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Research Article

Formulations of Topical Ointment for Wound Healing Activity using *Gynura procumbens* (Lour.) Merr. Leaves Extract

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ABSTRACT

Gynura procumbens (Lour.) Merr., an herb found in Southeast Asia and China, has been used in traditional medicine for treatment at a wide range of health ailments. Several biological activities were reported including wound healing activity. The previous studies revealed that ethanolic *G. procumbens* leaves extract contained chlorogenic acid as the major compound, which exhibited antioxidant, anti-inflammatory and wound healing potential. In this study, we aimed to develop topical formulations of *G. procumbens* extract and evaluate wound healing activity in mice. *G. procumbens* was extracted with 95% ethanol. Phytochemicals were investigated and quantified using thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) techniques. The analysis of total phenolic content (TPC), total flavonoid content (TFC) and diphenyl-picrylhydrazyl (DPPH) radical scavenging assay were performed by spectrophotometric techniques. Topical ointment of *G. procumbens* leaves extract for further pharmaceutical purposes was developed. The stability and shelf life of these formulations, as well as the crude extract, underwent evaluation in accordance with ASEAN guideline. Assessments were carried out at 0, 3, and 6 months under specified storage conditions. The effect of an ethanolic *G. procumbens* extract on wound healing activity in murine subjects was investigated. A full thickness excisional skin wound was generated on the shaved dorsum of eight-week-old C57BL/6 mice. *G. procumbens* ointment (0.5 or 2%w/w) or ointment base was applied once daily for 7 days. The wound size was monitored once a day for 14 days. Our results showed that ethanolic *G. procumbens* leaves extract contained total phenolic and total flavonoid at 43.80 ± 1.79 μg GAE and 132.67 ± 1.40 μg QE in 1 g extract, respectively. For DPPH radical scavenging activity, the extract exhibited IC_{50} of 181.70 ± 0.76 µg/mL. Formulations of topical *G. procumbens* ointment with 0.5% and 2% w/w extract were developed. Stability study revealed that *G. procumbens* ointment formulas and crude extract unstable under accelerated stored at 40°C ± 2°C/75% RH ± 5% RH in tightly closed container for 6 months. All phytochemical contents decreased approximately 4–27%. For *in vivo* wound healing study, topical *G. procumbens* ointment showed a good appearance and smooth texture when applied on dorsal back of mice but did not significantly accelerated wound closure compared to the ointment base-treated controls. Therefore, although *G. procumbens* extract trend to potential for antioxidant and wound healing activity, it is imperative to conduct further studies on the chemical degradation pathways that could compromise the potency and quality of drug products. Additionally, employing larger sample sizes in these studies is recommended to ensure accuracy and reliability of the results.

Keywords:
Gynura procumbens; Ointment; Stability; Wound healing activity.

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1. INTRODUCTION

The skin, recognized as the body's largest organ, consists of three primary layers: the epidermis, dermis, and hypodermis. This highly dynamic organ undergoes continuous renewal and contains a diverse array of specialized cells and structures. Its primary functions include serving as a barrier against pathogens, transmitting sensory information from the external environment, and contributing to the immune response to protect against diseases. Additionally, the skin is essential for thermoregulation, which is vital for maintaining homeostasis and supporting optimal physiological functions[1-3].

Wound care, an ancient practice, has experienced limited progress in effective treatments despite centuries of accumulated knowledge. Chronic and acute wounds pose significant global challenges, often resulting in impaired tissue repair and complications such as microbial infections. Chronic wounds, including pressure ulcers and diabetic ulcers, are particularly problematic due to their prolonged healing times and susceptibility to microbial colonization. Acute wounds, such as burns and cuts, also face risks of microbial infections. Efforts to develop effective treatments for both types of wounds have struggled to minimize side effects and costs. Ongoing research seeks to innovate therapeutic strategies to enhance wound healing outcomes and improve the quality of life for those affected[4,5].

Gynura procumbens (Lour.) Merr., commonly known as “longevity spinach” and known as “pra kham di khwai,” "pae-tam pueng," or "mumaeng sang" in Thai, is an herbaceous plant characterized by its climbing growth pattern. It is native to Southeast Asia and China, particularly found in regions such as China, Indonesia, Malaysia, Vietnam, and Thailand, where it thrives in diverse habitats including forested areas, sandy slopes, and as a creeper on shrubs or trees[6,7]. The leaves of *G. procumbens* have garnered attention in traditional medicine due to their various health benefits and are also commonly incorporated into culinary practices owing to their non-toxic plant[8]. *G. procumbens* is employed for the management of various health conditions including kidney disorders, fevers, skin conditions, hypertension, diabetes mellitus, hypercholesterolemia, gastrointestinal issues, migraines, and cancer[9-11]. Furthermore, in traditional Chinese medicine, it is applied topically to alleviate inflammation, particularly in the context of traumatic injuries, and is also used to address skin ailments caused by insect bites. Similarly, in Thailand, it is extensively used to reduce inflammation, alleviate rheumatic conditions, and combat viral infections[12-14].

The leaves of *G. procumbens* contains flavonoids and phenolics as major groups of substances, A previous study indicated that an ethanolic extract contained chlorogenic acid, nicotiflorin, and astragalin as major constituents[15] (Figure 1.). Additionally, kaempferol, quercetin, rutin, sterol glycosides, stigmasterol, saponins, tannins, terpenoids, essential oils, and other compounds were also identified[15-18].

G. procumbens has been the focus of numerous studies due to its antioxidant, anti-inflammatory, and wound healing properties. Particularly, its ethanolic extract from the leaves demonstrated excitatory effects on monocyte adherence to activated endothelial cells. This extract also reduces the expression of key proteins involved in monocyte-endothelial interaction, such as ICAM-1, VCAM-1, and MCP-1, by suppressing the NF-κB signaling pathway. Furthermore, *G. procumbens* extract decreases the secretion of inflammatory markers such as NO, TNF-α, and PGE2 by inhibiting AP-1 and NF-κB nuclear translocation, mediated through the downregulation of PI3K/Akt and MAPK signaling pathways[19-21]. Additionally, the ethanolic extract of *G. procumbens* demonstrated potent antioxidant properties and exhibited anti-inflammatory effects by modulating nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression in RAW264.7 macrophages stimulated with lipopolysaccharide (LPS)[22].

