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Original article

Baeckea frutescens L. Promotes wound healing by upregulating expression of TGF-β, IL-1 β, VEGF and MMP-2

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ABSTRACT

Baeckea frutescens L. has been traditionally used for treating snakebites and is known to possess antifebrile and hemostatic properties. These properties are closely related to wound healing. This study aimed to evaluate the wound healing properties of *B. frutescens* leaves extract (BFLE) in vitro and in vivo. The in vitro study focused on proliferation, migration, and expression of TGF-β, IL-1β, VEGF, and MMP-2 genes and proteins. The in vivo study included excisional wound healing, histology, and tensile strength studies. The ethanolic extract of *B. frutescens* (BFLE) was tested for its effects on proliferation and migration using keratinocytes (HaCaT) and fibroblasts (BJ) cells. Gene and protein expression related to wound healing were analyzed using real-time PCR and Western blot assays. The wound healing properties of BFLE were evaluated in vivo using Wistar albino rats, focusing on excisional wound healing, histology, and tensile strength studies. The BFLE displayed significant proliferative and migratory effects on keratinocytes and fibroblasts cells, while upregulating the expression of TGF-β, IL-1β, VEGF, and MMP-2 genes and proteins. BFLE also exhibited significant wound healing effects on Wistar albino rats' excisional wounds and improved the overall tensile strength. The results suggest that BFLE has strong wound healing properties, as demonstrated by its ability to increase keratinocytes and fibroblasts proliferation and migration, upregulate genes and proteins involved in the wound healing process, and improve wound healing rates and tensile strength. The findings of this study provide important insights into the potential use of *B. frutescens* as a natural wound healing agent.

1. Introduction

Wound healing is a process of replacing lost tissues and restoring their integrity following injury. Wound healing is a dynamic process involving four successive but overlapping stages, including hemostasis, inflammation, proliferative, and remodeling. The process of wound healing is characterized by platelet aggregation and the recruitment of inflammatory cells (Barrientos et al., 2008), the formation of granulation tissue, extracellular matrix (ECM) and collagen deposition, proliferation and migration of fibroblasts, keratinocytes, and endothelial cells (Enoch & Leaper, 2008), and angiogenesis (Veith et al., 2019). Several cytokines and growth factors, including transforming growth factor (TGF)-β, interleukins, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), are released during the inflammatory and proliferative phases of wound healing (Barrientos et al., 2008).

TGF-β, which is expressed by macrophages, fibroblasts, and keratinocytes (Branton & Kopp, 1999; Liarte et al., 2020), plays several roles

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in wound healing, including regulating cell migration and growth (Roberts & Sporn, 1993), stimulating angiogenesis, collagen synthesis and deposition, fibroblast and epithelial proliferation, granulation tissue development, and extracellular matrix deposition (Shady et al., 2022).

Interleukins, especially interleukin (IL)-1β, is a type of cytokine that is produced by different types of cells, such as neutrophils, monocytes/macrophages, keratinocytes, and fibroblasts (Ishida et al., 2008). IL-1β plays a major role in wound healing by alerting surrounding cells of barrier damage (Birincioğlu et al., 2016) and attracting neutrophils to the site to kill pathogens and remove debris (Barrientos et al., 2008).

VEGF, secreted by various types of cells, including endothelial cells, fibroblasts, smooth muscle cells, neutrophils, macrophages, and keratinocytes (Bao et al., 2009; Bae et al., 2015), acts as an endothelial cell mitogen, chemotactic agent, and inducer of vascular permeability, which is crucial for the angiogenesis process. VEGF is also critical to improve re-epithelialization and collagen deposition (Shady et al., 2022).

MMP-2, also known as gelatinase A, is involved in the degradation of extracellular matrix (ECM) (Girsang et al., 2019; Caley et al., 2015) and non-ECM molecules (Hattori et al., 2009). MMP-2 is also involved in cell migration and angiogenesis (Cabral-Pacheco et al., 2020), epithelialization, modeling of new connective tissues (Dai et al., 2021), and the regulation of the cell cycle (Cabral-Pacheco et al., 2020).

Medicinal plants have been used as an alternative medicine for various types of diseases, including degenerative diseases, diabetes, and cancer, for a long time. According to WHO, 80 % of the world's population relies on medicinal plants as their primary source of healthcare (WHO, 2022). Previous studies have reported the effectiveness of medicinal plants like *Calendula officinalis* (Givol et al., 2019), *Bergenia ciliate* (Faiz et al., 2023), and *Garcinia brasiliensis* (Souza et al., 2022) for wound treatments.

Many herbs and medicinal plants found in Malaysia are known to possess wound healing properties, including *Aloe vera* (Liang et al., 2021), *Curcuma longa* (Kumar et al., 2022), *Chromolaena odorata* (Oso et al., 2019), and *Centella asiatica* (Arribas-López et al., 2022).

In this study, the ethanolic extract of *Baeckea frutescens* was used to evaluate its wound healing properties. *B. frutescens* (Myrtaceae), locally known as 'cucur atap' in Malaysia, is a shrub with linear leaves and white flowers typically growing up to 1–2 m tall. *B. frutescens* is native to eastern Southeast Asia, New Guinea, and Australia. In Malaysia, this plant can be found on top of mountains, sandy coasts, and the Klang Gates Quartz Ridge (Navanesan et al., 2015).

B. frutescens has been traditionally used for massaging and aromatherapy (Navanesan et al., 2015; Setzer et al., 2004), as well as for the treatment of headaches, rheumatism, menstrual disorders, indigestion problems, and snakebites (Bich et al., 2004; Cheung & Li, 1980). The whole plant, except for the roots, possesses antibacterial, antifebrile, and haemostatic properties (WHO, 1990). This plant has also been reported to possess various biological activities, including antibacterial (Razmavar et al., 2014), anticancer (Ma et al., 2021; Shahruzaman et al., 2019), antifungal (Jiang et al., 2020), anti-inflammatory (Hani et al., 2010; Hou et al., 2017; Lin et al., 2021), antioxidant (Kamarazaman et al., 2022; Saad et al., 2021), and larvicidal activity (An et al., 2020).

Several bioactive constituents or groups of compounds have been reported to be responsible for the biological properties of *B. frutescens*, including meroterpenoids (Hou et al., 2017) and baeckeins F-I (Jia et al., 2014) for anti-inflammatory properties, flavonoids for antioxidant activities (Quang et al., 2008), and biflavonoid glycoside for the inhibition of lipid peroxidation (Kamiya and Satake, 2010). *B. frutescens* has also been reported to have several phytochemical constituents, including baekeins (Jia et al., 2011; Jia et al., 2014; Jia et al., 2016; Jia et al., 2020), baeckenones D–F (Nisa et al., 2016), baeckfrutones A–L (Qin et al., 2018), chromones, chromanones (Tsui & Brown, 1996), flavonoids (Hou et al., 2017; Hou et al., 2020), frutescone A–G (Hou et al., 2017), phloroglucinols (Ito et al., 2017), tasmanones (Tam et al., 2004), pinoquercetin (Zhong et al., 1997), sesquiterpenes (Tsui et al., 1996), and quercetin (Lu et al., 2008).

Rapid healing is a desirable outcome expected from wound occurrence, and the application of a substance that aids and accelerates the wound healing process is much appreciated to avoid infections or prolonged injury due to chronic wound occurrence. While the use of currently available wound healing management products such as bandages and treatment using creams and ointments had positive effects, the increasing prevalence of wound and chronic wound in the global scenario, high cost of synthetic drug and various side effects associated with drugs had prompted us to seek new alternative treatment from natural products.

Furthermore, traditional usages of this plant demonstrated that this plant had been used to treat snakebite and possesses hemostatic properties. Scientific evaluations of this plant also exhibited antibacterial, anti-inflammatory, and antioxidant properties, suggesting that this plant might have wound healing properties. Therefore, this study was conducted to evaluate the in vitro and in vivo wound healing properties of ethanolic extract from *B. frutescens*. In vitro part comprises cell cycle regulation, gene, and protein expression study, while in vivo part comprises dermal toxicity, excisional wound healing, tensile strength, and histology studies.

