Bael Leaf Oil Increases AQP3 Expression and Exerts Wound-Healing Effect in Human Immortalized Keratinocytes

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Abstract

Bael (*Aegle marmelos* L. Corrêa, Family Rutaceae) leaf oil has been reported to promote wound healing in keratinocyte cell models. The oil key components, according to GC-MS analysis, are trans-caryophyllene and limonene. In this study, we investigated the mechanism by which bael leaf oil and its key components aid wound healing by focusing on the water channel aquaporin-3 (AQP3), which is crucial for maintaining skin hydration and identifying the active component responsible for the activity. To this objective, keratinocyte (HaCaT) cells were grown in media containing 2% FBS and subjected to a wound healing assay. The expression of AQP3 mRNA in the cells was observed using RT-real-time PCR. The results demonstrated that trans-caryophyllene and bael leaf oil significantly stimulated keratinocytes to close the wound ($p<0.05$ *vs.* vehicle group) and enhanced the level of AQP3 mRNA expression. It suggests that trans-caryophyllene, at least among the components of bael leaf oil, enhances the wound healing action instigated by the bael leaf oil, and that this effect is associated with the water channel aquaporin-3.

Keywords: bael leaf oil, trans-caryophyllene, HaCaT, wound healing, aquaporin-3

Introduction

A wound is defined as any disruption of the skin layers that alters the skin structure and function. The healing process begins as soon as a wound occurs. During wound healing, haemostasis, inflammation, migration-proliferation, and maturation-remodeling, occur (Grada, Otero-Vinas, Prieto-Castrillo, Obagi, & Falanga, 2017; Stamm et al., 2016). Keratinocytes at the edge of the wound margin proliferate and migrate onto the wound bed in response to injury and the influence of growth factors and cytokines. Cell migration, proliferation, and differentiation are all involved in this process (Grada et al., 2017), and, because migration is thought to be the rate-limiting process during healing, migration assays are an important part of wound healing research (Stamm et al., 2016).

Aquaporin-3 (AQP3) is a member of the aquaporin water channel family and a water-transporting, and glycerol-transporting, and is a protein that plays an important role in several cellular functions. Water transport, facilitated by AQP3, is important in cell migration which accelerates wound healing. Furthermore, AQP3-mediated glycerol transport is involved in cell proliferation as well as skin hydration and elasticity (Martinotti, Pellavio, Laforenza, & Ranzato, 2019).

According to our findings, bael leaf oil significantly improved wound closure (Kesornnoi, Prangsaengtong, & Sitthithaworn, 2022). Bael leaf oil is a volatile oil extracted from the leaves of *Aegle marmelos* L. Corrêa, (Family Rutaceae) through water distillation. The major oil constituents, according to GC-MS analysis, are germacrene A (11.65%), germacrene B (3.59%), germacrene D (24.66%), trans-caryophyllene (14.86%),

linalool (3.99%), limonene (0.32%), and ocimenes (1.51%). Among these constituents, trans-caryophyllene and limonene have been shown to promote wound healing in animal models. Trans-caryophyllene has been shown to improve wound healing in skin wound excision models in rats and mice (Gushiken et al., 2022; Koyama et al., 2019). Ahmad, Khan, Ansari, and Ahmad (2014) demonstrated that limonene improves wound healing in alloxan-induced diabetic mice by lowering pro-inflammatory markers and chemokine expression. Limonene was also found to have anti-inflammatory properties in murine dermal inflammation and wound healing (d'Alessio, Mirshahi, Bisson, & Bene, 2014). The objective of the current study was to explore how bael leaf oil help wound healing by focusing on the water channel aquaporin-3 (AQP3), which is essential for maintaining skin hydration, and identifying whether trans-caryophyllene or limonene is responsible for the activity.

Methods and Materials

Materials

Chemical

Limonene and trans-caryophyllene were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Volatile oil

Mature fresh leaves of *A. marmelos* L. Corrêa were collected from the botanical garden of the Faculty of Pharmacy, Srinakharinwirot University, Nakonnayok, Thailand. The volatile oil was extracted by conventional water distillation. The oil was stored at -20°C until use.

Cell culture

Human epidermal keratinocyte (HaCat) cell culture was obtained from Cell Lines Service GmbH (Eppelheim, Germany) and maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated in a humidified 95% air/5% CO_2 atmosphere at 37°C. The cells were grown to 70-80% confluence for use in each experiment.