The prior research demonstrated that a 95% ethanol extract from *G. procumbens* was effectively enhanced wound healing in regular and diabetic mice by inducing the expression of angiogenic factors such as angiogenin (ANG), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) in various cell types including keratinocytes, endothelial cells, fibroblasts, and mast cells. Additionally, the extract stimulated proliferation in keratinocytes, fibroblasts, and endothelial cells[23].

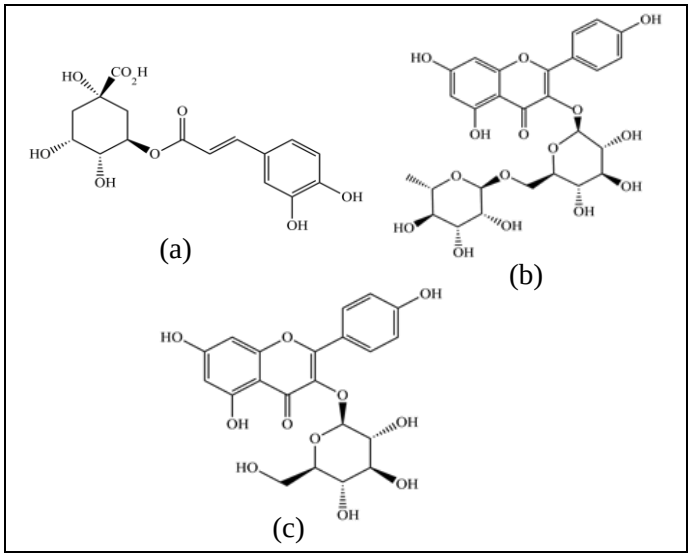

Figure 1. Chemical structures of the major compounds in *G. procumbens*.: Chlorogenic acid (a), Nicotiflorin (b), Astragalin (c).

Chlorogenic acid, the major compound, demonstrated potential for anti-inflammatory and wound healing properties. When formulated into an ointment, it exhibited notable efficacy in wound healing by accelerating wound closure, enhancing skin regeneration, stimulating cell growth, and increases levels of TNF-α during the initial inflammation phase. Additionally, it enhanced the expression of TGF-β1 and collagen IV synthesis while exhibiting strong antioxidant properties by elevating the levels of superoxide dismutase, catalase, and glutathione enzymes and reducing lipid peroxidation. Furthermore, chlorogenic acid significantly increase capillary-like tube formation in endothelial cells, promoting angiogenesis, and accelerates the fibroblastic and remodeling phases of wound healing[24-26].

In this study, the phytochemical composition of *G. procumbens* was investigated and quantified, with a specific focus on its wound healing activity. Chromatographic techniques, such as thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC), were performed for phytochemical analysis. Spectrophotometric methods were utilized to determine the total phenolic content, total flavonoid content, and diphenyl-picrylhydrazyl (DPPH) radical scavenging activity. Furthermore, the research involved the formulation of a topical application using *G. procumbens* leaves extract for potential pharmaceutical applications. The study aimed to optimize the formulation for enhanced wound healing efficacy. This involved assessing various parameters such as stability, shelf-life, and efficacy in promoting wound healing. The goal was to develop an effective topical treatment derived from *G. procumbens* for use in wound management.

2. MATERIALS AND METHODS

2.1. Plant materials and sample extraction

Fresh leaves of *G. procumbens* (Figure 2.) were purchased, the authentic specimen was identified using flora of China volume 20-21, page 541, 2011 and compared with herbarium specimens kept at the Forest Herbarium (BKF), Department of National park, Wildlife and Plant Conservation, Bangkok. Voucher specimens were identified by Dr. Sunisa Sangvirotjanapat, Sireeruckhachati Nature Learning Park, Mahidol University. The voucher specimens (PBM 006192) have been deposited at the Herbarium of Mahidol University.

2.2. Raw material extract preparation

Fresh leaves of *G. procumbens* underwent washing and subsequent drying under hot air at 50 °C for 3 days before being ground using a grinder with a sieve size of 20. The resulting leaves powder was then extracted with a 95% ethanol solvent at a ratio of 1 : 10 w/v for 3 days. This extraction processes were repeated three times, and the collected extract was then filtered and evaporated to dryness using a rotary evaporator under reduced pressure. The percentage yields of extracts were calculated, and all extracts were stored in desiccators until use.

2.3. Thin layer chromatographic (TLC) analysis of *G. procumbens* extract

The crude extract was dissolved in methanol at a concentration of 2 mg/mL. Subsequently, a 10 µL volume of the sample solution was applied as a band with a length of 5 mm. onto a precoated aluminum plate containing silica gel 60 GF254, using ethyl acetate : formic acid : methanol : water (20 : 0.5 : 2.5 : 2 v/v/v/v) as the solvent system. Chlorogenic acid, serving as the reference standard, was dissolved in methanol at a concentration of 1 mg/mL, and a 10 µl volume of the standard solution was applied in a similar manner. Each chromatogram was developed to a height of 8 cm. TLC plates were visualized under UV light at wavelengths of 254 and 366 nm, as well as under NP/PEG spraying reagent. The resulting TLC fingerprints were subsequently analyzed.

2.4. Quantitative analysis of chlorogenic acid by High Performance Liquid Chromatography (HPLC)

Both the crude extract and the standard chlorogenic acid were dissolved in methanol, achieving concentrations of 5 and 1 mg/mL, respectively, and then subjected to sonication for 30 minutes. Subsequently, the solutions underwent filtration using a nylon membrane filter with a pore size of 0.22 µm.