2. Material & methods

2.1. Collection of plant materials and extract preparation

Plant samples of *Baeckea frutescens* were collected in Setiu, Terengganu, Malaysia. The authenticity of the sample was confirmed by botanist Ms. Tan Ai Lee at the Forest Research Institute Malaysia (FRIM), and it was assigned the specimen number SBID: 044/20. The herbarium specimen was deposited at the Faculty of Pharmacy, Universiti Teknologi MARA Selangor, Malaysia. The name of the plant was checked with https://www.worldfloraonline.org on 16 Mei 2023, and the name was confirmed as the latest revised name. The ethanolic extract of *B. frutescens* (BFLE) was prepared using the method described in our previous paper (Kamarazaman et al., 2022).

2.2. Cell culture and maintenance

Immortalized human keratinocyte (HaCaT, Cat: 300493) and human dermal fibroblasts (BJ, Cat.: CRL-2522) cells were procured from American Type Culture Collection (ATCC). HaCaT cells were nurtured in Dulbecco's Modified Eagle's Medium (DMEM), while BJ cells were sustained in Eagle's Minimum Essential Medium (EMEM). Both DMEM and EMEM were supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin. The cells were maintained and incubated in a chamber under 95 % humidity and 5 % CO_2 at 37 °C. The cells were subcultured or seeded for experimentation when they attained 80–95 % confluency, with media replacement every three days at a minimum.

2.3. Proliferation assay

Proliferation assay was performed according to our previously described method (Kamarazaman et al., 2022). HaCaT and BJ cells were plated in 96-well plates (TPP, Switzerland) at a density of 5 x 10^3 cells/well. After a 24-hour incubation period, the cells were exposed to varying concentrations of BFLE (0–25 μg/ml for HaCaT and 0–50 μg/ml for BJ), chosen based on their respective cytotoxicity profiles (not shown). Following treatment, cells were allowed to proliferate for an additional 24, 48, or 72 h before undergoing MTT assay. The MTT assay was conducted following a modified version of the protocol outlined by Maryati et al. (2020). The culture media were replaced with 100 μl of fresh media containing 0.5 μg/ml MTT and incubated for 4 h. Subsequently, the media were aspirated, and DMSO was added to solubilize the purple formazan crystals generated by viable cell mitochondria. The resulting solution was purple in color and measured

Table 1
List of forward and reverse primers used in real time PCR assay.

Gene names	Forward primer	Reverse primer
TGF-β	CCTGGACACCAACTATTGCT	AAGTTGGCATGGTAGCCCTT
IL-1β	GCTGGAGAGTGTAGATCCCAA	CTTGAGAGGTGCTGATGTACC
VEGF	ACGAACGTACTTGCAGATGTGA	GCAGCGTGGTTTCTGTATCG
MMP-2	GCTGGAGACAAATTCTGGAGATAC	ACGACGGCATCCAGGTTATC
MMP-9	TGGAGGTTCGACGTGAAGGC	GCAGAAATAGGCTTTCTCTCGGTA
GAPDH	CACCCACTCCTCCACCTTTG	CCACCACCCTGTTGCTGTAG

spectrophotometrically at 570 nm. All experiments were performed in triplicate.

2.4. Migration assay

Migration assay was performed according to our previously described method (Kamarazaman et al., 2022). HaCaT and BJ cells were plated in 6-well plates (TPP, Switzerland) at densities of 1 x 10^6 cells/well and 2 x 10^5 cells/well, respectively. Upon reaching confluency, vertical scratches were made using a 200 µl micropipette tip. Following scratch induction, cells were rinsed twice with PBS to eliminate cellular debris. Subsequently, each well was treated with varying concentrations of BFLE (25, 20, 10, and 5 µg/ml for HaCaT and 10, 5, 2.5, and 1.25 µg/ml for BJ), while the positive control group treated with 10 µg/ml allantoin. After that, photographs of the scratch areas were captured and labeled as the 0-hour time point and the cells were then incubated in a CO_2 incubator. Further photographs were taken at 6 and 12 h for HaCaT cells, and at 16 and 24 h for BJ cells. Analysis of the scratch area images was conducted using Image J software, and the migration rate of each treatment was calculated by comparing the scratch areas at respective time points to that of the 0-hour baseline. The experiment was performed in triplicate and repeated independently three times.

2.5. Preparation of cell lysate for cell cycle analysis, Western blot, and qPCR

HaCaT and BJ cells were seeded in 6-well plates (TPP, Switzerland, Cat.: Z707902) at a density of 1 x 10^6 cells/well and 2 x 10^5 cells/well, respectively. The cells were then incubated at 37 °C in a CO_2 chamber for 24 h for HaCaT and 48 h for BJ to allow them to reach confluency. Once the cells reached confluency, the old media was discarded and replaced with fresh media containing different concentrations (5, 10, 20, and 25 µg/mL) of BFLE or 10 µg/mL of allantoin, which was used as a positive control.

For cell cycle analyses and Western blot assays, the cells were treated with these concentrations of BFLE, while for qPCR assays, BFLE was only treated at 25 µg/mL. The 6-well plates were then incubated in a CO_2 chamber for an additional 24 h before proceeding to the next step. After 24 h, all of the media were discarded, and the cells were washed with phosphate buffer solution (PBS), collected in a centrifuge tube by trypsinization, and washed twice by centrifugation at 500 x g using ice-cold PBS.

2.6. Western blot

Western blot was performed according to previously described method with some modifications (Shams et al., 2022). The previously prepared cell pellets were lysed by adding 100 µL of RIPA (radioimmunoprecipitation assay) lysis buffer (Thermofisher Scientific, USA, Cat.: 89900) containing 1 mM phenylmethanesulfonyl fluoride and protease inhibitor, sonicated, and centrifuged at 12,000 x g for ten minutes. The resulting supernatant was collected, and the protein concentration was quantified using the Bradford assay, as previously described by Kielkopf et al. (2020). Then, the cell lysates were added to 2x Laemmli dye buffer (4 % SDS, 5 % 2-mercaptoethanol, 20 % glycerol, 0.004 % bromophenol blue, 0.125 M Tris HCl) (Sigma-aldrich, USA, Cat.: S3401) and heated on a heat block at 95 °C for five minutes to denature the protein.

Protein samples (50 µg) were separated using sodium dodecyl sulfate–polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was then blocked with 5 % skim milk in Tris buffer saline/Tween 0.05 % (TBST) and washed with TBST three times for five minutes each time. The membrane was probed with mouse anti-human primary antibodies against TGF-β (Cat.: sc-130348), IL-1β (Cat.: sc-12742), VEGF (Cat.: sc-53462), MMP-2 (Cat.: sc-13595) and MMP-9 (Cat.: sc-21733) (1:500) (Santa Cruz, CA, USA) at 4 °C for 24 h.

After the incubation period, the membrane was washed three times with TBST and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:1000) (Santacruz, CA, USA, Cat.: sc-2005). Protein expression was enhanced by chemiluminescence with the ECL Substrate Kit (Ultra High Sensitivity) (Abcam, UK, Cat.: ab133409). The gel band of enhanced protein expression was viewed using the Gel Imaging System (Amersham Imager 600, UK), and the image was captured. β-actin was used as the loading control, and the relative protein expression was calculated using Image J software. The experiment was performed in three membrane replicates.

2.7. RNA extraction, cDNA synthesis and qPCR assay

RNA extraction, cDNA synthesis and qPCR assay was performed according to previously described method with some modifications (Shams et al., 2022). Total RNA was extracted from HaCaT and BJ cells using Qiagen's RNeasy Plus Mini Kit (Qiagen, Germany, Cat.: 74134) according to the manufacturer's instructions. RNA purity, calculated by the 260/280 ratio, was between 2.0 and 2.1. Subsequently, 2 µg of RNA were converted into single-stranded cDNA using the High-Capacity cDNA Kit (Applied Biosystems, USA, Cat.: 4368814), following the manufacturer's instructions. Real-time PCR was used to analyze mRNA expression, using SYBR Select Master Mix (Applied Biosystems, USA, Cat.: 4472908) and cDNA template, forward and reverse primers for GAPDH, TGF-β, IL-1β, VEGF, MMP-2 and MMP-9 genes. GAPDH was used as the internal control. The forward and reverse primers used are listed in Table 1.

