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Original article

Polyherbal formulation exerts wound healing, anti-inflammatory, angiogenic and antimicrobial properties: Potential role in the treatment of diabetic foot ulcers

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ABSTRACT

Diabetic foot ulcer (DFU) is a common and devastating complication in diabetic patients and is associated with an elevated risk of amputation and mortality. DFU remains a major therapeutic challenge due to poor understanding of its underlying pathogenesis. This complication is characterized by impaired wound healing; however, mechanisms causing this impairment are complicated and involve interactions between many different cell types and infections. In addition to other conventional DFU treatments, herbal foot baths are also common, although little is known about their mechanisms of action, and they contain a wide variety of herbal ingredients. In this study, we aimed to examine the effects of three polyherbal formulations consisting of medicinal plants used in traditional Thai herbal foot baths on wound healing, anti-inflammation, angiogenesis, and extracellular matrix modulation using high-concentration glucose-treated human keratinocytes, in addition to antibacterial evaluation. Our results showed that formulation 3 (F3) possessed the greatest potential to restore the impairment of keratinocytes caused by high glucose concentrations. We found that F3 could inhibit the growth of *Staphylococcus aureus*, accelerate wound healing, and upregulate the expression of TIMP-1, VEGF, and TGF-β, and downregulate the expression of TNF-α, IL-6, and MMP-9. Collectively, these data support the potential of F3 for therapeutic development in the treatment of DFU.

1. Introduction

Diabetes is a non-communicable disease (NCD) that has represented a major public health problem in countries around the world, including Thailand, for many years. The International Diabetes Federation (IDF) reported that, in 2017, there were approximately 425 million people with diabetes worldwide; this number is expected to increase to 629 million in the next 28 years (Cho et al., 2018). It is estimated that, presently, there are at least 5 million people with diabetes in Thailand. Each year, there are over 10,000 deaths due to diabetes-associated complications. According to the data of the Thai National Health Examination Survey (NHES V), the age-adjusted prevalence of diabetes in Thailand has risen sharply from 7.8% to 8.9% over the past 5 years (2009–2014), and it is likely to continue to increase (Aekplakorn et al., 2018). Despite the increasing number of people who are diagnosed and cured with diabetes, over 60% of these patients are unable to maintain their glucose levels in an optimal range. This results in a range of chronic complications, particularly in large blood vessels (e.g., blood vessels in the brain and heart) and small blood vessels

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(e.g., retinal and kidney capillaries), which can lead to death in diabetic patients.

Diabetic foot ulcer (DFU) is common complication in people with diabetes. A *meta*-analysis reported that the prevalence of chronic foot ulcers in diabetic patients was 6.3% globally (Zhang et al., 2017). Up to 25% of diabetic patients can develop foot ulcers (Singh et al., 2005). This foot complication is also a leading cause of loss or lower extremity amputation (LEA), including legs and feet. Data from the IDF reported that people with diabetes were at a 10–20-fold higher risk of amputation than non-diabetic people. It is believed that every 30 s, someone loses a leg or foot from diabetes (International Diabetes Federation, 2017); approximately 75–85% of patients in this group have a prior history of foot ulcers (Boulton et al., 2005). Moreover, the occurrence of chronic foot ulcers is a factor that increases the cost of care. A study in Western countries found that the cost of treating diabetic foot ulcers ranged from $1,000 to $17,000 per ulcer and could be as high as $66,000 in patients who required amputation (Raghav et al., 2018). Therefore, foot complications present a major problem affecting patients physically, mentally, economically, and socially (Vileikyte, 2001). Chronic wounds on the feet of diabetic patients are a complication that results from having persistently high blood glucose levels for an extended amount of time. Elevated blood glucose levels lead to destruction of the peripheral nervous system (peripheral neuropathy) and peripheral vascular disease (Clayton and Elasy, 2009, Noor et al., 2015). People with diabetes are more prone to foot injuries without being aware of them due to their abnormal peripheral sensory nervous system. The foot structure is also often deformed due to damage to the motor nervous system of the foot muscles combined with deterioration and narrowing of the blood vessels that supply the toes, causing the development of chronic wounds that are difficult to heal and are more likely to become infected. Due to these conditions, the wound healing process of the body is slower than normal in diabetic patients (Falanga, 2005). The mechanism underlying the abnormal wound healing process in diabetic patients is complex and involves the interaction of multiple cell types and involves chronic inflammation, impaired angiogenesis, abnormal proliferation and migration of keratinocytes, and abnormal extracellular matrix (ECM) remodeling (Brem and Tomic-Canic, 2007, Davis et al., 2018). In addition, foot ulcers in diabetic patients are often associated with infections, especially bacterial complications (Hirsch et al., 2008). For the effective and proper care of diabetic foot ulcers, the primary focus should be on addressing these underlying mechanisms.

Natural treatment with traditional Chinese and Thai herbal remedies are an option that may help to effectively care for and solve problems in this group of patients (Huang et al., 2015, Shuo et al., 2017). A review of research articles in Thailand studying the effectiveness of foot bathing with Thai herbs in treating foot complications in diabetic patients reported that most patients had improved foot sensation and a lower risk of pedicle insertion following treatment (Khumsub, 2018). However, there is a lack of knowledge on the bioactive mechanism of each constituent Thai medicinal plant in these foot baths. We attempted to develop a herbal medicinal formulation by determining the ingredients in the formulation according to the theory of traditional Thai medicine. The ingredients were selected from Thai herbs that have been used as ingredients in herbal foot bath recipes in the past, namely, *Centella asiatica*, *Curcuma longa*, *Zingiber cassumunar*, *Garcinia mangostana*, *Zingiber officinale*, *Eleutherine americana*, *Piper nigrum*, *Senna alata*, and *Areca catechu*. The purpose of the study was to evaluate the bioactivity of herbal extract formulations by targeting the mechanism of wound healing, anti-inflammation, antibacterial activity, and stimulation of blood vessel formation. The results obtained from this study will provide insight into the properties of Thai medicinal plants and aid in the development of suitable herbal formulations for the treatment of foot complications in diabetic patients.

