital, Suphanburi, Thailand)
- Dimethyl sulfoxide (DMSO) (Fisher Chemical, Fisher Scientific, UK)
- Dulbecco's modified eagle medium (DMEM) (Gibco BRL, Rockville, MD, USA)
- Ethanol (Merck, Germany)
- Fetal bovine serum (FBS) (Gibco BRL, Rockville, MD, USA.)
- GantrezTM S-97 with MW of 1500 kDa (Ashland, Surrey, UK)
- McFarland Standard (HimediaTM)
- Methanol (Honeywell, USA)
- Natural wild honey (Khaokho Talaypu Company, Thailand)
- Non-essential amino acid (Gibco BRL, Rockville, MD, USA.)
- Normal human fibroblasts (NHFs) (ATCC, Rockville, MD, USA)
- Penicillin-streptomycin (Gibco BRL, Rockville, MD, USA.)
- polyvinyl alcohol (PVA) with MW of 60,000 daltons and degree of hydrolysis ≥ 98 % (Merck, Germany)
- Tamarind seed xyloglucan (Megazyme, Bray, Co. Wicklow, Ireland)
- Tryptic soy broth (HimediaTM)

3.2 Equipment

- 15-mL centrifuge tube (CentristarTM Cap, Corning Incorporated Life science, Jiangsu, China)

- 24-wells Cell Culture plate (Costar®, Corning Incorporated, ME, USA)
- 50-mL centrifuge tube (Centristar™ Cap, Corning Incorporated Life science, Jiangsu, China)
- 6-wells Cell Culture plate (SPL Life Science Co., Ltd., Gyeonggi-do, Korea)
- 96-wells Cell Culture plate (Costar®, Corning Incorporated Life Sciences, Jiangsu, China)
- Aluminium foil
- Analytical balances (Model BSA3202S-CW, Sartorius)
- Autoclave (Model: HVE-50; HIRAYAMA)
- Differential scanning calorimetry (DSC) (Pyris Diamond, PerkinElmer, USA)
- Fourier-transform infrared spectroscopy (FTIR) (Nicolet iS5, Thermo Fisher Scientific, MA, USA)
- Freezer/Refrigerator at -20°C and -80°C
- Gloves
- High-performance liquid chromatography (HPLC) (Model 1220 Infinity II, Agilent)
- Hot air oven (Mettler)
- Humid CO₂ Incubator (37°C, 95% RH, 5% CO₂) (HERA Cell 240 Heraeus)
- Inverted microscope (Nikon® T-DH, Nikon, Tokyo, Japan)
- Laminar airflow (BIO-II-A)
- Magnetic stirrer and magnetic bar
- Mask
- Micropipette (2-20 µL, 20-200 µL, 100-1,000 µL, 1-10 mL)
- Micropipette tips (Corning®, Corning Incorporated, New York, U.S.A.)
- Microplate reader (VICTOR Nivo™ Multimode Plate Reader, PerkinElmer, Germany)
- Parafilm (Bemis, Wisconsin, U.S.A.)
- pH meter (Horiba compact-B212)
- Scanning electron microscope (SEM) (Tescan, Mira 3, Czech Republic)
- Thermometer
- UV-Visible Spectrophotometer (Model Biomate 6, Thermo Scientific)

3.3 Methods

3.3.1 Plant extraction

3.3.1.1 *B. rotunda* rhizomes

B. rotunda fresh rhizomes were cleaned, cut, and dried at 50 °C. Then, fine powder of dried *B. rotunda* was macerated with 70 % v/v of ethanol at room temperature. After 7 days, the filtrate was collected, and the solvent was removed using a rotatory evaporator.

3.3.1.2 *D. bulbifera* bulbils

D. bulbifera fresh bulbils were washed, sliced, and dried at 50 °C. Then, the dried pieces were ground, and the fine powder was macerated with 80 % v/v ethanolic solution at room temperature for 15 days. The crude was separated, and the filtrate was concentrated using a rotatory evaporator.

The extracts were kept in a well-closed container at 4 °C until use.

3.3.2 Characterization of the extracts

3.3.2.1 FT-IR

The FT-IR spectra of the extract were recorded over wavenumbers ranging from 400 – 4,000 cm⁻¹ using an ATR.

3.3.2.2 DSC

The thermal characteristics of the extracts were investigated using a differential scanning calorimeter. The extracts were heated at a rate of 10 °C/min from 25 – 400 °C.

3.3.2.3 Determination of percent yield

Dried extracts were weighted and the percent yields were calculated according to the following Eq. (1)

$$\% \text{ Yield} = \frac{W}{W_i} \times 100 \quad (1)$$

where W is the weight of the dried extract and W_i is the initial weight of the herb powder.

3.3.2.4 Standardization of the extracts using HPLC

Quantification of pinostrobin and pinocembrin from *B. rotunda* extract was performed using an HPLC with a UV visible detector as previously reported with some modification (72). Sample solutions were prepared by dissolving the extract in ethanol. The determination of the active compounds in the extract was performed using an HPLC connected to a C18 column (250 × 4.6 mm, 5 µm). The HPLC conditions were as follows: gradient elution with 0.15 % v/v of water-phosphoric acid and acetonitrile, a flow rate of 1.5 mL/min, and a running time of 62 min. The chromatograms were monitored at 285 nm.

Quantification of diosgenin from *D. bulbifera* extract was also evaluated using HPLC connected to a UV-visible detector. The content of the active compounds in the extract solution was examined using a C18 column (150 × 4.6 mm, 5 µm). The HPLC conditions were as follows: acetonitrile and water (90:10 v/v), a flow rate of 1 mL/min, and UV detection at 203 nm (73).

3.3.3 Biological activities of extracts

3.3.3.1 Antimicrobial activity

3.3.3.1.1 Minimum inhibitory concentration (MIC)

The MIC determination of plant extracts against *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) was performed according to a method described in the guidelines of Clinical Laboratory Standard Institute (CLSI) M7-A10 (2015) with some alterations. In brief, bacterial suspensions were prepared in TSB and incubated at 37 °C overnight. Then, the turbidity of bacterial suspensions was adjusted to give an equivalent to a 0.5 McFarland standard. Thereafter, the bacterial suspensions were diluted with TSB medium to contain bacteria approximately 1×10^6 CFU/mL. The extract samples were prepared in a TSB medium using a 2-fold serial dilution technique. The antibiotic solution (gentamycin solution) and blank TSB medium were used as the positive and negative controls, respectively. The extract solution with a volume equal to the volume of bacterial suspension was mixed, and the mixtures were incubated at 37 °C for 16 h. The MIC values of the

extracts were observed from the lowest extract concentration that could prevent the bacterial visible growth.

3.3.3.1.2 Minimum Bactericidal Concentration (MBC)

The MBC values of extracts against pathogenic bacteria were evaluated by a procedure similar to the MIC determination. The extract-treated bacterial suspensions causing no growth of the bacteria after incubation were sub-cultured on a TSA plate at 37°C for 16 h. The lowest concentration of the extract that showed no growth on the TSA plate was reported as the MBC (6). The untreated bacteria were used as a control.

3.3.3.2 Antioxidant activity

The effects of plant extracts on DPPH were assessed in 96-well plates, 40 µL of each extract with various concentrations were mixed with 160 µL of a DPPH solution (0.2 mM in ethanol)(74). Ascorbic acid was used as the standard antioxidant. The absorbance was measured at 520 nm after incubation for 30 min in the dark at room temperature. The percent inhibition of DPPH was calculated according to the equation demonstrated below (Eq. 2). The DPPH radical scavenging activities of plant extracts were reported as a concentration of 50% inhibition (IC₅₀).

$$\text{Inhibition (\%)} = \frac{A_0 - A}{A_0} \times 100 \quad (2)$$

Where A and A₀ are the absorbances of DPPH solution with and without extract, respectively.

3.3.3.3 Effects on cell viability, proliferation, and migration

3.3.3.3.1 Sample preparation

Both extracts were completely dissolved in DMSO and then diluted with DMEM containing 1 % penicillin/streptomycin (DMEM-). †

3.3.3.3.2 Cell viability

Cell viability on NHFs was determined by an MTT assay (49). In brief, NHFs were seeded into 96-well plates at a cell density of 1 x 10⁴ cells per well and then

incubated at 37 °C, 5 % CO₂ atmosphere in 100 µL of DMEM supplemented with 10 % FBS, 1% Glutamax, 1% non-essential amino acids and 1% penicillin/streptomycin (DMEM+) for 24 h. After incubation, DMEM+ was discarded and the sample solutions were added to each well. Then, NHFs were further incubated for 24 h. Next step, sample solutions were replaced with PBS pH 7.4 to wash out the sample residual. For cell viability measurement, 25 µl of MTT solution with a concentration of 2.5 mg/mL in PBS and 100 µl of DMEM+ were added into each well. After 4 h, the MTT solution and DMEM+ were removed and DMSO was filled. The absorbance at 550 nm was measured using a microplate reader and the percentage of cell viability was calculated as an Eq. (3).

$$\text{Cell viability (\%)} = \frac{\text{Abs. of the treated group}}{\text{Abs. of the untreated group}} \times 100 \quad (3)$$

3.3.3.3 Cell proliferation

NHF cells were seeded into a 96-well plate at the cell density of 5 x10³ cells per well and then incubated in an environment similar to the cell viability testing method. The extracts that improved cell viability were chosen to evaluate the proliferation of NHFs under long-term exposure to the extracts. Cell proliferation at 24, 48, and 72 h after treatment was measured using an MTT assay. The percentage of cell proliferation was calculated as Eq. (4).

$$\text{Cell proliferation (\%)} = \frac{\text{Abs. of the treated group}}{\text{Abs. of the untreated group}} \times 100 \quad (4)$$

3.3.3.4 Cell migration

The effect of extracts on NHFs migration was evaluated by *in vitro* scratch assay (75). NHF cells were seeded into a 6-well culture plate with 2×10⁵ cells per well and then incubated in DMEM+ . A Scratch area was created on NHF monolayer culture using a 200-µL micropipette tip. The medium was replaced with PBS pH 7.4 to gently remove the detached cells. Then, 3 mL of extract solutions were added to each well. The untreated and TGF (1 ng/mL) treated cells represented the negative and positive control groups, respectively. Images of scratch areas before and after 24-, 48- and 72-h incubation time were observed under an inverted microscope (4x). The percentage of wound closure was computed from the difference between gap areas before and after incubation following Eq. (5).

$$\text{Wound closure (\%)} = \frac{\text{Wound area (t}_0\text{)} - \text{Wound area (t)}}{\text{Wound area (t}_0\text{)}} \times 100 \quad (5)$$

Where t_0 is the wound area after immediate scratching and t is the wound area after incubation for 24, 48, and 72 h.

The extracts that had desirable properties for wound healing enhancement were selected to incorporate in dressing patches.

3.3.4 Preparation of herbal extract-loaded patches by experimental design

3.3.4.1 Physical crosslinking method

PVA with total concentrations of 15 - 20 % w/w and *B. rotunda* extract solution with a total concentration of 30 – 50 % w/w were used to fabricate herbal extract-loaded patches by a freeze-thaw method. Firstly, PVA powder was dissolved in pure water at 90 °C for 4 h. Next, 500 mg of *B. rotunda* extract was dissolved in 30 mL of 70 % ethanol. Then, ethanol was evaporated and the extract solution was mixed with PVA solution following formulations from the experimental design. The mixtures were poured into molds and maintained at room temperature for 1 h. The *B. rotunda* extract-loaded patches were prepared by freezing at -20 °C (18 h) and thawing at room temperature (6 h).

A Box-Behnken design with factors including PVA concentration, freeze-thaw cycle, and *B. rotunda* extract solution loading was chosen for this study. The experimental design consisted of set points in midst of each multidimensional cube border ($n = 12$) and repeated center points ($n = 5$). The polynomial quadratic equation generated from the experimental design was shown as Eq. (6).

$$Y_i = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{23}BC + b_{13}AC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 \quad (6)$$

Where A, B and C are the fabrication variables including PVA concentrations (15, 17.5 and 20 % w/w), freeze-thaw cycle (2, 3 and 4 cycles) and *B. rotunda* extract solution loading (30, 40 and 50 % w/w), respectively. The terms of Y_i ($i = 1$ to 6) are the measured responses (hydrogel patch properties) including tensile strength (Y_1), elongation (Y_2), Young's Modulus (Y_3), water content (Y_4) swelling (Y_5) and erosion (Y_6), respectively. The intercept value and the regression coefficients of each equation are presented as b_0 and b_1 to b_{33}

The optimized patches with the desirable properties including the highest swelling capacity, water content, elongation and the lowest erosion were prepared and characterized.

3.3.4.2 Thermal crosslinking method

Copolymers of methyl vinyl ether and maleic anhydride (Gan) with total concentrations of 15 - 18 % w/w and tamarind seed XG with total concentrations between 0.10 and 0.25 % w/w were mixed to prepare *D. bulbifera* extract-loaded patches by the thermal crosslinking method. First, Gan was dissolved in pure water at room temperature overnight. XG solution was prepared by stirring in hot water (50 °C) for 4 h. The mixtures of Gan and XG solutions following formulas from the experimental design were cast into the 2.5 x 2.5 cm² square mold and dried at room temperature for 3 days. Next step, the dried films were removed from molds and crosslinked at 130 °C for 30 min followed by the crosslinking method of Gan that was previously published (39). The hydrogel patches were prepared by immersion of the crosslinked film in 50 % w/w of honey solutions for 1 h.

The experimental design with the amounts of Gan and XG was performed for this study. The response surface modeling coupled with the full central composite design (CCD) was used to analyze and optimize the hydrogel patches. The 2 fabricated factors (input); A: Gan and B: XG, and the 6 hydrogel properties (output); Y₁: water absorption, Y₂: tensile strength, Y₃: Young's modulus, Y₄: elongation, Y₅: erosion, and Y₆: water content, were measured. A total of 13 experimental runs was statistically calculated by ANOVA with a p-value < 0.05. The quadratic equation of hydrogel properties created from the experimental model was given as Eq. (7).

$$Y_i = b_0 + b_1A + b_2B + b_{12}AB + b_{11}A^2 + b_{22}B^2 \quad (7)$$

The optimized hydrogel patches with the maximum swelling and maximum elongation were prepared and characterized.

D. bulbifera extract and honey solutions were embedded in optimized films. After maceration for 15 days, the *D. bulbifera* extract solution was concentrated to achieve half the volume. The mixture of *D. bulbifera* extract solution and honey with concentrations of 20% and 50% w/w was prepared and loaded in the optimized film.

The excess solution was removed using filter papers. The honey-loaded and unloaded hydrogel patches were prepared as controls.

3.3.5 Characterization of the optimized herbal extract-loaded patches

3.3.5.1 FT-IR

The FT-IR spectra of patches were collected using the method described in Section 3.3.2.1

3.3.5.2 Scanning electron microscopy (SEM)

Surface and cross-section morphologies of hydrogel patches were examined using a scanning electron microscope. The patch samples were dried by critical point drying (CPD) and then sputtered with a thin layer of gold prior to SEM observation.

3.3.5.3 Quantitative analysis of chemical substances in hydrogel patches using HPLC

To analyze the amounts of chemical substances incorporated into the hydrogel patches, the patches were soaked in 20 mL of absolute ethanol. The amounts of chemicals in each patch were determined using HPLC methods described previously in extract standardization (Section 3.3.2.4). The percentages of loading efficiency and loading capacity were calculated according to the following Eq. (8) and Eq. (9).

$$\% \text{ Loading efficiency} = \frac{\text{Amount of chemical substance}}{\text{An initial amount of chemical substance}} \times 100 \quad (8)$$

$$\% \text{ Loading capacity} = \frac{\text{Amount of chemical substance}}{\text{weight of patch}} \times 100 \quad (9)$$

3.3.5.4 *In vitro* release

The hydrogel patches were added to 10 mL of PBS pH 7.4 and constantly stirred at 37°C in an incubator shaker. An aliquot of 2 mL was removed at specific time points (0.5, 3, 6, 9, 24 h) and replaced by 2 mL of PBS to complete the initial volume (76). The amount released in each period was measured using HPLC methods described previously (Section 3.3.2.4). The percentage of extract release was calculated following Eq. (10).

$$\text{Release (\%)} = \frac{A_t}{A_i} \times 100 \quad (10)$$

Where A_i and A_t are amounts of initial extract and release after immersion in PBS.

3.3.5.5 Water content

The water content of the hydrogel patch was examined. The patches were weighed and dehydrated in a hot-air oven to achieve constant weight. The water contents of hydrogel patches were computed from the weight difference between the initial weight and the dried weight after heating as Eq. (11).

$$\text{Water content (\%)} = \frac{W_i - W_d}{W_i} \times 100 \quad (11)$$

Where W_d and W_i are the weights of dried and initial hydrogel patches, respectively.

3.3.5.6 Water absorption

The swelling properties of the hydrogel patches were assessed. The samples were weighed and soaked in pure water at 37 °C for 24 h. Superfluous water on the sample surface was gently wiped using filter paper before sample weighing (77). The percentage of water absorption was evaluated following Eq. (12).

$$\text{Water absorption (\%)} = \frac{W_s - W_i}{W_i} \times 100 \quad (12)$$

Where W_s and W_i are weights of swollen and the beginning patches, respectively.

3.3.5.7 Erosion

The hydrogel patches were weighed and submerged in pure water for 24 h. Next step, the moist samples were dried at 60 °C until their constant weights were obtained. The percentage of erosion was computed following Eq. (13).

$$\text{Erosion (\%)} = \frac{W_i - W_{t(d)}}{W_i} \times 100 \quad (13)$$

Where W_i and $W_{t(d)}$ are dried weights of the beginning patches and patches after water immersion, respectively.

3.3.5.8 Water vapor transmission rate (WVTR)

To assess the WVTR of the hydrogel patches, the patches were cut in a circle shape. The mounts of bottles filled with pure water were covered with circular hydrogel patches. Water vapor transmissions from the inside of the bottle through hydrogel patches were investigated (78). The beginning weights of bottles were

weighed (W_0) and then incubated at 37 °C, 75 % RH. After 24 h, the bottles were weighed again (W_t). Uncovered bottles and bottles covered with hydrogel patches without extract were used as control groups. The WVTR values of the hydrogel patches were calculated from the following Eq. (14).

$$WVTR = \frac{W_0 - W_t}{A} \quad (14)$$

where A is the area of bottle mouth.

3.3.5.9 Mechanical properties

The mechanical properties of hydrogel patches were assessed by tension test. Tensile strength, elongation at the breaking point, and Young's Modulus of hydrogel patches were measured using a texture analyzer. Briefly, the hydrogel patches were prepared into a strip and fixed under a 5-kg load cell. The strips were elongated at a constant speed of 1 mm/s until the breaking point. The mechanical properties including tensile strength, Young's Modulus, and elongation at the break of the hydrogel patches were calculated using Eq. (15) – (17), respectively (79).

$$\text{Tensile strength} = \frac{\text{Maximum load at breaking point}}{\text{Transverse section area}} \quad (15)$$

$$\text{Young's modulus} = \frac{\text{Stress}}{\text{Strain}} \quad (16)$$

$$\text{Elongation at break (\%)} = \frac{\text{Extension of length at breaking point}}{\text{Initial length}} \times 100 \quad (17)$$

3.3.6 Biological activities of optimized herbal extract-loaded patches

3.3.6.1 Antimicrobial activity

3.3.6.1.1 Qualitative method

The antimicrobial activity of the hydrogel patches was evaluated by disc diffusion assay. In brief, bacterial suspensions were prepared to the bacterial density of 1.5×10^8 CFU/mL using 0.5 McFarland standard. Next, 50 μ L of the bacterial suspensions were pipetted and spread onto TSA plates. The hydrogel patches were cut into circular discs with a diameter of 6 mm and then sterilized under UV light for 20 min on each side. Then, the hydrogel discs were gently put onto the surface of the bacterial plate. The plates were inverted and incubated at 37 °C for 24 h. The

antimicrobial activities of hydrogel patches were verified from the diameter of the clear zone around hydrogel discs (56, 78). The hydrogel patch without extract and medicated commercial dressings were used as control groups.