The PCR reaction was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, UK), with a cycle of 95 °C for 20 s (holding stage), followed by 40 cycles of 95 °C for three seconds and 60 °C for 30 s (cycling stage), and finally a cycle of 95 °C for 15 s, 60 °C for one minute, 95 °C for 15 s, and 60 °C for 15 s (melt curve stage). The specificity of amplification was confirmed by melting curve analysis. The expression level of each gene was calculated using the $2^{-\Delta\Delta CT}$ method, with GAPDH as the internal control. The experiment was performed with three replicates of each amplicon.

2.8. Animal handling

A total of 36 Wistar albino rats, weighing between 180–200 g, were used in the in vivo study. The rats were provided by the Laboratory Animal Facility & Management (LAFAM), UiTM Puncak Alam, Selangor, Malaysia and were randomized into groups upon receipt. They were housed and maintained at a constant temperature (22 ± 1 °C) under a

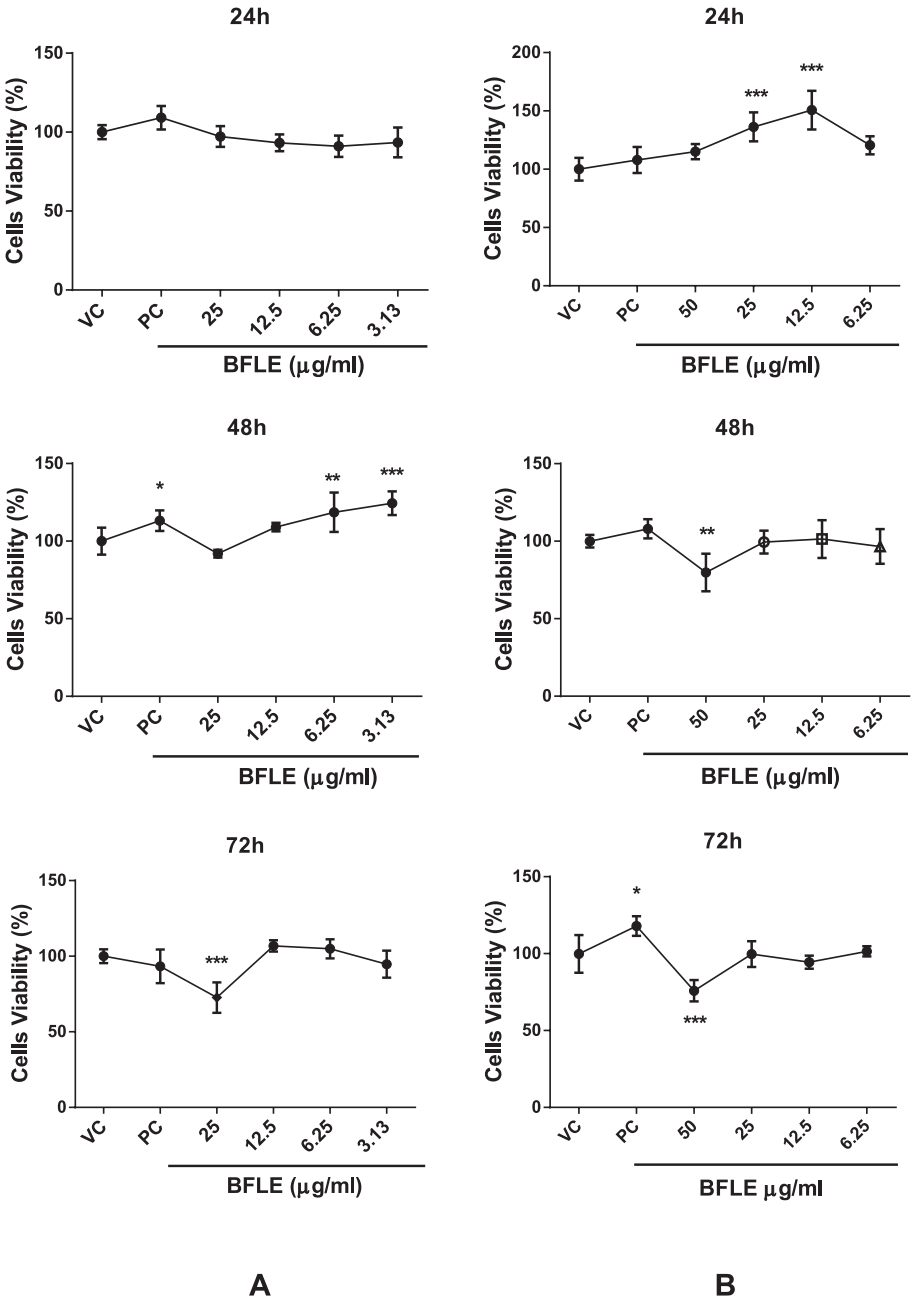

Fig. 1. A. HaCaT and **B.** BJ proliferation against BFLE at different concentrations for 24, 48, and 72 h. Allantoin at 10 µg/ml was used as positive control (PC). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs vehicle control (VC) group, n = 3 in each group.

12:12 h light/dark cycle, with ad libitum access to food and water. The rats were allowed to acclimatize to the laboratory environment for one week prior to starting the experiments. All methodologies used for the animal study were presented to and approved by the Institutional Ethical Committee (UITM CARE: 337/2021). All rats were housed in individual cages during the dermal toxicity and excisional wound healing experimental period. Three animals were used per group for the dermal toxicity study and six animals were used per group for the excisional wound healing study. Female rats were used for the dermal toxicity study, while male rats were used for excisional wound healing study. All rats were monitored for their weight and health status. Unhealthy rats were excluded from this study.

2.9. Dermal toxicity

An acute dermal toxicity study was conducted following the Organisation for Economic Co-operation and Development (OECD) Guideline (OECD, 2017). As suggested by the guideline, female rats were used in this study. Briefly, the midline dorsal area of rats (n = 3) was shaved and uniformly applied with 20 % (w/v) of BFLE. The control group (n = 3) was applied with glycerol, which was used as a solvent to dissolve BFLE. All rats were observed for changes in skin and fur, tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma for the first 30 min and periodically during the 24 h (OECD, 2017). After 24 h, the BFLE was removed and washed, and additional observations on skin irritation, inflammation, edema, or rash were performed (Faiz et al., 2023).

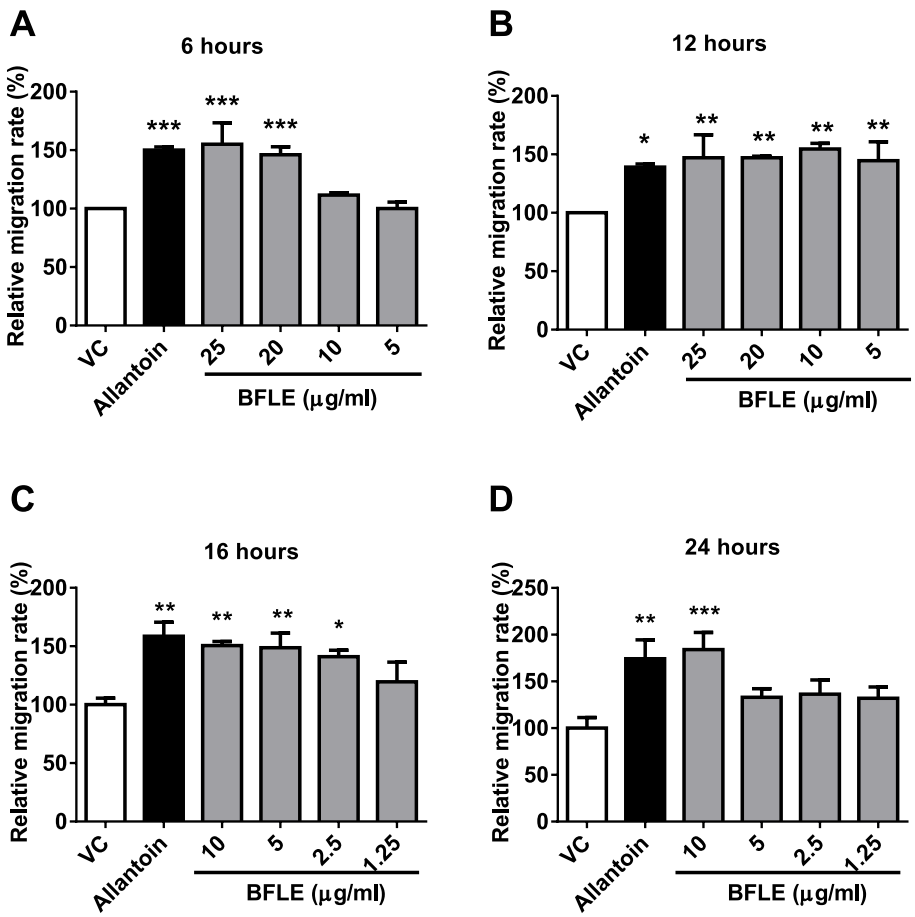

Fig. 2. Relative migration of HaCaT at **A.** 6 h, **B.** 12 h, and BJ at **C.** 16 h, **D.** 24 h when treated with BFLE at different concentrations. Allantoin at 10 µg/ml was used as positive control. $^{**}P < 0.01$, $^{***}P < 0.001$ vs vehicle control (VC) group, n = 3 in each group.

