Letters in Applied NanoBioScience
Open-Access Journal (ISSN: 2284-6808)

Article
Volume 14, Issue 4, 2025, 270
https://doi.org/10.33263/LIANBS144.270

Evaluation of the Potential In Vitro Effects of *Heterotrigona itama* Propolis Extract on Wound Healing in Human Dermal Fibroblast Cells

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Received: 12.05.2024; Accepted: 1.11.2025; Published: 20.12.2025

Abstract: Propolis, a substance produced by stingless bees, has a number of pharmacological and biological properties that have been known since ancient times. The aim is to investigate the active fractions of the propolis extract from the species Heterotrigona itama (H. itama) and their potential effects on cell viability, proliferation, and wound healing in human fibroblast cells. The crude propolis extract of H.itama-HB was fractionated by vacuum liquid chromatography (VLC) using different solvent systems to obtain 10 fractions, each separating polar and non-polar compounds. The fractions were eluted with hexane: Ethyl acetate (6:4). F3 is the most potent fraction as it proliferates cells faster than the control (29%). F3 showed cytotoxicity at higher concentrations; concentrations of 25 µg/mL or lower were considered safe. At 25 µg/mL F3, cell proliferation was significantly improved by 21 % of wound closure. Epidermal growth factor (EGF) was highest at 85 pg/mL, and collagen production was 314.4%, an amazing achievement. These results underline the benefits of propolis enriched with EGF and FGF as a natural, effective alternative for promoting skin regeneration and improving wound-healing outcomes. In summary, the propolis extract from *H. itama* (F3) exhibits the highest antioxidant activity and the greatest number of polyphenolic compounds, both of which have a positive effect on wound healing. It promotes cell proliferation, enhances normal wound healing, and increases collagen and growth factor production, including epidermal growth factor (EGF) and fibroblast growth factor (FGF).

Keywords: propolis; wound healing; cell viability; cell proliferation; epidermal growth factor (EGF); fibroblast growth factor (FGF); human skin fibroblasts (HSF11184).

1. Introduction

Propolis, a resinous substance produced in large quantities by stingless bees, including Heterotrigona itama, makes a major contribution to the ecological and cultural context. Propolis, known for its pleasantly sweet, floral taste and aroma, is not only a popular ingredient in traditional Malaysian cuisine but also possesses a variety of medicinal properties. These include antibacterial, anti-inflammatory, antifungal, anesthetic, antispasmodic, anti-ulcer, anticancer, and immunomodulatory effects, as well as remarkable wound-healing abilities. Studies have shown that its bioactive compounds, including flavonoids and terpenoids, are responsible for its strong antioxidant and anti-inflammatory properties. Propolis is often considered a waste product, but its importance as a valuable natural resource for health-related applications, especially in wound care, is increasingly recognized.

In recent years, the use of natural and traditional medicines in wound care has gained attention due to their therapeutic efficacy, cost-effectiveness, and bioavailability. Propolis has shown significant results when used in wound dressings. Dressings enriched with 1% ethanolic propolis extract (EEP) have shown superior antibacterial activity against common pathogens, including *Staphylococcus aureus* and *Escherichia coli* [1]. Similarly, combining propolis with polymers such as polyvinyl alcohol (PVA) to produce nanofiber wound dressings has been shown to improve wound closure rates by up to 68% within seven days [2]. These results demonstrate the growing interest in incorporating natural substances such as propolis into modern wound care solutions.

There is a need for effective, accessible wound-healing treatments for skin injuries. As the body’s largest organ, the skin is an important barrier against external influences. Yet millions of people worldwide, and over 8.2 million in the United States alone, suffer from chronic wounds that do not heal within the typical 4–6-week period. These wounds can have a significant impact on quality of life and healthcare costs, especially for patients with chronic diseases such as cancer or diabetes. Although previous research highlights propolis's potential as a therapeutic agent for various wound types, further studies are needed to determine the most effective formulation and dosage. Recent studies using in vitro and in vivo models have helped to clarify the biological and pharmacological properties of propolis from stingless bees. However, the specific effects of *H. itama* propolis on the viability and migration of human skin fibroblasts, which are crucial for wound healing, remain unclear. The present study aims to determine the optimal extraction solvent for maximizing constituent recovery from *H. itama* propolis and to investigate the effect of antioxidant content on wound closure activity in human skin fibroblast cells. Understanding the cellular-level effects will be key to using propolis as a reliable, natural solution in modern wound care. Therefore, in this study, the properties of the active compounds in H. itama propolis extract on wound-closure activities in human skin fibroblast cells are investigated. Therefore, it is crucial to determine the optimal propolis dosage for wound closure, specifically the effects of Heterotrigona itama propolis on the viability of human skin fibroblasts. Stingless bee propolis is a promising natural solution for wound healing, but its medicinal properties require further investigation.

