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Evaluation of Cytotoxicity and Migratory Effect of Aqueous Extract of *Baeckea frutescens* Lin. on Keratinocytes

Ihsan Safwan Kamarazaman[1,2,3], Mohd Kamal Nik Hasan[2], Nur Nazihah Adnan[3], Siti Sarah Khotib[3], Sandra Maniam[4], Hasseri Halim[1,3*]

[1]*Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA Selangor, Puncak Alam Campus, 42300, Bandar Puncak Alam, Selangor, Malaysia.*
[2]*Natural Products Division, Forest Research Institute Malaysia (FRIM), 52109 Kepong, Selangor, Malaysia*
[3]*Faculty of Pharmacy, Universiti Teknologi MARA Selangor, Puncak Alam Campus, 42300, Bandar Puncak Alam, Selangor, Malaysia.*
[4]*Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), Serdang, 43400, Malaysia.*

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ABSTRACT

Wounds, especially chronic wounds, have become a global problem affecting the quality of life, economic welfare, and productivity. Like fibroblast and endothelial cell migration, Keratinocyte migration has been associated with the wound-healing properties of certain compounds, fractions, or plant extracts. This study aimed to investigate the cytotoxicity and migration effect of the aqueous extract of *Baeckea frutescens* (BFAE) on immortalized human keratinocytes (HaCaT). Cytotoxicity was assessed using the MTT tetrazolium reduction assay, and the viability of HaCaT treated with 7.81–1000 µg/ml BFAE was determined. The migratory effect of BFAE at concentrations of 0, 1, 5, and 10 µg/ml was determined by scratching assay, and the comparison of migration rate between 0 and 12 hours was evaluated. The results indicate that BFAE is not cytotoxic when treated at concentrations of 200 µg/ml and below, when it has a cell viability greater than 80 %, compared to the untreated cells. BFAE also showed a migratory effect on HaCaT when it increased the migration of HaCaT by 1.3-fold when treated with 1 and 5 µg/ml BFAE, respectively. These results demonstrate the wound-healing properties of BFAE, which may be a very good candidate for a plant-derived wound-healing product.

INTRODUCTION

Wounds may occur to all regardless of age and gender, but children and the elderly are more susceptible to injury [1]. Individuals with diabetes or obesity are at increased risk for chronic wounds [2]. There are several risk factors for injury, such as accidents, medical conditions, and daily activities. An acute wound heals through natural healing mechanisms, but disruptions in the healing process cause the wound not to heal promptly, leading to chronic wounds [3]. Chronic wounds undergo the same healing process as

* Corresponding author. *E-mail address*: hasseri2945@uitm.edu.my

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acute wounds, but the transition from the inflammatory to the proliferative phase was hindered due to sustained inflammatory response [4]. Wound healing can progress to non-healing, over-healing, or inadequately healing wounds such as venous ulcers, keloid scars, and dehiscence [5].

Chronic non-healing wounds cause numerous complications and discomforts and can potentially lead to disability, amputation, and poor quality of life [6]. Chronic non-healing wounds impose an enormous financial burden on society, not only through the economic impact on the healthcare system but also by reducing productivity [7]. Remarkably, a study by Lindholm and Searle (2016) found that nurses spend over 60 % of their time on wound care [8]. The situation is exacerbated by the aging population and the increasing prevalence of diseases such as obesity, diabetes, and cardiovascular diseases, suggesting that the global incidence of chronic wounds is expected to increase [9]. Surprisingly, non-healing wounds have been shown to have a comparable impact on mortality to cancer [10].

The wound healing process is a dynamic and multifaceted event that includes several overlapping but distinct steps such as hemostasis, inflammation, proliferation, and remodeling phases. During these phases, various biological and molecular events take place, such as coagulation, infiltration of inflammatory cells, formation of granulation tissue, angiogenesis, re-epithelialization, revascularization, migration and proliferation of fibroblasts, keratinocytes and endothelial cells, collagen deposition, and scar formation [11]. The proliferative phase, characterized by the formation of granulation tissue, re-epithelialization, and neovascularization, is one of the most important phases of wound healing. Parameters that have been most frequently studied in the proliferative phase include fibroblast and keratinocyte migration and proliferation. Wound healing aims to restore intact tissue in the shortest possible time, reduce discomfort and pain, and leave a minimal scar on the skin [5]. Therefore, focusing on restoring tissue integrity is a key factor that prevents delayed healing and stimulates immediate healing in both acute and chronic wounds [12].