2. Material and methods

2.1. Plant material

Thai medicinal plants (9 samples), including *C. asiatica*, *C. longa*, *Z. cassumunar*, *G. mangostana*, *Z. officinale*, *E. americana*, *P. nigrum*, *S. alata*, and *A. catechu*, were chosen for developing the herbal formulations and were collected from gardens or purchased from local markets. All plants were botanically authenticated, and their voucher specimens were deposited in the Department of Botany, Faculty of Science, Chulalongkorn University. The scientific names of the plants, parts used, plant authentication numbers, and percentage yield of their extracts are provided in Table 1.

2.2. Plant preparation and extraction

The plant materials were washed, dried in a hot air oven at 65 °C, and finely ground in a mechanical grinder. The extraction of the dried powder (80 g) was performed via the maceration method using ethanol (800 ml) as a solvent. After extraction, the solvent was removed using a Rotary evaporator and the dried residues were prepared as a 100 mg/mL stock solution by re-dissolving the residues in dimethyl sulfoxide (DMSO) and sterilizing the solution through a 0.2-μm pore syringe. The resulting extracts were stored at −20 °C and protected from light until use.

2.3. Cell culture

Human keratinocyte cell line (HaCaT) was used as a skin cell model in this study. Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C in a humidified incubator with 5% CO_2. Upon reaching approximately 80–90% confluence, the cells were sub-cultured in fresh medium to maintain their exponential growth or seeded to perform the experiments.

2.4. Cell viability assay

The cytotoxicity of the plant extracts on the HaCaT cell line was determined to obtain the optimal concentrations of plant extracts to be used in the study. The cytotoxic effect was evaluated by measuring the percentage of cell viability using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. HaCaT cells (5×10^3 cells) were seeded into a 96-well plate with DMEM medium and grown for 24 h. Then, the cells were treated with different concentrations of plant extract and incubated for 24 h. MTT reagent (20 μl) was added to each of the wells and incu-

Table 1
Scientific name, parts used, plant authentication number, and percentage extraction yield of Thai plants.

Scientific name	Parts used	Authenticate number	% Yield (w/w)
Centella asiatica (L.) Urb	Leave	016426 (BCU)	4.3
Curcuma longa Linn	Rhizome	013396 (BCU)	18.2
Zingiber cassumunar Roxb	Rhizome	013701 (BCU)	6.6
Garcinia mangostana Linn	Peel	016436 (BCU)	11.8
Zingiber officinale Roscoe	Rhizome	016425 (BCU)	5.3
Eleutherine americana (Aubl.) Merr	Rhizome	016530 (BCU)	3.5
Piper nigrum L.	Seed	016428 (BCU)	7.6
Senna alata (L.) Roxb	Leave	016298 (BCU)	12.0
Areca catechu Linn	Fruit	016434 (BCU)	6.7

bated for 3 h. The culture medium was removed and absolute DMSO (100 µl) was added to dissolve the purple formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Enspire, Perkin-elmer, USA). The percentage cell viability was calculated by comparison with the control (untreated cells). The concentration of plant extracts that maintained the rate of the survival over 80% were considered appropriate concentrations for use in this study.

2.5. Plant formula preparation

Three formulations of Thai herbal extracts (F1–F3) were prepared from the nine types of Thai herbal extracts tested for the optimum concentration range in the previous step. In each formulation, the composition was developed according to the theory of Thai traditional medicine that consists of the main drug, a secondary drug, and a synergistic drug (Table 2). Herbs that acted as the main drugs were selected from herbs that were reported to have wound-healing properties. Herbs that acted as secondary drugs were selected from medicinal herbs that were reported to have anti-inflammatory and antimicrobial effects. Herbs that acted as synergistic drugs were selected from herbs that were reported to have an effect on blood vessel stimulation.

2.6. Wound scratch assay

The study was conducted using a wound scratch assay, which is a technique for studying cell migration. HaCaT cells was grown under high glucose concentration (50 mM) to simulate diabetic conditions. Initially, the cell content and glucose concentration required to simulate diabetic conditions were tested. After determining the necessary cell volume and glucose concentration, all three formulations were used at their ideal concentrations based on the results of the previous experiment. The wound scratch assay was performed under high glucose concentrations. The cells were spaced (ulcers) by scraping directly onto the cell site to form an incision across the center of the cell plate. The lesions were then photographed at different time intervals with a phase-contrast camera, and the width of the scraped lesions were measured using ImageJ software (National Institutes of Health, USA). The results are expressed as percentage of wound healing (% wound closure), which was calculated by comparing the change in wound width at each measurement time with the starting width of the lesion (Muniandy et al., 2018).

2.7. Real-time quantitative PCR analysis (qRT-PCR)

HaCaT cells were seeded in six-well plates at 4×10^5 cells per well with high concentrations of glucose (50 mM) and the appropriate concentrations of formulations for 6 h at 37 °C in a humidified incubator with 5% CO_2. The total RNA was then extracted by adding TRIzol reagent to the cell well trays. The solution was then aspirated into 1.5-ml microtubes, chloroform was added, and the solution mixed using a vortex mixer. The tubes were centrifuged at high speed (120,000*g*) at 4 °C for 15 min to stratify the solution. The transparent layer became separated. The RNA-containing layer was placed in a new microtube containing isopropanol to precipitate the RNA. The solution was mixed in the ampoule and stored at −20 °C for 30 min. The tube was then centrifuged at 120,000*g* at 4 °C for 15 min. The supernatant was removed, ethanol was added, and the tubes were centrifuged at 7,500*g* at 4 °C for 5 min, resulting in the formation of RNA precipitate. The solution was left to dry at room temperature for 1 h. The RNA precipitate was then dissolved with RNase-free water and the resulting total-RNA solution was used to measure the RNA concentration using a NanoDrop spectrometer (Thermo Scientific, USA). The RNA was converted to complementary DNA (cDNA) by reverse transcription using the AccuPower RT Premix kit (Bioneer, South Korea). The cDNA was used as a sample for real-time PCR. The real-time PCR assay was performed using AccuPower 2X GreenStar™ qPCR Master Mix (Bioneer) in Exicycler™ 96 (Bioneer) using gene-specific primers (Table 3). The mRNA expression level was calculated via the delta-delta Ct method using GAPDH as an internal control (Rangsinth et al., 2021).