2. Materials and Methods

All solvents were purchased from Merck, Germany. The chemicals used in the cell culture assay were fetal bovine serum (FBS), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Sircol collagen assay kit (Biocolor Assays, Ireland). Using assay kits (DL-EGF-Hu and DL-FGF2-Hu, DIDevelop, Wuhan, China), the level of secreted epidermal growth factor (EGF) and fibroblast growth factor (FGF) was determined.

A modified methanol extraction technique was used to extract the propolis from *Heterotrigona itama*. First, crude propolis samples were collected from rubber smallholdings in Bukit Berangan, Kuala Nerus, Terengganu, frozen at -10°C, ground into powder, and stored in a desiccator before use. The ground propolis samples were then macerated overnight in acetone, filtered, and the remaining sample was immersed in 70% methanol until the filtrate turned colorless. The extracts were dried at 45°C using a rotary evaporator. The propolis extract sample was subjected to Vacuum Liquid Chromatography (VLC) to obtain distinct fractions. Initially, 10 g of propolis extract was evenly spread onto silica slurry. A thin layer of sand acid was applied atop the VLC chamber to maintain silica levels during solvent eluent addition. Subsequently, 100 mL of organic solvent was introduced into the VLC chamber, and the pump was activated to elute the first fraction through the silica slurry. The resulting first fraction was transferred to an Erlenmeyer flask. This fractionation process was repeated 10 times using various eluted of solvent systems using hexane, ethyl acetate, and methanol with increasing polarity, resulting in fractions with its ratios, (v:v); F1 (80:20), F2 (70:30), F3 (60:40), F4 (50:50), F5 (20:80) with (Hexane: Ethyl acetate), and F6 (90:10), F7 (80:20), F8 (70:30), F9 (60:40), F10 (50:50) with (Ethyl acetate: Methanol). Each fraction was concentrated under vacuum using a rotary evaporator and subsequently stored in a freezer (4°C) until further analysis.

The obtained fractions were tested on Human skin fibroblasts (HSF1184) at concentrations ranging from 3.125 to 100 µg/mL to determine the safe concentration of the propolis samples for all assays. Cells were initially seeded overnight in 96-well plates and subsequently exposed to varying concentrations of the extract, which was diluted in medium. After a 24-h incubation period, MTT (Invitrogen, USA) solution was added to each well and incubated for 4 h. The resulting MTT formazan was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), and the color development was measured at 570 nm. The experiment was conducted in six replicates, with untreated cells serving as controls. The inhibitory concentration at 50% (IC_{50}) and safe concentrations were determined from a graph. This methodology was also employed to identify the active fraction capable of promoting cell proliferation beyond normal levels.

The selected fractions were further investigated for their ability to enhance cell proliferation in HSF1184. Cells were exposed to F2 (50 µg/mL), F3 (25 µg/mL), F4 (12.5 µg/mL), and F5 (25 µg/mL) for 24 h, after which the cell concentration was determined using the MTT assay.

HSF1184 cells were seeded in six-well plates and allowed to grow until a 100% confluent monolayer formed. A cross was carefully scored into the middle of each well by using a sterile 1-ml pipette tip to form two vertical scratches. After scratching, the wells were rinsed twice with phosphate-buffered saline (PBS), then filled with fresh medium containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), and the samples were tested. The cells were cultured for 24 h, and photographs of cell migration and wound closure were taken using an inverted microscope. The calculation of wound closure as a percentage was performed using this equation (1) [3]:

$$\text{Wound closure (\%)} = (Wa - Wb)/Wa \times 100 \qquad (1)$$

Where Wa is the initial scratch wound, and Wb is the scratch wound at a given time interval.