3.3.6.1.2 Quantitative method

To measure the bacterial reduction after exposure to the hydrogel patches, the bacterial suspensions with a density of 1.5×10^5 CFU/mL were diluted from a density of 1.5×10^8 CFU/mL, and then 0.2 mL of bacterial suspensions were mixed with 1.8 mL of TSB. The 50 mg of hydrogel samples were added to the bacterial suspensions and incubated at 37 °C for 24 h. Next, the absorbances of the bacterial suspensions were evaluated at 600 nm using a UV–Visible spectrophotometer (56, 70, 78). The bacterial suspension without a sample was prepared as a control. The antibacterial activity was computed from the absorbances at a wavenumber of 600 nm of bacterial suspension between treated and untreated groups using the following Eq. (18).

$$\text{Antibacterial activity (\%)} = \frac{\text{Abs.of control} - \text{Abs.of sample}}{\text{Abs.of control}} \times 100 \quad (18)$$

Where Abs. of control and Abs. of the sample are the absorbance of the untreated and treated bacterial suspensions, respectively.

3.3.6.2 Infiltration bacterial test

First, the bacterial suspension was incubated at 37 °C overnight. Then, 10 µl of the bacterial suspensions with a density of 1.5×10^8 CFU/mL were carefully dropped in the center of the hydrogel patches that were placed on a TSA plate and incubated at 37 °C overnight. The bacterial suspensions directly placed on agar plates were used as a positive control. Three pieces of agar from 3 positions including agar from the area under the hydrogel patch, agar with bacteria, and agar that was far from the hydrogel patch (negative control) were taken to incubate again in TSB at 37 °C. After incubation for 30 min, the bacterial growth was observed at various intervals using a spectrophotometer (70).

3.3.6.3 Antioxidant activity

The free radical scavenging activity of the hydrogel patch was examined using a DPPH assay. First, each sample was weighed (50 mg), immersed in 3 mL of 0.1 mM of DPPH ethanolic solution, and then incubated at room temperature under dark conditions. The DPPH solution without a hydrogel patch was represented as a control. At various time intervals, the absorbance of the treated solution was measured at 520 nm using a UV–Visible spectrophotometer (56, 78). The antioxidant activity was computed in percentage according to the following Eq. (19).

$$\text{Antioxidant activity (\%)} = \frac{\text{Abs.of control} - \text{Abs.of sample}}{\text{Abs.of control}} \times 100 \quad (19)$$

3.3.6.4 Cell viability, proliferation, and migration of the hydrogel patches

3.3.6.4.1 Cell viability

The cytotoxicity of hydrogel patches was studied on NHFs by indirect cytotoxicity and MTT assay. Before the investigation, the hydrogel samples were sterilized under UV light for 1 h on each side and then submerged in DMEM and vibrated at 37 °C for 24 h to obtain sample solutions. The NHF cells were cultured in 96-well plates and incubated using the same method described previously in the cell viability test of plant extracts (Section 3.3.3.2). After incubation, the culture medium was removed, and sample solutions were added. After incubation again for 24 h, the sample solutions were discarded and the viability of NHF was assessed using an MTT assay (56, 80). The OD at the wavelength of 550 nm was measured. The viability of NHFs was calculated using the following Eq. (20).

$$\text{Cell viability (\%)} = \frac{\text{Abs. of the treated group}}{\text{Abs. of the untreated group}} \times 100 \quad (20)$$

3.3.6.4.2 Cell proliferation

For the proliferation assay, the sample solutions were produced by soaking the patches in a culture medium same as the cell viability test (Section 3.3.6.4.1). The NHFs were seeded in a 96-well plate before being incubated with the sample solution for 24, 48, and 72 h. Then, the medium was replaced with an MTT solution in

DMEM+ and further incubated for 4 h. The OD at 550 nm was measured, and the percentage of cell proliferation was calculated according to Eq. (21).

$$\text{Cell proliferation (\%)} = \frac{\text{Abs.of the treated group}}{\text{Abs.of the untreated group}} \times 100 \quad (21)$$

3.3.6.4.3 Cell migration

The effect of the hydrogel patch on NHFs migration was assessed by *in vitro* scratch assay. The sample solutions were prepared by immersion of patches in the culture medium as previously described (Section 3.3.6.4.1). Cell seeding and scratch area on cell monolayer were generated as mentioned above (Section 3.3.3.3.4). Thereafter, 3 mL of sample solutions were added to each well covering the cells. Images of the beginning scratch areas and areas after treatment for 24, 48, and 72 h were observed under an inverted microscope (4x) (80). The percentage of wound closure was evaluated from the difference between scratch areas before and after treatment following Eq. (22).

$$\text{Wound closure (\%)} = \frac{\text{Wound area (t}_0\text{)} - \text{Wound area (t)}}{\text{Wound area (t}_0\text{)}} \times 100 \quad (22)$$

Where t_0 is the wound area after immediate scratching and t is the wound area after treatment for 24, 48, and 72 h.

3.3.7 Stability study

To analyze the stability study, the hydrogel patches were sterilized under UV radiation. Then, the patches were stored in well-closed and light protective packages before being kept at 5, 25 and 40 °C according to ICH guidelines. The physicochemical stabilities were evaluated depending on the periods of stability study (0, 1, 2, 3 and 6 months).

3.3.8 Statistical analysis

All experimental data were measured in triplicate (N=3). The data were shown as mean $\pm$ standard deviation (SD). The significant differences between groups were analyzed using a one-way analysis of variance (ANOVA). The significant difference was considered at $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Appearance and characterization of plant extracts

The *B. rotunda* extract exhibited a sticky feature and had yellow-brown color, while the *D. bulbifera* extract was red-brown powder as shown in **Figure 9** (A and B).

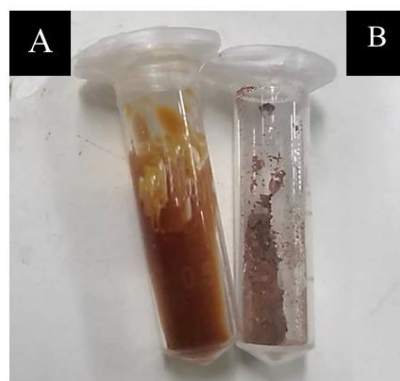


Figure 9 The appearances of *B. rotunda* (A) and *D. bulbifera* (B) extracts.

4.1.1 FT-IR, DSC and percent yield of the plant extracts

The FT-IR spectrum of *B. rotunda* extract is shown in **Figure 10**. The distinct peaks were observed at around 1500 – 1700 and 1150 cm^{-1} representing C=O stretching and C–O stretching, respectively. The results were similar to *B. rotunda* extracted with ethanol that was previously reported (81).

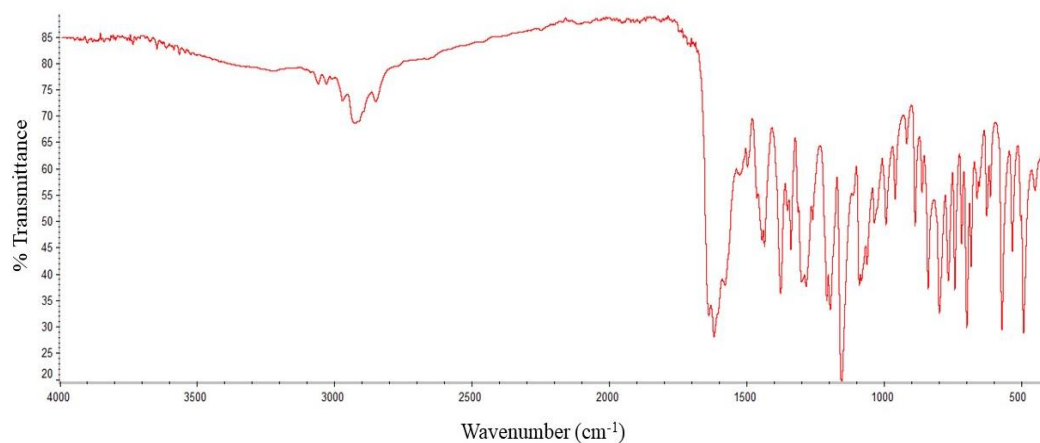


Figure 10 FTIR spectrum of *B. rotunda* extract.

Figure 11 shows the FT-IR spectrum of *D. bulbifera* extract. The obvious peaks appeared at approximately 1050 and 1600 cm^{-1} that are attributed to C–O stretching and C=C stretching, respectively.

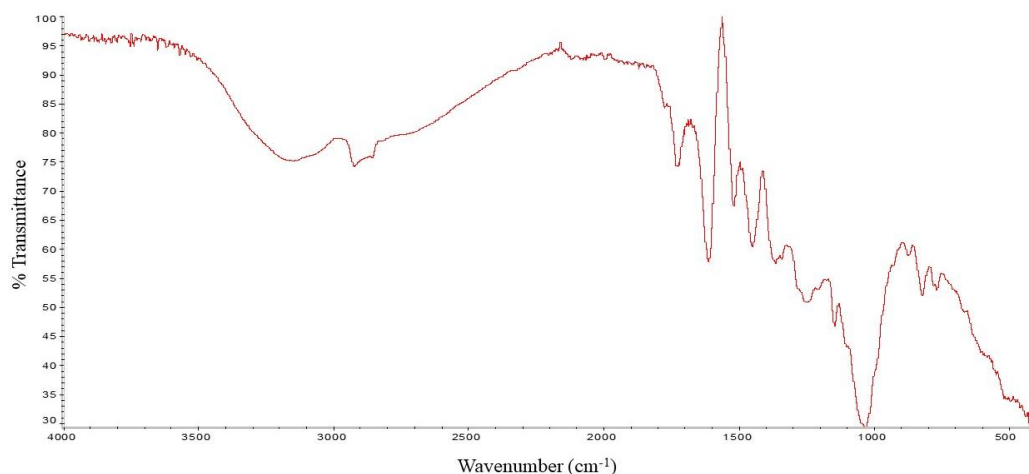


Figure 11 FTIR spectrum of *D. bulbifera* extract.

The DSC thermogram of *B. rotunda* extract is shown in **Figure 12**. Three endothermic peaks were observed. The dehydration peak appeared at approximately 94 °C. The peak at about 232 °C was the melting temperature (T_m) of the extract. The peak at about 330 °C might be the degradation temperature.

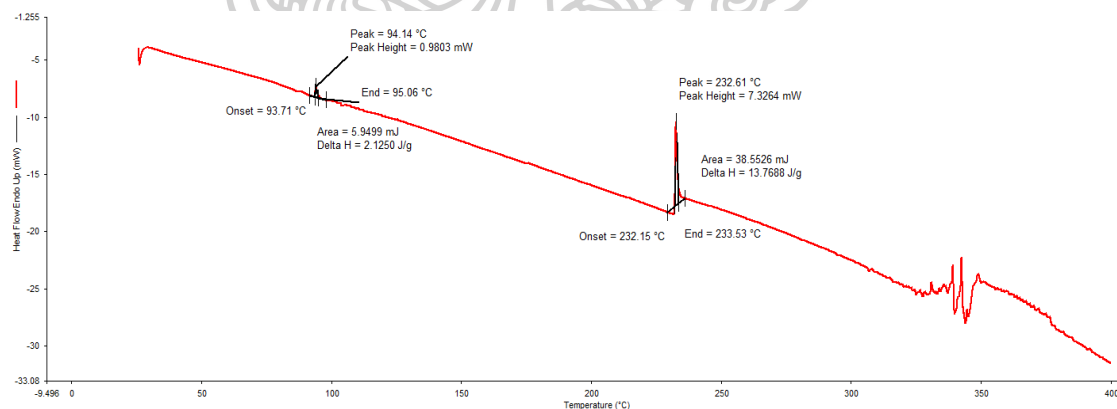


Figure 12 DSC thermograms of *B. rotunda* extract.

The endothermic peak of *D. bulbifera* extract is exhibited in **Figure 13**. Only one peak was observed at approximately 128 °C which might be the melting point of the extract. The degradation temperature of *D. bulbifera* extract could not be seen.

The percent yields of the plant extracts were computed from the percent weight ratio of the dried extract and the plant powder. The percent yield of *B. rotunda*

extracted with 70 % v/v ethanol for 7 days was 3.61 %. The *D. bulbifera* extracted with 80 % v/v ethanol for 15 days has a slightly higher percent yield of 7.47 %.

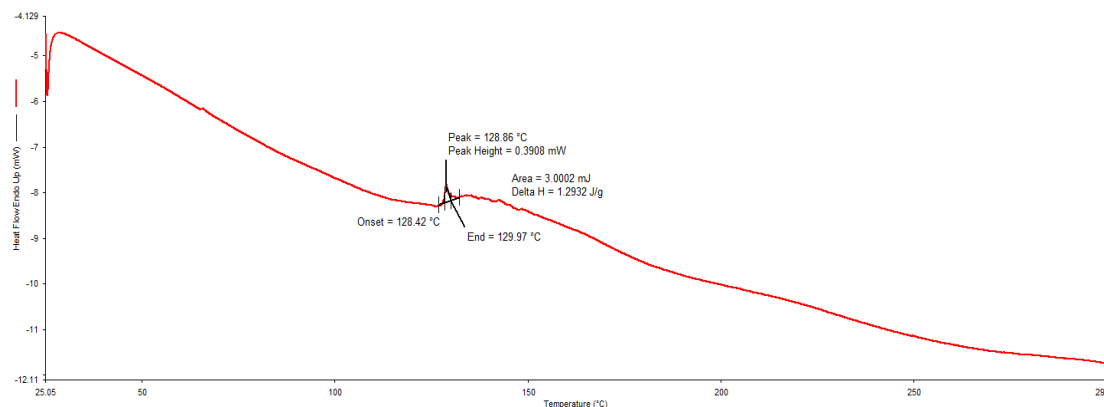


Figure 13 DSC thermograms of *D. bulbifera* extract.

4.1.2 Standardization of the extracts using HPLC

4.1.2.1 *B. rotunda* extract

The active substances, pinostrobin and pinocembrin, were analyzed by HPLC with UV detector operated at 285 nm. The HPLC chromatograms showed the peaks of pinostrobin and pinocembrin at the retention time of approximately 34 and 16 min (Figure 14). The crude extract was composed of 29.86 ± 0.19 and 3.70 ± 0.02 % w/w of pinostrobin and pinocembrin, respectively.

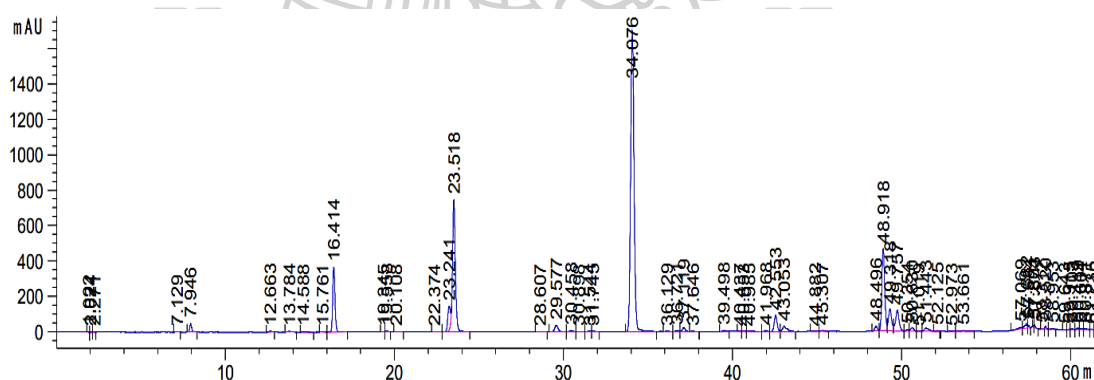


Figure 14 The HPLC chromatogram of *B. rotunda* extract.

4.1.2.2 *D. bulbifera* extract

Figure 15 shows that diosgenin could not be separated from *D. bulbifera* crude extract whereas the pure substance appeared at around 17.5 min.

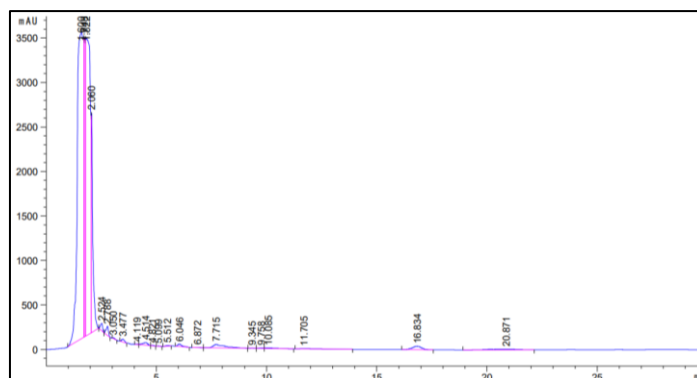


Figure 15 The HPLC chromatogram of *D. bulbifera* extract.

4.2 Biological activities of extracts

4.2.1 Antimicrobial activities

The antibacterial activities of *B. rotunda* and *D. bulbifera* extracts against *S. aureus* and *E. coli* were assessed. Gentamycin solution was used as a positive control. The MIC data of *B. rotunda* extract against both bacteria could not be observed because the extract could not be completely dissolved in TSB (**Table 1** and **Table 2**). *B. rotunda* extract exhibited bactericidal activity against *S. aureus* with the minimum concentration (MBC) of 156.25 ± 0.00 $\mu\text{g/mL}$. On the other hand, *D. bulbifera* extract was not able to inhibit both bacterial growths.

Table 1 Antimicrobial activities of *B. rotunda* and *D. bulbifera* extracts against *S. aureus*.

Samples	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>B. rotunda</i>	N/A	156.25 ± 0.00
<i>D. bulbifera</i>	> 5,000	> 5,000
gentamycin	13.02 ± 5.64	39.06 ± 0.00

Table 2 Antimicrobial activities of *B. rotunda* and *D. bulbifera* extracts against *E. coli*.

Samples	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>B. rotunda</i>	N/A	> 5,000
<i>D. bulbifera</i>	> 5,000	> 5,000
gentamycin	39.06 ± 0.00	156.25 ± 0.00

4.2.2 Antioxidant activity

The antioxidant property is one of the important points that could enhance the wound healing process by reducing free radicals from oxidative stress. The antioxidant activities of *B. rotunda*, *D. bulbifera*, and positive control; ascorbic acid are shown in **Figure 16**. Both plant extracts exhibited antioxidant activities against DPPH radicals.

The IC₅₀ indicates the fifty percent inhibition of the substance. The IC₅₀ of extracts and positive control are listed in **Table 3**. *D. bulbifera* extract had a lower IC₅₀ value indicating a higher antioxidant activity than *B. rotunda* extract. Ascorbic acid was employed as a positive control and showed the lowest IC₅₀ (5.90 µg/mL).

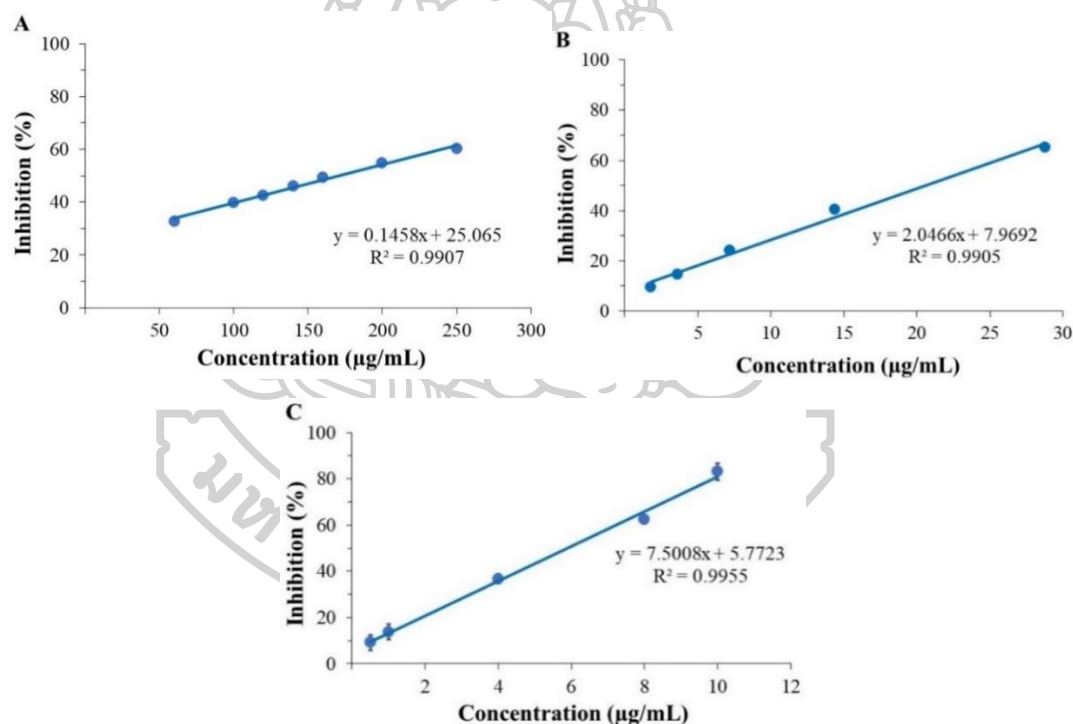


Figure 16 Antioxidant activities of (A) *B. rotunda* extract, (B) *D. bulbifera* extract and (C) ascorbic acid.