2.8. Antibacterial evaluation

The broth dilution technique and the CLSI M07-A9 standard test method were used to quantitatively evaluate antibacterial activity. Each herbal formulation solution was prepared at an initial concentration of 10 mg/mL. The extracts were diluted with culture medium to two-fold serial dilutions, with final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, and 0.019 mg/mL. The concentrations were tested against *Staphylococcus aureus* (ATCC strain 25923) in liquid medium (broth) at 37 °C for 24 h. Bacterial growth (minimum inhibitory concentration [MIC]) was determined based on the minimum concentration that prevented bacterial growth or cloudy medium. The turbidity-free culture medium was then spread on an agar plate to determine the minimum bactericidal concentration (MBC) based on the lowest sterile concentration where no bacterial growth was observed on the petri dish.

2.9. Statistical analysis

The results are presented as means ± standard error of the mean (SEM) from at least three independent experiments and were analyzed using SPSS version 16.0 software. One-way ANOVA with Tukey's honest significant difference post-hoc test was used for the evaluation of statistical significance. A p-value <0.05 was considered statistically significant.

Table 2
Formulations of Thai herbal extracts used in the study.

	Plant	Formulations		
		F1	F2	F3
Main drugs	*Curcuma Longa* Linn	●	●	●
	Zingiber cassumunar Roxb	●	●	●
	Garcinia mangostana Linn	●		
	Eleutherine americana (Aubl.) Merr		●	
	Areca catechu Linn			●
Secondary drugs	*Centella asiatica* (L.) Urb	●	●	●
	Senna alata (L.) Roxb	●	●	●
Synergistic drugs	*Zingiber officinale* Roscoe	●	●	●
	Piper nigrum L.	●	●	●

Table 3
Primer sequences used in real-time PCR.

Genes	Sequence of Primer (5′−3′)	Tm (°C)	PCR product (bp)
MMP-9_F	CCTTGTGCTCTTCCCTGGAG	59.4	112
MMP-9_R	CGACTCTCCACGCATCTCTG	59.2	
TIMP-1_F	GTTTTGTGGCTCCCTGGAAC	57.9	149
TIMP-1_R	GTCCGTCCACAAGCAATGAG	57.6	
TNF-α_F	GCCCATGTTGTAGCAAACCC	58.3	203
TNF-α_R	CTGATGGTGTGGGTGAGGAG	59.2	
IL-6_F	CACAGACAGCCACTCACCTC	59.5	125
IL-6_R	GCCTCTTTGCTGCTTTCACA	57.2	
VEGFA_F	CTCCACCATGCCAAGTGGTC	60.2	105
VEGFA_R	GCAGTAGCTGCGCTGATAGA	58.4	
TGF-β_F	CCCTGGACACCAACTATTGC	57.2	164
TGF-β_R	GTCCAGGCTCCAAATGTAGG	56.7	
GAPDH_F	ACATCGCTCAGACACCATGG	58.7	94
GAPDH_R	ACCAGAGTTAAAAGCAGCCCT	57.4	

3. Results

3.1. Different single plant extracts and polyherbal formulations exhibited dose-dependent cytotoxicity in human keratinocytes

The results of the MTT assay for single-plant extracts revealed that, when comparing the results of all nine herbs, *C. longa* rhizome extract and *G. mangostana* peel extract exhibited the highest cytotoxicity. The maximum concentration of both extracts that resulted in cell survival greater than 80% was 5 μg/mL, while *C. asiatica*, *E. americana*, and *S. alata* extracts required up to 100 μg/mL. The remaining four plant extracts had maximum optimal concentrations of 10 μg/mL (Fig. 1).

Once the optimal maximum concentration of a single plant extract was determined, the mixed formulation was prepared. Each herbal extract was prepared in the form of a stock solution of 100 mg/mL in DMSO solvent and mixed in equal proportions according to the types of herbs specified in the formulation (F1, F2, and F3), as shown in Table 2. A new concentrated solution of the formulation was obtained, with a concentration of each extract of 14.29 mg/mL. When tested, the highest final concentration was 14.29 μg/mL.

The toxicity of the three formulations was then assessed at final concentrations of 0.14, 0.36, 0.71, 1.43, 3.57, 7.14, and 14.29 μg/mL. The survival of the treated cells after 24 h was compared with cells in the control group that were not exposed to herbal extracts. The maximum concentration of all herbal formulations that resulted in cell survival greater than 80% was 1.43 μg/mL (Fig. 2).

3.2. Polyherbal formulations accelerated wound healing in high-concentration glucose-treated human keratinocytes

The wound-healing ability of optimal concentrations of the formulations was evaluated using a wound scratch assay on HaCaT cells initially to determine the optimal cell count for study with this technique. The number of cells cultured per well was tested in 12-well culture trays as follows: 300,000, 350,000, 400,000, 450,000, 500,000, and 550,000 cells. The results showed that 400,000 cells per well was an appropriate density as it yielded a cell density of approximately 95% of the total area 24 h after the cells appeared in the wells.

After determining the ideal number of cells per well, the optimal glucose concentration to simulate diabetic conditions in the cells was determined by experimenting with glucose solutions at various concentrations (5–50 mM). HaCaT cell cultures were voided (ulcerated) by directly scraping the cell site. Microscopic images were taken at 0, 12, 15, 18, and 24 h to measure the width of the scraped wound. The results indicated that the percentage of wound closure tended to decrease as the glucose concentration increased. At 50 mM glucose, there was a significant reduction in wound healing compared with other concentrations 12 h after glucose addition (Fig. 3A). Therefore, a glucose concentration of 50 mM was determined as the optimal concentration to simulate diabetic conditions; a glucose concentration of 5 mM was used to simulate normal conditions in the control group.