Confluent cells were maintained hard in serum-free media for 2 h before treatment with F3 at selected concentrations (6.25, 12.5, and 25 µg/mL). The incubation period was extended to 24 h. The Sircol collagen assay kit (Biocolor Assays, Ireland) was used to determine the amount of soluble collagen secreted into the solution. About 100 µL of the concentrated medium was mixed with Sircol dye reagent (1 ml) and incubated using an orbital shaker for 30 min. Then, the samples were centrifuged (9.3g; 10 min), followed by dissolving the collagen pellet containing dye in NaOH solution (1mL; 0.5 M). The absorbance of the samples was determined at 570 nm using a microplate reader.

For EGF and FGF assessment, the confluent cells were first preincubated in serum-free media for 2 h before treatment with F3 at different doses of 6.25, 12.5, and 25 µg/mL. After the collection of the spent medium, cell debris was removed by centrifugation. Commercial assay kits (DL-EGF-Hu and DL-FGF2-Hu, DIDevelop, Wuhan, China) were used to determine EGF and FGF concentrations. The assay kit contained a pre-coated EGF-specific antibody microtiter plate. Standard solutions or samples were added to the wells containing biotin-conjugated EGF-specific antibody preparation. Horseradish peroxidase (HRP)-conjugated avidin was then added and incubated. Only the wells containing EGF, biotin-conjugated antibody, and enzyme-conjugated avidin changed color upon addition of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. The color change was halted by adding a sulfuric acid solution, and the optical density (OD) was measured at 450 nm ± 10 nm using a spectrophotometer. The EGF concentration in the samples was estimated by assessing the OD of the samples with the reference curve.

Descriptive statistical analysis was performed to examine and summarize the data collected in this study. All analyses were performed in Microsoft Excel, which provided sufficient functionality for the study's scope and objectives.

3. Results and Discussion

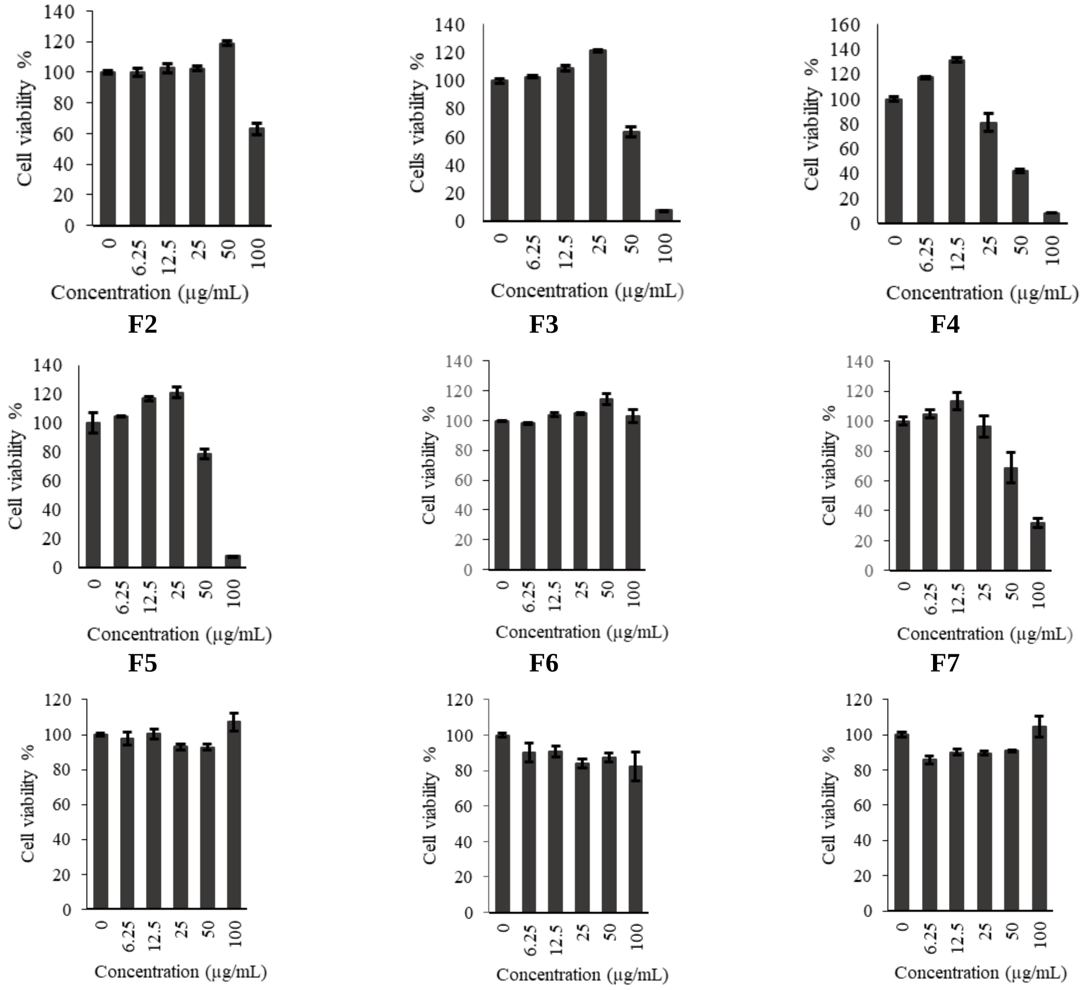
3.1. Cytotoxicity effect of heterotrigona itama propolis fractions on human skin fibroblast (HSF1184).