Cell viability assay (WST-1 assay)

The test compounds were dissolved in DMSO to a stock concentration of 50 mg/mL, and stored at -20°C until used. The compounds were diluted to appropriate concentrations in culture media, and the final DMSO concentration was 0.8%(v/v). The growth-modulating effects of the volatile oil on HaCat cells were determined by WST-1 assay kit (Merck, Darmstadt, Germany). The cells were plated into each well of a 96-well plate (1.5×10^4 cells/well) and maintained in the culture for 24 hr in a humidified atmosphere (37°C, 5% CO_2). The culture medium was removed and replaced with medium containing the test compounds at concentrations ranging from 1.56 μg/mL to 200 μg/mL, and the vehicle alone served as a control (DMSO, 0.8% (v/v) in media). The cells were incubated for 24 hr and 48 hr at 37°C and 5% CO_2 WST-1 reagent was introduced at each incubation time point. The viable cells were determined, after incubation for 2 hr, by measuring the absorbance at 450 nm.

Wound healing assay

HaCat cells were either directly seeded into a silicone culture-insert in a 35 mm dish (ibid, GmbH, Germany), with the silicone insert removed before treatment to create an incision-like gap, or were seeded onto

24-well plates at a density of 2.5×10^5 cells/well and grown until they reached confluence in a monolayer, at which point they were scratched with a sterile 200 µL pipette tip. Then, the cells were treated with test compounds diluted in 2% FBS medium with a final DMSO content of less than 0.1% and incubated for a further 24 hr and 48 hr to allow time for covering into the cell-free area (Kadam, Vandana, & Kaushik, 2020). The vehicle without the test compounds served as a control. A microscope was used to inspect the data, and images were taken with a digital camera connected to the microscope and computer system (Nikon Eclipse). Nikon NIS Element Imaging was used to quantify the wound area covered by the cells and the percentage of wound closure at each time point. The percentage of wound closure was calculated with this equation:

$$\%\text{wound closure} = [(A_{t=0\,h} - A_{t=\Delta\,h})/A_{t=0\,h}] \times 100\%$$

where $A_{t=0h}$ is the area of the wound measured immediately after scratching and $A_{t=\Delta_h}$ is the area of the wound measured h hours after the scratch was performed (Liang, Park, & Guan, 2007).

Reverse Transcription-Real-time-PCR

Total RNA was isolated from HaCat cells using RNeasy Mini Kits (Qiagen, Germany). Reverse transcriptase was used to reverse transcribe total RNA (2 ng) and synthesize complementary DNA (cDNA) in a 20-µL reaction (qScript cDNA SuperMix, QuantaBio, USA). cDNA for the AQP3 gene was amplified using PerfeCTa SYBR Green FastMix (QuantaBio, USA). The cycle conditions were as follows: 15 sec of denaturation at 95°C, 30 sec of annealing at 60°C, 15 sec of extension at 72°C, and 30 sec at the specific melting temperature. At the end of each cycle, the SYBR Green fluorescence was measured. The mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used to normalize the AQP3 gene. Cycle threshold (Ct) values were used to calculate fold changes in gene expression using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). The following primers were used (Boury-Jamot et al., 2006).

AQP3 forward: 5'-ACCCTCATCCTGGTGATGTTT-3';

AQP3 reverse: 5'-TCTGCTCCTTGTGCTTCACAT-3'

GAPDH forward: 5'-AGCCACATCGCTCAGACAC-3';

GAPDH reverse: 5'-GCCCAATACGACCAAATCC-3'.

Statistical analysis

The experiment was performed in triplicate. Data were reported as mean ± S.D. Statistical significance was assessed by the student's *t*-test and *p*-values < 0.05 were significant.

Results

Effect of cell viability

The WST-1 assay was used to determine the conditions that allow cells to survive. The assay methodology is based on mitochondrial dehydrogenases in cells converting the tetrazolium salt WST-1 to formazan. The more cells that are alive, the more active the mitochondrial dehydrogenases are, and thus the more formazan dye is produced. Cell growth that was not significantly decreased, or exceeded that of the control group was considered viable. The concentrations that allowed the number of viable cells equivalent to those of the control group (treated with DMSO) (Figure 1 and Table 1) were labeled as a non-proliferative dose. The concentrations that allowed

the cell number to increase dramatically as compared with the increase in the control were labeled the proliferative dose and the concentrations that caused cell numbers to be significantly lower than those of the control were labelled as being toxic doses.