Once the cell volume and the appropriate glucose concentration had been determined, the wound healing ability of varying concentrations of formulation was tested. Cells were cultured in 12-well trays, with each well containing 400,000 cells, a final glucose concentration of 50 mM, and final formulation extract solution concentration of 0.14, 0.71, or 1.43 μg/mL. Cells receiving only glucose at a concentration of 5 mM was the control group. Cells in each well were scraped to form a gap (wound), and the width of the scraped wound was measured at 0, 6, 12, and 24 h. Wound healing (percentage of wound closure) of cells in the different test groups was observed at 12 h, as this was the shortest time that cells treated with only 50 mM glucose showed a significant reduction in the percentage of wound healing. As shown in Fig. 3B–C, cells in the 50 mM glucose group showed a significant reduction in the percentage of wound healing compared with cells in the control group (26% vs 52%, respectively; $p < 0.05$). In contrast with the glucose-treated group, it was found that the herbal formulations were able to significantly induce wound healing. Among the tested formulations, F2 showed the highest efficacy in a dose-dependent manner, with increased wound healing up to 50% at a concentration of 1.43 μg/mL. Moreover, F1 and F3 were found to increase the percentage of wound healing up to 40% at concentrations as low as 0.14 μg/mL. With increasing concentrations of the formulations, the percentage of wound healing was reduced; however, the percentage wound healing remained higher than the control group.

3.3. Polyherbal formulations ameliorated inflammation in high-concentration glucose-treated human keratinocytes

To assess the anti-inflammatory activity of the three formulations, the gene expression of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) were measured using qRT-PCR in human keratinocytes under simulated diabetes conditions (50 mM glucose) with or without the addition of herbal formulations (0.14 μg/mL or 1.43 μg/mL). The cells that received only glucose at a concentration of 5 mM were used as the control.

As shown in Fig. 4A–B, the cells cultured under simulated diabetes conditions showed an approximately 1.6-fold increase in TNF-α gene expression and approximately 6-fold increase in IL-6 gene expression, which was significantly higher compared with the control group. The results indicated abnormalities in the inflammatory mechanism of cells under simulated diabetes conditions. However, following treatment with the herbal formulations, it was found that some formulations were able to modify the expression of genes that were altered during diabetes simulation, which returned close to their baseline level of expression, as in the control group. Among the three formulations, it was found that F1 was most effective in reducing the expression of both inflammatory genes, the levels of which were significantly reduced close to levels observed in the control cells. F2 was able to significantly reduce IL-6 gene expression at 0.14 μg/mL, and F3 was able to significantly reduce TNF-α gene expression at 0.14 μg/mL and 1.43 μg/mL.

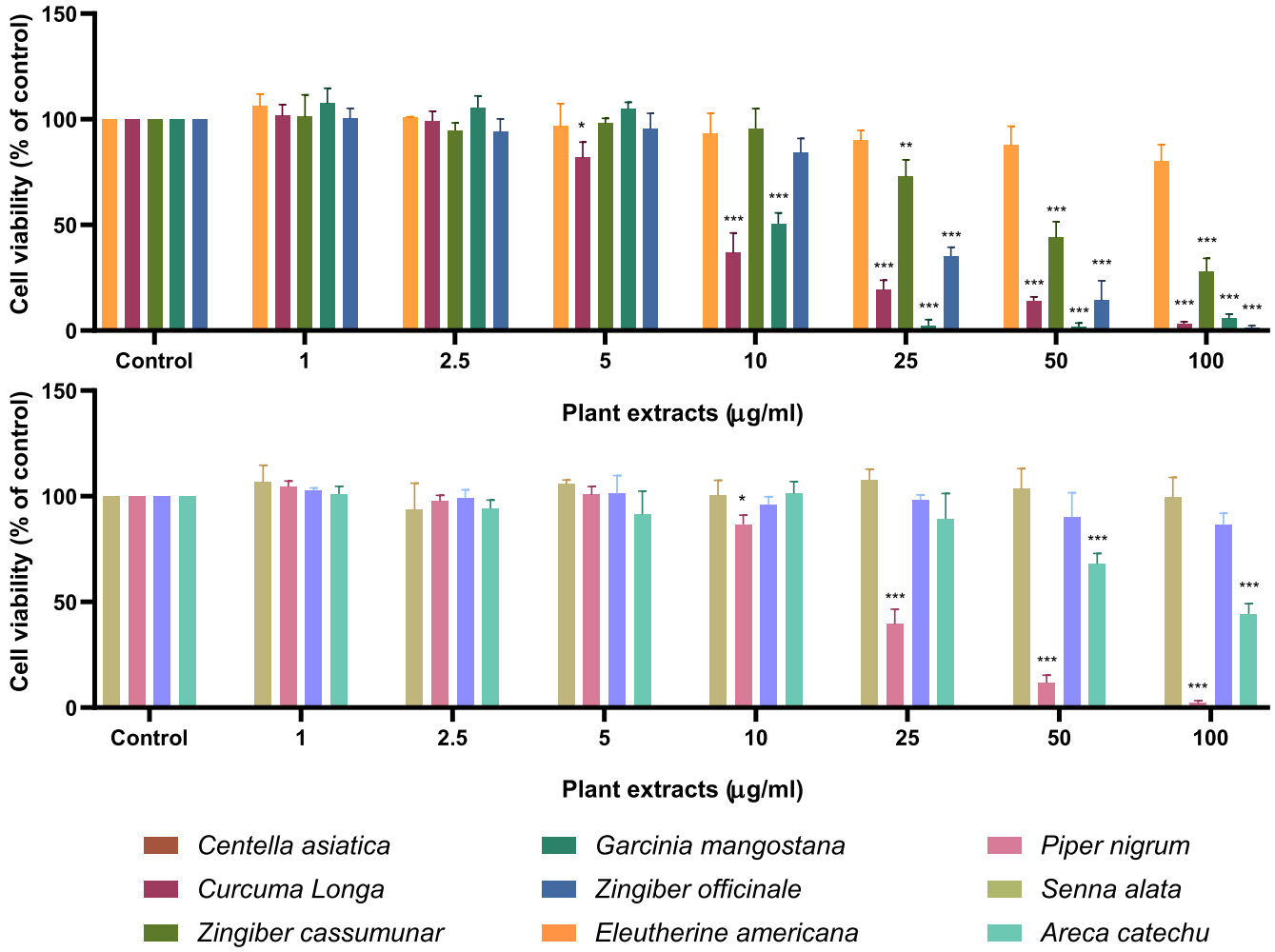

Fig. 1. Dose-dependent cellular toxicity in human keratinocytes after exposure to nine different plant extracts. Cell viability was tested via MTT assay in HaCaT cells treated with ethanol extracts of each plant at various concentrations for 24 h. Data are expressed as means ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