The cytotoxicity of *Heterotrigona itama* fractions was evaluated to determine the inhibitory concentration (IC_{50}) and safe concentrations in fibroblast cells. As summarized in Table 1, fractions F2, F3, F4, and F5 showed dose-dependent cytotoxicity, with F4 being the most cytotoxic (IC_{50} = 44.87 µg/mL). In contrast, fractions F6, F8, F9, and F10 did not converge for IC_{50} values and exhibited minimal cytotoxicity at concentrations up to 100 µg/mL, suggesting potential safety for further applications. Among the active fractions, F3 demonstrated significant cytotoxicity ($p < 0.01$) at concentrations ≥ 50 µg/mL, but was considered safe at ≤ 25 µg/mL.

Table 1. Cytotoxicity of *Heterotrigona itama* propolis fractions (IC_{50} and safe concentrations).

Fraction	IC_{50} (µg/mL)	Safe concentration (µg/mL)	Cytotoxicity notes
F2	102.6	< 50	Mild cytotoxicity at 100 µg/mL($\rho < 0.05$ vs control)
F3	53.63	< 25	Strong cytotoxicity at ≥50 µg/mL($\rho < 0.01$)
F4	44.87	< 12.5	Dose-dependent cytotoxicity ($\rho < 0.01$)
F5	63.22	< 25	Moderate effect ($\rho < 0.05$)
F6	Not converged	Not available	No toxicity observed
F7	71.96	< 25	Cytotoxicity at 50-100 µg/mL($\rho < 0.05$)
F8	Not converged	>100	No significant cytotoxicity
F9	Not converged	>100	Minimal effect
F10	Not converged	>100	Minimal effect

Cell viability is an essential parameter for assessing cell damage, including cell death or growth inhibition, as illustrated in Figure 1, which shows the cytotoxic effects of *Heterotrigona itama* fractions at different dosages. Of note, administration of F9 and F10 resulted in a slight decrease in cell viability, with no discernible adverse effects. The maintenance of cell viability upon exposure to F6, F8, F9, and F10 suggests that these fractions do not pose a risk to cell health and are thus potential candidates for drug development without harming human somatic cells. However, higher doses of F9 and F10 resulted in a slight decrease in cell viability, suggesting the possibility of adverse effects. Therefore, further studies are needed to determine the safe and effective concentrations of these chemicals for therapeutic development.



F2 **F3** **F4**

F5 **F6** **F7**

F8 **F9** **F10**

Figure 1. Cytotoxic effects of different extractions of *Heterotrigona itama* fractions (F2-F10) at different concentrations of implemented dosages.