2.10. Excision wound healing

The rats were anesthetized by intraperitoneal injection of a ketamine/xylazine cocktail consisting of 80 mg/kg of ketamine and 12.5 mg/kg of xylazine. The fur on the dorsal area of each rat was shaved 3 cm down from the neck and between the shoulder blades using a pet shaver. The skin was disinfected with 70 % ethanol, and two wounds were created in each rat by punching the dorsal area of the skin using an 8 mm biopsy punch (Kai Medical, Japan). The wounds were then treated as follows (n = 6):

Group 1: Vehicle (100 % glycerol).
Group 2: Positive control (10 % povidone-iodine).
Group 3: 10 % (w/v) BFLE.
Group 4: 5 % (w/v) BFLE.
Group 5: 2.5 % (w/v) BFLE.

The extract with a concentration of 2.5 % (w/v) was chosen because BFLE shown to be effective in the in vitro test at a low concentration and showed a slight cytotoxic effect at a higher concentration. This is supported by other studies where the in vivo effect of 2.5 % (w/w) plant extracts (Azis et al., 2017; Fikru et al., 2012), was used as the lowest concentration of the test material, so the same concentration was used as the lowest concentration in this study. The remaining concentrations were increment of that concentration (two times).

The rats were allowed to recover from anesthesia and were returned to their cages. The wounds were monitored, photographed, and measured using a ruler and graph paper on days 0, 3, 7, 10, and 14. The bedding was changed daily as a hygienic precautionary step to avoid contamination of the wounds. The percentage of wound closure was calculated using the following formula:

% wound closure = Where A_0 = area of the wound at day 0; A_t = area of the wound at the measured time.

On day 14, all rats were sacrificed in a CO_2 chamber, and the dorsal skin area was removed and preserved in 10 % formalin for histological analysis and in saline water for tensile strength analysis.

2.11. Tensile strength

Tensile strength analysis was carried out using a tensiometer (TA. XTplus Texture Analyser, Stable Micro Systems, UK) according to the previously described method with some modifications (Pensara et al., 2020). On day 14, two ends of a 5x2 cm^2 area of the wounded rat's skin were longitudinally gripped with the wound area at the center by the two arms of the tensiometer. The tensiometer was set to apply force to the skin by pulling at a rate of 1.667 mm/second until the skin ruptured. The force applied by the tensiometer was measured by the machine and recorded.

2.12. Histopathology

Histopathological analysis full-thickness wound samples were performed according to previously described method with some modifications (Masson-Meyers et al., 2020). The full-thickness wound samples, comprising epidermis, dermis, and subcutaneous tissue, were sequentially dehydrated with 75 %, 85 %, 95 %, and 100 % ethanol. The samples were then embedded in paraffin and sectioned at 4 µm thickness using a microtome. The tissue sections were stained with Haematoxylin and eosin (H&E) and Masson's trichrome staining. The tissue slides were photographed using a microscope (Leica DM 3000 LED, Germany)

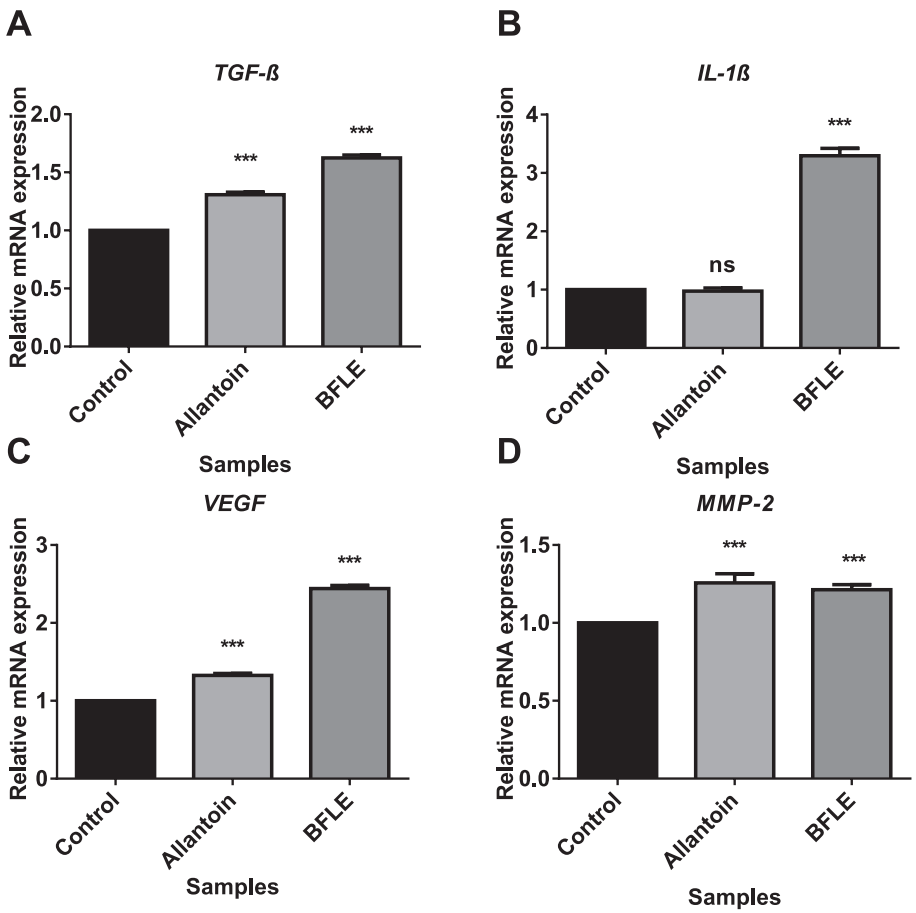

Fig. 3. Relative mRNA expression of **A.** TGF-β, **B.** IL-1β, **C.** VEGF, and **D.** MMP-2 genes in HaCaT treated with BFLE. Allantoin is used as a positive control. Results were expressed as mean ± SD expression of respective genes relative to untreated control (NC). ns; not significance, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group, n = 3 in each group.

attached to a camera (Leica ICC50 E, Germany) and subsequently analyzed.

2.13. Statistical analysis

The results were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 7 software, employing one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test for assessing differences between groups. A P-value below 0.05 was considered statistically significant. For the excision wound healing study, the required number of rats to achieve statistically significant differences was determined using standard power calculations, with $\alpha = 0.05$ and a power of 0.8.

3. Results

3.1. BFLE treatment increases proliferation of keratinocytes and fibroblasts

Treatment of BFLE against keratinocytes (HaCaT) at 6.25 and 3.13 µg/ml for 48 h have significantly increased ($P < 0.01$) their proliferation rate by 9.06 ± 2.72 and 18.61 ± 12.72 %, respectively, compared to vehicle control. Meanwhile, treatment of BFLE against fibroblasts (BJ) at 25 and 12.5 µg/ml for 24 h has significantly increased ($P < 0.001$) by 36.33 ± 12.47 and 50.73 ± 16.57 %, respectively, compared to vehicle control (Fig. 1).