4.2.3 Cell viability, proliferation, and migration

4.2.3.1 *B. rotunda* extract

The viability of NHFs exposed to *B. rotunda* extract solution was evaluated using an MTT assay. Viability of the untreated fibroblasts was employed as a control

and regarded as 100 % viability. Viabilities of NHFs after incubation with 0.025 – 150

µg/mL of *B. rotunda* extract for 24 h are shown in **Figure 17**. Viabilities of NHFs treated with *B. rotunda* extract solutions with a concentration between 0.025 – 10 µg/mL were not significantly different compared with the control. The *B. rotunda* extract at the concentrations of ≤ 10 µg/mL exhibited non-toxicity. On the contrary, the *B. rotunda* extract at the concentrations ≥ 50 µg/mL showed significant toxicity to fibroblast cells with the cell viability less than 40 %. Therefore, the *B. rotunda* extract with a concentration between 0.1 – 10 µg/mL was selected for intensive studies.

Table 3 Antioxidant activities of *B. rotunda* extract, *D. bulbifera* extract and ascorbic acid.

Sample	IC ₅₀ (µg/ml)
<i>B. rotunda</i>	171.02
<i>D. bulbifera</i>	20.54
Ascorbic acid	5.90

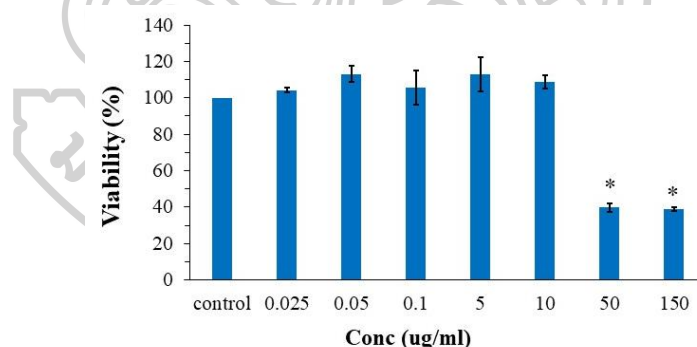


Figure 17 Viability of NHFs after incubation with 0.025 – 150 µg/mL of *B. rotunda* extract. * p-value < 0.05 compared with untreated cells (control group).

The NHF proliferations after *B. rotunda* extract treatment for 24, 48 and 72 h are presented in **Figure 18**. After incubation for 24 h, all samples demonstrated no toxicity. Moreover, the extract concentration between 0.5 – 5 µg/mL could significantly promote NHF growth. Test sample with 10 µg/mL inhibited NHF

proliferation after 48-h treatment. In addition, test samples except 1 $\mu\text{g/mL}$ significantly suppressed NHF increase after 72-h incubation.

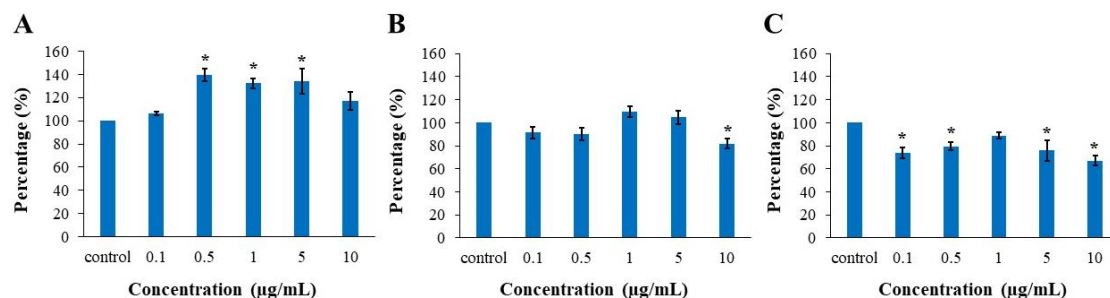
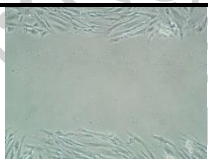
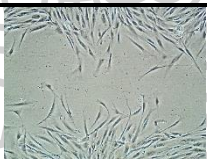
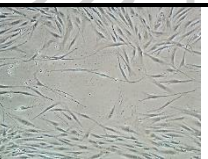
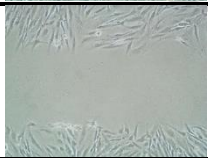
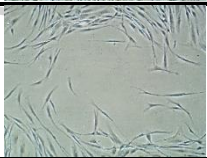
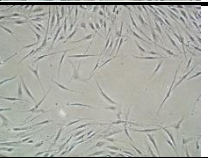
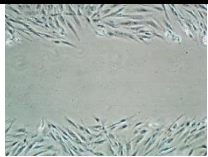
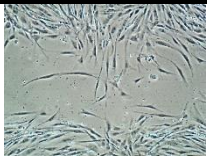
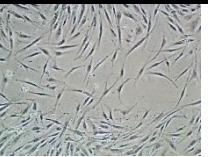


Figure 18 Proliferation of NHFs after incubation with 0.1 – 10 $\mu\text{g/mL}$ of *B. rotunda* extract at (A) 24-, (B) 48-, and (C) 72 h. * p-value < 0.05 compared with untreated cells (control group).

From NHF viability and proliferation data, *B. rotunda* extract with a concentration of 1 $\mu\text{g/mL}$ was chosen for the migration test. The TGF- β 1 with a concentration of 1 ng/mL was used as the positive control. The scratch areas of untreated and treated NHFs are shown in **Table 4**.

Table 4 Migration images of NHFs treated with *B. rotunda* extract and controls.

Samples	Migration test			
	0h	24h	48h	72h
Control				
<i>B. rotunda</i> extract				
TGF- β 1				

The percentage of wound closure calculated from the difference in scratch areas before and after treatment is presented in **Figure 19**. The migrations of NHFs treated with *B. rotunda* were not significantly different from untreated cells. The

TGF- β 1 significantly enhanced NHFs migration at 24 and 48 h whereas the result was not different from other groups at 72 h.

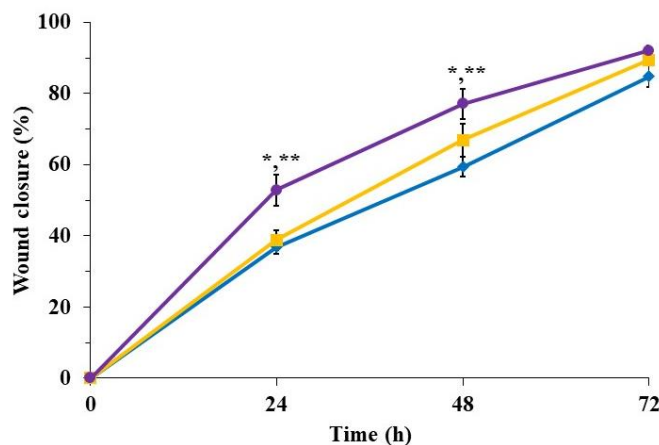


Figure 19 The wound closures of scratch areas of (■) control (untreated cells) and treated cells with sample solutions: (●) TGF- β 1 (positive control) and (◆) *B. rotunda* extract. p-value < 0.05 compared to untreated cells (*) and *B. rotunda* extract (**) at the same treatment time.

4.2.3.2 *D. bulbifera* extract

The viability of NHFs after 24-h incubation with *D. bulbifera* extract solution was investigated using MTT assay. The test procedure was performed similar to the test of *B. rotunda* extract described above. Growth of NHFs treated with *D. bulbifera* extract solution increased at the high doses of the extract (**Figure 20**). Significantly enhanced cell viability was observed when the cells were treated with *D. bulbifera* extracts solution at the initial concentration of 100 μ g/ml. The results explained the positive effects of the *D. bulbifera* extract solution on NHF viability. Whereas the *D. bulbifera* extract at the concentrations below 50 μ g/mL were selected for further studies due to precipitation of the extract at high concentrations, which may disturb the enlargement and movement of NHF cells.

The proliferation of NHFs treated with *D. bulbifera* extract is displayed in **Figure 21**. The *D. bulbifera* extract could not inhibit NHF growth compared with the control. Moreover, the extract with a concentration of 10 μ g/mL significantly enhanced cell proliferation. Therefore, the *D. bulbifera* extract at the concentration of 10 μ g/mL was used for the migration study.

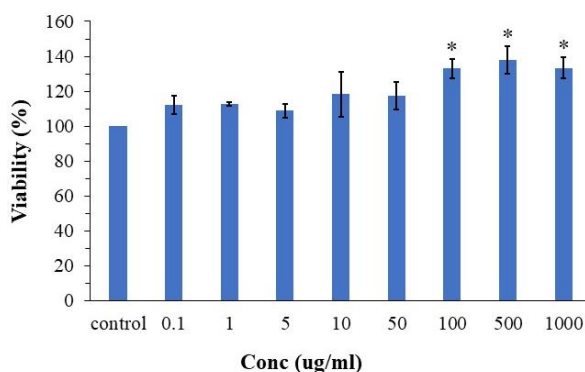


Figure 20 Viability of NHFs after incubation with 0.1 – 1,000 µg/mL of *D. bulbifera* extract. * p-value < 0.05 compared with untreated cells (control group).

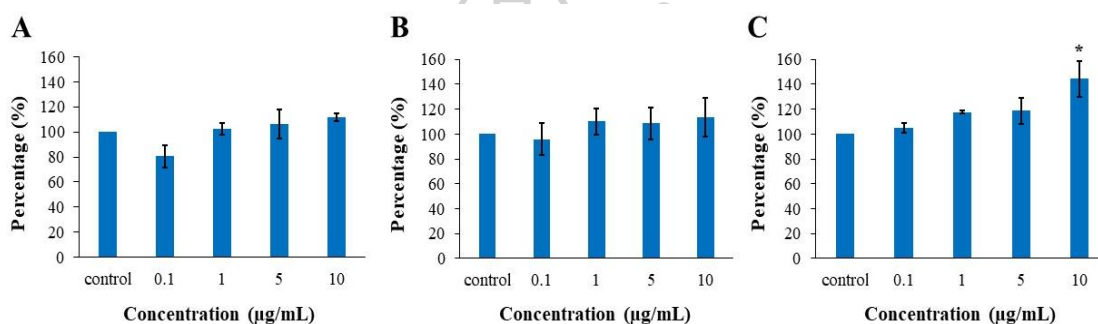


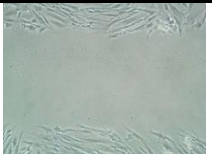
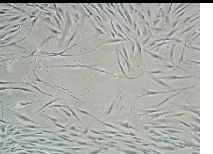
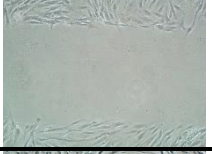
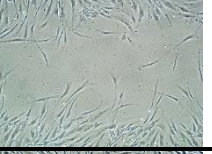
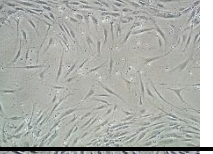
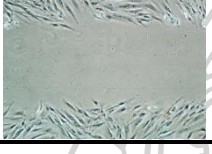
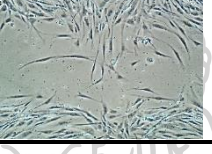
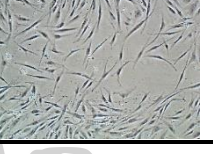
Figure 21 The Proliferation of NHFs after incubation with 0.1 – 10 µg/mL of *D. bulbifera* extract at (A) 24-, (B) 48-, and (C) 72 h. * p-value < 0.05 compared with untreated cells (control group).

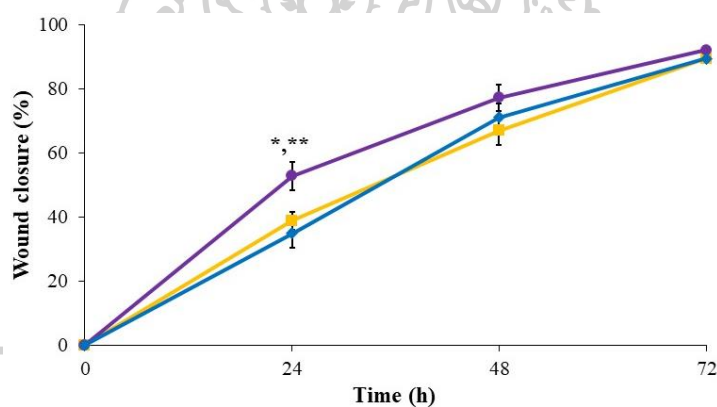
The effect of *D. bulbifera* extract on NHF migration is exhibited in **Table 5** and **Figure 22**. The *D. bulbifera*-treated group had a higher tendency to increase wound closure compared with the control but they were not significantly different at all incubated periods. The TGF-β1 (positive control) significantly enhanced NHFs movement at 24 whereas the results were not different from other groups at 48 and 72 h.

4.3 Hydrogel patches fabricated by the physical crosslinking method

The *B. rotunda* extract-loaded PVA hydrogel patches were designed by experimental analysis. A Box-Behnken model with 3 factors and 3 levels was selected for the physical crosslinking method. Seventeen designed experiments with 3 fabrication variables and 6 observed responses are shown in **Table 6**.

Table 5 Migration images of NHFS treated with *D. bulbifera* extract and controls.

Samples	Migration test			
	0h	24h	48h	72h
Control				
<i>D. bulbifera</i> extract				
TGF-β1				

**Figure 22** The wound closures of scratch areas of (■) control (untreated cells) and treated cells with sample solutions: (●) TGF-β1 (positive control) and (◆) *D. bulbifera* extract. p-value < 0.05 compared to untreated cells (*) and *B. rotunda* extract (**) at the same treatment time.

Multiple regression analysis ($p < 0.05$) was performed on 6 response data to generate polynomial quadratic equations (**Table 7**). The effects of three variables including FT cycle, PVA and *B. rotunda* concentrations on considered responses are presented as coefficients of various terms. A positive value of the coefficient indicates an enhancement effect, whereas a negative one represents a diminution effect on hydrogel patch responses. The suitable equation was deliberated from the coefficient of determination (R^2).

Table 6 The designed experiments with 3 fabrication variables and 6 observed responses.

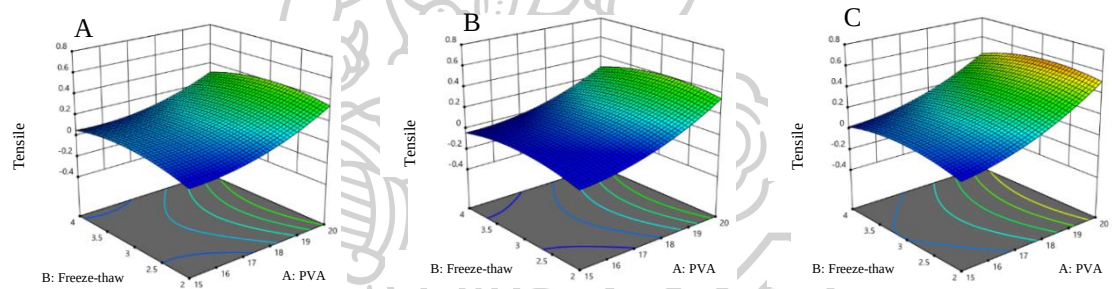
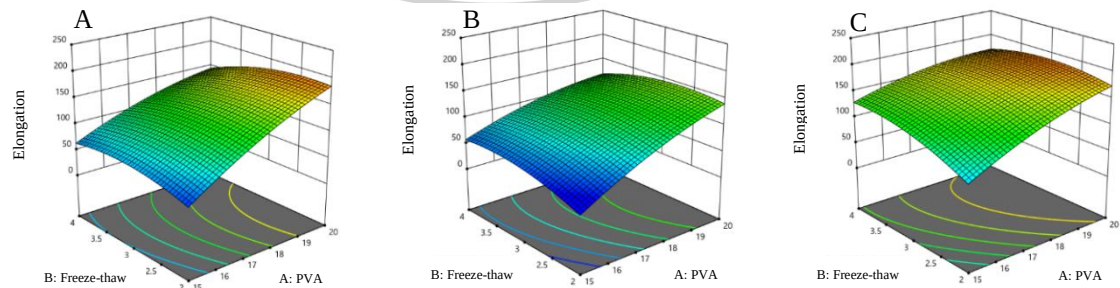
Run	Variables			Observed responses					
	A: PVA (%)	B: FT (cycles)	C: Extract (%)	Y ₁ : Tensile strength (MPa)	Y ₂ : Elongation (%)	Y ₃ : Young's modulus (MPa)	Y ₄ : Water content (%)	Y ₅ : Water absorption (%)	Y ₆ : Erosion (%)
1	20	3	50	0.638	195.99	0.350	69.39	108.59	15.40
2	15	3	30	0.028	62.70	0.048	81.40	48.55	46.46
3	17.5	3	40	0.136	117.72	0.139	77.29	110.63	31.39
4	17.5	2	50	0.141	150.20	0.130	72.41	123.72	35.78
5	15	2	40	0.018	37.94	0.042	80.84	57.24	48.23
6	20	4	40	0.199	105.37	0.216	70.77	90.97	14.47
7	17.5	4	30	0.121	101.90	0.161	76.19	83.65	21.03
8	17.5	2	30	0.073	123.16	0.086	76.19	120.38	45.81
9	20	2	40	0.297	118.48	0.269	70.59	100.00	24.05
10	17.5	3	40	0.129	128.04	0.146	77.33	106.88	31.07
11	20	3	30	0.483	208.15	0.291	73.29	104.22	17.91
12	15	4	40	0.019	79.63	0.033	79.11	80.71	28.53
13	17.5	4	50	0.153	167.42	0.143	73.37	120.80	27.89
14	15	3	50	0.024	90.09	0.033	79.53	63.16	45.73
15	17.5	3	40	0.129	111.64	0.161	76.06	108.57	33.94
16	17.5	3	40	0.120	99.52	0.146	77.87	102.70	34.87
17	17.5	3	40	0.111	104.45	0.131	76.92	100.55	30.95

All 17 formulation of *B. rotunda* extract-loaded PVA hydrogel dressings were prepared according to the experimental designed formula. Tensile strength (Y₁) values of hydrogel patches were 0.018 – 0.638 MPa. From the response surface plot for Y₁ (**Figure 23**) and polynomial equation for Y₁ (**Table 7**), results show that PVA content and loading content of *B. rotunda* extract affected the strength of hydrogel patches. The positive values of b₁ and b₃ presented the strength of the dressing enhanced when PVA concentration and extract loading increased. Moreover, the concentration of PVA had more impact on the strength than the extract concentration. In contrast, negative values of b₂ showed a slight decrease in effect on hydrogel strength.

Elongation at the breaking point of experimental dressing (Y₂) was in the range of 37.94 - 208.15 %. The coefficient values of A (b₁ = 44.70), B (b₂ = 3.07) and C (b₃ = 13.47) presented multiple effects on elongation. From the response surface plot in **Figure 24**, all fabrication factors influenced elongation. Moreover, compared with the loading content of the extract and the FT cycle, the PVA content had the highest impact on the elongation.

Table 7 Polynomial equations created from Box-Behnken design.

$Y_i = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{23}BC + b_{13}AC + b_{11}A^2 + b_{22}B^2 + b_{33}C^2$						
Coefficients	Y ₁	Y ₂	Y ₃	Y ₄	Y ₅	Y ₆
	Tensile strength (MPa)	Elongation (%)	Young's Modulus (MPa)	water content (%)	Water absorption (%)	Erosion (%)
b ₀	0.1250	112.27	0.1448	77.09	105.87	32.44
b ₁	0.1910	44.70	0.1212	-4.61	19.27	-12.14
b ₂	-0.0046	3.07	0.0033	-0.0738	-3.15	-7.74
b ₃	0.0314	13.47	0.0088	-1.55	7.43	-0.8013
b ₁₂	-0.0248	-13.70	-0.0108	0.4775	-8.13	2.53
b ₂₃	0.0398	-9.89	0.0188	-0.5075	-2.56	-0.4450
b ₁₃	-0.0090	9.62	-0.0152	0.2400	8.45	4.22
b ₁₁	0.0897	-11.68	0.0226	-0.2020	-27.32	-2.44
b ₂₂	-0.0815	-15.24	-0.0277	-1.56	3.69	-1.19
b ₃₃	0.0785	38.64	0.0129	-0.9895	2.59	1.37
p-value	0.0132	0.0211	0.0007	< 0.0001	0.0082	0.0003
R ²	0.8869	0.8685	0.9540	0.9872	0.9025	0.9636

**Figure 23** Response surface plots for the optimization of the tensile strength (Y₁); (A) C = 30, (B) 40, and (C) 50 % w/w.**Figure 24** Response surface plots for the optimization of elongation (Y₂); (A) C = 30, (B) 40, and (C) 50 % w/w.