In contrast to F6, F8, F9, and F10, these fractions exhibited cytotoxic properties. F2 showed cytotoxicity at a concentration of 100 µg/mL, resulting in a 37% decrease in cell viability. The cytotoxic effect of fraction F2 from Heterotrigona itama has an IC_{50} value of 102.6 µg/mL, with a recommended safe concentration of less than 50 µg/mL. In comparison, F3 exhibited greater cytotoxicity than F2, with 36% and 93% decreases in cell viability at 50 µg/mL and 100 µg/mL, respectively. The IC_{50} value for F3 was 53.63 µg/mL, and concentrations below 25 µg/mL were considered safe. F4 exhibited dose-dependent cytotoxicity starting at a concentration of 25 µg/mL(29% inhibition) and increasing to 58% and 92% at 50 µg/mL and 100 µg/mL, respectively. The IC_{50} value for F4 was 44.87 µg/mL, and concentrations below 25 µg/mL were considered safe. F5 has an IC_{50} value of 63.22 µg/mL, and the safe concentration is less than 25 µg/mL. No IC_{50} and safe concentration data are available for F6. Similarly, F7 showed dose-dependent cytotoxicity, starting at 50 µg/mL (31.2% inhibition) and increasing to 68.4% at 100 µg/mL. The IC_{50} value for F7 was 71.96 µg/mL, and a safe concentration was established at 25 µg/mL or less. There are no convergent IC_{50} values for F8, F9, and F10, but they require concentrations greater than 100 µg/mL to inhibit. The safe concentrations for F8, F9, and F10 are not given. The low IC_{50} values observed for F2, F3, F4, and F7 indicate their strong antioxidant activity, which may be responsible for their significant cytotoxic effects [4]. Furthermore, the antioxidant activity of a plant extract is often associated with its content of phenols, flavonoids, alkaloids, and terpenoids [5]. However, it should be noted that these components may also contribute to the extract's cytotoxicity. In addition, the F1 fraction was excluded from further processing due to its non-polar nature, which could limit its potential antioxidant activity.

The study investigated the proliferative activity of fractions F2, F3, F4, and F5 in HSF1184 cells and showed that they are able to promote cell proliferation, in contrast to fractions F6-F10, which showed no such effect. At a concentration of 25 µg/mL, F3 increased cell viability by 21%, while F5 increased it by 20%. The most potent effect on cell proliferation was observed with F4, which increased cell viability by 31% at a dose of 12.5 µg/mL. Subsequent evaluation of these fractions in HSF1184 cells confirmed their potential for tissue regeneration and wound healing. The unique components of stingless bee propolis have shown promising antioxidant and proliferative activities, making it a viable natural source for new drug development. However, further research is needed to identify the specific chemical molecules responsible for these effects and to evaluate their safety and efficacy in vivo.

3.2. Cell proliferation assay.

In the cytotoxicity assay (Figure 2), fractions F2, F3, F4, and F5 were found to promote the proliferation of skin fibroblast cells at the predicted doses. Specifically, F2 and F3 increased cell viability by 11% and 29%, respectively, at a concentration of 25 µg/mL, while F4 increased cell viability by 20% at a dosage of 12.5 µg/mL. These results indicate that an active fraction of *Heterotrigona itama* propolis has the potential to promote cell proliferation during wound healing. Among these fractions, F3 proved to be the most effective with a 29% increase in cell proliferation.

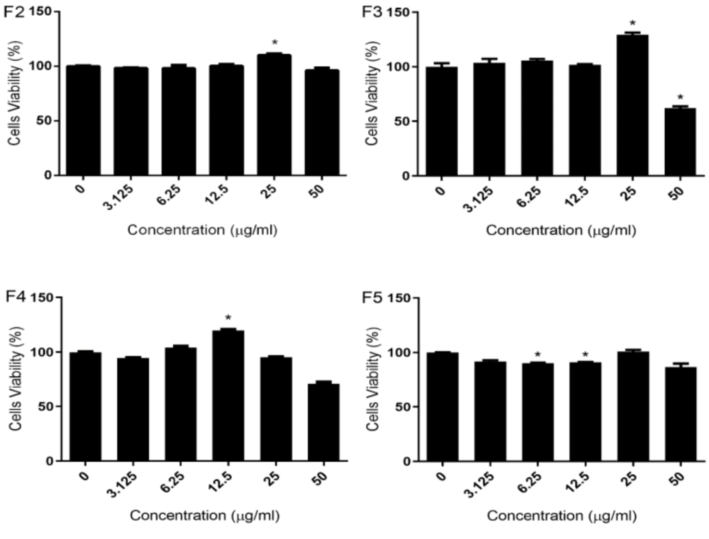


Figure 2. Cell proliferation of human dermal fibroblast cells, HSF1184 cells, using different extraction (F2, F3, F4, and F5) at different concentrations.