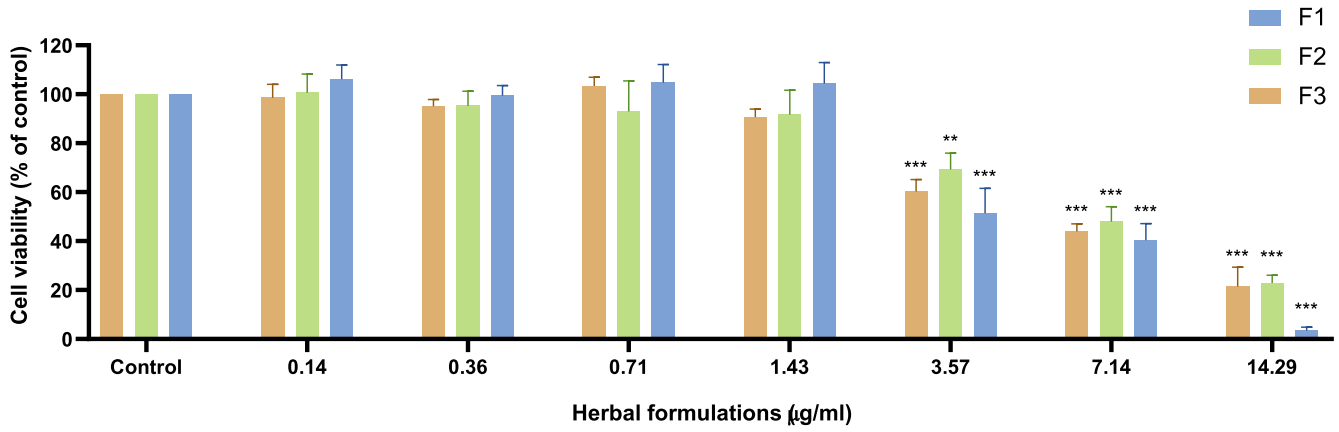

Fig. 2. Dose-dependent cellular toxicity in human keratinocytes following exposure to three different herbal formulations. Cell viability was tested via MTT assay in HaCaT cells treated with each herbal formulation at various concentrations for 24 h. Data are expressed as means ± SD, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control.

3.4. Polyherbal formulations promoted angiogenesis in high-concentration glucose-treated human keratinocytes

To investigate the angiogenesis-inducing capacity of the three formulations, changes in the expression of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) genes were measured at the RNA level by qRT-PCR in human keratinocytes under simulated diabetic conditions (50 mM glucose) with or without herbal formulations. The changes in expression under these conditions were compared with the control cells, which received only 5 mM glucose.

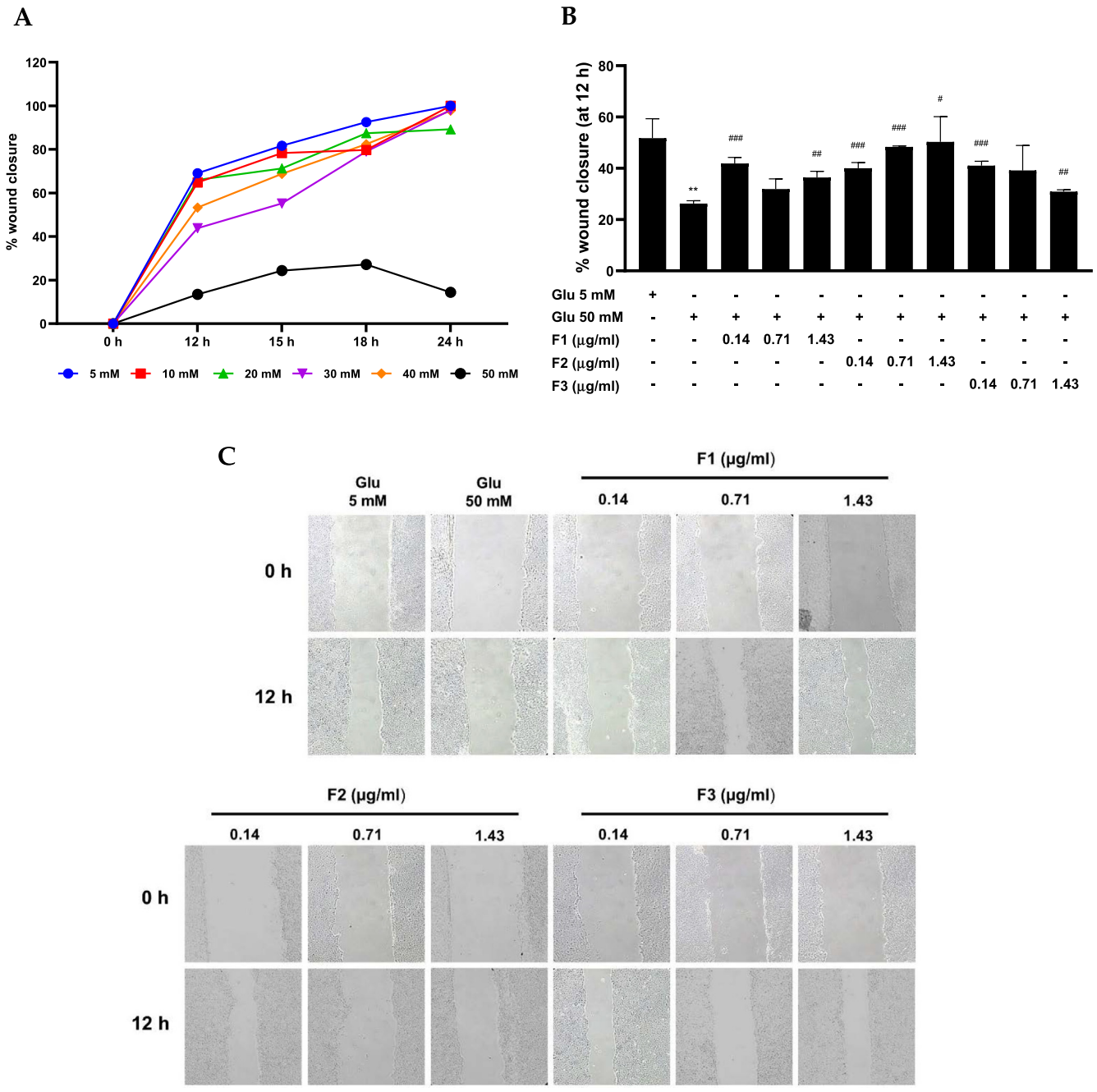

Fig. 3. Effects of glucose and three different herbal formulations on *in vitro* scratch-wound healing assay in human keratinocytes. (A) Percentage of wound closure following exposure to various concentrations of glucose at different time intervals. (B) Percentage of wound closure following exposure to various concentrations of three different herbal formulations under high glucose conditions (50 mM) for 12 h. (C) Representative images from wound scratch assay taken the cells at different time points and under different treatments. Data are expressed as means ± SD, $^{**}P < 0.01$ vs. normal glucose conditions (5 mM); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. high glucose conditions (50 mM).