Young's Modulus of dressing (Y_3) was reported in the range of 0.033 – 0.350 MPa (**Table 6**). From the polynomial equation explained in **Table 7**, the positive coefficients of A ($b_1 = 0.1212$), B ($b_2 = 0.0033$) and C ($b_3 = 0.0088$) exhibited an increased effect on Young's Modulus. When PVA concentration, extract loading and number of FT cycles were increased, Young's Modulus was also higher (**Figure 25**). The augmentation of Young's Modulus is related to the higher strength of dressings (Y_1) caused by high PVA concentration and *B. rotunda* extract loading.

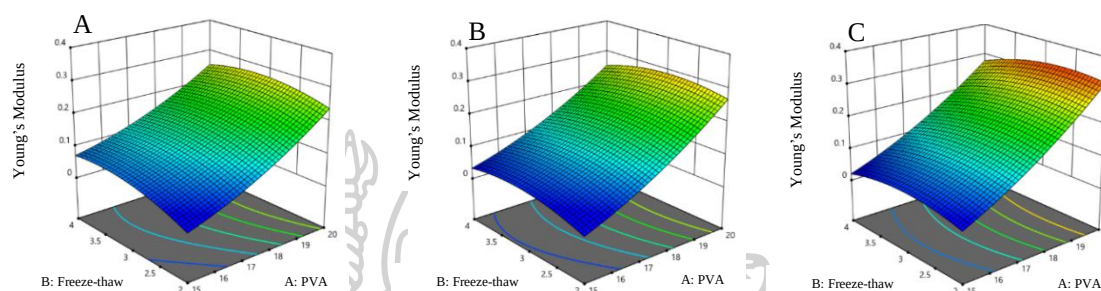


Figure 25 Response surface plots for the optimization of Young's Modulus (Y_3); (A) $C = 30$, (B) 40 and (C) 50 % w/w.

The water content of hydrogel patch preserves a moist environment at the wound bed and may lead to improvement of the healing process. Water content (Y_4) of the *B. rotunda* extract-loaded hydrogel patch was determined to be in the range of 69.39 – 81.40 % (**Table 6**). The polynomial equation and response surface plot for Y_4 are generated and described in **Table 7** and **Figure 26**.

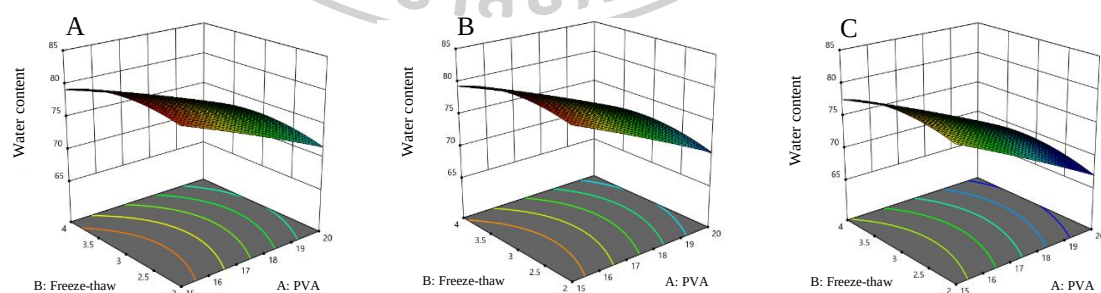


Figure 26 Response surface plots for the optimization of water content (Y_4); (A) $C = 30$, (B) 40 and (C) 50 % w/w.

It was confirmed that all 3 fabrication factors decreased moisture content. Water content reduction was predominantly affected by PVA concentration (A), followed by extract loading (C) and freeze-thaw cycle (B), respectively

Water absorption property illustrated wound exudate absorption of hydrogel patch. Water absorption values of patches (Y_5) were 48.55 – 123.72 %. The response surface plot and polynomial equation for Y_5 are illustrated in **Figure 27** and **Table 7**. The positive values of b_1 (19.27) and b_3 (7.43) showed that the water absorption improved when the PVA content and the loading content of the extract was increased. While negative values of b_2 (-3.15) showed a decrease effect (FT cycle) on the water absorption property of hydrogel patches.

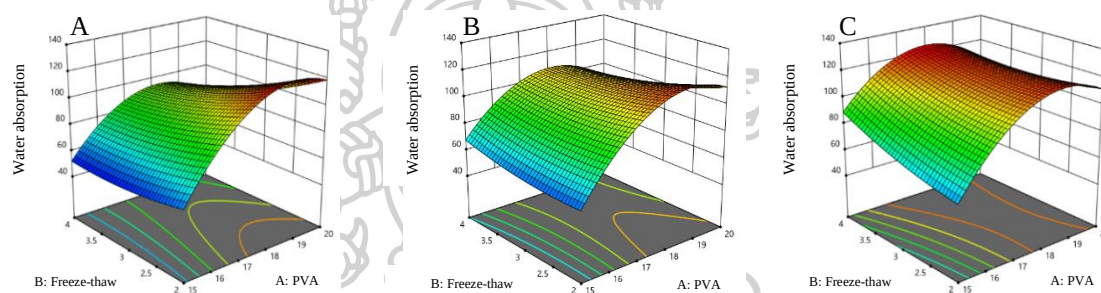


Figure 27 Response surface plots for the optimization of water absorption (Y_5); (A) C = 30, (B) 40 and (C) 50 % w/w.

The erosion property describes the crosslinking proportion of raw material in the hydrogel patch. Erosions of the patches (Y_6) were in the range of 14.47 – 48.23 %. The polynomial equation and response surface plot of erosion are presented in **Table 7** and **Figure 28**. All fabrication factors distinctly influenced the erosion of the hydrogel patch. PVA concentration (A) exhibited a more eminent impact followed by FT cycle (B) and *B. rotunda* extract loading (C), respectively. These results corresponded to tensile strength and Young's Modulus data that higher PVA concentration, higher *B. rotunda* extract loading and greater cycles of FT provided the higher strength patch structure with lower erosion.

The optimized fabrication variables of *B. rotunda* extract-loaded PVA hydrogel patches were as follows: A (PVA concentration) = 17.07 % w/w, B (FT cycle) = 3.86 cycles and C (*B. rotunda* solution loading) = 50.00 % w/w (1.00 % w/w of dried extract). The predicted properties of the optimized hydrogel patch were Y_1

(tensile strength) = 0.130 MPa, Y_2 (elongation) = 159.75 %, Y_3 (Young's Modulus) = 0.114 MPa, Y_4 (water content) = 74.36 %, Y_5 (swelling) = 120.65 % and Y_6 (erosion) = 30.71 %.

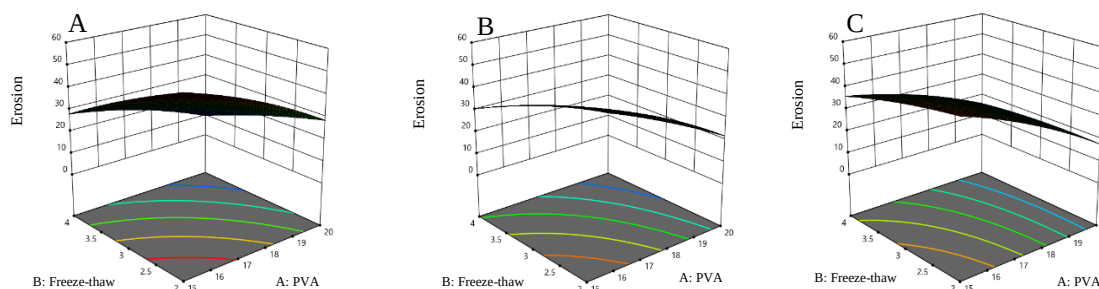


Figure 28 Response surface plots for the optimization of erosion (Y_6); (A) $C = 30$, (B) 40 and (C) 50 % w/w.

The optimized hydrogel patch was prepared and then characterized. The physical properties and biological activities of *B. rotunda* loaded and unloaded PVA hydrogel patches were compared. Images of *B. rotunda* loaded and unloaded PVA hydrogel patches are shown in **Figure 29**. The unloaded patch was colorless, while the *B. rotunda* loaded patch had a light-orange color.

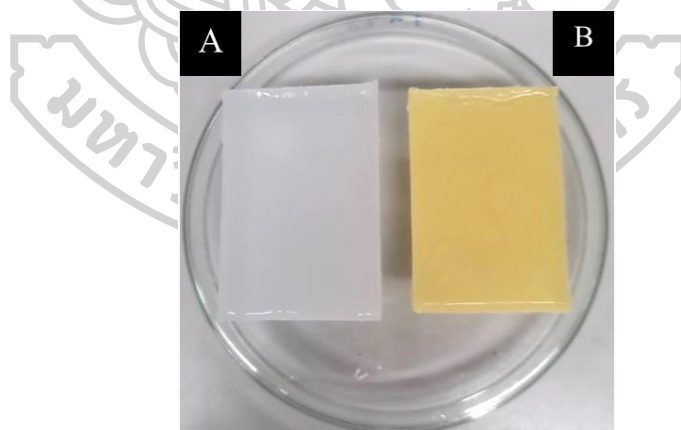


Figure 29 Images of (A) unloaded and (B) *B. rotunda* loaded hydrogel patches.

4.3.1 FT-IR spectra

The spectra of *B. rotunda* loaded and unloaded hydrogel patches are presented in **Figure 30**.

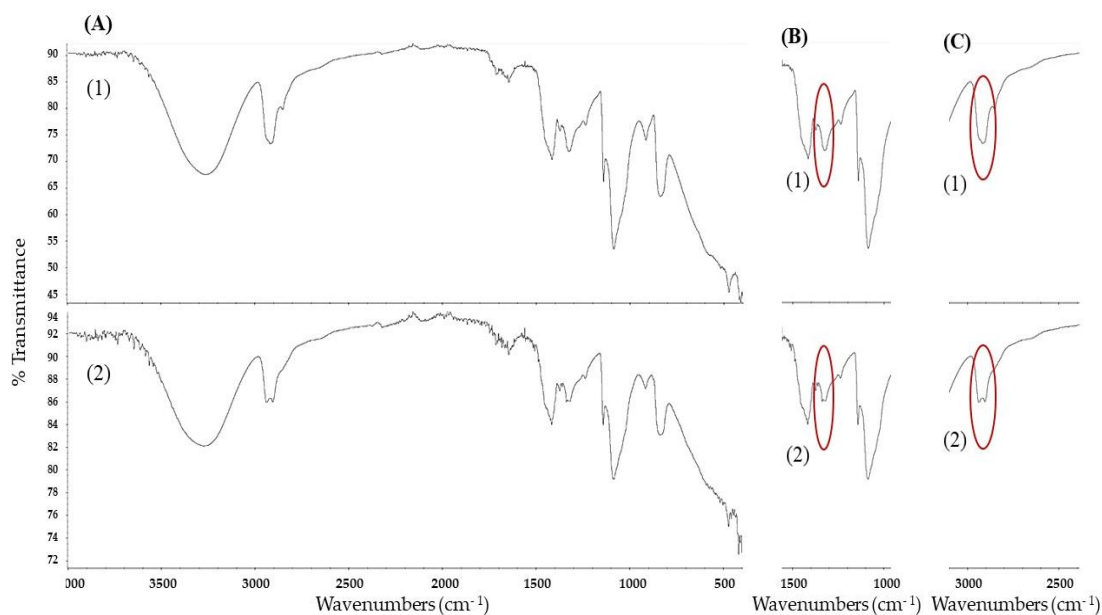


Figure 30 (A) FT-IR spectra of (1) unloaded and (2) *B. rotunda* loaded hydrogel patches. Enlarged FT-IR spectra at approximately (B) 1300 – 1400 and (C) 2900 cm^{-1} .

The broad peaks of loaded and unloaded hydrogel patches at approximately 3300 – 3400 cm^{-1} were the O–H group which is the main functional group in PVA. After the *B. rotunda* extract was loaded into the PVA hydrogel patch, the new peaks were observed at around 1300 – 1400 and 2900 cm^{-1} that was signified C–O and C–H bonds, respectively. Therefore, the new bands confirmed the crosslinking between PVA and *B. rotunda* extract after FT cycles.

4.3.2 Photographs and SEM images

The hydrogel patches were dried by the CPD method. Briefly, the water in the hydrogels was replaced with ethanol. The hydrogel patches were cut into trapezium pieces and then soaked in concentration series of ethanolic solvents starting from 35 to 100 % w/w (**Figure 31**). In the end, sample pieces were removed from absolute ethanol and dried.

The dried samples were sputtered with gold and observed using SEM. The SEM images of *B. rotunda* loaded and unloaded hydrogel patches are presented in **Tables 8** and **9**.

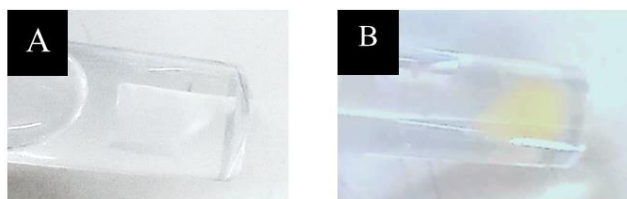


Figure 31 Images of unloaded (A) and *B. rotunda* loaded (B) hydrogel patches in absolute ethanol for the CPD method.

Table 8 The surface images of *B. rotunda* loaded and unloaded hydrogel patches observed from SEM.

Samples	X 1,000	X 20,000	X 50,000
The <i>B. rotunda</i> loaded hydrogel patch			
The unloaded hydrogel patch			

Surface morphologies of *B. rotunda* loaded and unloaded hydrogels were different, especially observation at a high magnifying power. The surface of unloaded hydrogels contained PVA networks with large pores, while the *B. rotunda*-loaded hydrogel structures resembled PVA networks covered with the extract. Internal structures of *B. rotunda*-loaded PVA hydrogels were similar to the unloaded PVA hydrogel whereas the networks of *B. rotunda*-loaded PVA hydrogels were slightly smaller than the unloaded hydrogels, which might be due to the crosslinking between the polymer observed from FT-IR spectra.

4.3.3 Pinostrobin or Pinocembrin analysis

The chemical structures of pinostrobin and pinocembrin are displayed in **Figure 32**. Pinostrobin and pinocembrin incorporated in hydrogel patches were

analyzed using the HPLC technique. The percentages of LE and LC were measured and calculated. The percents of LE and LC of pinostrobin were 95.40 ± 0.58 and 0.25 ± 0.00 %, respectively. The LE and LC of pinocembrin were 99.26 ± 2.68 and 0.04 ± 0.00 %, respectively. The release of extract from the *B. rotunda* extract-loaded hydrogel patch is demonstrated in **Figure 33**.

Table 9 The cross-section images of *B. rotunda* loaded and unloaded hydrogel patches observed from SEM.

Samples	X 1,000	X 20,000	X 50,000
The <i>B. rotunda</i> loaded hydrogel patch			
The unloaded hydrogel patch			

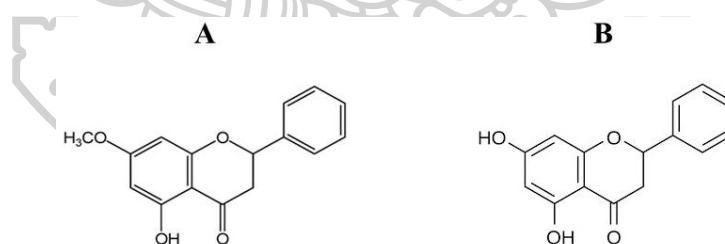


Figure 32 Chemical structures of (A) pinostrobin and (B) pinocembrin.

B. rotunda extract was rapidly released at the initial time with 3.06 ± 0.13 , 7.48 ± 0.64 and 9.57 ± 0.35 % at 0.5, 3 and 6 h, respectively. Moreover, the release was slower since 9 h and the release was 11.02 ± 0.10 % at 24 h.

4.3.4 Water content, water absorption, erosion and WVTR

The physical properties of *B. rotunda* loaded and unloaded hydrogel patches including water content, water absorption, erosion and WVTR were characterized and

are presented in **Table 10**. The water content of the *B. rotunda* loaded hydrogel patch was 72.58 ± 0.69 % which was significantly lower than the unloaded patch. The water absorption property of the hydrogel patch demonstrated exudate displacement from the wound bed. Absorption of *B. rotunda* extract loaded hydrogel patch resembled higher than unloaded whereas the difference was not significant. The erosion of both hydrogel patches was not statistically different. Thus, *B. rotunda* extract loaded, and unloaded patches consisted of a similar crosslinking hydrogel network.

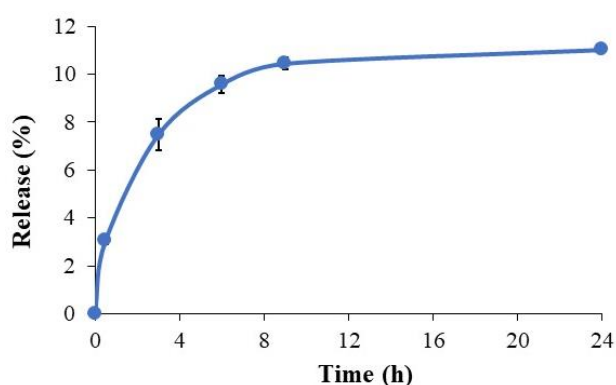


Figure 33 Release curve of extract from *B. rotunda* extract loaded hydrogel patch.

Table 10 Water content, water absorption, erosion and WVTR of *B. rotunda* loaded and unloaded hydrogel patches.

Samples	Water content (%)	Water absorption (%)	Erosion (%)	WVTR (g/m ² /day)
<i>B. rotunda</i> extract loaded hydrogel patch	$72.58 \pm 0.69^*$	111.48 ± 6.52	25.12 ± 1.29	$1,463.64 \pm 31.82^*$
unloaded patch	75.64 ± 0.86	100.33 ± 5.57	28.67 ± 1.97	$1,251.52 \pm 80.07$

* p -value < 0.05 compared with unloaded patch

WVTR of the hydrogel patch was also evaluated using a bottle filled with water. The vapor evaporated through the hydrogel patches that covered the bottle mouths are shown in **Figure 34**. The *B. rotunda* extract-loaded hydrogel patch illustrated significantly higher WVTR than the unloaded hydrogel patch.

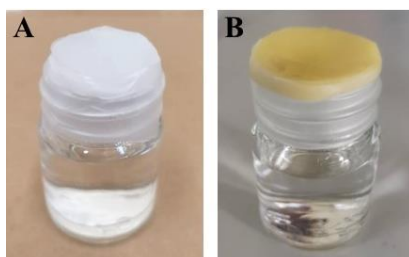


Figure 34 Photographs of bottles covered with hydrogel patches for WVTR assay; (A) unloaded and (B) *B. rotunda* extract loaded hydrogel patches.

4.3.5 Mechanical properties

The strength and expansion abilities of hydrogel patches were investigated using a texture analyzer. The sample strips were fixed between two grips under the load cell and then stretch until they were broken (**Figure 35**). The mechanical values including tensile strength, Young's Modulus and elongation at the breaking point are shown in **Table 11**.

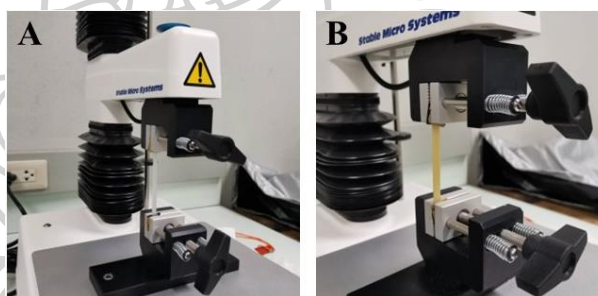


Figure 35 Photographs of the tensile test; (A) unloaded and (B) *B. rotunda* extract loaded hydrogel patches.

Table 11 Mechanical properties of the hydrogel patches.