3.3. Effect of Heterotrigona itama propolis extract on fibroblast cells in the wound scratch assay.

A wound scratch test is a laboratory technique used to assess a cell's ability to migrate into and close a wound. The healing ability of the cells is assessed by observing wound closure and scoring the cells according to a predetermined system, including measuring the distance between the wound edges and assessing the extent of cell migration into the wound area. Fraction F3 was used for in vivo wound-healing assays. The images in Figure 3 show that F3 accelerated wound healing compared to the untreated wound, as evidenced by faster wound closure within 12 h of treatment, and by almost complete closure at 24 h in the F3-treated group (25 µg/mL). At the same time, a significant gap remained in the control group. In addition, the results showed that F3 (25 µg/mL) promoted cell proliferation 29% faster than the control group, indicating a positive effect on cell growth and division (Figure 4). These results suggest that F3 may have therapeutic potential to improve wound healing and increase cell proliferation.

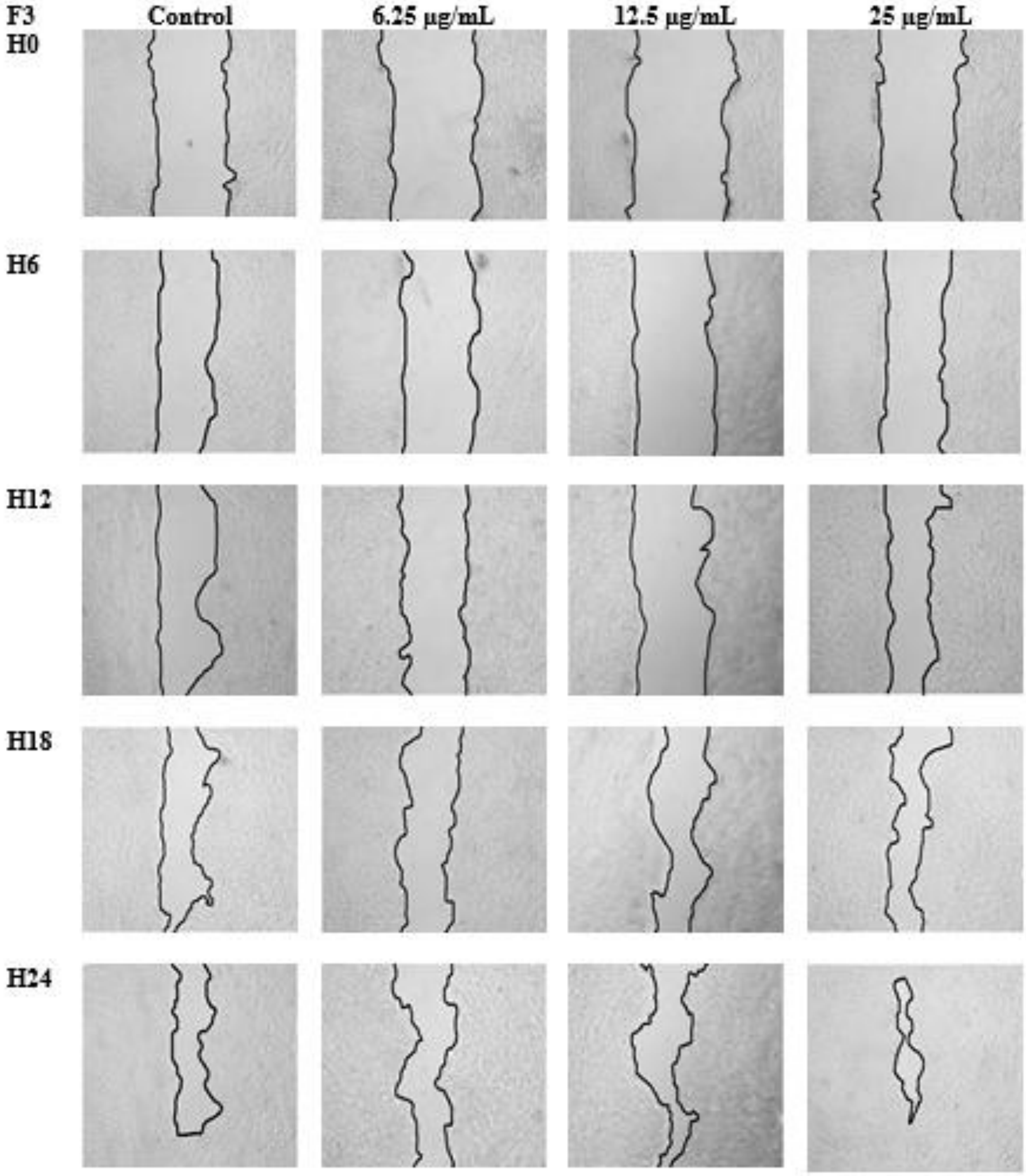


Figure 3. Pictures of scratch assay of wound closure of human dermal fibroblast cells after being treated with different concentrations of *H. itama* propolis fraction (F3) for 24 hours of observation.

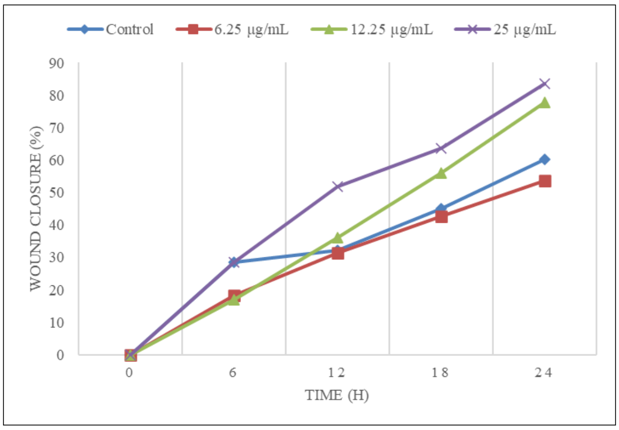


Figure 4. Graph representing wound closure over time using different concentrations of fraction F3.

The wound-healing assessment is a valuable tool for investigating the cellular and molecular mechanisms underlying wound healing and for screening potential therapeutic agents that could improve this process. Wound healing involves a few cell and biological systems that can be divided into several phases, including hemostasis, inflammation, proliferation, and remodeling [6]. Effective control of cell proliferation and migration is crucial for optimizing tissue regeneration. Various strategies can be employed, such as the use of growth factors or other signaling molecules that promote cell proliferation and migration, or the use of scaffolds or matrices that mimic the extracellular matrix, to create a favorable environment for cell growth and migration. In wound-healing assessments, cell monolayers usually respond to injury or damage by combining proliferation and migration, driven by disruption of cell-cell contacts and increased growth factor levels [7].