The World Health Organization (WHO) reports that approximately 80 % of the world's population depends on herbal plants as a primary source of medicine [13]. Many herbs, such as *Aloe vera*, *Azadirachta indica*, *Calendula officinalis*, *Centella asiatica*, *Chromolaena odorata*, and *Curcuma longa*, have been used for wound healing [14]. Herbs and traditional medicines have become very popular in recent decades thanks to their effectiveness, low cost, and minimal side effects [15]. *Baeckea frutescens* L., also known as ‘cucur atap’ in Malaysia, is an evergreen small shrub belonging to the Myrtaceae family. This heath-like shrub is characterized by small, white, single flowers with round petals and needle-like leaves that contain angular and flattened seeds. *B. frutescens* usually live and grow on moist soils, sandy coasts, low hills, and sheltered places at high altitudes, such as mountains, quartz ridges, or open grass (grasslands) [16,17]. Several efficacy claims have been made for *B. frutescens*, including treating influenza, sunstroke, headache, fever, hemorrhagic dysentery, and irregular menstrual cycles [18]. Several studies have reported this plant's cytotoxic, antibacterial, anti-inflammatory, antipyretic, and diuretic effects [19,20,21].

The present study aims to investigate the cytotoxic and migratory effects of the aqueous extract of *B. frutescens* (BFAE). The study's results will justify using this plant for wound healing to develop a plant-derived wound healing product.

EXPERIMENTAL

Preparation of Aqueous Extract of *Baeckea frutescens* (BFAE)

Fresh leaves of *B. frutescens* were collected from Setiu, Malaysia, and authenticated and given voucher number KLU 47909 by the Institute of Biological Sciences, Faculty of Medicine and Health Science, University Putra Malaysia. The leaves were dried in the oven at 40 °C and ground to a fine powder with a

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grinder machine. The leaf extract was prepared by soaking a 200 g grounded sample of *B. frutescens* leaves in 3.4 L distilled water at room temperature for 48 hours before filtering through the Whatman filter paper. The resultant infusion was lyophilized by freezing at -80 °C for 48 hours before being freeze-dried for 48 hours. The lyophilized extract was stored at -20 °C for further use.

Cell Culture

The immortalized human keratinocyte cells (HaCaT cells) (PCS-200-011; A.T.C.C., Manassas, VA, U.S.A.) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, U.S.A.) containing 10 % fetal bovine solution (FBS), penicillin/streptomycin (1:100; Sigma), and 4 mM L-glutamine in a CO2 incubator at 37 ◦C.

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The MTT assay was conducted using a similar method performed by Muniandy et al. (2018), with some minor modifications [22]. One of the cell-based assays, the tetrazolium reduction assay, uses 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA) as a measure of cell viability. Cells were seeded at 1 x 10^4 cells/well in a 96-well plate and incubated for 24 hours. After incubation, the old media were removed and replaced with 180 µl of media with or without various concentrations of BFAE (7.81, 15.62, 31.25, 62.5, 125, 200, 250, 300, 500, and 1000 µg/mL). After 24 hours, 20 µl of MTT reagent (5 mg/ml) is added to each well and incubated for another 4 hours. Then, the medium is removed, and the formazan product is dissolved in 50 µl of dimethyl sulfoxide (DMSO). Finally, absorbance values are measured at 570 nm using a microplate reader (Infinite M200) and recorded.

Scratching Assay

The migration assay was performed on 6-well plates following the method described by Governa et al. (2019) with some modifications [23]. Three horizontal lines were marked behind each well of the 6-well plate before seeding the cells. HaCaT cells were then seeded into the 6-well plates at a density of 1 x 10^6 cells/well on 6-well plates, and the cells were allowed to grow until they reached confluency. Then, the cells were scratched vertically with 200-µl pipette tips at three locations in each well. Then, the cells were washed twice with phosphate buffer solution (PBS) to remove cell debris, and the cells were treated with or without BFAE at concentrations of 1, 5, and 10 µg/mL on HaCaT. The gap size in the area where the marker line and scratch line meet was observed, and images of these areas were taken at 0 hours. The migration rate in these areas was observed over the next 12 hours, and the images were taken. Images of the cells were analyzed using Image J software. The migration rate of HaCaT treated with BFAE was calculated by comparing the size of the areas at 12 hours with those at 0 hours in the respective cells and treatments.