Figure 2. *G procumbens* (Lour.) Merr.

Phytochemical analysis of the *G. procumbens* leaves extract was carried out utilizing a Shimadzu LC-20 series equipped with a diode array detector. For quantitative analysis, a Purospher® STAR RP-18 endcapped LiChroCART® column (4.6 mm i.d. × 250 mm, 5 µm) was employed. Chromatographic separation was achieved through gradient elution[27] using a mixture of 0.25% acetic acid in water and acetonitrile (100:0 to 0:100 %v/v) in 65 minutes, at constant flow rate of 1 mL/min. Ultraviolet (UV) detection was conducted at 340 nm with an injection volume of 20 μL. Quantification of the sample was performed by measuring the integrated peak area, and the content was calculated using the calibration curve generated by plotting peak area against concentration of the respective standard, chlorogenic acid.

2.5. Determination of total phenolic content (TPC)

The total phenolic content (TPC) was assessed utilizing the Folin–Ciocalteau method[28]. *G. procumbens* extract (1 mg/mL in 95 % ethanol) and seven different concentrations of standard gallic acid (ranging from 6.25 to 150 µg/mL in 95% ethanol) were prepared. Subsequently, 25 µL of either the standard or plant extraction solution was added into each well, followed by mixing with 100 µl of 25% (v/v) Folin–Ciocalteau reagent. After a 5-minute incubation period, 100 µL of 7.5% (w/v) sodium carbonate was added and the mixture was left to stand in darkness for 1 hour at room temperature. The resulting, blue-colored solution was subjected to absorbance measurement at 765 nm using a microplate reader, with each sample analyzed in triplicate. The total phenolic content was determined based on the standard curve of gallic acid and expressed as milligrams of gallic acid equivalent (GAE) per gram of extract (mg GAE/g extract).

2.6. Determination of total flavonoid content (TFC)

The total flavonoid content was determined through the aluminum chloride colorimetric assay[28]. *G. procumbens* extract (1mg/mL in 95% ethanol) and seven concentrations of standard quercetin (ranging from 10 to 500 µg/mL in 95% ethanol) were prepared. Each well received 100 µL of deionized water (DI), followed by the addition of 25 µL of either the standard or plant sample solution. Subsequently, 10 µL of 5% (w/v) sodium nitrite solution was added, and the mixture was allowed to stand for 5 minutes. Afterward, 15 µL of 10% (w/v) aluminum chloride solution was added, followed by 50 µL of 1 M sodium hydroxide and another 50 µL of deionized water (DI). The absorbance was measured at 510 nm using microplate reader. The flavonoid content was then calculated using the standard curve of quercetin and expressed as milligram quercetin equivalent (QE) per gram of extract (mg QE/g extract).

2.7. Determination of antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method

The antioxidant capacity of *G. procumbens* extracts was evaluated through the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay[28]. This method involved assessing the ability of plant extracts, chlorogenic acid, and a standard solution (ascorbic acid) to neutralize free radicals using the DPPH radical scavenging method. Specifically, 100 µL of various concentrations of the extract (ranging from 15.62 to 1,000 µg/mL), chlorogenic acid (ranging from 0.78 to 50 µg/mL), or standard solution (ranging from 0.78 to 50 µg/mL) were combined with 100 µL of DPPH in methanol solution (0.2 mM). Following a 30-minute incubation period, the absorbance of each solution was measured at 517 nm using a microplate reader. The IC_{50} value was determined from the linear equation derived from the plot correlating sample concentration and inhibition percentage. Each experiment was conducted in triplicate.

2.8. Development of *G. procumbens* topical ointment

2.8.1 Solubility of the extracts

The ethanolic *G. procumbens* extracts, each weighing 1 mg, were dissolved in various solvents including distilled water, ethanol solution with concentrations ranging from 10 to 90% v/v, absolute ethanol, propylene glycol, and polyethylene glycol, all at ambient temperature. Solubility assessments were conducted in accordance with the quality control methods for medicinal plant materials outlined by the World Health Organization (WHO)[29].

2.8.2 Preparation of G. procumbens ointment

G. procumbens extract ointment was prepared by the incorporation of *G. procumbens* extract into PEG

Table 1. The compositions of *G. procumbens* ointment

Composition	Function	Formulation (%w/w)					
		F1	F2	F3	F4	F5	F6
PEG 400	Ointment base	39.5	49.5	59.5	69.5	79.5	89.5
PEG 4000	Ointment base	60	50	40	30	20	10
G. procumbens extract	API	0.5	0.5	0.5	0.5	0.5	0.5

400 at the concentration of 0.5 or 2% w/w, then a mixture of PEG 400 and PEG 4000 was melted on a hot plate by heating to 60°C. The mixture was then removed from the hot plate and was stirred continuously until it congealed[30] and kept at room temperature for 24 hours to observe its stability and consistency. The compositions of the formulations were presented in Table 1.

2.9. Stability test of *G. procumbens* topical ointment

Accelerated studies/testing was undertaken stability to established shelf-life of a finished product.

Testing frequency: 0, 3 and 6 months.

Storage conditions: Three batches of *G. procumbens* leaves extract and ointment were kept in glass bottle and sealed with aluminum foil for 6 months at accelerated condition: 40 ± 2°C / 75 ± 5% RH for generic products according to ASEAN guideline on stability study of traditional medicines[31].

2.9.1 Physical Appearance

The ointment formulations underwent evaluation for their organoleptic properties and uniformity post-packaging. Visual inspection involved assessing the physical attributes such as texture, color, odor, and smoothness to ensure consistency and quality.

2.9.2 Determination of Viscosity

The viscosity of all formulated was performed in a cone and plate type of rotational viscometer. Viscometer condition was set at shear rates of 5 (1/s), temperature of 30 ± 0.5°C, sensor C35/2, Gap of 0.104 mm; volume of 0.4 ml.