As shown in Fig. 5A–B, cells cultured under simulated diabetes conditions showed a significant reduction in TGF-β gene expression of approximately 80% (0.2-fold decrease). There was no significant reduction in the gene expression of VEGF in the diabetes cells compared with cells in the control group. This observation indicates an abnormality of angiogenesis under the simulated diabetes conditions. The expression of these abnormal genes was modified upon treatment with herbal formulations and returned close to the expression levels observed in cells in the control group.

Among the three formulations, F3 was most effective in stimulating angiogenesis. Concentrations of 0.14 µg/mL and 1.43 µg/mL were found to significantly increase the gene expression of both VEGF and TGF-β to levels greater than or close to those observed in the control cells. F2 was able to increase VEGF gene expression at a concentration of 1.43 µg/mL; however, F1 was unable to significantly increase VEGF gene expression. Furthermore, it was found that both F1 and F2 were able to significantly restore the gene expression of TGF-β to levels similar to those observed in the control cells.

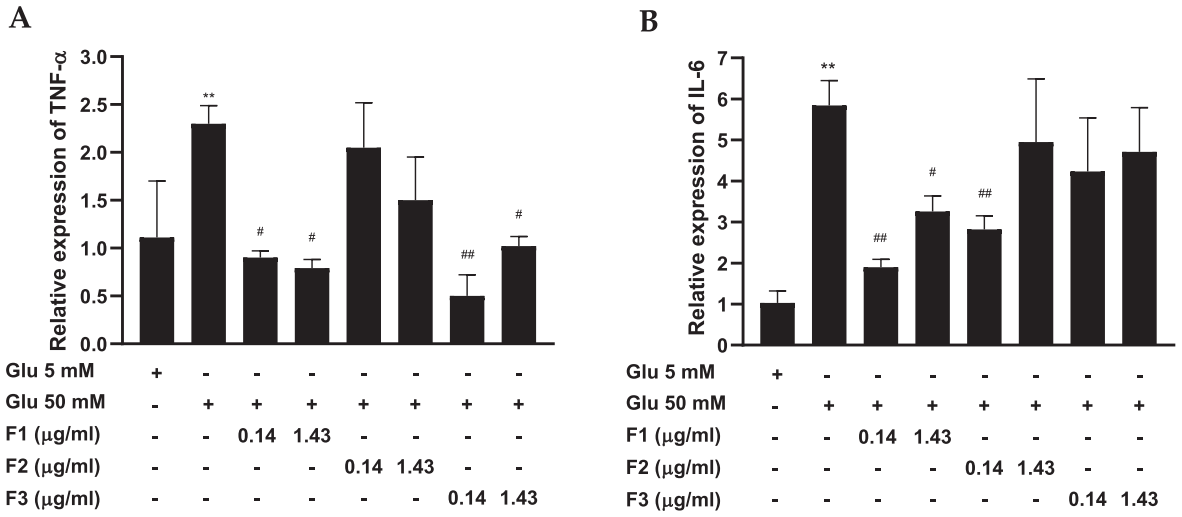

Fig. 4. Effects of three different herbal formulations on gene expression of inflammatory cytokines in human keratinocytes. The qRT-PCR analysis of (A) TNF-α and (B) IL-6 mRNA expression in HaCaT cells exposed to various concentrations of three different herbal formulations under high glucose conditions (50 mM) for 6 h. Data are expressed as means ± SD, $^{**}P < 0.01$ vs. normal glucose conditions (5 mM); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. high glucose conditions (50 mM).

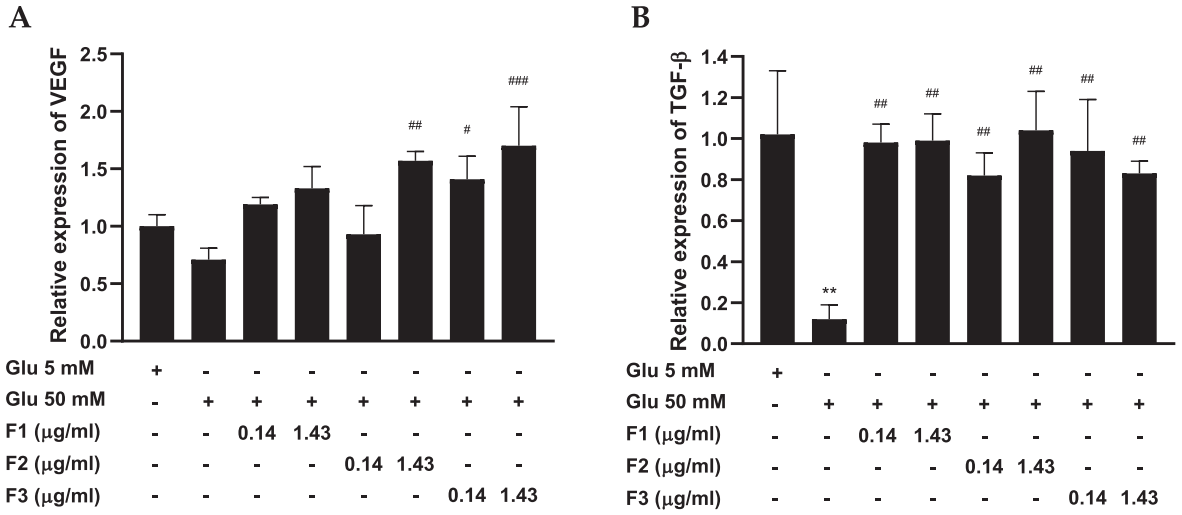

Fig. 5. Effects of three different herbal formulations on gene expression of angiogenic factors in human keratinocytes. The qRT-PCR analysis of (A) VEGF and (B) TGF-β mRNA expression in HaCaT cells exposed to various concentrations of three different herbal formulations under high glucose conditions (50 mM) for 6 h. Data are expressed as means ± SD, $^{**}P < 0.01$ vs. normal glucose conditions (5 mM); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. high glucose conditions (50 mM).