Statistical Analysis

Data are expressed as means ± standard deviation (SD) of three independent experiments, and the data shown in the figures are from a representative group of experiments. Statistical analysis was performed using Student's t-test to assess differences between groups. The value of $p < 0.05$ indicates the significance of the statistic.

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RESULTS

Viability Assay

Assay on the viability of HaCaT treated with different concentrations of BFAE demonstrated that the viability of HaCaT was reduced to about 50, 45, and 20 % when treated with 250, 300, and 500 µg/ml of BFAE, respectively (Fig.1). Morphological assessment on HaCaT exhibits a reduced number of monolayer cells that appeared to cover the surface of the flask when treated with BFAE at concentrations of 250–1000 µg/ml (Fig. 2).

Fig. 1. A graph of the percentage of cell viability against concentration was plotted. Statistical analysis of $p < 0.05$ was determined by one-way ANOVA followed by Bonferroni's post hoc. * showed $p < 0.05$ ($n = 4$).

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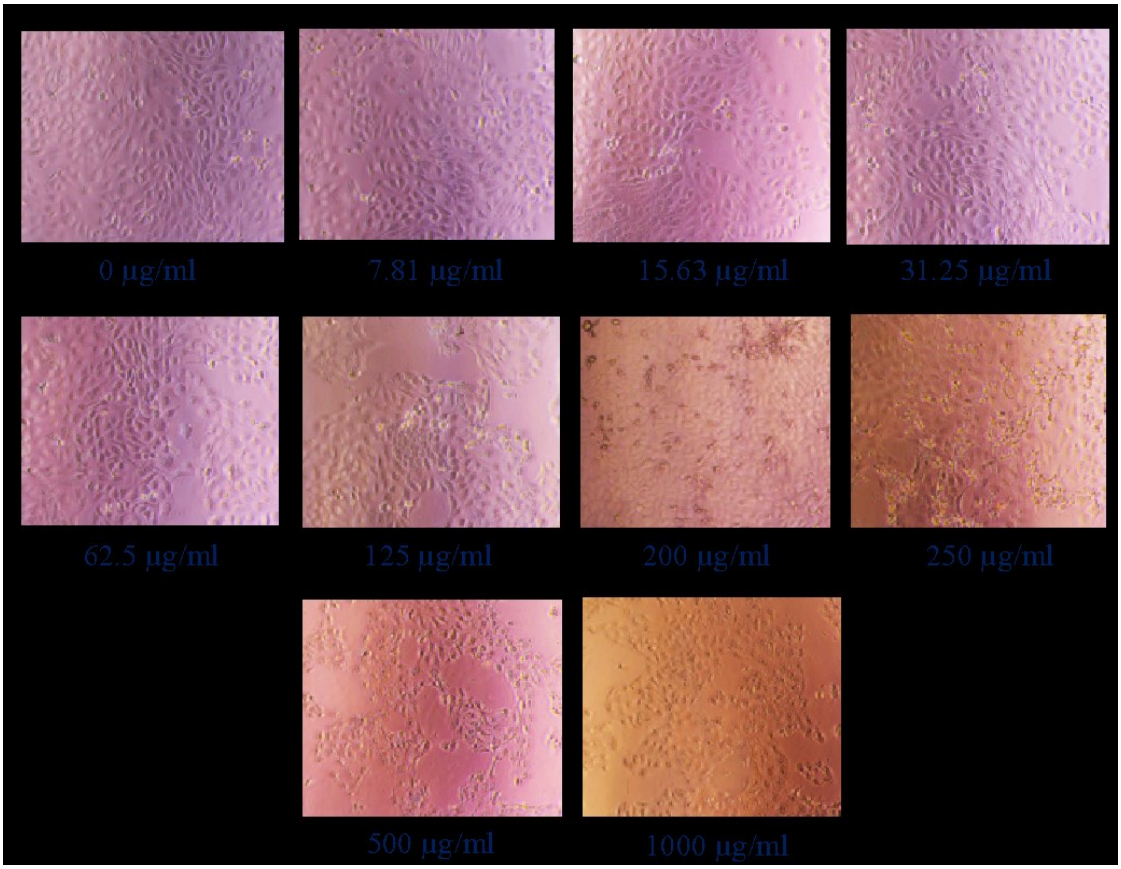