Samples	Young's Modulus (MPa)	Tensile Strength (MPa)	Elongation (%)
<i>B. rotunda</i> extract loaded hydrogel patch	$0.095 \pm 0.005^*$	$0.145 \pm 0.016^*$	$189.30 \pm 13.17^*$
unloaded patch	0.075 ± 0.004	0.039 ± 0.006	56.42 ± 5.75

* p -value < 0.05 compared with unloaded patch

The *B. rotunda* extract-loaded hydrogel dressing exhibited higher tensile strength, Young's Modulus and elongation than the unloaded patch. Thus, it was proved that *B. rotunda* extract was able to enhance the strength and elongation of the PVA hydrogel patch.

4.3.6 Biological activities

4.3.6.1 Antimicrobial activities

The antimicrobial activity of the *B. rotunda* extract-loaded hydrogel patch was determined by qualitative and quantitative methods. For the qualitative method, a disc diffusion assay was selected to investigate antimicrobial activities against *S. aureus* and *E. coli*. The unloaded hydrogel patch and Aquacel™ Ag+ Extra were used as the negative and positive control. Photographs of the inhibition zone of hydrogel and control discs (6-mm diameter) against *S. aureus* and *E. coli* are shown in **Figures 36** and **37**, respectively. An inhibition zone against *S. aureus* was observed around the extract-loaded hydrogel discs with a diameter of 7.33 ± 0.58 mm, while the clear zone of the extract-loaded hydrogels against gram-negative *E. coli* could not be noticed according to the results by Jitvaropas (6). The unloaded hydrogel disc could not inhibit both bacterial growths. In addition, Aquacel™ Ag+ Extra exhibited both bacterial growth inhibitions with clear zones of 13.67 ± 1.53 mm against *S. aureus* and 10.00 ± 0.00 mm against *E. coli*.

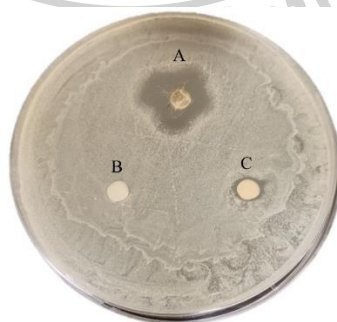


Figure 36 The photographs of inhibition zones against *S. aureus*; (A) Aquacel™ Ag+ Extra disc, (B) unloaded hydrogel disc and (C) *B. rotunda* extract loaded hydrogel disc.

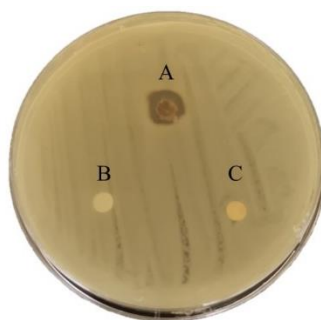


Figure 37 The photographs of inhibition zones against *E. coli*; (A) Aquacel™ Ag+ Extra disc, (B) unloaded hydrogel disc and (C) *B. rotunda* extract loaded hydrogel disc.

For the quantitative method, 50 mg of each hydrogel patch was soaked in the bacterial suspension. After treatment for 24 h, the result showed no inhibition of unloaded hydrogel against both bacteria, whereas the *B. rotunda* extract loaded hydrogel clearly inhibited both *S. aureus* and *E. coli* growths. The turbidities of bacterial suspensions at 600 nm significantly decreased after being treated with the *B. rotunda* extract-loaded hydrogels (**Figures 38 and 39**). The *B. rotunda* extract-loaded hydrogel could inhibit *S. aureus* and *E. coli* growths with the percent inhibition of 49.76 ± 3.82 % and 20.13 ± 2.75 %, respectively.

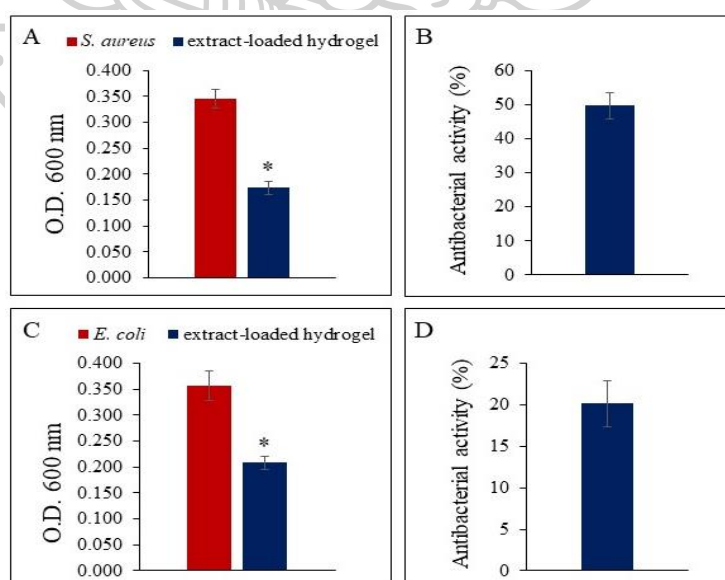


Figure 38 Antimicrobial activities of *B. rotunda* extract-loaded hydrogel patch against *S. aureus* (A and B) and *E. coli* (C and D) *p-values < 0.05 compared with bacterial suspensions.

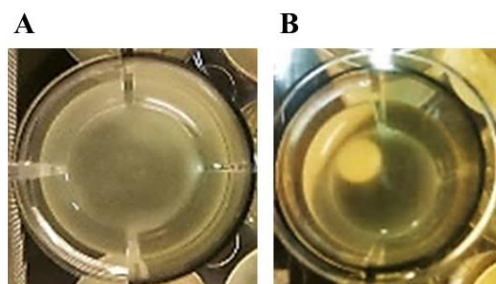


Figure 39 Antimicrobial activities of *B. rotunda* extract-loaded hydrogel patch against *S. aureus*; (A) bacterial suspension and (B) bacterial suspension treated with *B. rotunda* extract-loaded hydrogel patch.

4.3.6.2 Infiltration bacterial test

The property of hydrogel patches to be a barrier of wound bed from outside bacterial infection was investigated by infiltration test. **Figure 40 (A)** and **(B)** show the ability of the hydrogel patches to protect from *S. aureus* infiltration. The results confirmed inhibition of *S. aureus* infiltration in both groups, *B. rotunda* extract-loaded hydrogel patch, and the unloaded hydrogel patch. As shown in **Figure 40 (B)**, the turbidity of TSB incubated with agar pieces under the hydrogel patches was lower than those incubated with agar piece containing the bacteria and was not significantly different from the control agar piece without bacteria. Moreover, the *B. rotunda* extract-loaded hydrogel patches distinctly inhibited *E. coli* infiltration (**Figure 40 (C)** and **(D)**). After incubation in broth for 4 h, the broth incubated with agar piece containing *E. coli* showed the highest turbidity. On the other side, the turbidity of the broth incubated with the agar piece under the *B. rotunda* extract-loaded hydrogel patch was not significantly different from the control. For the broth incubated with the agar piece under an unloaded hydrogel patch, slight turbidity could be shown due to the *E. coli* overgrowth and colony expansion out of the patch as shown in **Figure 40 (C)**.

4.3.6.3 Antioxidant activity

The antioxidant activities of the hydrogel patches were assessed using a DPPH assay (**Table 12**) and the results are shown in **Figure 41**. The unloaded PVA hydrogel patches had low antioxidant activity. On the contrary, the antioxidant activity of the *B.*

rotunda extract-loaded hydrogel patches at 0.5-, 1-, 2-, 4- and 6-h treatment were 18.56 ± 3.48 , 59.18 ± 2.34 , 70.36 ± 1.71 and 75.00 ± 0.79 80.27 ± 0.96 %, respectively. The antioxidant activity after incubation significantly increased when incubation time increased. The antioxidant property of the dressing patch is advantageous for the wound healing process as the antioxidants help to maintain intracellular redox balance and normal inflammatory phase. Moreover, it also protects the endothelial cell, keratinocyte, and fibroblast dysfunction from traumatic oxidative stress (82, 83).

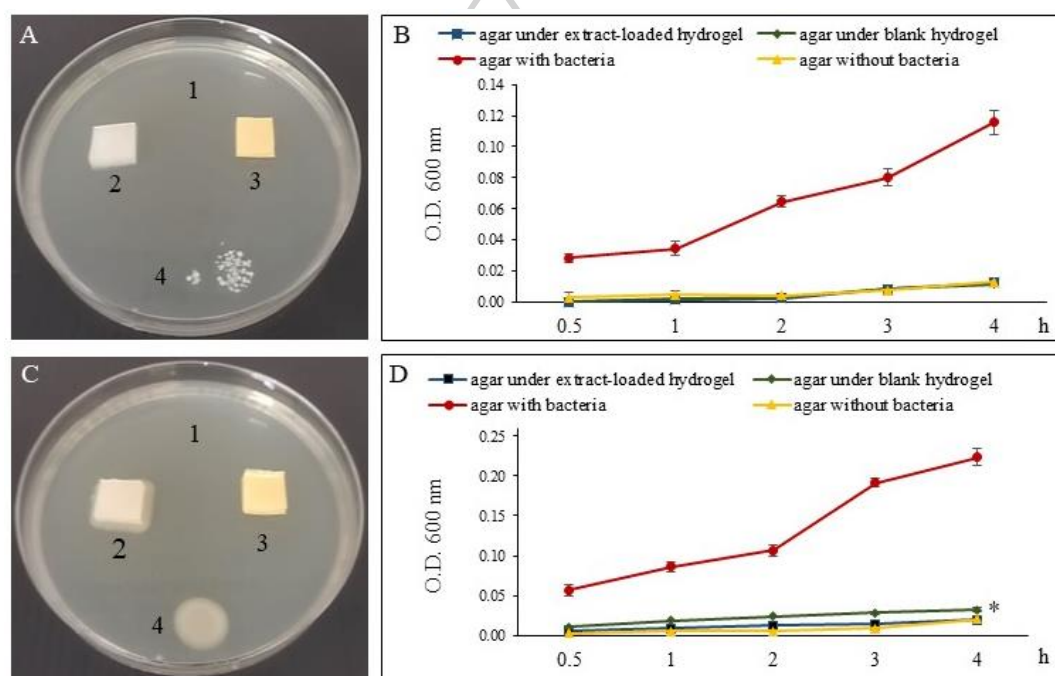


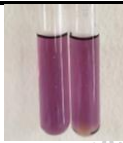
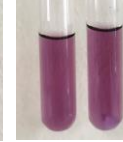
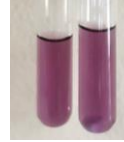
Figure 40 Infiltration test against *S. aureus* (A and B) and *E. coli* (C and D). Photographs of the agar plates (A and C); (1) no treatment, (2) blank hydrogels with bacterial suspension dropped on the surface, (3) the extract-loaded hydrogels with bacterial suspension dropped on the surface, and (4) agar with bacterial suspensions. The absorbances of bacterial growth (*S. aureus* and *E. coli* (B and D)) from the 4 different positions on an agar plate at different time points.

4.3.6.4 Cell viability, proliferation and migration

The NHF viability after exposure to *B. rotunda* loaded and unloaded hydrogel patches was investigated using the indirect method and MTT assay. Untreated NHFs

signified a control and were regarded as 100 % of viability. The *B. rotunda* loaded and unloaded hydrogel patches were immersed in DMEM- to prepare sample solutions at concentrations of 2.5 - 150 mg/mL.

Table 12 Antioxidant activity of the hydrogel patches. The left tubes are the control, and the right tubes are the experimental groups.

Samples	Incubation Time (h)					
	0	0.5	1	2	4	6
<i>B. rotunda</i> extract loaded hydrogel patch						
unloaded patch						

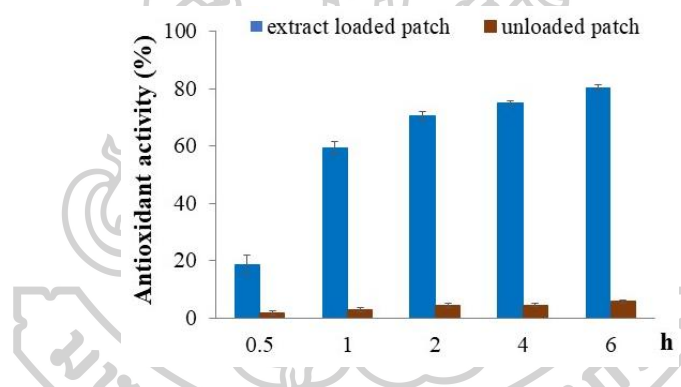


Figure 41 Antioxidant activities of *B. rotunda* loaded and unloaded hydrogel patches.

The viability percentage of *B. rotunda* loaded hydrogel patch at concentrations of 100 and 150 mg/mL was lower than 80 % and poisonous to NHFs (**Figure 42**). Among non-toxic groups (with a percent viability of more than 80 %), the viability values of *B. rotunda*-loaded hydrogel patches at concentrations of 25 and 50 mg/mL and unloaded hydrogel patches at a concentration of 150 mg/ml were significantly different compared with the untreated group.

Fibroblast augmentation after incubation with test samples was investigated using the indirect method and MTT assay similar to the viability test. **Figure 43** manifests the proliferation of treated NHFs at 24, 48 and 72 h. The experimental results related to the viability test that sample solutions at concentrations ≥ 25 mg/mL

exhibited a lower tendency of proliferation compared with the control. The proliferation of more than 80 % was statistically analyzed. The NHF proliferation of unloaded hydrogel patches at concentrations ≥ 5 mg/mL was not significantly different compared with the control group. Moreover, the test solution of unloaded hydrogel patch at a concentration of 2.5 mg/mL presented significantly higher proliferation at 48- and 72-h due to the effect of PVA material. The NHF proliferations of *B. rotunda*-loaded hydrogel patches at concentrations 2.5 and 5 mg/mL were not significantly different compared with the untreated group. Nevertheless, the proliferation of the cells after treated with the sample solution at a concentration of 2.5 mg/mL was lower than those treated with the unloaded hydrogel patch due to the presence of *B. rotunda* extract.

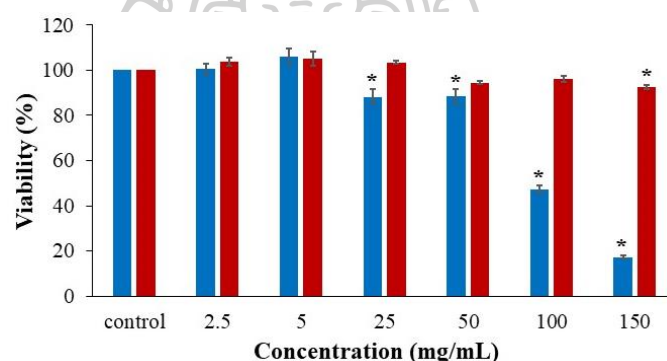


Figure 42 Viability of fibroblast cells after incubation with extract loaded and unloaded hydrogel patches for 24 h; (■) *B. rotunda* extract loaded patch and (■) unloaded patch. * p-value < 0.05 compared with the control group (untreated cells).

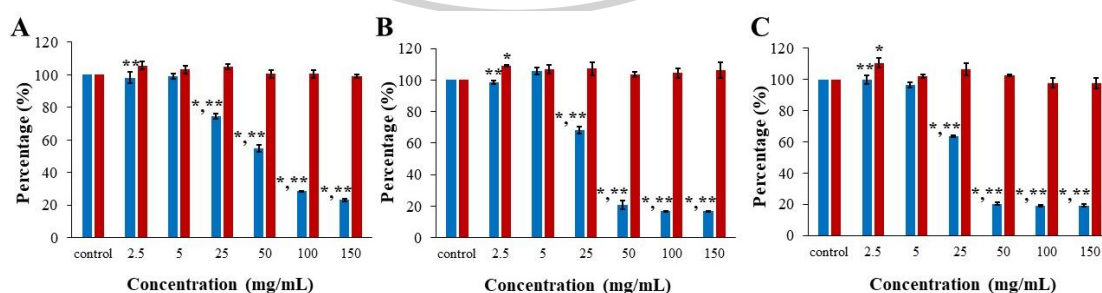
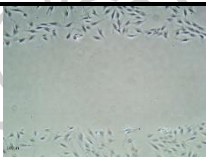
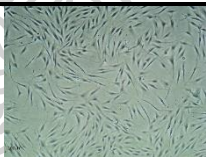
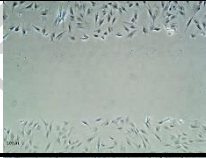
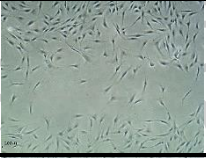
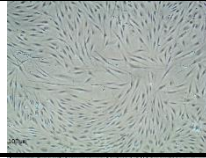


Figure 43 Proliferation of fibroblast cells treated with *B. rotunda* extract-loaded and unloaded PVA hydrogel patches; (■) *B. rotunda* extract-loaded patch and (■) unloaded patch after incubation for (A) 24, (B) 48 and (C) 72 h. *, ** p-values < 0.05 compared with control and unloaded patch at the same concentration.

The migration of hydrogel patch-treated NHFs was appraised by a scratch assay. *B. rotunda* loaded and unloaded hydrogel patches soaked in DMEM- at a concentration of 2.5 mg/mL were chosen for migration test. Untreated NHFs were denoted as the negative control. The images of cell migration were recorded every 24 h (**Table 13**). The NHF movements were calculated from the area at that time compared with the beginning area and presented as percentages of wound closure (**Figure 44**). Wound closure of *B. rotunda* loaded and unloaded hydrogel patches at 24-h treatment were nearly equal to untreated NHFs. The closures of all groups at 48- and 72-h were statistically analyzed. The data showed the significantly higher NHF mobilization of both hydrogel patches at 72 h compared with the control. In addition, the unloaded hydrogel patch also significantly narrowed the scratch area compared with the *B. rotunda* loaded hydrogel patch at 48- and 72-h after incubation.

Table 13 Movement images of NHFs treated with *B. rotunda* extract loaded and unloaded hydrogel patch.

Samples	Migration test			
	0h	24h	48h	72h
Control				
<i>B. rotunda</i> loaded hydrogel patch				
unloaded hydrogel patch				

4.3.7 Stability test

The *B. rotunda* extract-loaded PVA hydrogel patches were packed in aluminum foil bags (**Figure 45**) and kept at 5, 25 and 40 °C. The PVA hydrogel patch without extract was also evaluated as a control.

The color of *B. rotunda* extract-loaded PVA hydrogel patches at all temperature conditions was changed after 2 months. The photographs of *B. rotunda*

extract-loaded PVA hydrogel patches at the beginning and after 6-month storage are presented in **Figure 46**. After stability testing for 6 months, the patches kept at 5 °C were more transparent and their color changed from light orange to light yellow. The patches stored at 25 °C for 6 months were opaque and slightly the same color. Moreover, storage at 40 °C for 6 months, the patches were opaque, and the color converted to dark orange.

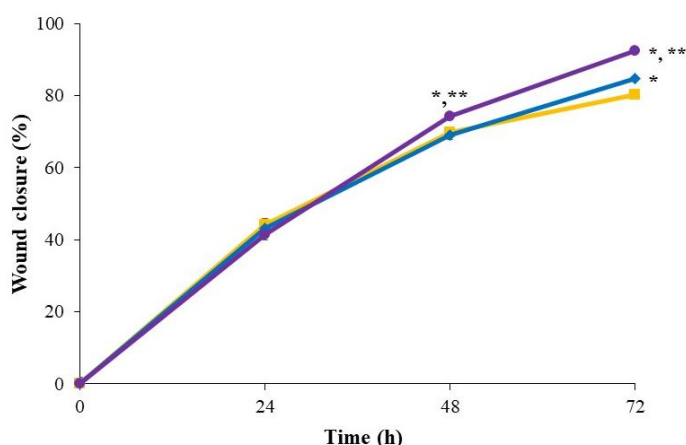


Figure 44 The wound closures of scratch areas of (□) control (untreated cells) and treated cells with sample solutions of hydrogel patches: (●) unloaded and (◆) *B. rotunda* extract-loaded hydrogel patch. p-value < 0.05 compared to untreated cells (*) and *B. rotunda* extract-loaded hydrogel patch (**) at the same treatment time.



Figure 45 The *B. rotunda* extract loaded PVA hydrogel patches in aluminum foil bags for the stability study.

4.3.7.1 Pinostrobin and pionocembrin analysis

The remaining percentages of *B. rotunda* extract-loaded hydrogel patches after were kept at various temperatures were displayed in **Figure 47**. From **Figure 47 (A)**, pinostrobin content in the hydrogel patch kept at 40 °C was lower than other conditions that might be the degradation of pinostrobin at high temperatures.