3.5. Polyherbal formulations modulated regulatory proteins for extracellular matrix remodeling in high-concentration glucose-treated human keratinocytes

To investigate the astringent activity of the three formulations, changes in the mRNA expression of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor matrix metalloproteinase 1 (TIMP-1) were evaluated via qRT-PCR under simulated diabetes conditions (50 mM glucose) in human keratinocytes with or without herbal formulations. The changes in mRNA expression were compared with the control cells, which received only 5 mM glucose.

As shown in Fig. 6A–B, cells cultured under simulated diabetes conditions showed an approximately 7-fold increase in MMP-9 gene expression and an approximate 0.2-fold decrease (i.e., 80% reduction) in TIMP-1 gene expression as compared with cells in the control group. These findings indicated malfunction of the wound healing mechanism in the simulated diabetes cell model. However, when the cells were treated with herbal formulations, some formulations were found to modify the abnormal gene expression and restore the expression to levels similar to those observed in the control group.

Among the three formulations, F3 showed the most potent inhibitory effect on MMP-9 gene expression, reducing MMP-9 gene expression to levels close to those of control cells at a concentration of 1.43 μg/mL. F1 reduced MMP-9 gene expression somewhat, to a level that was still higher than that of the control cells. F2 did not significantly reduce the gene expression of MMP-9. All herbal formulations were found to significantly increase the gene expression of TIMP-1. F2 and F3 were the most potent regarding their ability to increase TIMP-1 gene expression to levels close to those observed in control cells at concentrations of 1.43 μg/mL and 0.14 μg/mL, respectively. However, when the concentration of F3 was increased to 1.43 μg/mL, it did not significantly increase gene expression. F1 increased the gene expression of TIMP-1 somewhat, but expression levels remained lower than those of the control cells.

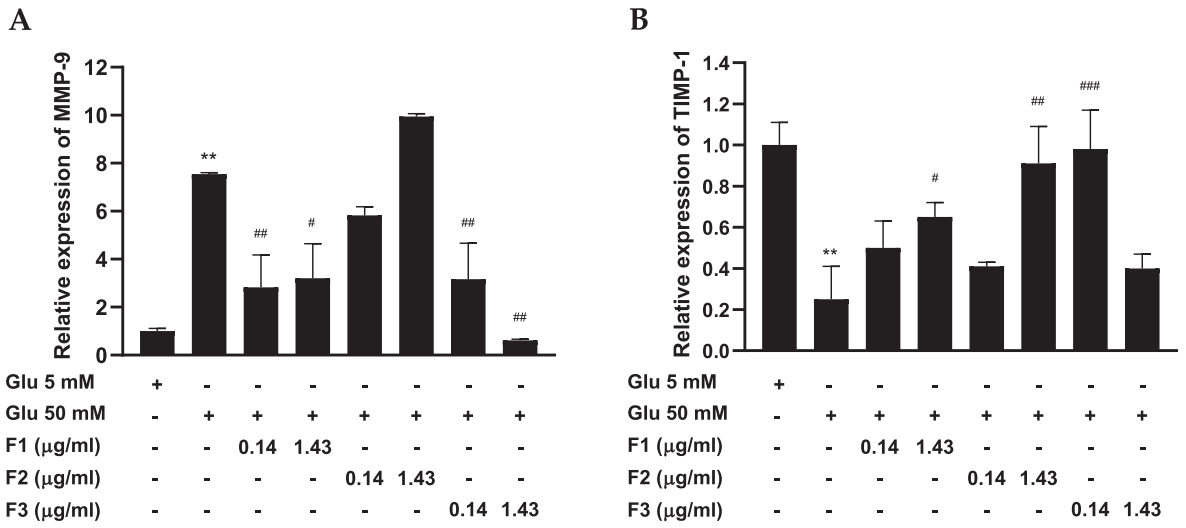

Fig. 6. Effects of three different herbal formulations on gene expression of regulatory proteins for extracellular matrix remodeling in human keratinocytes. The qRT-PCR analysis of (A) MMP-9 and (B) TIMP-1 mRNA expression in HaCaT cells exposed to various concentrations of three different herbal formulations under high glucose conditions (50 mM) for 6 h. Data are expressed as means ± SD, $^{**}P < 0.01$ vs. normal glucose conditions (5 mM); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. high glucose conditions (50 mM).

3.6. Polyherbal formulations possessed antibacterial activity against the skin pathogen Staphylococcus aureus

All formulations exhibited antibacterial properties (Table 4). F1 was found to be the most effective against *S. aureus* when considering both MIC and MBC. Although all formulations were able to inhibit bacterial growth at the lowest concentration (MIC) of 0.156 mg/mL, F1 was more effective at killing the bacteria (MBC) than F2 and F3. Regarding the mode of action (Mogana et al., 2020), only F2 possessed bactericidal activity against *S. aureus*, as indicated by an MBC/MIC ratio > 4, whereas the other two formulations showed only bacteriostatic activity against *S. aureus* (MBC/MIC $\leq$ 4).

4. Discussion

The purpose of this research was to develop an herbal formulation as an alternative treatment for foot complications in diabetic patients. We developed three different formulations from herbal extracts that were common ingredients in herbal foot bath recipes in sub-district health-promoting hospitals in Thailand. The composition of all formulations was primarily constructed following the theory of traditional Thai medicine, which consisted of two herbs (*C. longa* and *Z. cassumunar)* functioning as the main drugs, two herbs (*C. asiatica* and *S. alata*) functioning as the secondary drugs, and two herbs (*Z. officinale* and *P. nigrum)* functioning as the synergistic drugs. Then, one additional herb acting as a main drug component was added to each formulation: *G. mangostana* to F1, *E. americana* to F2, and *A. catechu* to F3. The formulation structure with a single different herb may be able to provide a clearer understanding of the effectiveness of each herbal formulation developed in this study through the evaluation of anti-inflammatory activity, angiogenesis, ECM modulation, wound-healing activity, and antibacterial properties.