Fig. 2. Cellular morphology of keratinocytes in different concentrations of BFAE was observed under an ECLIPSE Ts2 microscope (10 x magnification).

Scratching Assay

Fig. 3 shows the image taken before and after 12-hour exposure to BFAE. The length of the adjacent wound margin was calculated at the designated fixed point, and the differences (length before treatment minus the length after treatment) were calculated. The experiment was performed in triplicates (n = 3).

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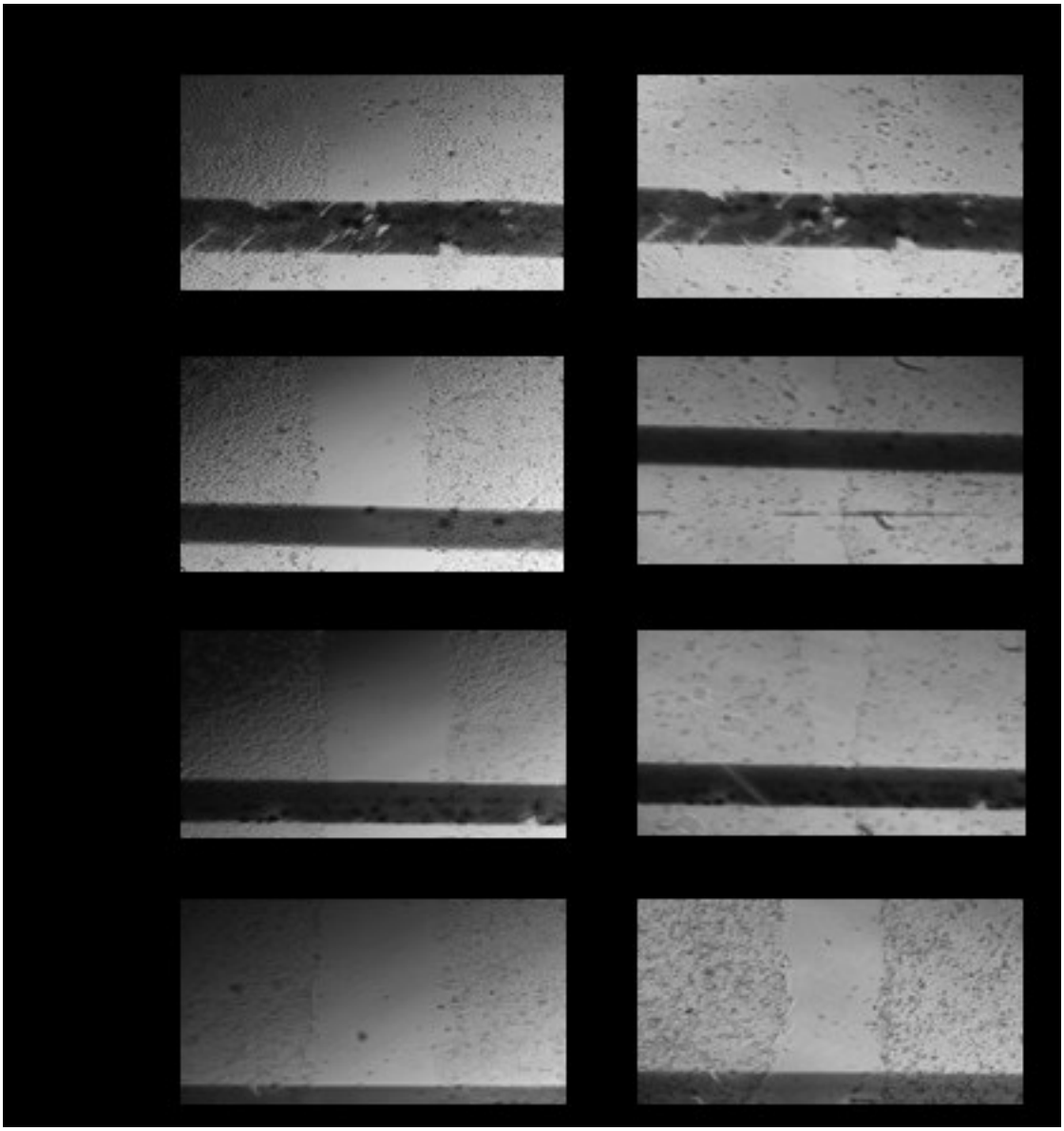