2.9.3 Determination of microbial contamination

The presenting of microorganisms including bacteria, yeast and mold in the product was determined through microbial enumeration tests and tests for specified microorganisms. These microbial assessments were conducted by the academic services division in Department of Microbiology on 0 and 6 months of accelerated stability testing. The methods employed adhered to the guidelines outlined in the United States Pharmacopoeia 43 - NF 38[32] based on the general tests and assays entitled “Microbial Examination of Nonsterile Products: Microbial Enumeration tests <61> and Tests for Specified Microorganisms <62>. The acceptance criteria for microbiological quality of the product were determined according to the general information <1111>, titled “Microbiological Examination of Nonsterile Product: Acceptance Criteria for Pharmaceutical Preparations and Substances for Pharmaceutical Use. The acceptance criteria for cutaneous use are as follows:

- Total Aerobic Microbial Count (TAMC) ≤ 2×10^2 cfu/g or cfu/mL
- Total Yeast and Mold Count (TYMC) ≤ 2×10^1 cfu/g or cfu/mL
- Absence of *Pseudomonas aeruginosa* in 1 g or 1 mL
- Absence of *Staphylococcus aureus* in 1 g or 1 mL

This acceptance criteria were compiled with the Acceptance Criteria for Microbiological Quality of Herbal Drug Preparations for the product type: transdermal patches and topical preparations for intact skin, e.g., creams, lotions, ointments, solutions, powders, etc. as specified in the Limits for Microbial Contamination <10.5> of the Thai Herbal Pharmacopoeia 2021[33]. According to the attachment at the end of the announcement from the Herbal Product Committee concerning the quality control methods and specific requirements for herbal products, as well as the principles, methods, and conditions related to the certification of herbal product analysis results (B.E. 2564) by the Ministry of Public Health[34], herbal products intended for skin application must be free of *Clostridium* spp. in 1 g of sample. Therefore, an assay for *Clostridium* spp. was also conducted. Furthermore, the absence of *Candida albicans* must be verified for products intended for use as cosmetics, as specified in the Ministry of Public Health Announcement Regarding the Specifications of Cosmetics Prohibited from Manufacturing, Importing, or Selling, B.E. 2559[35].

2.9.4 Stability of phytochemical compounds in G. procumbens crude extract and ointment

The *G. procumbens* crude extract and ointment underwent testing for stability under identical frequency and storage conditions as the products. This involved assessing the stability of phytochemical compounds, including total phenolic content, total flavonoid content, and antioxidant activity using the DPPH radical scavenging method. Additionally, determination of marker compound chlorogenic acid, was conducted using the HPLC method.

2.10. *In vivo* wound healing activity of *G. procumbens* ointment

2.10.1. Preparation of G. procumbens ointments.

Ointments incorporating *G. procumbens* at concen- trations of 0.5 and 2% w/w were formulated utilizing a water-soluble ointment base (Figure 3.) The prepara- tion of the ointment base followed the established formula 5 as described previously. Both the

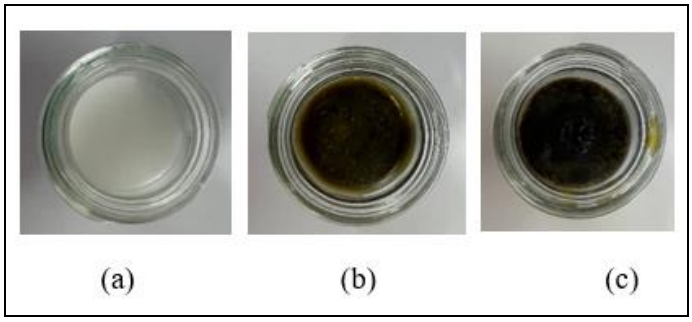

Figure 3. Physical appearance and texture of ointment; Ointment base (a), 0.5% *G. procumbens* ointment (b), 2% *G. procumbens* ointment (c).

0.5 and 2% *G. procumbens* ointments were freshly prepared and subsequently stored at 2−8 °C until their intended use.

2.10.2. Animal

Eight-week-old male and female wild-type (WT) C57BL/6 mice were used.

2.10.3. Skin wound Model

Six mice were divided into three groups: 0.5% *G. procumbens* treated, 2% *G. procumbens* treated, and ointment base-treated control. To investigate the wound healing activity of the *G. procumbens* ointments, a 4 mm diameter full thickness excisional skin wound was generated on the shaved dorsum of the animals using a biopsy punch under isoflurane anesthesia. After hemostasis (a few minutes after biopsy), 10 - 20 mg of 0.5% or 2% *G. procumbens* ointment or ointment base was applied once daily (every 24 h) for 7 days (day 0 to day 6 postinjury). Elizabethan collars were used to prevent animal licking. Wounds were left open, and the wound size was measured daily for 14 days (day 0 to.day 13 postinjury) using calipers and FiJi software Wound images were taken using a Nikon COOLPIX B500 digital camera.

2.11. Statistical analysis

All results were expressed as means ± SD of three replicated determinations. The statistical differences among treatment were determined one-way analysis of variance (ANOVA) by SPSS for Window 18.0. A statistical probability (*p* value) less than 0.05 indicated a statistically significant difference between groups.

3. RESULTS AND DISCUSSION

3.1. Plant materials and sample extraction

The extract of *G. procumbens* leaves extract obtained by maceration with 95% ethanol, presented as dark green color sticky paste with characteristic odor (Figure 4.). The percentage yield was 16.72% w/w**.**

3.2. Thin layer chromatographic (TLC) analysis

The components of the ethanolic extract of *G. procumbens* were analyzed using TLC fingerprinting. Detection was performed under UV light at 254 nm (Figure 5a) and 366 nm (Figure 5b), as well as using natural product - polyethylene glycol (NP/PEG) spraying reagent (Figure 5c). Fluorescent spots were detected under UV light at 366 nm after the plate was sprayed with NP/PEG reagents. The extract exhibited bands at the same Rf value and color as chlorogenic acid (Figure 5b, c) at Rf = 0.22.