3.4. Production of collagen, epidermal growth factor (EGF), and fibroblast growth factor (FGF).

The wound-healing process significantly depends on growth factors, which are integral during the initial phases of tissue repair. These growth factors induce fibroblasts to migrate to the wound site, where they actively participate in collagen synthesis [8, 9]. The influence of the F3 fraction of propolis on collagen production and growth factor content at various dosages is illustrated in Figure 5 and Table 2. At a concentration of 6.25 µg/mL, a notable 226.2% increase in collagen production was observed relative to the control group, with even greater increases of 244.5% and 314.4% at concentrations of 12.5 µg/mL and 25 µg/mL, respectively. These findings indicate that propolis, particularly the F3 fraction, may enhance epithelial regeneration and promote tissue repair by stimulating collagen synthesis. Fibroblasts are responsible for producing collagen and elastin, which furnish structural support to developing tissues [10]. Consequently, the presence of the F3 fraction positively influenced the proliferation phase of open wounds, leading to substantial enhancements in epidermal growth factor (EGF) and fibroblast growth factor (FGF) production, especially at concentrations of 12.5 µg/mL and 25 µg/mL. EGF levels reached approximately 43.9 pg/mL and 85 pg/mL, respectively, whereas FGF production increased significantly to 118.5 pg/mL at 25 µg/mL, compared to 39.2 pg/mL in the control. Likewise, concentrations of 6.25 µg/mL and 12.5 µg/mL of the F3 fraction resulted in FGF levels of approximately 42.5 pg/mL and 47.7 pg/mL, respectively, which were slightly higher than those in untreated wounds. These results imply that propolis can enhance the overall healing process by stimulating cellular proliferation and angiogenesis, primarily through increased production of EGF and FGF [11].