Fig. 3. Scratch wound healing assay shows the images of HaCaT cells before and after treatment with BFAE.

Fig. 4 demonstrates the relative migration of HaCaT cells after 12-hour exposure to BFAE. The relative migration was calculated by comparing the length of treated HaCaT cells with untreated control. Treatment with 1 µg/mL and 5 µg/mL of BFAE improved the migration of HaCaT cells. BFAE at 1 and 5 µg/mL concentrations has caused a 1.3-fold increase in cell migration. Meanwhile, at 10 µg/mL, the extract has shown a reduced migration of HaCaT cells. Cells treated with concentrations of 1 and 5 µg/mL promote migration, while concentrations of 10 µg/mL showed inhibitory activity towards the migration of cells.

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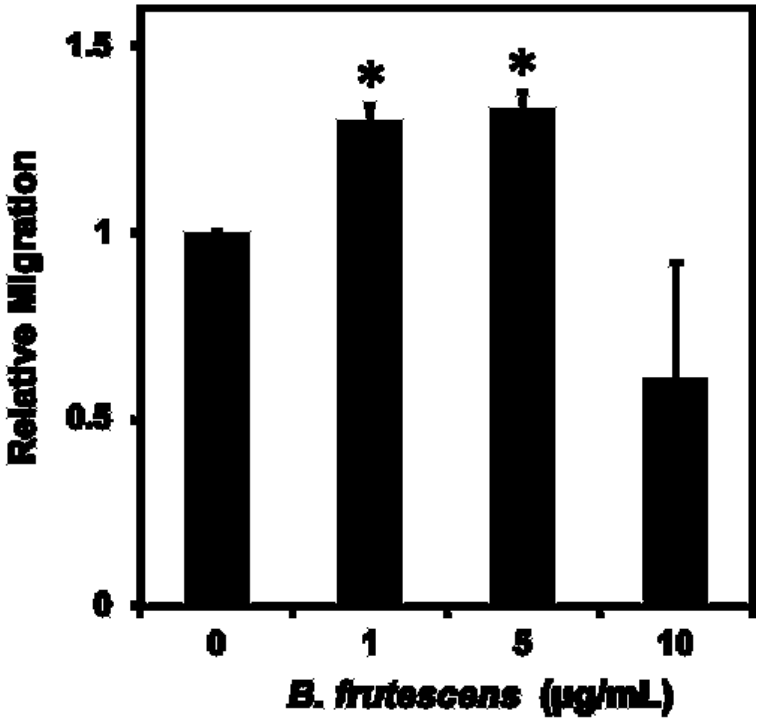

Fig. 4. Relative migration was assessed by wound healing scratch assay in HaCaT cells after exposure to BFAE. Each point represents the mean ± SD of three independent experiments. *$p < 0.05$ versus untreated control.

DISCUSSION

Wound healing and repair are necessary for restoring skin integrity after wounds, which if uncontrolled, lead to chronic wounds that cause discomfort to affected individuals. To contribute to the development of research in wound healing and repair, the aqueous extract of *Baeckea frutescens* (BFAE) was used to investigate the cytotoxicity and migratory effect of this plant extract on keratinocytes, which play an essential role in wound healing and repair.