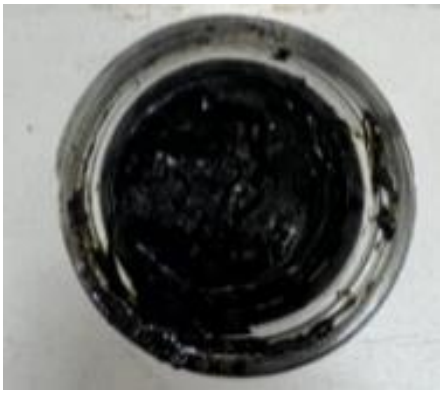

Figure 4. The appearance of 95% ethanolic *G. procumbens* leaves extract

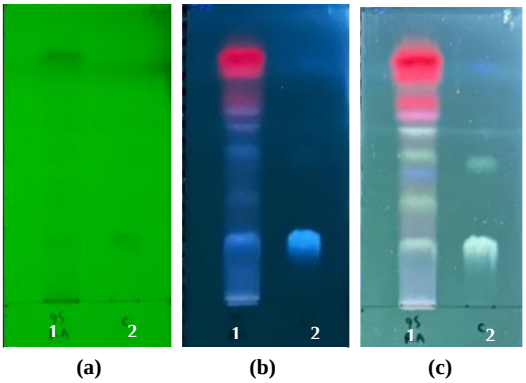

Figure 5. TLC chromatograms of ethanolic *G. procumbens* extract (1), Chlorogenic acid (2) observed under (a) UV at 254 nm, (b) UV at 366 nm, (c) Spray with Natural products-polyethylene glycol (NP/PEG) reagents and observed under UV 366 nm.

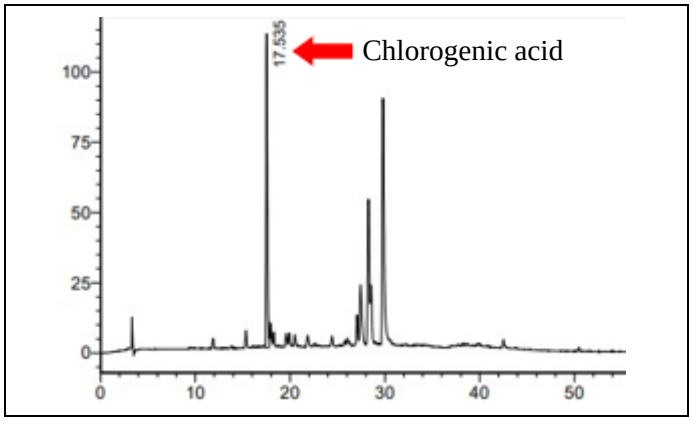

Figure 6. HPLC chromatogram of 95% ethanolic extract of *G. procumbens.*

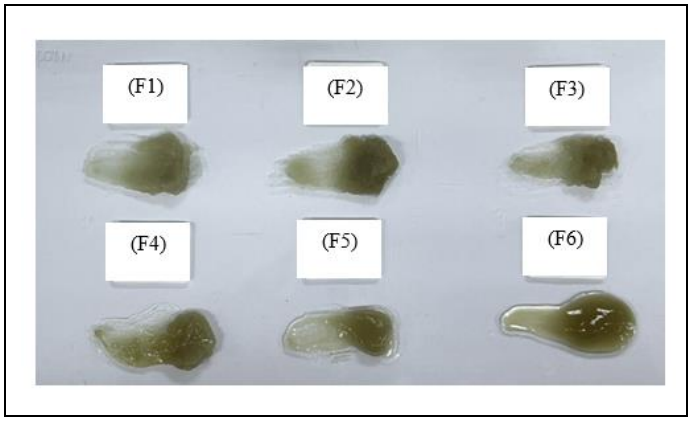

Figure 7. Physical appearances of *G. procumbens* ointment formulas 1-6 (F1-F6).

3.3. Quantitative analysis of chlorogenic acid in *G. procumbens* extract by HPLC

The concentration of chlorogenic acid in *G. procumbens* extract was found to be 5.26 ± 0.02 mg/g, with a retention time at 17.53 minutes (Figure 6.).

3.4. Determination of total phenolic and total flavonoid contents

The result of total phenolic and total flavonoid contents of ethanolic *G. procumbens* leaves extract were 43.80 ± 1.79 mg GAE and 132.67 ± 1.40 mg QE in 1 g extract, respectively (Table2)

3.5. Determination of antioxidant activity using DPPH radical scavenging method

The result of *in vitro* antioxidant activities assessed through free radical scavenging using DPPH is presented in Table 2. The ethanolic extract of *G. procumbens* and chlorogenic acid exhibited significant DPPH radical scavenging activity with an IC_{50} of 181.70 ± 0.76 and 8.61 ± 0.26 µg/mL, respectively. Ascorbic acid used as a standard, demonstrated potent scavenging activity with IC_{50} values of 4.70 ± 0.15 and µg/mL.

3.6. Development of *G. procumbens* topical ointment

3.6.1. Solubility of the extracts

The results exhibited that the extract was freely soluble in 90% v/v ethanol and absolute ethanol, soluble in 70, 80% v/v ethanol, propylene glycol and polyethylene glycol, and sparingly soluble in 50 and 60% v/v ethanol. It demonstrated slightly soluble in 30 and 40% v/v ethanol, and very slightly soluble in 10, 20% v/v ethanol, as well as distilled water.