3.2. BFLE treatment increases migration rate of keratinocytes and fibroblasts

Migration rate of keratinocytes (HaCaT) treated with BFLE at 25 and 20 µg/ml for six hours have significantly increased ($P < 0.01$) by 55.23 ± 18.38 and 46.45 ± 6.93 %, respectively, compared to vehicle control while migration rate of HaCaT treated with BFLE at 25, 20, 10, and 5 µg/ml for 12 h have significantly increased ($P < 0.001$) by 47.23 ± 19.82, 47.04 ± 1.41, 54.53 ± 4.95 and 44.34 ± 16.26 %, respectively, compared to vehicle control.

Meanwhile, the migration rate of fibroblasts (BJ) treated with BFLE at 25, 20 and 10 µg/ml for 16 h have significantly increased ($P < 0.05$) by 58.45 ± 12.02, 48.67 ± 12.45 and 41.34 ± 5.66 %, respectively, compared to vehicle control while their migration when treated by BFLE at 10 µg/ml has significantly increased ($P < 0.001$) by 84.22 ± 18.38 %, compared to vehicle control.

Allantoin, which was used as positive control at concentration of 10 µg/ml, has significantly increased migration rate of HaCaT treated with BFLE for 6 and 12 h by 50.42 ± 2.83 and 39.45 ± 2.83 %, compared to vehicle control while it has significantly increased BJ migration by 58.37 ± 12.02 and 74.33 ± 20.23 %, when applied for 16 and 24 h, compared to vehicle control, respectively (Fig. 2).

3.3. BFLE treatment increases TGF-β, IL-1β, VEGF and MMP-2 gene expressions

Treatment of BFLE at 25 µg/mL on HaCaT cells (Fig. 3) demonstrated significant upregulation ($P < 0.001$) of TGF-β, IL-1β, VEGF and MMP-

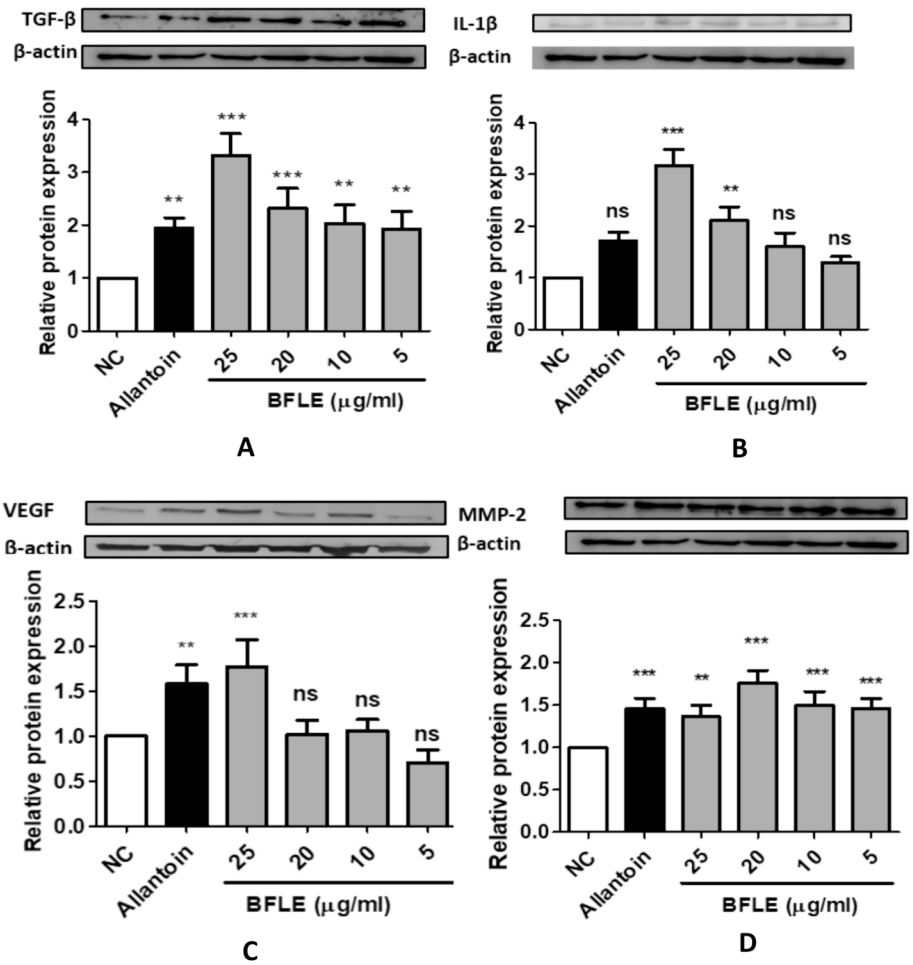

Fig. 4. Relative protein expression of **A.** TGF-β, **B.** IL-1β, **C.** VEGF and **D.** MMP-2 protein in HaCaT treated with BFLE. Allantoin is used as a positive control. Results were expressed as mean ± SD expression of respective proteins relative to untreated control (NC). ns; not significance, $^{**}P < 0.01$, $^{***}P < 0.001$ vs normal control (NC) group, n = 3 in each group.

2 genes expression with 1.62 ± 0.03, 3.30 ± 0.13, 2.44 ± 0.04, 1.21 ± 0.03 fold increase, compared to untreated control, respectively. Allantoin, the positive control used in this study, also demonstrated the upregulation of TGF-β, VEGF and MMP-2 gene expression with 1.31 ± 0.02, 1.33 ± 0.03, 1.25 ± 0.06-fold increase compared to untreated control, respectively. However, allantoin did not significantly affect the IL-1β gene expression.

Meanwhile, treatment of BFLE at 25 μg/ml on BJ cells did not significantly affect TGF-β, VEGF and MMP-2 genes expression. At the same time, IL-1β gene expression was significantly downregulated ($P < 0.01$) by 0.71 ± 0.07-fold compared to control. Treatment with 10 μg/mL allantoin also did not alter the expression level of TGF-β, IL-1β and VEGF genes in BJ. Only MMP-2 gene expression was significantly upregulated by allantoin with 1.62 ± 0.52-fold increase compared to control.

3.4. BFLE treatment increases TGF-β, IL-1β, VEGF and MMP-2 protein expression

Treatment of BFLE at various concentrations on HaCaT cells (Fig. 4) increased TGF-β, IL-1β, VEGF, and MMP-2 protein expression in a dose-dependent manner.

HaCaT treated with BFLE at 25, 20, 10, and 5 μg/mL significantly increased ($P < 0.01$) TGF-β protein expression by 3.32 ± 0.41, 2.32 ± 0.38, 2.03 ± 0.35 and 1.92 ± 0.34 fold, compared to untreated control, respectively. HaCaT treated with BFLE at 25 & 20 μg/mL significantly increased ($P < 0.01$) IL-1β expression by 3.12 ± 0.32, 2.11 ± 0.26-fold, respectively. HaCaT treated with BFLE at 10 and 5 μg/mL were not significantly affecting IL-1β protein expression compared to untreated control.

For VEGF protein expression, only BFLE at 25 μg/mL significantly increased ($P < 0.001$) HaCaT protein expression with 1.77 ± 0.30-fold increase compared to untreated control.

Meanwhile, MMP-2 expression of HaCaT treated with BFLE at 25, 20, 10, and 5 μg/mL significantly increased ($P < 0.01$) by 1.36 ± 0.14, 1.76 ± 0.15, 1.50 ± 0.16 and 1.46 ± 0.11-fold, respectively. Allantoin, which was used as positive control, significantly increased ($P < 0.01$) TGF-β, VEGF, and MMP-2 protein expression by 1.95 ± 0.19, 1.58 ± 0.22 and 1.46 ± 0.12-fold, respectively.

3.5. BFLE treatment reduces the size of excisional wounds in rats

Wound healing rates of Wistar albino rat skin were observed and depicted in Fig. 5A. Treatments of BFLE at different concentrations (2.5 %, 5 %, and 10 % w/v) exhibited improvements and reductions in lesion sizes compared to the normal control (glycerol), especially on days 3 and 7. Treatment with 10 % iodine (w/v) also exhibited a reduction in lesion size compared to that of the normal control. The size of each wound was measured using graph paper, and the size of each lesion is demonstrated in Fig. 5B.