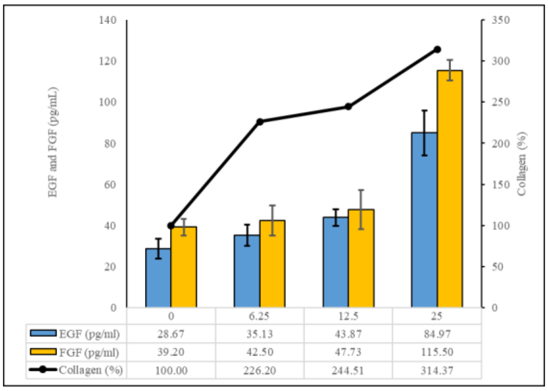


Figure 5. The amount of collagen, EGF, and FGF produced when treated with different concentrations of fraction F3 of *H. itama*.

Table 2. Impact of F3 on collagen and growth factor production.

Dose (µg/mL)	Collagen production (% of control)	EGF (pg/mL)	FGF (pg/mL)	Statistical significance
Control	100%	21.5	39.2	–
6.25	226.2%	28.4	42.5	$p < 0.05$
12.5	244.5%	43.9	47.7	$p < 0.01$
25	314.4%	85.0	118.5	$p < 0.001$

Propolis is known for its high tolerability, minimal allergenicity, and non-toxicity, making it an excellent candidate for burn therapy [12]. Several studies have shown that propolis creates a favorable biochemical environment that promotes re-epithelialization, as evidenced by quantitative and qualitative analyses of collagen expression and degradation in the wound matrix [13]. Collagen, the predominant protein in connective tissue, plays a critical role in the synthesis of new proteins during the wound healing process. While traditionally the rate of turnover has been thought to be slow, recent research has emphasized the dynamic nature of collagen synthesis, which is influenced by factors such as tissue type and age. Influencing collagen metabolism during wound healing is important, given variations in collagen production and degradation that can affect tissue collagen content, underscoring the role of propolis in this process. In light of these promising results, the development of F3 within topical delivery systems such as ointments or hydrogels could enhance its therapeutic efficacy. Ointments are recognised for their occlusive properties, which facilitate the absorption of active ingredients, and such formulations must be non-irritating to ensure safe application to the skin [14]. At the same time, hydrogels provide a moist environment that promotes wound healing and can enable sustained release of the active compound, thereby preserving the therapeutic effect in the wound area [15]. The favourable rheological characteristics of hydrogel formulations suggest that they may serve as stable and effective carriers for F3, with non-cytotoxicity being an essential safety criterion.

4. Conclusions

The use of propolis from *Heterotrigona itama* is a promising, natural, and practical approach to wound healing. F3 fraction exhibited the highest antioxidant activity and contained bioactive chemicals that contributed to a higher total flavonoid content compared to total phenolic compounds. Moreover, the F3 fraction demonstrated the potential to promote wound healing through mechanisms such as increased cell proliferation, collagen synthesis, and growth factor production. Formulating F3 as an ointment or hydrogel could exploit its potent wound-healing properties by enabling effective, sustained delivery to the wound site. However, the optimal dosage of propolis may vary depending on factors such as wound type, age, gender, and the patient's health status. Further research is needed to determine the appropriate dosage for different populations and wound types. Nevertheless, further research and clinical studies are needed to optimize these formulations and validate their safety and efficacy.

Author Contributions

Investigation, N.A.; formal analysis, N.A., F.A.A.M., N.W.M., H.F.I., and N.I.Y.; methodology, N.A.; Writing – original draft preparation, N.A. and N.I.Y.; conceptualization, N.A.; validation; N.A. and F.A.A.M.; resources, N.A.; supervision, N.A., and F.A.A.M; writing-review and editing; funding acquisition, N.A. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

This research was funded by the MINISTRY OF HIGHER EDUCATION OF MALAYSIA under the Fundamental Research Grant Scheme (FRGS), grant number FRGS/1/2016/TK02/UMT/01/1.

Acknowledgments

The authors express their high appreciation to the Faculty of Ocean Engineering Technology, University Malaysia Terengganu, for providing the facilities.

Conflicts of Interest

The authors declare no conflict of interest.

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