Moreover, the storage at low temperature (5 °C) showed higher stability of pinostrobin than others. The stability of pinocembrin related to pinostrobin (**Figure 47 (B)**). The content of pinocembrin storage at high temperatures was lower than in other conditions.

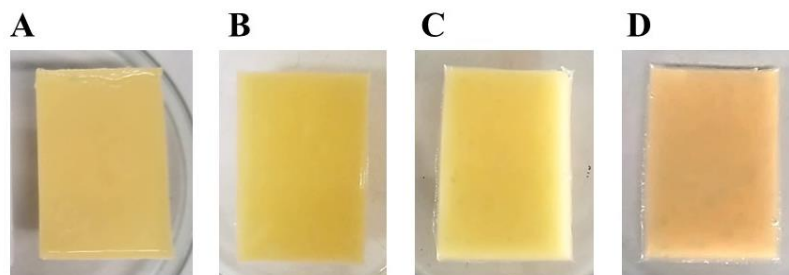


Figure 46 Images of *B. rotunda* extract loaded PVA hydrogel patches; (A) at the beginning, (B) at 5 °C for 6 months, (C) at 25 °C for 6 months and (D) at 40 °C for 6 months.

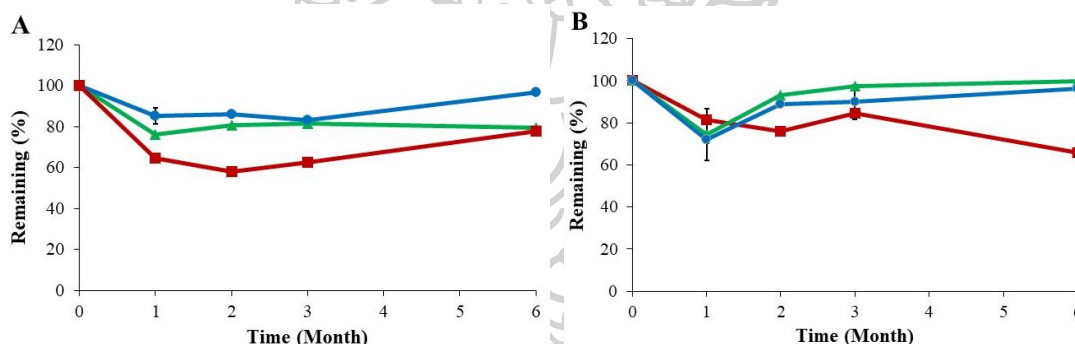


Figure 47 Stability of (A) pinostrobin and (B) pinocembrin at various temperature conditions; (●) 5 °C, (▲) 25 °C and (■) 40 °C.

4.3.7.2 Water content, water absorption, erosion, WVTR and mechanical properties

The physical properties of *B. rotunda* extract-loaded hydrogel patches are presented in **Figure 48 (A-G)**. The water content of hydrogel patches kept at all conditions including 5, 25 and 40 °C slightly decreased after 6 months. WVTR of hydrogel patches kept for 6 months was comparable to the initial patches. In addition, the *B. rotunda* extract-loaded hydrogel patches storage at all conditions showed a decrement in its water absorption, erosion, and elongation. Other physical properties including tensile strength and Young's Modulus, the results showed that its properties were higher than the beginning patches.

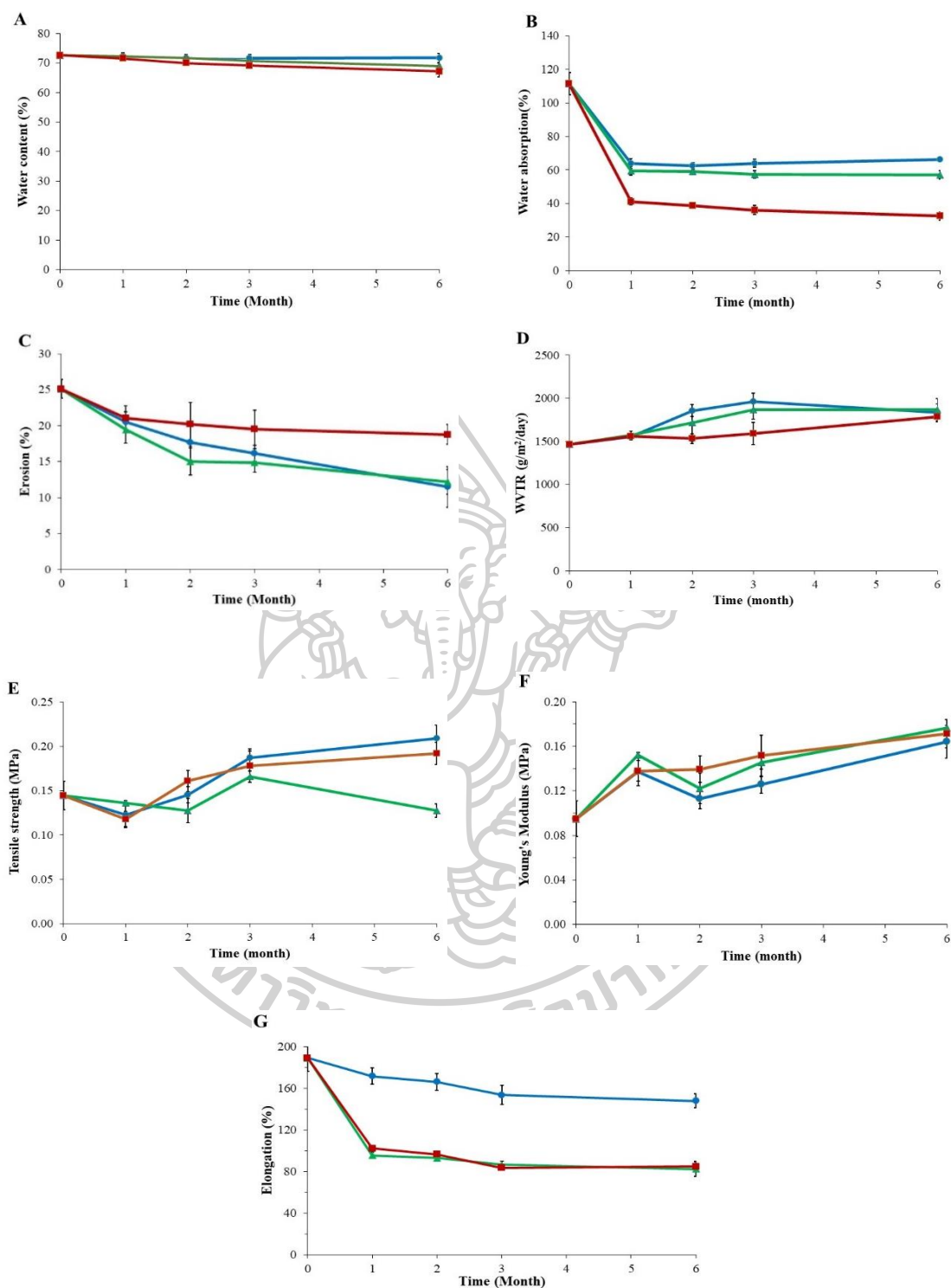


Figure 48 Stability of *B. rotunda* hydrogel patches: (A) Water content, (B) water absorption, (C) erosion, (D) WVTR, (E) Tensile strength, (F) Young's Modulus and (G) Elongation of hydrogel patches kept at (●) 5°C, (▲) 25 °C and (■) 40 °C.

4.4 Hydrogel patches fabricated by the thermal crosslinking method

The Gan and XG hydrogel patches were designed and optimized using response surface methodology coupled with the full CCD. The 2 independent variables (input), including A: Gan and B: XG, and the 6 experimental responses (output), including Y₁: water absorption, Y₂: tensile strength, Y₃: Young's modulus, Y₄: elongation, Y₅: erosion, and Y₆: water content, are shown in **Table 14**. A total of 13 experimental runs were statistically calculated by ANOVA with a p-value < 0.05.

Table 14 The full CCD of the hydrogel patches and measured values (output) of the six responses.

$Y_i = b_0 + b_1A + b_2B + b_{12}AB + b_{11}A^2 + b_{22}B^2$							
Factors		Responses					
A: Gan (%)	B: XG (%)	Y ₁ : Water Absorption (%)	Y ₂ : Tensile Strength (MPa)	Y ₃ : Young's Modulus (MPa)	Y ₄ : Elongation (%)	Y ₅ : Erosion (%)	Y ₆ : Water Content (%)
18.62	0.175	505.35	0.184	0.067	284.45	5.44	45.27
18.00	0.100	912.42	0.071	0.015	360.20	26.29	44.01
14.38	0.175	265.64	0.134	0.108	230.60	16.67	49.22
16.50	0.281	247.17	0.405	0.242	210.75	0.00	46.75
15.00	0.250	130.93	0.241	0.176	194.10	6.90	45.52
15.00	0.100	623.50	0.051	0.030	370.15	24.14	50.22
16.50	0.175	532.74	0.290	0.101	273.85	18.33	50.74
16.50	0.175	411.39	0.306	0.125	308.15	25.00	50.91
16.50	0.175	430.18	0.332	0.068	301.65	21.21	50.75
18.00	0.250	269.17	0.302	0.165	246.28	0.00	48.18
16.50	0.175	405.25	0.267	0.099	299.63	24.24	50.81
16.50	0.069	798.35	0.112	0.028	420.15	24.24	46.75
16.50	0.175	439.03	0.327	0.084	255.45	15.42	50.23

The relationship between independent variables and output responses is described as coefficients of various terms in the polynomial equations (**Table 15**). A positive coefficient value expresses an enhancement effect, while a negative one

explains a decreasing effect on the hydrogel patch responses. The coefficient of determination (R^2) is considered for the appropriate equation.

Table 15 The experimental equations created for various hydrogel patch responses.

$Y_i = b_0 + b_1A + b_2B + b_{12}AB + b_{11}A^2 + b_{22}B^2$						
Coefficient	Y ₁	Y ₂	Y ₃	Y ₄	Y ₅	Y ₆
b ₀	443.72	0.3043	0.0954	287.75	20.84	50.69
b ₁	95.77	0.0189	−0.0106	14.80	−2.58	−1.14
b ₂	−239.41	0.1044	0.0749	−73.26	−9.73	−0.0660
b ₁₂	−37.67	0.0101	0.0009	15.53	−2.26	2.22
b ₁₁	−21.64	−0.0833	−0.0077	−13.56	−4.21	−1.72
b ₂₂	46.99	−0.0335	0.0160	15.40	−3.67	−1.97
p-value	0.0002	0.0001	0.0002	0.0001	0.0018	<0.0001
R ²	0.9502	0.9565	0.9535	0.9568	0.9062	0.9887

The three-dimensional response surface plots show that the contents of Gan and XG significantly affected all output values (**Figure 49**). The water absorption values of the experimental hydrogel patches were 130.93–912.42%. The water absorption of the hydrogel patches was affected by the increment of Gan content and the decrement of XG content (**Figure 49 (A)**). The amounts of Gan and XG affected the strength of the hydrogel patches (**Figure 49 (B)**). The positive values of b₁ and b₂ showed that patch strength was enhanced when Gan and XG amount increased. Moreover, the amount of XG showed a more eminent effect on patch strength than Gan. The surface response graph of Young's modulus is shown in **Figure 49 (C)**. The negative values of b₁ showed a descent effect of Gan content on Young's modulus, on the contrary, the positive value of b₂ indicated that Young's modulus improved when XG content increased. Elongation values at the breaking point of the hydrogel patches were in the range of 194.10–420.15%. The polynomial equation and response surface graph for elongation are demonstrated in **Table 15** and **Figure 49 (D)**, respectively. The data indicated that elongation enhanced when Gan content increment, while XG content decrement.

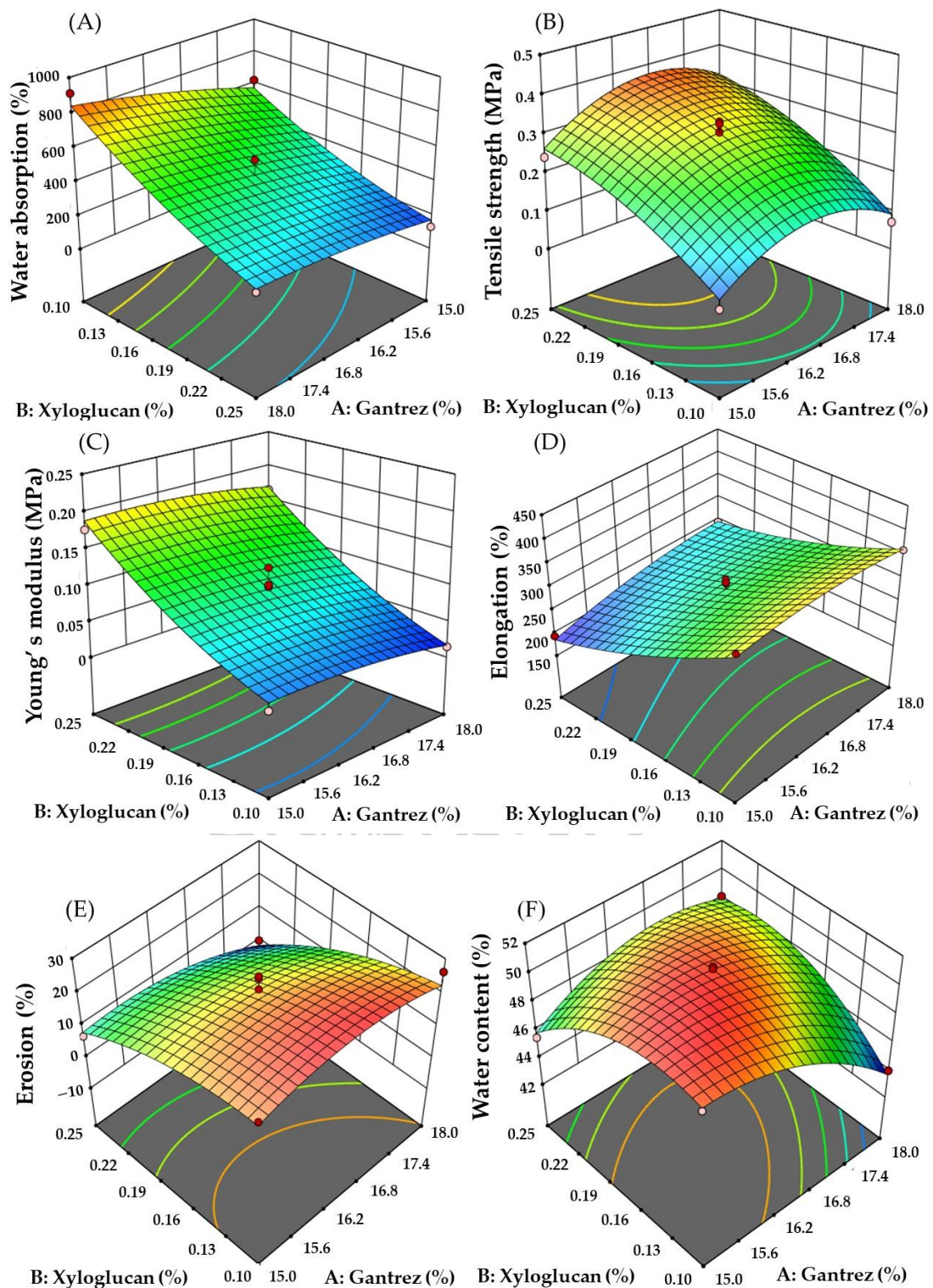


Figure 49 The 3D response surface plots of outputs: (A) water absorption, (B) tensile strength, (C) Young's Modulus, (D) elongation, (E) erosion, and (F) water content.

From the response surface plot in **Figure 49 (E)**, the data showed both Gan and XG amounts were affected by the decreasing erosion. Moreover, XG content exhibited a more considerable effect than Gan. The effects of Gan and XG on the water content of the hydrogel patches are presented in **Figure 49 (F)**. Both Gan and XG indicated decreased effects on water content.

The experimental design was the optimization of Gan and XG contents for the hydrogel patch with criteria for maximum water absorption and maximum elongation. The optimization of the hydrogel patch formulation was 17.78% w/w of Gan and 0.1% w/w of XG. The predicted values of water absorption, tensile strength, Young's Modulus, elongation, erosion, and water content were 827.97%, 0.114 MPa, 0.021 MPa, 365.97%, 23.58%, and 44.68%, respectively. The optimized films which composed 17.78% w/w of Gan and 0.1% w/w of XG are presented in **Figure 50**. The thermal crosslinking led to a color change of the films without changing the dimensions and the size of the films ($2.26 \times 2.26 \times 0.07 \text{ cm}^3$). However, the weight of the films decreased by approximately $9.61\% \pm 1.40\%$ due to the moisture evaporation during the thermal crosslinking process. After thermal crosslinked films were immersed in 50% w/w of honey solution for 1 h, their physical properties were determined. The results showed that water absorption, tensile strength, Young's Modulus, elongation, erosion, and water content of the optimized hydrogel patch were $824.20\% \pm 8.76\%$, $0.114 \pm 0.005 \text{ MPa}$, $0.019 \pm 0.003 \text{ MPa}$, $371.12\% \pm 10.60\%$, $21.23\% \pm 2.72\%$, and $44.80\% \pm 1.17\%$, respectively.

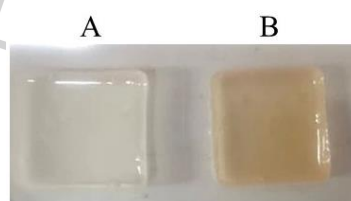


Figure 50 The optimized films (A) before and (B) after thermal crosslinking.

For the extract embedding to the optimized film, sodium hydroxide solution was included in the optimized mixture. Then, the mixture was poured into molds and dried at room temperature for 3 days. After thermal crosslinking, the dried films were soaked in 50% w/w of honey and the mixture of *D. bulbifera* extract and honey with concentrations of 20% and 50% w/w, respectively. The hydrogel patch which had

been soaked in distilled water was used as a control. The hydrogel patches incorporated with 50% w/w of honey (honey-loaded Gan/XG), the mixture of *D. bulbifera* extract and honey with concentrations of 20% and 50% w/w (*D. bulbifera* extract/honey-loaded Gan/XG), and the unloaded hydrogel patch (Gan/XG) are shown in **Figure 51**. The Gan/XG hydrogel patch was soft, transparent, and colorless. Embedding of honey and *D. bulbifera* extract into the thermally crosslinked hydrogel patches by absorption led to the change in the color of the hydrogel patches to light brown and light orange colors due to the color of honey and the *D. bulbifera* extract.

As compared to the hydrogels loaded with water, the hydrogels loaded with honey alone and the honey/extract mixture exhibited a somewhat lower swelling due to the higher viscosity of the mixture, resulting in a lower penetration into the hydrogels compared to water. This led to a slight change in the dimension and weight of the hydrogel patches. The size of the hydrogel patch was $3.05 \times 3.22 \times 1.08 \text{ cm}^3$ after water loading, whereas the sizes were $3.02 \times 3.18 \times 1.04 \text{ cm}^3$ and $3.01 \times 3.16 \times 1.02 \text{ cm}^3$, respectively, for the hydrogels incorporated with honey and the honey/extract mixture. The weights of the hydrogel patches increased by $792.11\% \pm 9.49\%$ after immersion in the water, whereas the weight changes of the hydrogel patches were $778.38\% \pm 7.15\%$ and $754.05\% \pm 5.41\%$ after immersion in honey and the *D. bulbifera* extract/honey mixture.

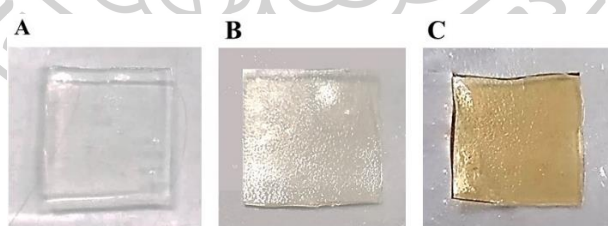


Figure 51 The hydrogel patches: (A) Gan/XG, (B) honey-loaded Gan/XG, and (C) *D. bulbifera* extract/honey-loaded Gan/XG.

4.4.1 FT-IR spectra

The thermal crosslinking of Gan/XG film was proved by the FT-IR technique. **Figure 52** exhibits the FT-IR spectra of XG powder, Gan powder, and the crosslinked Gan/XG film. After thermal crosslinking at 130°C for 30 min, the new spectra of the

crosslinked Gan/XG film arose at approximately $1100\text{--}1300\text{ cm}^{-1}$ (in the red ellipse), which denoted the bands of the C-O stretching bond.