The size of the wound on Wistar albino rat skin was significantly reduced ($P < 0.01$) by 44.19 ± 9.44 %, 43.90 ± 6.02 %, and 43.91 ±

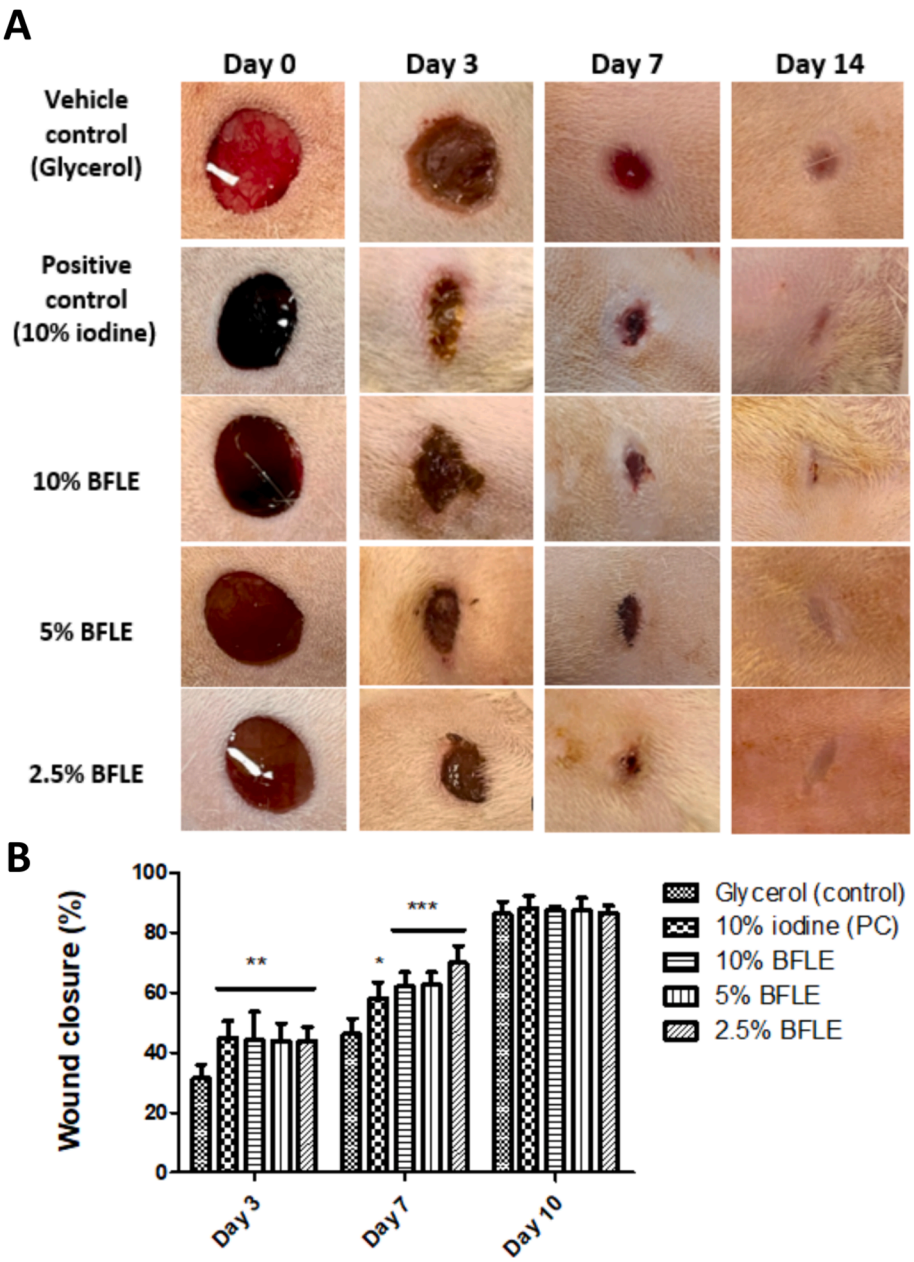

Fig. 5. **A**. Pictures of representative wounds and **B.** Percentage of wound closure (%) in rats treated with glycerol (control), 10 % iodine (positive control), 10, 5 and 2.5 % (w/v) BFLE for 14 days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control group, n = 6 in each group.

4.62 % on day 3, in the groups that applied 10 %, 5 %, and 2.5 % (w/v) of BFLE, respectively, as compared to the control group, which only reduced the lesion size by 31.45 ± 4.63 %. Meanwhile, on day 7, the size of the wound on Wistar albino rat skin was significantly reduced ($P < 0.001$) by 62.00 ± 4.97 %, 62.73 ± 4.19 %, and 69.94 %, respectively, as compared to the control group, which only reduced the lesion size by 46.19 ± 5.24 %. The positive group (10 % iodine) also significantly reduced wound size with 44.97 ± 5.66 % and 58.26 ± 5.31 % of reduction on day 3 and day 7, respectively.

3.6. Histology evaluation of wounds treated by BFLE

Histological examination of the wounds after 14 days revealed that the epidermis of the rats' skin had developed with the formation of hair follicles, and there was a decrease in the appearance of inflammatory cells in the positive control group (10 % w/v iodine), 5 % and 2.5 % (w/v) BFLE groups (Fig. 6). The thickness of the epidermis was also evident in the positive control and BFLE-treated groups compared to the vehicle control group. In Masson's trichrome-stained histological samples, collagen deposition and arrangement was markedly improved in the group treated with 10 % (w/v) iodine and 5 % (w/v) BFLE compared to the other groups.

3.7. BFLE treatment enhances the tensile strength of healed rat wounds

The results of the tensile strength (Table 2) indicate that the rats' skin treated with 2.5 % (w/v) BFLE showed the highest tensile strength, followed by 5 % (w/v) BFLE, and both concentrations showed a significant improvement ($P < 0.05$) in tensile strength compared to the control group. Rats' skin treated with 10 % (w/v) BFLE and 10 % (w/v) iodine groups, which were used as positive controls, also showed a slight increase in tensile strength compared to the control group, but it was not significant.