In the present study, human keratinocytes (HaCaT) cultured in a medium containing a high glucose concentration (50 mM) were used as a cell model of a diabetic wound. Following treatment, we found that this cell model demonstrated an impaired wound healing process, angiogenesis, and induced inflammation through increased gene expression of MMP-9, TNF-α, and IL-6, decreased gene expression of TIMP-1 and TGF-β, and no significant change in VEGF gene expression. All the observed mechanistic changes corresponded to the real conditions under which diabetic wound healing is impaired, thereby substantiating the use of this cell model and experimental conditions for the study of DFU (Pradhan et al., 2009, Baltzis et al., 2014, Li et al., 2019, Patel et al., 2019).

Inflammatory mediators are known to play a key role in the wound healing process. Abnormally increased levels of inflammatory mediators due to chronic inflammation can delay wound healing and are associated with the development of chronic ulcers in diabetic patients (Baltzis et al., 2014, Patel et al., 2019). The anti-inflammatory properties of herbal formulations were analyzed through gene expression analysis. F1 was found to be the most effective formulation for anti-inflammation by through its ability to reduce the expression of both TNF-α and IL-6 inflammatory mediators and restore them to levels observed in normal control cells. F2 and F3 also appeared to exhibit anti-inflammatory properties; both formulations were able to alter the gene expression of either TNF-α or IL-6.

Angiogenesis is one of the important steps in the wound healing process, with VEGF and TGF-β acting as a stimulus. Platelet-derived TGF-β plays a crucial role in the regulation of the early wound healing process (Hozzein et al., 2015). TGF-β has been shown to stimulate keratinocyte proliferation, keratinocyte migration, and collagen production (Braga Gomes et al., 2014), and restore the decreased VEGF expression of keratinocytes in diabetic wounds (Blakytny and Jude, 2006). VEGF is a pro-angiogenic factor and its levels influence the wound healing process by regulating both vasculogenesis and angiogenesis (Patel et al., 2019). However, TGF-β and VEGF levels have been found to be abnormal in diabetic patients, leading to delayed wound healing (Baltzis et al., 2014, Patel et al., 2019). Therefore, the ability of herbal formulations to stimulate blood vessel formation was also evaluated. The results showed that F3 was the most effective formulation for inducing angiogenesis as it was able to stimulate the release of angiogenic factors (VEGF and TGF-β) at levels that were close to or higher than the levels in normal cells.

During the remodeling phase of wound healing, new ECM components must be synthesized while old ones must be degraded.

Table 4
Antibacterial activity of three different herbal formulations against *S. aureus.*

Formula forms	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC ratio
F1	0.156	0.313	2.006
F2	0.156	0.625	4.006
F3	0.156	0.625	4.006

This process is largely controlled by the activity of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs); both MMP-9 and TIMP-1 are considered to be main mediators of the wound healing process (Falanga, 2005, Blakytny and Jude, 2006, Pradhan et al., 2009). Abnormalities in this enzyme and inhibitor (elevated MMP-9 and decreased TIMP-1) can lead to an imbalance in the formation and breakdown of the ECM, resulting in delayed wound healing (Baltzis et al., 2014, Suryavanshi and Kulkarni, 2017, Patel et al., 2019). We evaluated the ability of herbal formulations to modulate ECM remodeling and found that F3 was the most effective formulation in this regard. F3 was able to induce convergent cell motility across the simulated gap (wound), reduced the expression of MMP-9, and increased the expression of TIMP-1 close to levels observed in normal control cells.

Diabetic wounds are often associated with a bacterial infection, which can delay wound healing and intensify the wound (Hirsch et al., 2008). In this study, herbal formulations were tested against the pathogenic skin bacterium *S. aureus*. The results showed that F1 was the most effective antibacterial formulation due to the low concentration needed to kill all bacteria (MBC). The strength of F1 could be attributed to its constituent *G. mangostana* peel extract, which is well known for its potent antibacterial activity against several gram-positive and gram-negative bacterial species (Pedraza-Chaverri et al., 2008, Chomnawang et al., 2009, Tatiya-Aphiradee et al., 2016). Nevertheless, F2 and F3 were also considered to possess satisfactory antibacterial activity and had MIC values identical to that of F1.

The findings of this study are supportive of our developed herbal formulations, particularly F3, which have the potential to be further developed as an alternative treatment for DFU. Among the formulations, F3 containing *A. catechu* showed the highest capacity to act on multiple mechanisms related to wound healing process, including stimulating cell movement, modifying the expression of an enzyme and its inhibitor involved in the formation and breakdown of ECM, and upregulating the expression of angiogenic factors. Moreover, F3 was only slightly less effective than the other formulations regarding anti-inflammatory and antibacterial activity. As *A. catechu* was the only plant unique to F3 that could be responsible for the observed effects, further studies are needed to explore the properties of *A. catechu* extract.

5. Conclusions

Our study revealed the potential role of polyherbal formulations, which consisted of seven Thai plant extracts and were developed based on traditional Thai medicinal concepts, in the alternative treatment of DFU. Considering the complexity of the wound healing process, the developed herbal formulations were evaluated regarding multiple mechanisms involved in wound healing and we found that F3 exhibited the most potent ability to promote diabetic wound repair and suppress pathogenic bacterial infection of the skin. This study provides evidence supporting the efficacy of polyherbal formulations containing *C. longa*, *Z. cassumunar*, *C. asiatica*, *S. alata*, *Z. officinale*, *P. nigrum*, and *A. catechu* as promising alternatives in the treatment of diabetic wounds. Further studies on preclinical and clinical models, in addition to the effects of mixtures, are necessary to validate and improve the beneficial therapeutic outcomes of these formulations in DFU treatment.

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Author contributions

Conceptualization, T.T. and A.P.; Investigation, S.C., M.S. and S.I.; Data curation, validation and visualization, S.C. and A.P.; Writing – Original Draft Preparation, S.C. and A.P.; Writing – Review & Editing, S.C., T.T. and A.P.; Supervision, T.T. and A.P.; Resources, T.T. and A.P.; Funding acquisition, T.T. and A.P. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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