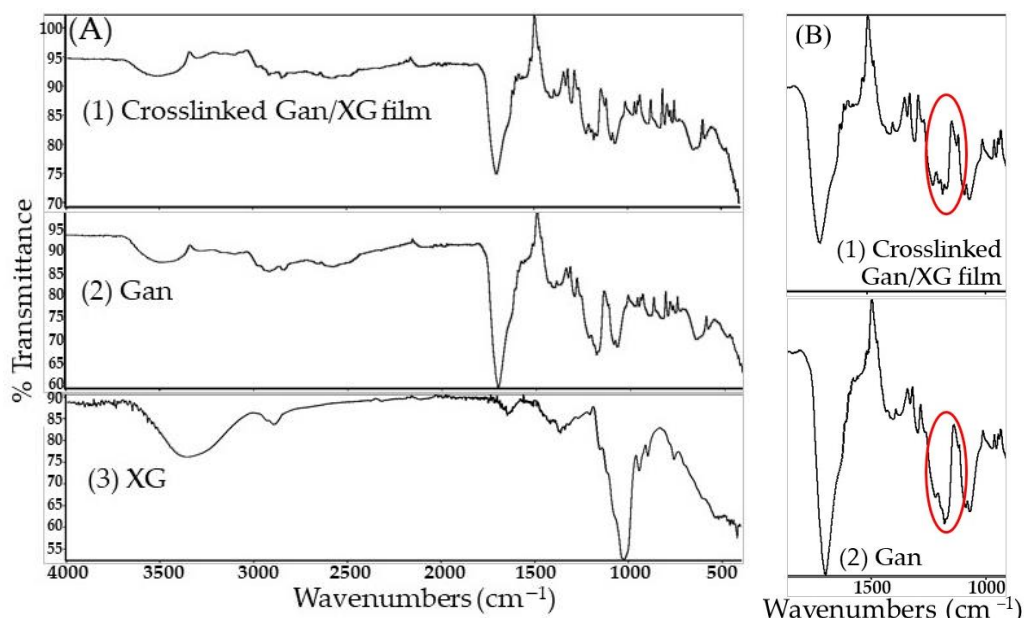


Figure 52 (A) The FT-IR spectra of (1) the crosslinked Gan/XG film, (2) Gan powder, and (3) XG powder. (B) Enlarged FT-IR spectra of (1) the crosslinked Gan/XG film and (2) Gan powder. Red ellipses show the new spectra after crosslinking of Gan/XG film compared with Gan powder.

4.4.2 SEM images

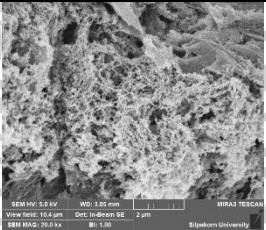
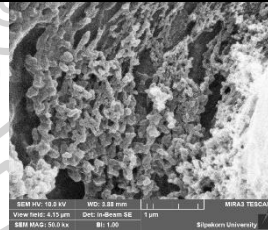
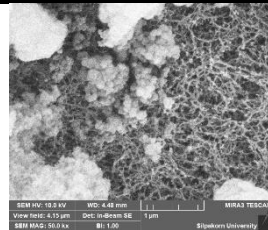
The hydrogel patches were dried by the CPD method same as the drying technique for physically crosslinked hydrogel patches describe above in section 4.3.2. On the contrary, the structure collapsed after the drying process. Thus, the images of thermal crosslinked hydrogel patch morphologies presented in **Tables 16** and **17** might be different from the factual morphologies.

Surface morphologies of both Gan/XG and *D. bulbifera* extract/honey-loaded Gan/XG were smooth. The collapse at the middle point of each patch was observed at the high magnifying power. The cross-section structures of Gan/XG and *D. bulbifera* extract/honey-loaded Gan/XG exhibited dense networks might be due to the drying method. However, the crosslinking of polymers in hydrogel patches was ensured.

Table 16 The surface images of *D. bulbifera* and honey loaded and unloaded hydrogel patches observed from SEM.

Samples	X 1,000	X 20,000	X 50,000
Gan/XG			
<i>D. bulbifera</i> / honey-loaded Gan/XG			

Table 17 The cross-section images of *D. bulbifera* and honey honey-loaded and unloaded hydrogel patches observed from SEM

Samples	X 20,000	X 50,000
Gan/XG		
<i>D. bulbifera</i> /honey- loaded Gan/XG		

4.4.3 Water content, water absorption and WVTR

Table 18 presents the water content, water absorption and WVTR of the hydrogel patches. The water content of honey-loaded Gan/XG was nearly the same as

D. bulbifera extract/honey-loaded Gan/XG, whereas the unloaded hydrogel patch had much more water content than the others.

Table 18 The physical properties of the hydrogel patches.

Hydrogel Patches	Water Content (%)	Water Absorption (%)	WVTR (g/m ² /day)
Gan/XG	89.24 ± 0.71	300.41 ± 13.45	N/A
honey-loaded Gan/XG	48.64 ± 0.60	466.89 ± 8.48 *	516.51 ± 12.86
<i>D. bulbifera</i> extract/honey-loaded Gan/XG	45.35 ± 1.18	650.19 ± 21.30 *,**	576.62 ± 32.52

p-value < 0.05 compared to Gan/XG (*) and honey-loaded Gan/XG (**).

Water absorption of the loaded and unloaded hydrogel patches is presented in **Table 18** and **Figure 53**. All hydrogel patches could absorb high amounts of water due to the super swelling property of Gan, as previously reported (35). Both honey-loaded and *D. bulbifera* extract/honey-loaded hydrogel patches manifested significantly higher water absorption compared with the unloaded hydrogel patch. In addition, the water absorption of *D. bulbifera* extract/honey-loaded Gan/XG was significantly higher than honey-loaded Gan/XG.

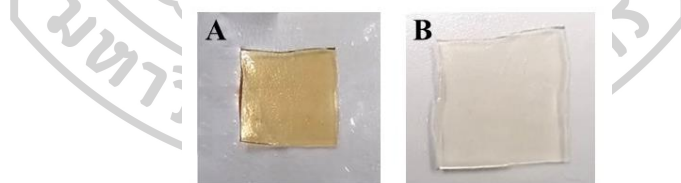


Figure 53 Photographs of *D. bulbifera* extract/honey-loaded Gan/XG; (A) initial patch and (B) swelling patch.

The transmissibility of water vapor through the hydrogel patches is shown in **Figure 54**. Water vapor transmission rates of both the loaded hydrogel patches were 516.51 and 576.62 g/m²/day (**Table 18**), which correlated to the semi-permeable film dressing property (27). The WVTR of the unloaded hydrogel patch could not be measured because it was not able to completely cover the bottle mouth for 24 h. The

high-water evaporation from the unloaded hydrogel patch was the reason that affected the patch contraction.

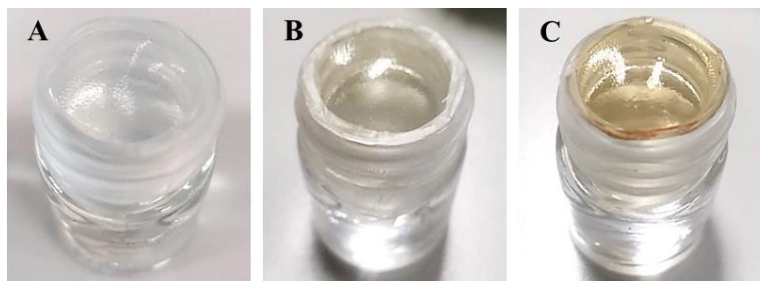


Figure 54 Photographs of bottles covered with hydrogel patches for WVTR assay; (A) Gan/XG, (B) honey-loaded Gan/XG and (C) *D. bulbifera* extract/honey-loaded Gan/XG.

The images of the tensile test of both loaded Gan/XG using a texture analyzer are shown in **Figure 55**. Young's Modulus, tensile strength, and percent of elongation were observed (**Table 19**). Honey loaded in the Gan/XG patch significantly increased Young's Modulus, tensile strength, and elongation compared with the unloaded Gan/XG patch, expressing greater stiffness, strength, and extensibility.

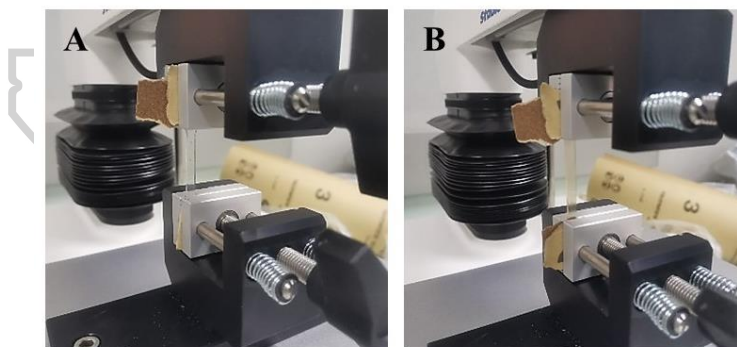


Figure 55 Photographs of tensile test; (A) honey-loaded Gan/XG and (B) *D. bulbifera* extract/honey-loaded Gan/XG.

4.4.4 Mechanical properties

The results of the *D. bulbifera* extract/honey-loaded hydrogel patch demonstrated that the combination of *D. bulbifera* extract and honey affected significantly higher tensile strength and elongation than the unloaded hydrogel patch,

which explained the higher strength and elasticity of the hydrogel patch. Furthermore, *D. bulbifera* extract and honey loading enhanced the extensibility of the hydrogel patch more than honey loading only. The addition of *D. bulbifera* extract could promote the elasticity of the patch more than honey alone.

Table 19 Mechanical properties of the hydrogel patches.

Hydrogel Patches	Young's Modulus (MPa)	Tensile Strength (MPa)	Elongation (%)
Gan/XG	0.271 ± 0.010	0.146 ± 0.009	59.47 ± 4.14
honey-loaded Gan/XG	$0.349 \pm 0.022 *$	$0.229 \pm 0.018 *$	$131.01 \pm 7.55 *$
<i>D. bulbifera</i> extract/honey-loaded Gan/XG	$0.253 \pm 0.015 **$	$0.243 \pm 0.027 *$	$191.18 \pm 14.88 **$

p-value < 0.05 compared to Gan/XG (*) and honey-loaded Gan/XG (**).

4.4.5 Biological activities

4.4.5.1 Antimicrobial activities

The antimicrobial activities of hydrogel patches against *S. aureus* and *E. coli* were assessed by qualitative and quantitative methods same as the hydrogel fabricated by the physical crosslinking method. Agar disc diffusion was used to observe the clear zone of the 6-mm sample disc. The results indicated no inhibition against both bacteria from all hydrogel discs, while Aquacel™ Ag+ Extra presented clear zones of 13.67 ± 0.58 and 10.00 ± 0.00 mm against *S. aureus* and *E. coli*, respectively (**Figure 56** and **57**).

Fifty mg of hydrogel patches were soaked in bacterial suspensions for the quantitative antimicrobial method. The antimicrobial activities against *S. aureus* are shown in **Figure 58** (A and B). After treatment for 24 h, all treated bacterial suspensions were more clear than the untreated group. Moreover, the percent of antibacterial activities of all treated groups were not significantly different. For the *E. coli* assay, the antimicrobial activities of all hydrogel-treated groups are presented in **Figure 58** (C and D). The results were correlated with the antimicrobial test of *S.*

aureus. The activities of all treated groups were not significantly different. The antibacterial activities of all loaded and unloaded Gan/XG might be due to the surface adsorption ability of hydrogel that affected the abnormal growth of bacteria (**Figure 59**).

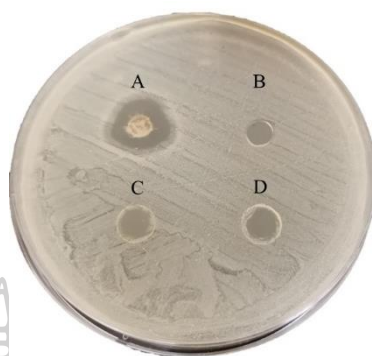


Figure 56 The photographs of inhibition zones against *S. aureus*; (A) Aquacel™ Ag+ Extra disc, (B) Gan/XG disc and (C) *D. bulbifera* and honey/loaded disc (D) honey-loaded Gan/XG disc.

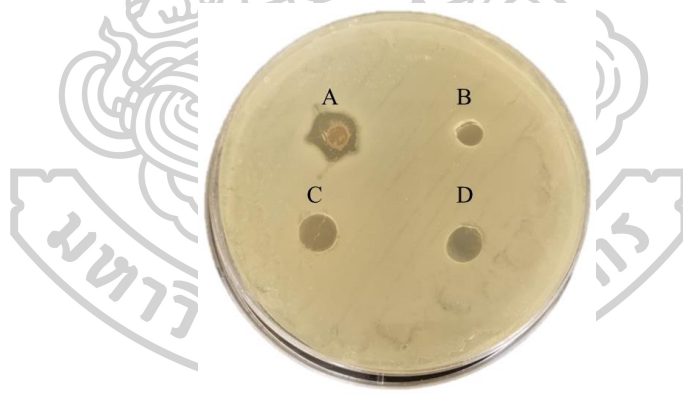


Figure 57 The photographs of inhibition zones against *E. coli*; (A) Aquacel™ Ag+ Extra disc, (B) Gan/XG disc and (C) *D. bulbifera* and honey/loaded disc (D) honey-loaded Gan/XG disc.

4.4.5.2 Infiltration bacterial test

The performance of the hydrogel patch as a guard that could protect the wound bed against infection from external bacteria was evaluated by the infiltration test. **Table 20** and **Figure 60** illustrate the ability of hydrogel patches to prevent *S. aureus* and *E. coli* infiltration. **Table 20** shows bacterial growths after incubation on

TSA overnight. The colonies of *S. aureus* and *E. coli* were perceived on agar plates denoted the positive control, while bacterial growths on the top surface of hydrogel patches were not observed.

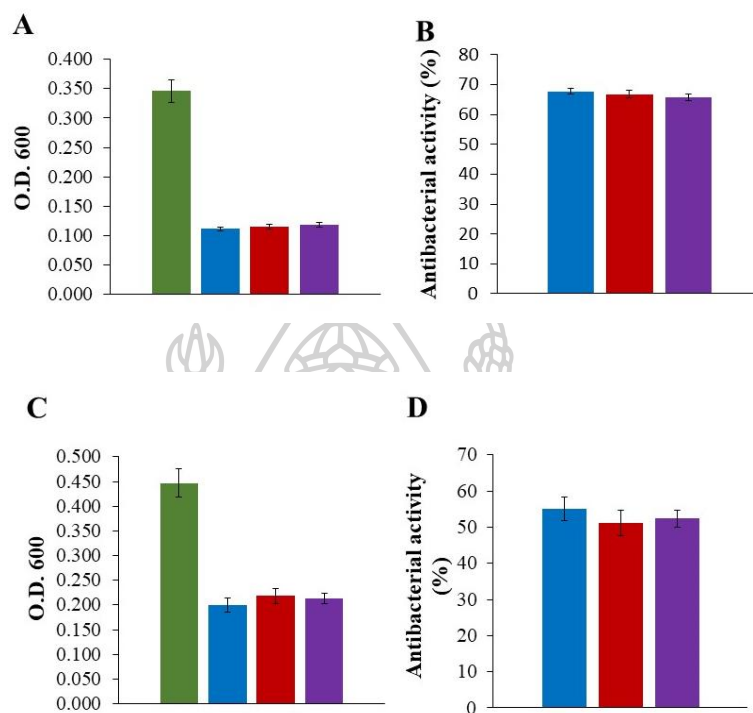


Figure 58 Antimicrobial activities: (A and B) *S. aureus* and (C and D) *E. coli* of hydrogel patches: (■) Gan/XG hydrogel patch, (■) honey-loaded Gan/XG hydrogel patch, and (■) *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patch. The (■) bacterial suspension (negative control).

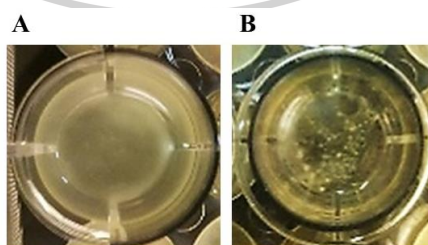


Figure 59 Antimicrobial activities against *S. aureus*; (A) untreated suspension and (B) *D. bulbifera* extract/honey-loaded Gan/XG treated suspension.

As shown in **Figure 60** (A), the turbidity values of TSB incubated with agar pieces under all hydrogel patches at 4 h were lower than those incubated with agar

containing *S. aureus* and were not significantly different from the control (agar without bacteria). Furthermore, all hydrogel patches distinctly obstructed *E. coli* infiltration from the outside environment (**Figure 60 (B)**). The turbidity values of the TSB incubated with the agar pieces under loaded and unloaded hydrogel patches at 4 h were not significantly different from the control. TSB incubated with the positive control displayed time-dependent augmentation.

Table 20 Infiltration test of the hydrogel patches against *S. aureus* and *E. coli*.

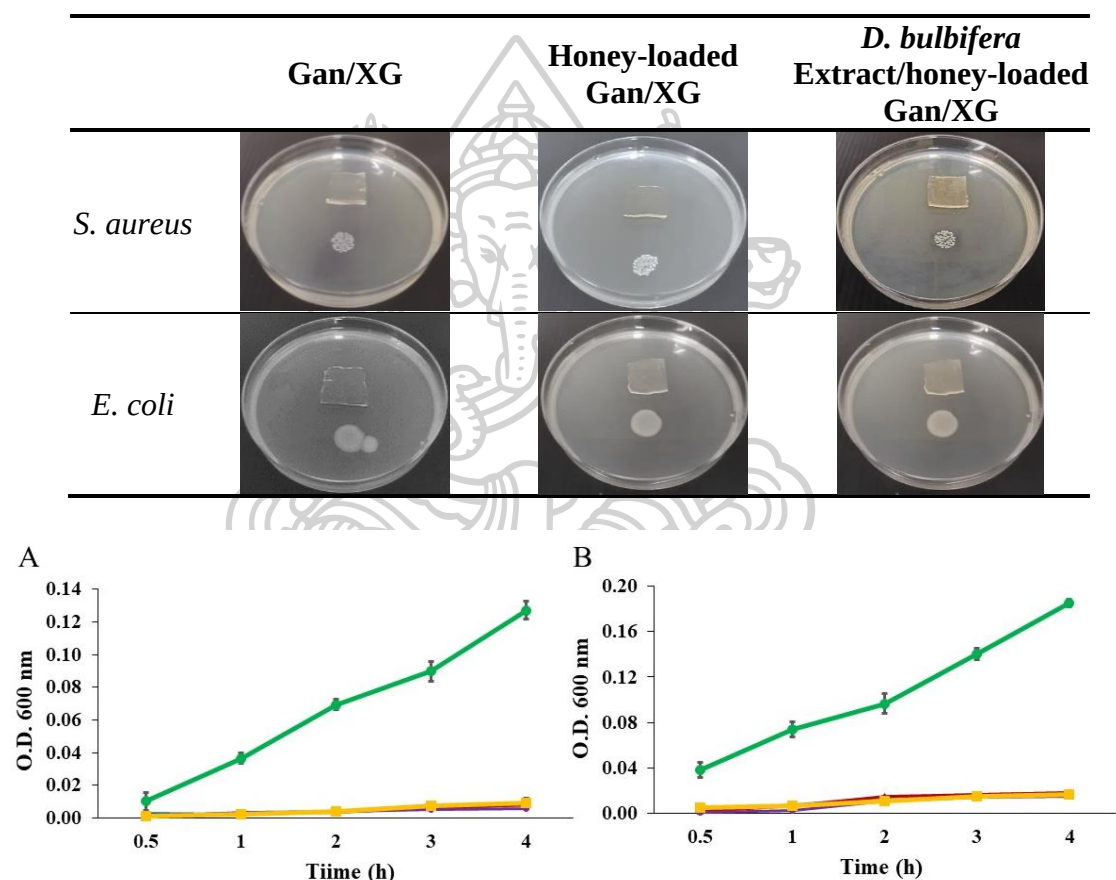


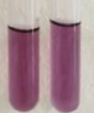
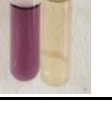
Figure 60 Infiltration bacterial test against bacteria: (A) *S. aureus* and (B) *E. coli*. Turbidity of TSB incubated with: (■) agar without bacteria, (★) agar with bacteria, (●) Gan/XG hydrogel patch, (▲) honey-loaded Gan/XG hydrogel patch, and (◆) *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patch.