3.6.2. Preparation of G. procumbens ointment

Physical appearances of *G. procumbens* ointment were presented in Figure 7. All 6 ointment formulations exhibited consistent characteristics, with no phase separation or sedimentation. They retained good color and aroma, attributable to the nature herbs used. However, formulas 1-4 demonstrated moderately to low absorption when applied to the skin and did not feel sticky, stiff, patchy, or irritating. They were malleable, smooth, and could be easily wiped off. In contrast, formulation F6 was too liquid and soft, resulting in poor adherence to the skin. Consequently, formulation F5 was selected for further study. *G. procumbens* extract was incorporated into the F5 ointment base at concentration of 0.5 and 2% w/w for stability studies and *in vivo* wound healing activity assessment.

3.7. Stability test of *G. procumbens* topical ointment

3.7.1. Physical appearance

Physical characteristics of prepared ointment base, 0.5 and 2% *G. procumbens* ointment were presented in Figure 8. All formulation demonstrated good appearance with green color. The texture was homogenous. None of them showed color change during study period. No phase separation was observed throughout the study period.

3.7.2. Viscosity

The viscosity of the ointment base, along with the 0.5 and 2% w/w *G. procumbens* ointments, significantly decreased after 3 months of accelerated storage conditions (Table 3.).

Table 2. Total phenolic content, total flavonoid content, and antioxidant activities of *G. procumbens* leaves extracts

	Result
DPPH (IC_{50}, µg/mL)	181.70 ± 0.76
Total phenolic content (mg GAE/g extract)	43.80 ± 1.79
Total flavonoid content (mg QE/g extract)	132.67 ± 1.40

Data are expressed as mean ± SD (n=3)
GAE = gallic acid equivalent; QE = quercetin equivalent.

3.7.3. Microbial contamination

All samples, including the ointment base, 0.5 and 2% *G. procumbens* ointment stored under accelerated conditions for 0 and 6 months, met the requirements outlined. Specifically, the total aerobic microbial count (TAMC) was found to be <10 cfu/g, the total yeast and mold count (TYMC) <10 cfu/g, and there was an absence of *P. aeruginosa*, *S. aureus* in 1 g of the sample

Furthermore, *Clostridium* spp. and *C. albicans* were also absent in 1 g of the sample.

Suitability test

According to the ingredients and excipients of the product, there might be antimicrobial activity present in the formulation. Therefore, the compendial microbial examination tests must be verified using the suitability test to assess the residual antimicrobial activity of the product, which must be neutralized under test conditions. The referenced microorganisms were spiked into the product, and the amount of those microorganisms must be recovered within the acceptable range of 50 - 200% recovery for microbial enumeration, and the referenced microorganism must be detected for the specified microorganism tests, to ensure that the results achieved are truly representative. All results of the suitability tests have passed for all products, confirming the validity of the test results, and ensuring their reliability.

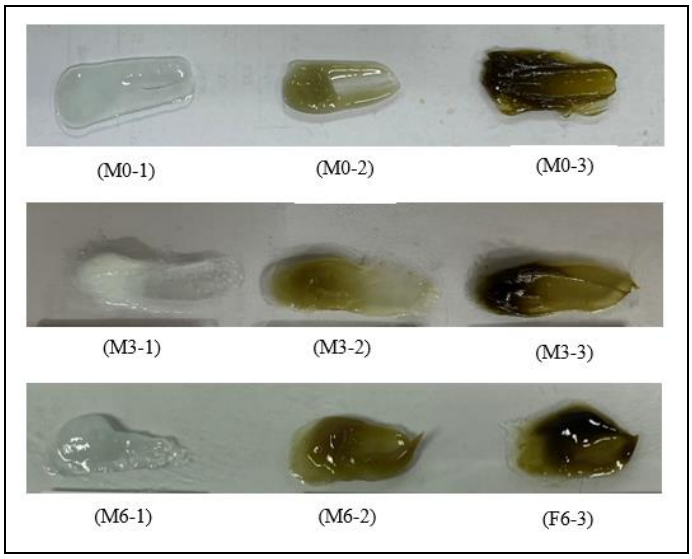

Figure 8. Physical appearances of ointments under accelerated storage condition; Ointment base at 0 month (M0-1), 0.5% *G. procumbens* ointment at 0 month (M0-2), 2% *G. procumbens* ointment at 0 month (M0-3), ointment base at 3 months (M3-1), 0.5% *G. procumbens* ointment at 3 months (M3-2), 2% *G. procumbens* ointment at 3 months (M3-3), ointment base at 6 months (M6-1), 0.5% *G. procumbens* ointment at 6 months (M6-2), 2% *G. procumbens* ointment at 6 months (M6-3).

3.7.4. Stability of phytochemical compounds in *G. procumbens* crude extract

G. procumbens leaves extract underwent storage under accelerated conditions following ASEAN guidelines on stability study and shelf-life of traditional medicines. After a 3-month storage period, the levels of all bioactive components and DPPH free radical scavenging activity in the extract were maintained at approximately 94 - 98% of their initial content. Specifically, the remaining amounts of chlorogenic acid, total flavonoid, total phenolic, and free radical scavenging activity were measured at 94.94, 98.13, 98.08, and 98.99% of their original amounts, respectively (Table 4.).

After 6 months of storage, the level of all active components within the extract (Table4.) declined by approximately 17 - 21%. The remaining concentrations of chlorogenic acid, total flavonoid, total phenolic and free radical scavenging activity were 78.88, 79.96, 83.11 and 82.24% of the initial levels, respectively. This suggested that the *G. procumbens* extract exhibited instability over time.

3.7.5 Stability of phytochemical compounds in *G. procumbens* ointment

After 3 months, both 0.5 and 2% w/w *G. procumbens* ointments maintained bioactive component contents and DPPH free radical scavenging activity at approximately 80 to 99% of their initial levels. Specifically, in the 0.5% w/w ointment, chlorogenic acid, total flavonoid, total phenolic, and free radical scavenging activity remained at 97.75, 99.34, 99.44, and 85.90% of their original amounts, respectively. Meanwhile, the 2% w/w ointment showed remaining levels of chlorogenic acid, total flavonoid, total phenolic, and DPPH free radical scavenging activity at 91.74, 97.31, 96.88, and 82.74% of their initial amounts, respectively (Table 5.).