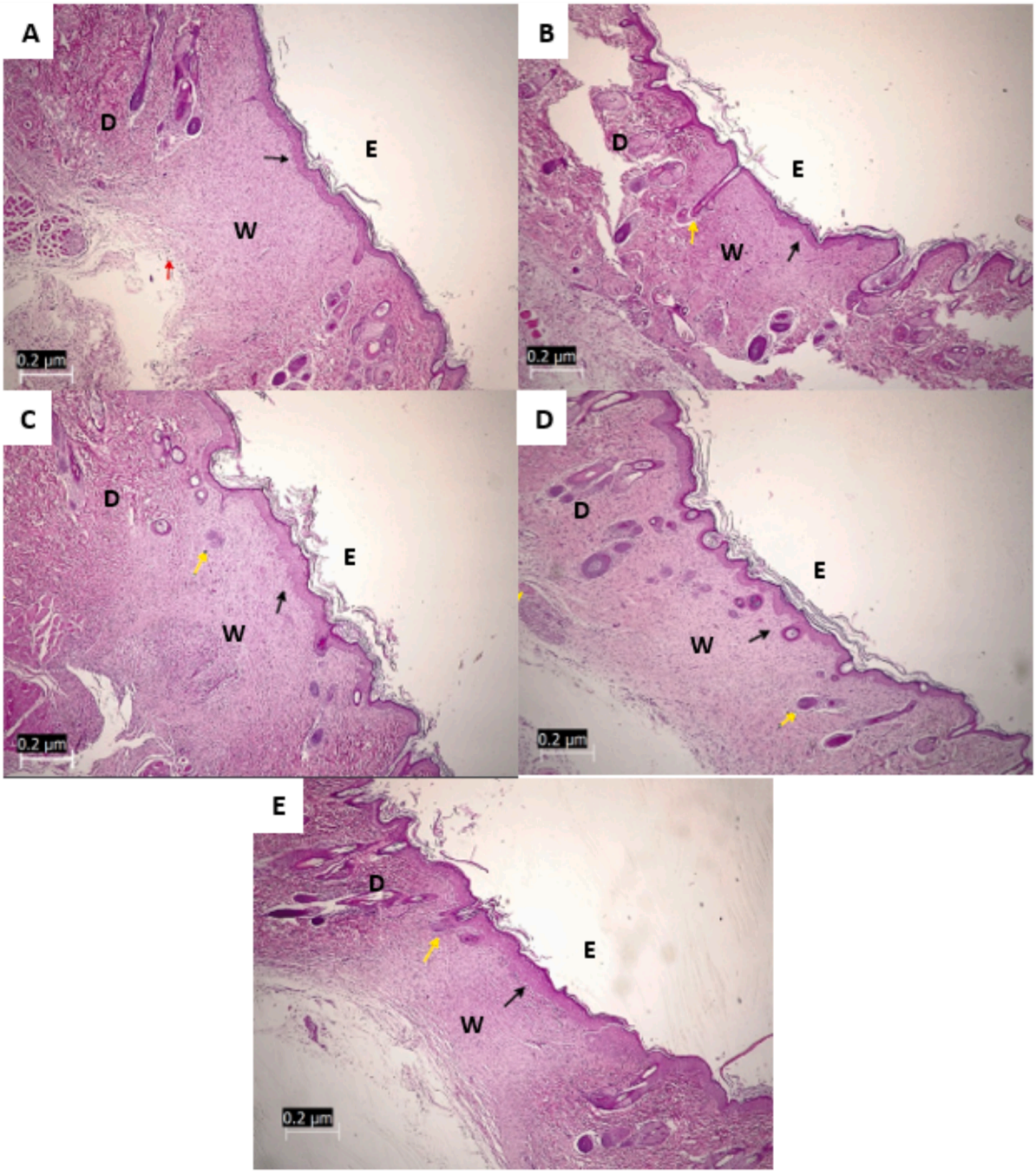

Fig. 6. Representative photomicrographs from different rats' wound samples on day 14 treated with **A.** Glycerol (vehicle control), **B.** 10 % (w/v) iodine, **C.** 10 % (w/v) BFLE, **D.** 5 % (w/v) BFLE, and **E.** 2.5 % (w/v) BFLE. The histological slides were stained with hematoxylin and eosin stain. The red arrow indicates inflammatory cells, the yellow arrow indicates hair follicles, and the black arrow indicates the epithelial layer. E = epithelium, D = dermis, and W = wound area. Images under microscope were captured at 40x magnification.

4. Discussion

The wound healing process involves many sequential and dynamic yet overlapping steps, such as hemostasis, inflammation, proliferation, and remodeling. The process is characterized by platelet aggregation, extracellular matrix deposition, cytokine and growth factor infiltration, granulation tissue formation, angiogenesis, re-epithelialization, proliferation, and migration of fibroblasts, keratinocytes, and endothelial cells, as well as collagen deposition (Singh et al., 2017; Tonnesen et al., 2000).

The search for wound healing candidates has become crucial in recent decades due to the alarming rate of wound prevalence, particularly chronic wounds, as well as the increasing cost of wound healing treatment. *Baeckea frutescens* leaves extract (BFLE) was used in this study based on traditional and pharmacological properties in antibacterial, antifebrile, and hemostatic properties, as well as its use in the treatment of snake bites, which is closely related to wound healing.

The aim of this study was to demonstrate the wound healing properties of BFLE in vitro and in vivo. The in vitro study was performed on the migration and proliferation of fibroblasts and keratinocytes, two types of cells that are predominantly found in the dermal and epidermal layers of the skin. The wound-healing effect of BFLE was then investigated using the expression of genes and proteins of various cytokines and growth factors such as TGF-β, VEGF, IL-1β and MMP-2. To

Table 2
Tensile strength of healed rat wounds in rats treated with glycerol (control), 10 % iodine (positive control), 10, 5 and 2.5 % (w/v) BFLE for 14 days. *$P < 0.05$, **$P < 0.01$ (comparison to the control group). The results were analyzed statistically using a one-way analysis of variance (ANOVA).

Group	Tensile Strength (g/mm^2), 14th Day
Glycerol (Control)	162.57 ± 17.02
10 % iodine (Positive)	178.93 ± 27.30
10 % BFLE	195.34 ± 7.14
5 % BFLE	206.46 ± 5.06*
2.5 % BFLE	216.09 ± 20.62**

n = 6 Wistar albino rats per group, values are represented as mean ± sd.

corroborate the results of this study, an in vivo study was also conducted to demonstrate the ability of BFLE to heal the excisional wound of Wistar albino rats, increase overall tensile strength and improve collagen fiber deposition.

In this study, BFLE has been shown to have a proliferative and migratory effect on fibroblasts and keratinocytes, as demonstrated in Figs. 1 & 2. Proliferation and migration are two main events that occur during the proliferative stage of wound healing, involving many types of cells, including fibroblasts, keratinocytes, endothelial cells, and epithelial cells. These events were induced by cytokines and growth factors such as TGF-β and VEGF. This data is supported by the upregulation of TGF-β and VEGF in gene and protein expression, which is evident in keratinocytes (HaCaT) and fibroblasts (BJ) (Figs. 3 and 4).

TGF-β promotes cell growth, proliferation, and migration (Lichtman et al., 2016) and also plays a crucial role in other stages of wound healing, including extracellular matrix deposition, angiogenesis, collagen synthesis, and deposition (Shady et al., 2022). TGF-β also regulates the inflammatory response by inducing the expression of other cytokines and leukocytes (Pakyari et al., 2013). VEGF, on the other hand, plays a role as an endothelial cell mitogen and proliferation factor (Brekken et al., 2001; Shima et al., 1995), chemotactic agent, and inducer of vascular permeability (Brekken et al., 2001). VEGF also has a unique function in wound healing, including angiogenesis, epithelialization, and collagen deposition (Bao et al., 2009).

The ability of BFLE to enhance the expression of TGF-β and VEGF genes and proteins demonstrates the wound healing properties of *B. frutescens*. These findings are in agreement with previous studies that have demonstrated the role of TGF-β and VEGF in expediting the process of wound healing (Kim et al., 2013; Zhang et al., 2022; Zhong et al., 2022). Recent study demonstrated the increased genes expression of TGF-β and VEGF by licorice extract responsible for the wound healing effect of the extract (Assar et al., 2021). The study showed that the enhancement of VEGF genes expression has remarkable effect on wound healing by enhancing angiogenesis and collagen deposition thus improved the epithelialization rate. Meanwhile, TGF-β were proposed to enhance the cells response to the normal regulatory factors at the site of injury (Assar et al., 2021).

Another finding of the present study is that BFLE treatment on keratinocytes (HaCaT) and fibroblasts (BJ) has induced the upregulation of IL-1β gene and protein expressions (Fig. 3B and 4B). This result is in contrast with a previous report where the IL-1β expression level was reduced in extracts possessing wound healing properties in order to control the prolongation of inflammatory phase thus avoiding the occurrence of chronic wound (Nazir et al., 2021). However, the differences might be due to the different types of cells used between this and that study, where they used RAW264.7 cell lines in their study. Moreover, this study focuses on the early phase of wound healing, in which the inflammatory response actually facilitates the wound healing process. Some other studies reported that IL-1β overexpression was apparent in cases of chronic wound healing, which was more crucial in the later days or weeks after wound occurrence (Raziyeva et al., 2021).

MMP-2 expression, on the other hand, has been found to be upregulated in keratinocytes (HaCaT) and fibroblasts (BJ) treated by BFLE (Fig. 3D & 4D). MMP-2 plays a major physiological role in the tissue remodeling process (Lechapt-Zalcman et al., 2006), especially in ECM remodeling, and plays a crucial role in keratinocytes migration and re-epithelialization (Wu et al., 2016). This finding suggests that BFLE plays a crucial role in ECM remodeling, migration, and re-epithelialization during the wound healing.

The in vivo part of this study was performed on Wistar albino rats using different concentrations of BFLE and iodine as positive control. The application of BFLE did not trigger biting or scratching on the wound area, and all the rats applied with BFLE did not cause any sign toxicity and irritation or rats. The excisional wound was about 8 mm^2 in size induced, and the different concentrations of BFLE or 10 % (w/v) were applied to the wound, and the progression of wound closure or wound contraction from the edge of the wounds were monitored during 14 days of study.

The results indicate that treatment with different concentrations of BFLE reduced the wound size in Wistar albino rats compared to the group that only applied glycerol (control group). The group treated with 5 % (w/v) BFLE showed the most significant reduction in wound size, demonstrating the wound healing properties of BFLE. In addition, tensile strength analysis showed that the rats' skin treated with 5 % (w/v) BFLE had the highest tensile strength compared to the other groups. Despite the wound lesions contracting in all groups by day 14, the differences in tensile strength suggest that the group treated with 5 % (w/v) BFLE may have a denser and more organized collagen fibril network formed during the remodeling stage of wound healing.