4.4.5.3 Antioxidant activity

The antioxidant activities at 0.5-, 1-, 2-, 4-, and 6-h of experiments of the hydrogel patches are revealed in **Table 21** and **Figure 61**. The results showed that the

antioxidant activities of the honey-loaded Gan/XG and the unloaded patch were almost the same after being incubated for 0.5, 1, and 2 h. In addition, the honey-loaded hydrogel patch exhibited significantly higher antioxidant properties compared with the unloaded patch after being incubated for 6 h, which was related to the antioxidant activity of the previously reported honey-loaded dressings (56, 78). The antioxidant ability was enhanced when the incubation period was increased due to the liberation of honey from the hydrogel patch. The complete release of honey from honey-loaded Gan/XG occurred within 3 h.

Table 21 Antioxidant activity of the hydrogel patches. The left tubes are the control, and the right tubes are the experimental groups.

Samples	Incubation Time (h)					
	0	0.5	1	2	4	6
Gan/XG						
Honey-loaded Gan/XG						
<i>D. bulbifera</i> extract/honey-loaded Gan/XG						

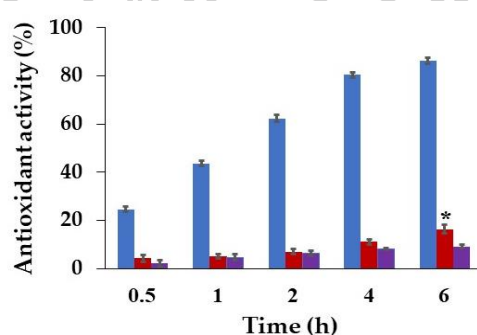


Figure 61 Antioxidant activities of (■) Gan/XG hydrogel patches, (■) honey-loaded Gan/XG hydrogel patches, and (■) *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patches after incubation for 0.5, 1, 2, 4, and 6 h. * p-value < 0.05 of honey-loaded Gan/XG compared with Gan/XG at the same concentration.

The antioxidant activity of the *D. bulbifera* extract/honey-loaded Gan/XG after being incubated for 1 h was a significant time-dependent enhancement. The data of the *D. bulbifera* extract/honey-loaded Gan/XG conformed to a previous study showing that the ethanolic extract of *D. bulbifera* bulbils exhibited antioxidant activity by the DPPH radical scavenging assay (43). Antioxidant patches are the alternative for the treatment of difficult-to-heal wounds because the antioxidants help to manage wound oxidative stress and balance the level of reactive oxygen species (84-86).

4.4.5.4 Cell viability, proliferation and migration

The viability of NHFs exposed to the loaded and unloaded hydrogel patches after a 24-h treated period was determined using the MTT assay. Untreated NHFs were denoted as the control and were considered as having 100% viability. The NHF remainders of all treatment groups with concentrations of 0.5, 1.0, and 2.5 mg/mL higher than 80% were non-toxic to NHFs. Besides, the viability of NHFs treated with the sample solution of the *D. bulbifera* extract/honey-loaded Gan/XG and the unloaded Gan/XG at the concentration of 1 mg/mL was significantly higher than the control (**Figure 62**). The results of the *D. bulbifera* extract/honey-loaded hydrogel patch agreed with the previous study of *D. bulbifera* ethanolic extract with concentrations from 1 to 100 $\mu\text{g/mL}$ that presented low toxicity (43).

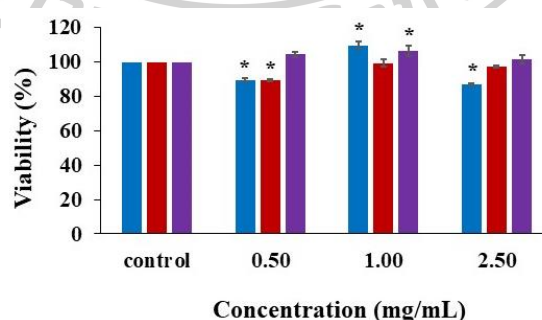


Figure 62 Viability of NHFs after 24 h incubated with (■) Gan/XG hydrogel patches, (■) honey-loaded Gan/XG hydrogel patches, and (■) *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patches. * p-value < 0.05 compared to untreated cells (control group).

The NHF augmentations after long-term contact with the sample solutions were investigated by the proliferation assay (**Figure 63**). The proliferation values higher than 80% were statistically appraised compared with the control. The honey-loaded Gan/XG and the *D. bulbifera* extract/honey-loaded hydrogel patches with a concentration of 1 mg/mL increased cell enlargement after 24-h incubation, whereas only the *D. bulbifera* extract/honey-loaded hydrogel patch significantly increased proliferation compared with the control. On the other hand, the *D. bulbifera* extract/honey-loaded hydrogel patch with a concentration of 2.5 mg/mL discouraged fibroblast proliferation after a long period of incubation.

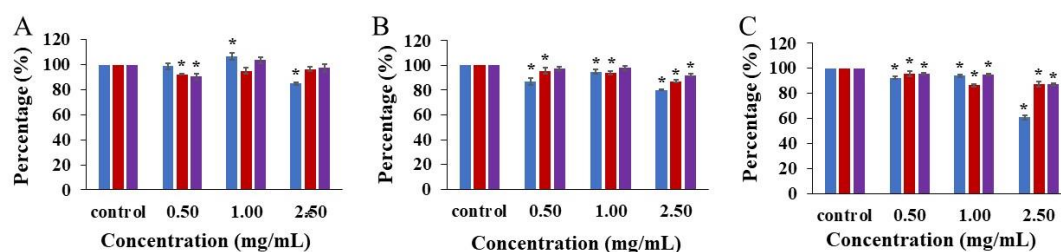


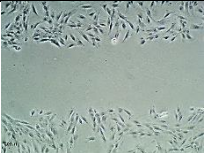
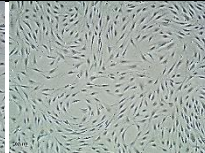
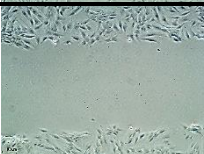
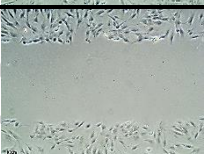
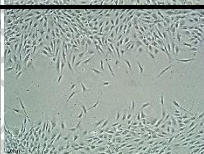
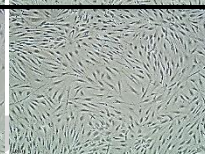
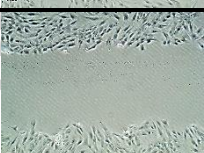
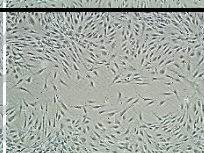
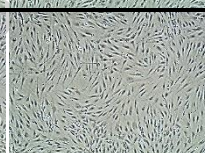
Figure 63 Proliferation of NHFs after (A) 24, (B) 48, and (C) 72 h incubated with (■) Gan/XG hydrogel patches, (■) honey-loaded Gan/XG hydrogel patches, and (■) *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patches. * p-value < 0.05 compared to untreated cells (control group).

The sample solution at a concentration of 1 mg/mL was selected for the NHF migration test because of its high viability and proliferation effects. **Table 22** presents images of the *D. bulbifera* extract/honey-loaded Gan/XG-, the honey-loaded Gan/XG-, and the unloaded Gan/XG-treated NHFs at 0, 24, 48, and 72 h. Untreated NHFs were used as a baseline.

Figure 64 presents the recovery of gap areas after exposure to the sample solutions of hydrogel patches for 24, 48, and 72 h. The scratched areas of all groups decreased after 24 h of the incubation period. The scratch areas decreased more than 50% after incubation for 48 and 72 h and were statistically evaluated. The percentage recovery of the scratched area at 72 h of all sample solutions and the control group was significantly higher than at 48 h. Moreover, the percentage recovery at 48 h of all treated groups was significantly different than the untreated NHFs. Both the honey-

loaded Gan/XG and the unloaded Gan/XG hydrogel-treated groups could significantly recover the scratched area compared with the untreated group after being incubated for 72 h. The percentage recovery of the NHFs treated with the honey-loaded Gan/XG and unloaded Gan/XG hydrogel patches was observed to be significantly higher than the *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patch-treated group.

Table 22 Movement images of NHFs treated with *D. bulbifera* extract/honey-loaded Gan/XG, honey-loaded Gan/XG, and Gan/XG hydrogel patches at the beginning and after treatment for 24, 48, and 72 h.

Samples	Migration Test			
	0 h	24 h	48 h	72 h
Control				
<i>D. bulbifera</i> extract/honey-loaded Gan/XG				
honey-loaded Gan/XG				
Gan/XG				

From the migration data, the honey-loaded and the unloaded Gan/XG hydrogel patches stimulated NHF movement within 24 h of incubation. The percentage would closure of the honey-loaded Gan/XG related to earlier research that reported the migration improvement encouraged by honey (87). In addition, the high percentage recovery of the unloaded hydrogel patch might be due to the high efficiency of XG to stimulate fibroblast migration, as previously described (66). The *D. bulbifera* extract/honey-loaded Gan/XG patch significantly improved fibroblast movement within 48 h of treatment.

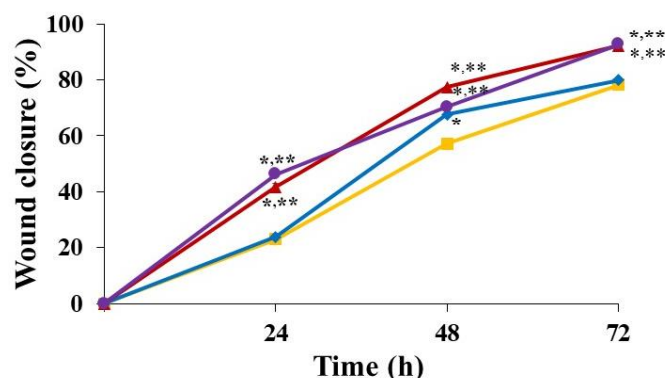


Figure 64 The wound closures of gap areas of (■) control (untreated cells) and treated cells with sample solutions of hydrogel patches: (●) Gan/XG, (▲) honey-loaded Gan/XG, and (◆) *D. bulbifera* extract/honey-loaded Gan/XG. p-value < 0.05 compared to untreated cells (*) and *D. bulbifera* extract/honey-loaded Gan/XG (**) at the same treatment time.

4.4.6 Stability study

The *D. bulbifera* extract/honey-loaded, honey-loaded and unloaded Gan/XG hydrogel patches were kept at various conditions for stability test. All types of hydrogel patches were not stable at 25 and 40 °C. The hydrogel patches at 25 °C were infected with bacteria and mold within 1 month because they were composed of more natural materials that were easily contaminated. The appearance of patches at 40 °C changed to dark color and viscous liquid within 6 months. The honey-loaded and *D. bulbifera*/honey-loaded Gan/XG hydrogel patches kept at 5 °C for 6 months are shown in **Figure 65**. Both loaded patches after stability exhibited dark color than the initial patches. The stabilities of hydrogel patches are shown in **Figure 66**.

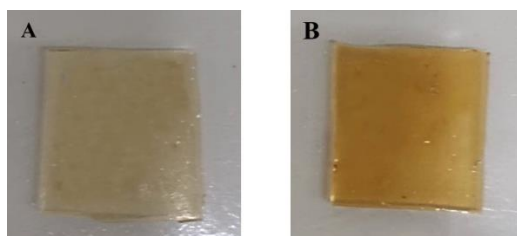


Figure 65 Photographs of hydrogel patches; (A) honey-loaded Gan/XG and (B) *D. bulbifera* extract/honey-loaded Gan/XG after storage at 5 °C for 6 months.

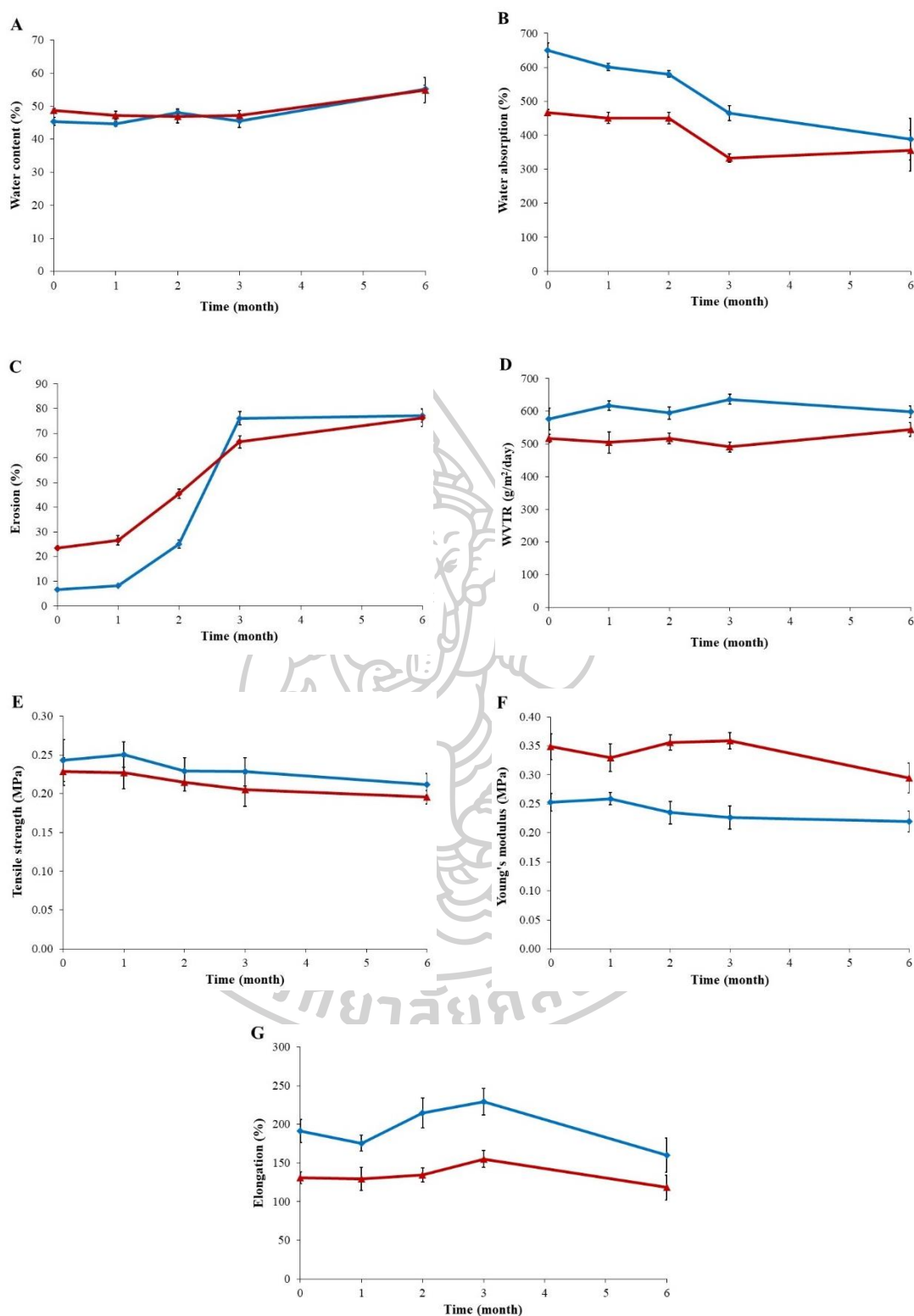


Figure 66 Stability of (▲) honey-loaded and (◆) *D. bulbifera*/honey-loaded hydrogel patches: (A) water content, (B) water absorption, (C) erosion, (D) WVTR, (E) tensile strength, (F) Young's Modulus and (G) elongation of hydrogel patches kept at 5 °C.

The stability of honey-loaded Gan/XG and *D. bulbifera*/honey-loaded Gan/XG hydrogel patches kept at 5 °C for 6 months was investigated. The results showed that the water content and WVTR of both hydrogel patches were nearly the same as the initial patches. Whereas, water absorption, tensile strength, Young's Modulus and elongation were lower tendency represented the frail patches. Moreover, the erosion result of both hydrogel patches supported the water absorption, tensile strength, Young's Modulus and elongation data. The higher changes in the erosion of both hydrogels exhibited unstable patches that had lower strength than the beginning patches.



CHAPTER 5

CONCLUSIONS

5.1 Plant extraction, characterization, and biological activities

5.1.1 *B. rotunda* extract

B. rotunda extract, obtained from the rhizomes of *B. rotunda* by extraction with 70 % v/v ethanol at room temperature for 7 days, exhibited a sticky feature and had yellow-brown color. The percent yield of the extraction was 3.61 % w/w calculated from the dried powder. The FT-IR spectrum showed that the extract contained major peaks of C=O and C–O functional groups. The melting point of the extract was approximately 232 °C and the degradation temperature was around 330 °C. Pinostrobin and pinocembrin which are important active substances were analyzed using HPLC. The contents of pinostrobin and pinocembrin in the extract were 29.86 ± 0.19 and 3.70 ± 0.02 % w/w, respectively. The *B. rotunda* extract had an antimicrobial activity against *S. aureus* with an MBC value of 156.25 ± 0.00 µg/mL. In addition, it exhibited antioxidant activity with an IC₅₀ value of 171.02 µg/mL. Moreover, it had no toxicity on NHF cells at the extract concentrations in the range of 0.025 – 10 µg/mL. The extract at the concentrations of 0.5 – 5 µg/mL could be able to promote NHF growth after 24-h treatment. Nevertheless, the extract did not affect the migration of NHF cells.

5.1.2 *D. bulbifera* extract

D. bulbifera extracted from bulbils using 80 % v/v ethanol at room temperature for 15 days exhibited red-brown powder. The percent yield of the extraction was 7.47 % w/w. From the FT-IR spectrum, the extract exhibited main peaks of C=C and C–O functional groups. The melting point of the extract was around 128 °C and the degradation temperature was not observed. The *D. bulbifera* extract did not show antimicrobial activity against *S. aureus* and *E. coli* in the broth dilution assays. Whereas it presented a strong antioxidant activity with an IC₅₀ value of 20.54 µg/mL. Furthermore, it manifested no toxicity against NHFs at the extract concentrations ranging between 0.1 – 1,000 µg/mL. The extract with a concentration

of 10 µg/mL was able to enhance the growth of NHF cells after 72-h treatment. The extract at a concentration of 10 µg/mL exhibited a slight effect on the NHF migration. However, the result was not significantly different compared to the control.

5.2 *B. rotunda* extract loaded hydrogel patch fabricated by a physical crosslinking method

The *B. rotunda* extract-loaded hydrogel patches were created and optimized using a Box-Behnken design with 3 factors and 3 levels. The 17 experiments were prepared and observed. The optimized hydrogel patch was obtained with a PVA concentration of 17.07 % w/w, an FT cycle of 3.86 cycles, and a *B. rotunda* extract concentration of 50.00 % w/w (1.00 % w/w of dried extract). The FT-IR spectra and SEM images showed the presence of the extract in the hydrogel structure. The *B. rotunda* extracts enhanced the vapor permeability, strength, and flexibility of the hydrogel patch. Moreover, the *B. rotunda* extract-loaded hydrogel patch exhibited antimicrobial activity against *S. aureus* and *E. coli*. Besides, it could be a barrier to bacterial infusion. Furthermore, the hydrogel patch had antioxidant activity, providing the ability to improve the healing process by reducing traumatic oxidative stress. Importantly, the *B. rotunda* extract-loaded hydrogel patch enhanced NHF migration at 72-h treatment.

5.3 *D. bulbifera* extract and honey-loaded Gan/XG hydrogel patch fabricated by a thermal crosslinking method

The Gan/XG hydrogel patches were generated and optimized using a full central composite design. Thirteen experiment runs were prepared and measured. The optimized Gan/XG film was composed of Gan at a concentration of 17.78 % w/w and XG at a concentration of 0.1 % w/w. The FT-IR spectra indicated the crosslinked structure of Gan and XG in the optimized film. *D. bulbifera* and honey were embedded into the optimized film to prepare hydrogel patches. SEM images of *D. bulbifera* extract/honey-loaded and unloaded Gan/XG also showed the crosslinked structure of the Gan/XG hydrogel patches. The honey and *D. bulbifera* extract/honey-loaded Gan/XG hydrogel patches exhibited high absorbent activity, strength, and flexibility. Moreover, the hydrogel patches could protect the wound area from outside

bacteria. They also had a strong antioxidant activity which could improve the healing process by reducing oxidative stress. Besides, honey and the *D. bulbifera* extract/honey-loaded hydrogel patches were able to enhance the migration of NHF cells.



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