The present study investigated the potential migratory effect of the BFAE on HaCaT (keratinocytes). Before determining the migratory effect of BFAE, the cytotoxicity of BFAE on HaCaT was first determined by MTT assay. The cytotoxicity or viability assay was performed by treating HaCaT with different concentrations of BFAE (7.81–1000 µg/mL) and then observing the morphological changes under the microscope. When observed under the microscope, the keratinocytes showed features of cell death at concentrations ranging from 200 to 1000 µg/ml. Some of the cells appeared fragmented and floating. The possible reason for this could be the death of keratinocytes. The relative number of dead cells was then determined by MTT assay. In the MTT assay, the formation of purple crystal formazan indicated that viable cells were present. This was due to converting orange MTT to purple formazan by mitochondrial dehydrogenase in the living cells. The results showed that BFAE exhibited some degree of toxicity at concentrations above 250 µg/ml when it reduced the viability of HaCaT by more than 50 %. At 500–1000 µg/ml concentrations, the number of living cells after treatment with BFAE was less than 20 %. However, microscopic observations showed a confluence-like shape of keratinocytes treated with 500–1000 µg/mL

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BFAE. This was probably because the cells had undergone necrosis and were already dead but still attached to the flask. This result showed that BFAE can be safely used at concentrations below 250 µg/mL.

Migration and proliferation of various types of cells, including macrophages, keratinocytes, fibroblast, and endothelial cells, marked the progression of wound healing. During the proliferative phase, fibroblasts migrate and proliferate to the wound matrix and serve as a scaffold for keratinocyte migration and proliferation [24]. Keratinocyte migration and proliferation are crucial during the re-epithelialization process in wound healing [25]. Under the influence of various growth factors and cytokines, keratinocytes proliferate from the wound edges and form hyperproliferative epithelia. These cells then migrate forward on the wound to facilitate the contraction of the wound and finally close the wound [26,27]. In the non-healing margins of diabetic foot ulcers, keratinocytes lack migratory activity, excessive proliferation, and incomplete differentiation [28,29].

In the present study, the scratching assay was performed to determine the migration of HaCaT. This study showed that the concentrations of 1 µg/mL and 5 µg/mL promoted migration, whereas the 10 µg/mL concentration had an inhibitory effect on cell spreading. The migratory effect of BFAE has shown that this extract has wound-healing properties. Other studies have reported that the enhanced proliferation and migration of keratinocytes by the polysaccharides of *Gracilaria lemaneiformis* can accelerate the wound healing process [30]. The study conducted by Mazumdar et al. (2021) demonstrated that the ethanolic extract of *Annona reticulata*, which induced the migration and proliferation of fibroblasts and keratinocytes, can improve wound healing in diabetic rats [31]. A recent study by Ratanachamnong et al. (2023) showed that the water extract of kaffir lime (*Citrus hystrix*) improved the migration and proliferation ability of keratinocytes and fibroblasts [32].

B. frutescens is rich in flavonoids, flavanones, sesquiterpenes, phloroglucinols, chromones, and chromanones [33]. Flavonoids are known to promote the wound-healing process [34]. Flavonoids have strong antioxidant properties and can scavenge free radicals that harm the wound-healing process [35]. Polyphenols also promote rapid re-epithelialization of acute wounds [36]. B. frutescens has also been reported to contain myricetin-3-O-rhamnoside and quercetin-3-O-alpha-L-rhamnoside. These compounds have been reported to improve wound healing in rat models by promoting re-epithelialization, fibroblast proliferation, and collagen regeneration [37]. It was suggested that these compounds are responsible for the keratinocyte migratory effect of *B. frutescens.*

CONCLUSION

The aqueous extract of *Baeckea frutescens* has shown cytotoxic effects at concentrations of 250 µg/mL and above. This plant extract is safe at concentrations of 200 µg/ml and below, where cell viability was more than 80 % compared to untreated cells. This plant extract also showed a perfect migration effect of keratinocytes when treated at concentrations of 1 and 5 µg/mL, indicating the wound-healing properties of this plant. The results of this study indicate that this plant is suitable for developing plant-derived wound-healing products. However, further study on the effect of this plant on animal models is suggested to confirm its efficacy.

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AUTHOR'S CONTRIBUTION

HH and SM designed the experiment. NNK and SSK carried out the experiment. ISK and MKNH involved in writing the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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