This observation is also supported by histological data, which shows that Masson's trichrome stain density was highest in the group treated with 5 % (w/v) BFLE (Fig. 7). The use of plant extracts has been showcased on many occasions as their effectiveness for excisional wound contraction, increased the skin's tensile strength and improved the overall morphology of the full-thickness wound in rats have been demonstrated in many studies. A study performed by Ghosh et al. (2019) found that an ointment containing leaves extract of *Aegialitis rotundifolia* showed an increase of wound contraction, collagen formation and improved wound skin histology. A more recent study performed by Coutinho et al. (2021) demonstrated that a wound healing cream formulated with *Kalanchoe pinnata* improved the wound healing time in excision wounds in rats and showed better re-epithelialization rate and denser collagen fiber in rats applied with the cream.

In our previous study, ethanolic extract of *B. frutescens* leaves has been demonstrated to increase the proliferation and migration rates of fibroblasts and keratinocytes (Kamarazaman et al., 2022). These proliferative and migratory effects of BFLE were deemed to be caused by the phytochemicals present in the extract, such as tannin and flavonoids, which were found to possess antioxidants (Beninger & Hosfield, 2003; Rex et al., 2018), anti-inflammatory (Shao & Bao, 2019) and wound healing effect (Rex et al., 2018).

Result of LCMS from that study also found that myricetin, myricetin 3-Oalpha-L-rhamnoside, quercetin-3-O-alpha-L-rhamnoside, 6-methylquercetin 7-O-b-D-glucopyranoside, pinoquercetin and 5,7-dihydroxy-6,8-dimethylflavanone has been tentatively identified base on their *m/z* masses. Myricetin and its derivative, myricetin-3-O-rhamnoside were postulated to be attributed for their proliferative and migratory activity based on studies that were performed previously (Elshamy et al., 2020; Moghadam et al., 2017). Another study also found that myricetin-3-O-rhamnoside and quercetin-3-O-alpha-L-rhamnoside isolated from *Pistacia lentiscus* improve wound healing in rats model by enhancing re-epithelization, fibroblasts proliferation, collagen regeneration (Elloumi et al., 2022).

5. Conclusion

The ethanolic extract from *Baeckea frutescens* or'cucur atap' leaves has been found to be effective in improving the migration and proliferation of keratinocytes (HaCaT) and fibroblast (BJ) cells. It enhances

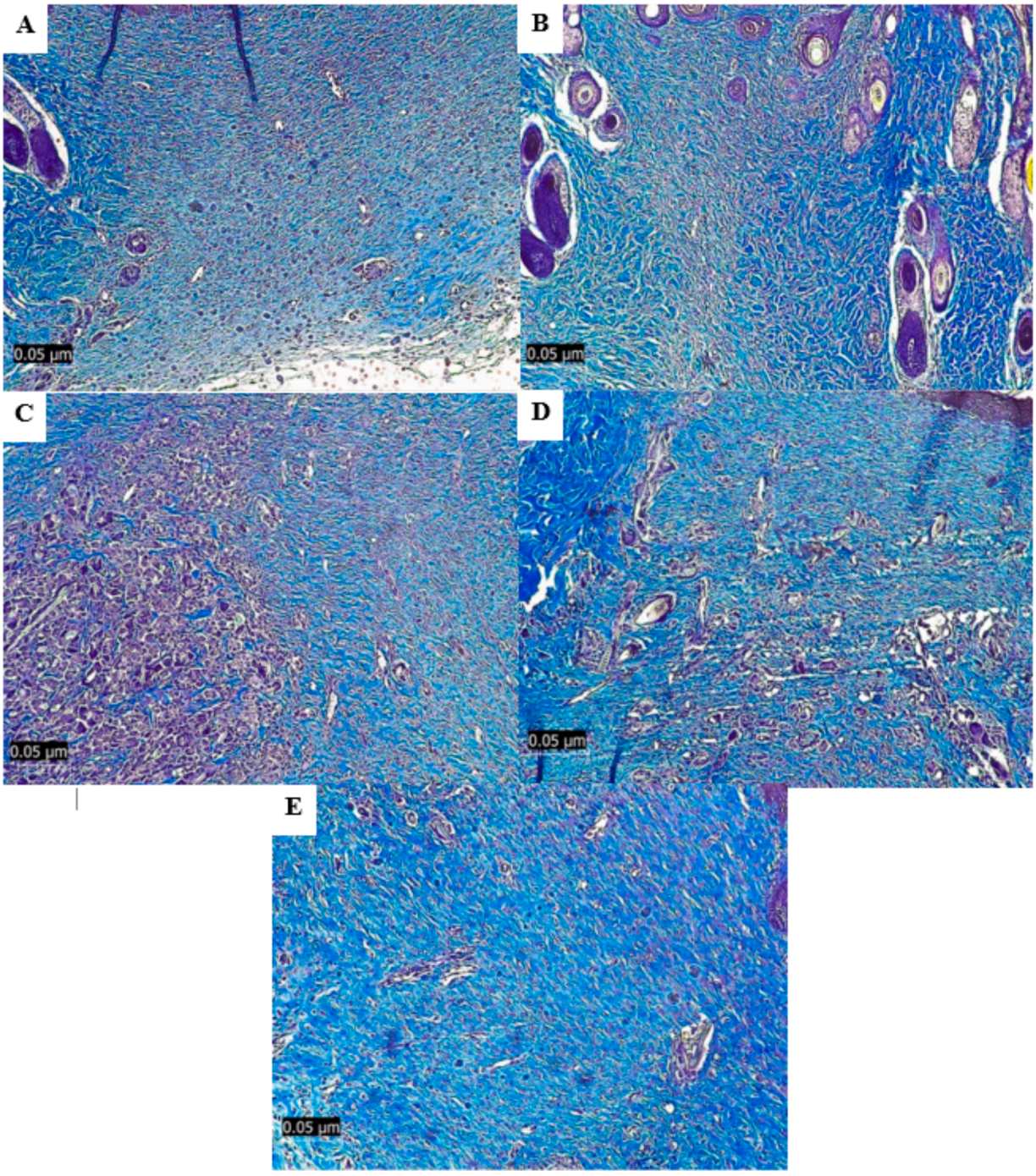

Fig. 7. Representative photomicrographs from different rats' wound samples on day 14 treated with **A.** Glycerol (vehicle control), **B.** 10 % (w/v) iodine, **C.** 10 % (w/v) BFLE, **D.** 5 % (w/v) BFLE, and **E.** 2.5 % (w/v) BFLE. The histological slides were stained with Masson's trichrome stain. E = epithelium, D = dermis, and W = wound area. Images under microscope were captured at 100x magnification.

the expression of TGF-β, VEGF, and MMP-2 genes and proteins involved in various stages of wound healing cascades, such as proliferation, migration, angiogenesis, re-epithelialization, vascularization, and ECM remodeling. In vivo experiments have demonstrated that *B. frutescens* also improves excisional wound healing rates, collagen deposition, and skin tensile strength of Wistar albino rats. This study confirms the wound healing properties of *B. frutescens*, which support its traditional use.

CRediT authorship contribution statement

Ihsan Safwan Kamarazaman: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Ling Sui Kiong:** Data curation, Formal analysis, Validation. **Mohd Kamal Nik Hasan:** Data curation, Formal analysis, Validation. **Norlia Basherudin:** Data curation, Formal analysis, Validation. **Nur Aini Mohd Kasim:** Data curation, Formal analysis, Validation. **Aida Azlina Ali:** Investigation, Supervision, Writing – review & editing. **Salfarina Ramli:** Conceptualization, Methodology, Writing – review & editing. **Sandra Maniam:** Conceptualization, Investigation, Writing – review & editing. **Richard Johari James:** Conceptualization, Validation, Writing – review & editing. **Pornchai Rojsitthisak:** Conceptualization, Validation, Writing – review & editing. **Hasseri Halim:** Conceptualization, Funding

acquisition, Supervision, Validation, Writing – review & editing, Project administration.

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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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2024.102110.

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