Table 3. Viscosity of ointments under accelerated storage condition for 0, 3, 6 months.

Name of sample	Study periods	Viscosity (mPa·s)
Ointment base	0 month (initial)	16,536.67
	3 months	11,210.23
	6 months	10,370.94
0.5% *G. procumbens* ointment	0 month (initial)	23,536.67
	3 months	14,366.67
	6 months	10,250.19
2% *G. procumbens* ointment	0 month (initial)	25,043.33
	3 months	17,216.67
	6 months	13,350.24

After 6 months of storage, both ointments experienced a decrease in active component contents within a range of 4 - 27%. In the 0.5% w/w ointment, the remaining levels of chlorogenic acid, total flavonoid, total phenolic, and free radical scavenging activity were 96.63, 73.18, 91.15, and 82.99% of the original amounts, respectively. Similarly, the 2% w/w ointment showed remaining levels of chlorogenic acid, total flavonoid, total phenolic, and free radical scavenging activity at 84.17, 79.41, 83.11, and 79.63% of their initial amounts, respectively (Table 5.). These findings suggested that the prepared *G. procumbens* ointments exhibited instability over the storage period.

To ensure product quality, stability studies were conducted following the ASEAN guidelines for traditional medicine. Testing was performed at recommended intervals and storage conditions suitable for establishing the stability profile of the finished product under accelerated conditions, with assessments conducted at 0, 3, and 6 months under standard storage conditions of 40 ± 2°C / 75 ± 5% RH. Accordingly, our experiment adhered to this testing frequency and storage condition.

Throughout the 6-month duration, both the 0.5 and 2% w/w ointments containing *G. procumbens* maintained their physical appearance similar to the beginning of the study. Microbiological analysis indicated that bacterial, yeast, and mold levels remained within acceptable limits, with no detection of specified microorganisms such as *P. aeruginosa*, *S. aureus*, *C. albicans*, and *Clostridium* spp. However, there were significant changes observed in the concentrations of bioactive components, including chlorogenic acid, total phenolic content, total flavonoid content, and DPPH free radical scavenging activity. As per the ASEAN guideline on stability studies for drug products[36], a "significant change" is delineated by a 5% deviation in assay from its initial value. For the 0.5% w/w *G. procumbens* ointment, there was an observed decline ranging from approximately 4-27% in initial content, while the 2% w/w *G. procumbens* ointment exhibited a decrease of approximately 16-21% in initial content. Additionally, the crude extract of *G. procumbens* displayed a decrease of 17-21% in initial content. Identifying factors contributing to this instability is crucial, and chemical stability of the compound could be a key factor[37] leading to changes exceeding the 5% threshold in the percentage of the active compound.

Hence, it is recommended to thoroughly explore the degradation pathways of *G. procumbens* extract before proceeding with the development of a topical formulation. This empirical investigation is essential to ensure the stability of the extract, a fundamental requirement for the subsequent formulation process and the overall efficacy of the topical product.

Table 4. Chlorogenic acid, total phenolic content, total flavonoid content, and DPPH free radical scavenging activity in *G. procumbens* crude extract under accelerated storage condition.

Sample	Analytical Method	Study periods of Accelerated condition			Percentages of remained compound	
		0 month (initial)	3 months	6 months	3 months	6 months
CE	HPLC	5.14 ± 0.01*	4.88 ± 0.001*	4.06 ± 0.002*	94.94%	78.99%
	TFC	224.45 ± 1.18	220.25 ± 1.81	179.46 ± 1.21*	98.13%	79.96%
	TPC	42.10 ± 0.06*	41.29 ± 0.03*	34.99 ± 0.41*	98.08%	83.11%
	DPPH	127.44 ± 0.39	128.73 ± 1.59	150.07 ± 0.15*	98.99%	82.24%

Data are expressed as mean ± SD (n=3)

*in the same row indicated significantly different ($p < 0.05$);

CE =*G. procumbens* crude extract, HPLC = High Performance Liquid Chromatography of chlorogenic acid (mg/g), TFC = Total flavonoid content (mg QE/g extract), TPC = Total phenolic content (mg GAE/g extract), DPPH = 2,2-diphenyl-1- picrylhydrazyl assay (IC_{50} µg/mL).

Table 5. Chlorogenic acid, total phenolic content, total flavonoid content, and DPPH free radical scavenging activity in 0.5 and 2% w/w *G. procumbens* ointment under accelerated storage condition

Sample	Analytical Method	Study periods of Accelerated condition			Percentages of remained compound	
		0 month (initial)	3 months	6 months	3 months	6 months
0.5GP	**HPLC**	2.67 ± 0.01*	2.61 ± 0.03	2.58 ± 0.001	97.75%	96.63%
	TFC	199.15 ± 1.22	197.83 ± 0.50	145.74 ± 0.93*	99.34%	73.18%
	TPC	37.28 ± 0.29	37.07 ± 0.52	33.98 ± 0.25*	99.44%	91.15%
	DPPH	160.32 ± 0.30*	182.93 ± 0.33*	187.59 ± 0.35*	85.90%	82.99%
2GP	**HPLC**	4.36 ± 0.01*	4.00 ± 0.04*	3.67 ± 0.01*	91.74%	84.17%
	TFC	209.16 ± 0.81*	203.54 ± 2.34*	166.10 ± 1.83*	97.31%	79.41%
	TPC	40.96 ± 0.10*	39.68 ± 0.03*	34.04 ± 0.13*	96.88%	83.11%
	DPPH	150.13 ± 0.32*	176.04 ± 0.49*	180.71 ± 0.44*	82.74%	79.63%

Data are expressed as mean ± SD (n=3)
*in the same row indicated significantly different ($p < 0.05$);
0.5GP = 0.5% w/w *G. procumbens* ointment, 2GP = 2% w/w *G. procumbens* ointment, HPLC = High Performance Liquid Chromatography of chlorogenic acid (mg/g), TFC = Total flavonoid content (mg QE/g extract), TPC = Total phenolic content (mg GAE/g extract), DPPH = 2,2-diphenyl-1-picryhydrazyl) assay (IC_{50} µg/mL).

3.8. *In vivo* wound healing activity of *G. procumbens* ointments

To assess the efficacy of *G. procumbens* ointment in promoting wound healing *in vivo*, mice were subjected to a single excisional wound, 4 mm in diameter, on their shaved dorsal skin. Treatment groups received either 0.5 or 2% *G. procumbens* ointment, while a control group was treated with ointment base alone. Ointments were applied once daily for 7 days, with wound closure monitored for 14 days. Although the ointment displayed favorable visual characteristics and a smooth texture, both 0.5 and 2% *G. procumbens* formulations showed no significant difference in wound appearance (Figure 9.) and did not notably hasten wound closure compared to the control group treated solely with ointment base.

It is well-established that a sample size larger than necessary provides a better representation of the population and yields more accurate results[38]. Therefore, increasing the sample size for enhanced accuracy is recommended for future studies.

4. CONCLUSION

The study aimed to develop a topical ointment using *G. procumbens* extracts, capitalizing on its rich content of phenolic and flavonoid compounds for their antioxidant and wound healing properties. Our investigation of *G. procumbens* extract included TLC fingerprinting and HPLC analysis, revealing chlorogenic acid content at 5.26 ± 0.02 mg/g, and total phenolic and flavonoid contents of 43.80 ± 1.79 mg GAE and 132.67 ± 1.40 mg QE per 1 gram of extract, respectively. The extract exhibited significant

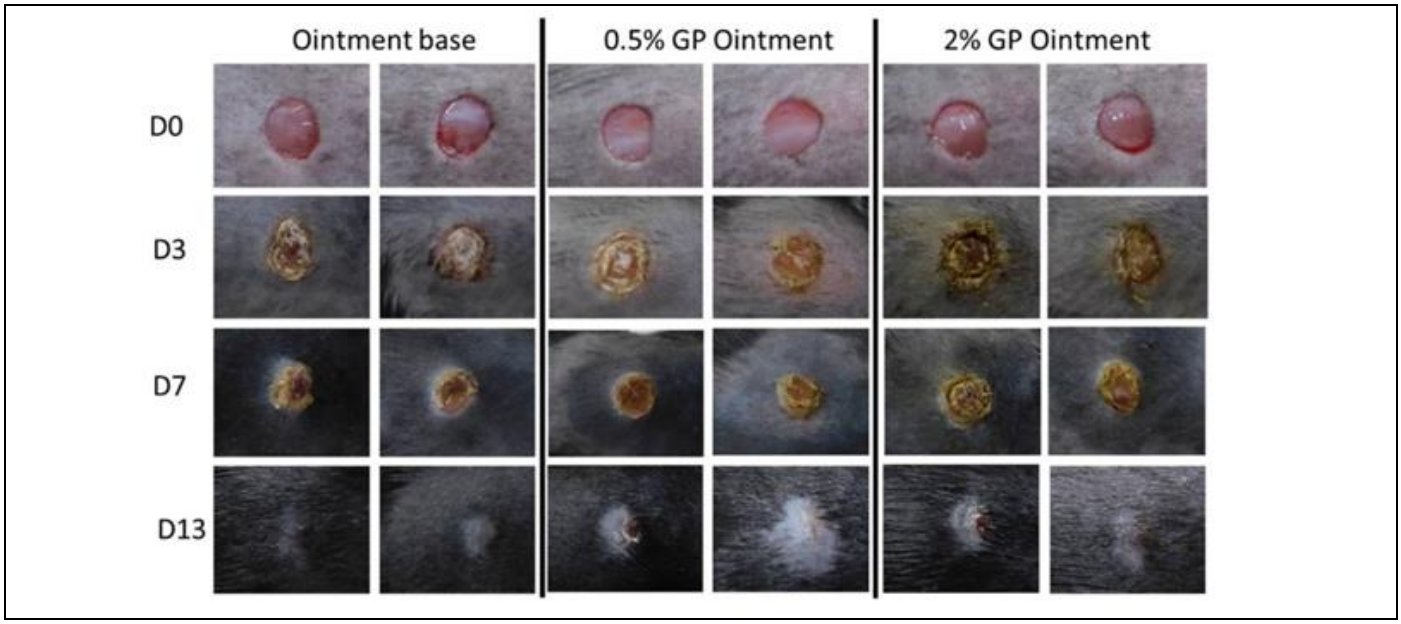

Figure 9. The effect of *G. procumbens* ointment on *in vivo* wound healing; Macroscopic wound appearance over 14 days (n = 2).

antioxidant activity with an IC_{50} value of 181.70 ± 0.76 µg/mL in the DPPH assay.

Ointment formulation (formula 5) containing *G. procumbens* extract, showed optimal characteristics for skin absorption, with ease of application, non-sticky texture, and pliable consistency, prompting further investigation at 0.5 and 2% w/w concentrations for stability and wound healing efficacy.

Stability testing over six months, following ASEAN guideline, indicated significant declines (4 - 27% for 0.5% w/w, 16 - 21% for 2% w/w) in bioactive components, likely due to chemical degradation pathways such as hydrolysis, oxidation, and interactions with excipients. Further investigation into these pathways is recommended for formulation efficacy and quality.

In vivo assessment of the ointment showed favorable appearance and texture, but not demonstrated notable improvement in wound healing compared to controls. Increasing sample sizes in future studies is advised for enhanced result accuracy and reliability.

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Conflict of interest

The authors do not have any conflict of interest.

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Ethics approval

This research was approved by Faculty of Pharmacy, Mahidol University-Institutional Animal Care and Use Committee under project number PYR 009/2023.

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