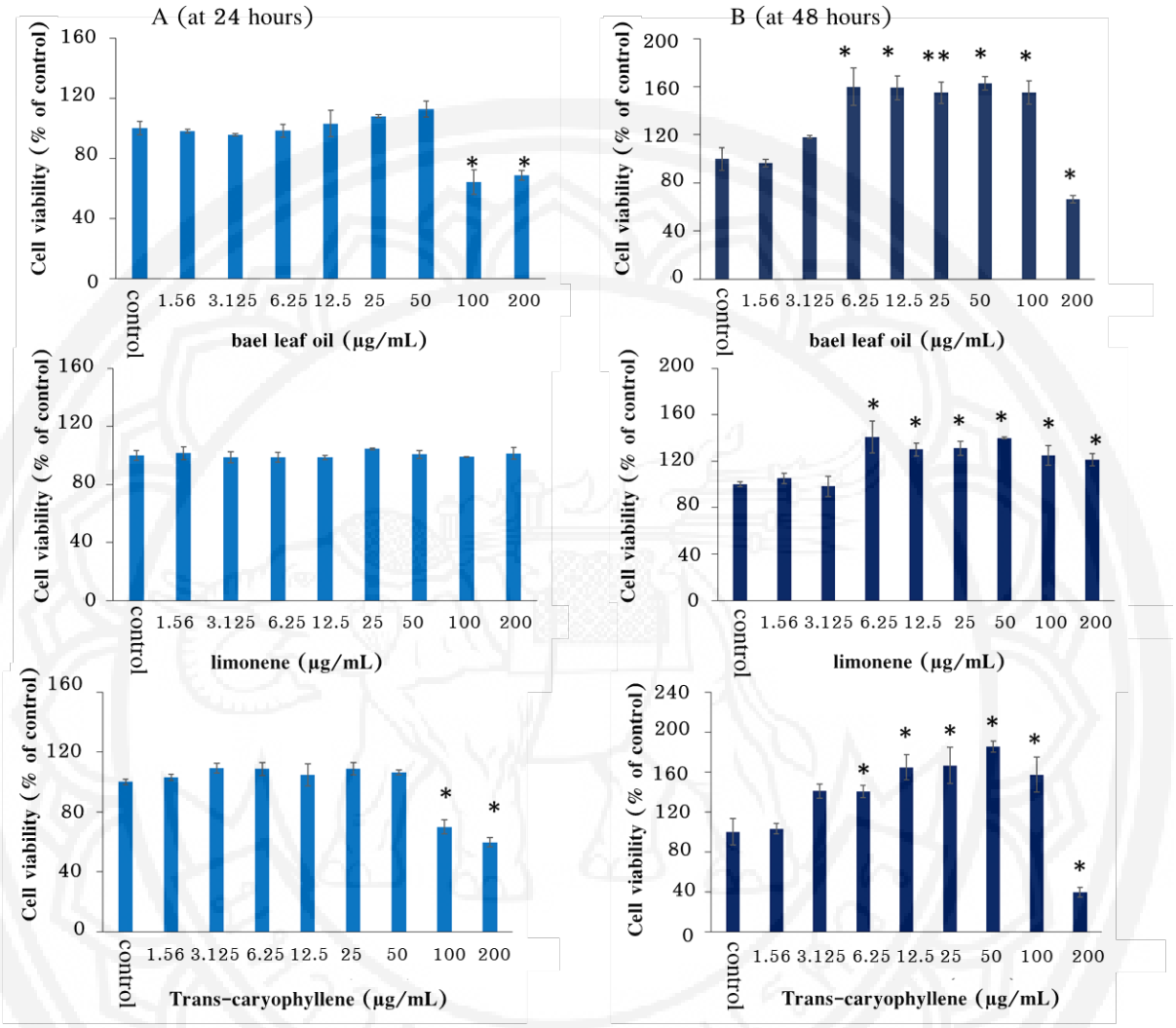

Figure 1 Effect of bael leaf oil, limonene, and trans-caryophyllene on HaCaT cell viability at 24 (A) and 48 (B) hours

Table 1 Effect of bael leaf oil, limonene, and trans-caryophyllene on cell characteristics

Sample	Incubation time (hour)	Non-toxic dose	
		Non-proliferative dose (µg/mL)	Proliferative dose (µg/mL)
Bael leaf oil	24	0-50	-
	48	0-3.125	6.25-100
Limonene	24	0-200	-
	48	0-3.125	6.25-200
Trans-caryophyllene	24	0-50	-
	48	0-3.125	6.25-100

Effect on wound healing

The wound healing experiment was conducted to examine the migration effect on cell models to cover the wound. For the oil, the assay was conducted at the concentrations (5, 25, and 50 µg/mL) which were determined to be non-proliferative doses. Trans-caryophyllene was 14.86% in the oil and limonene was 0.32% in the oil. Their concentrations were adjusted to correspond with those found in the oil. As a result, when the cells were treated with the oil at concentrations of 5, 25, and 50 µg/mL, the concentrations of limonene were 0.02, 0.1, and 0.2 µg/mL, and trans-caryophyllene were 0.8, 4, and 8 µg/mL. In comparison to the control, the test compounds at the concentrations used in the study tended to facilitate wound closure, but not in a dose-dependent manner, and the percentage of wound closure increased when the cells were incubated for a longer period (Figure 2).

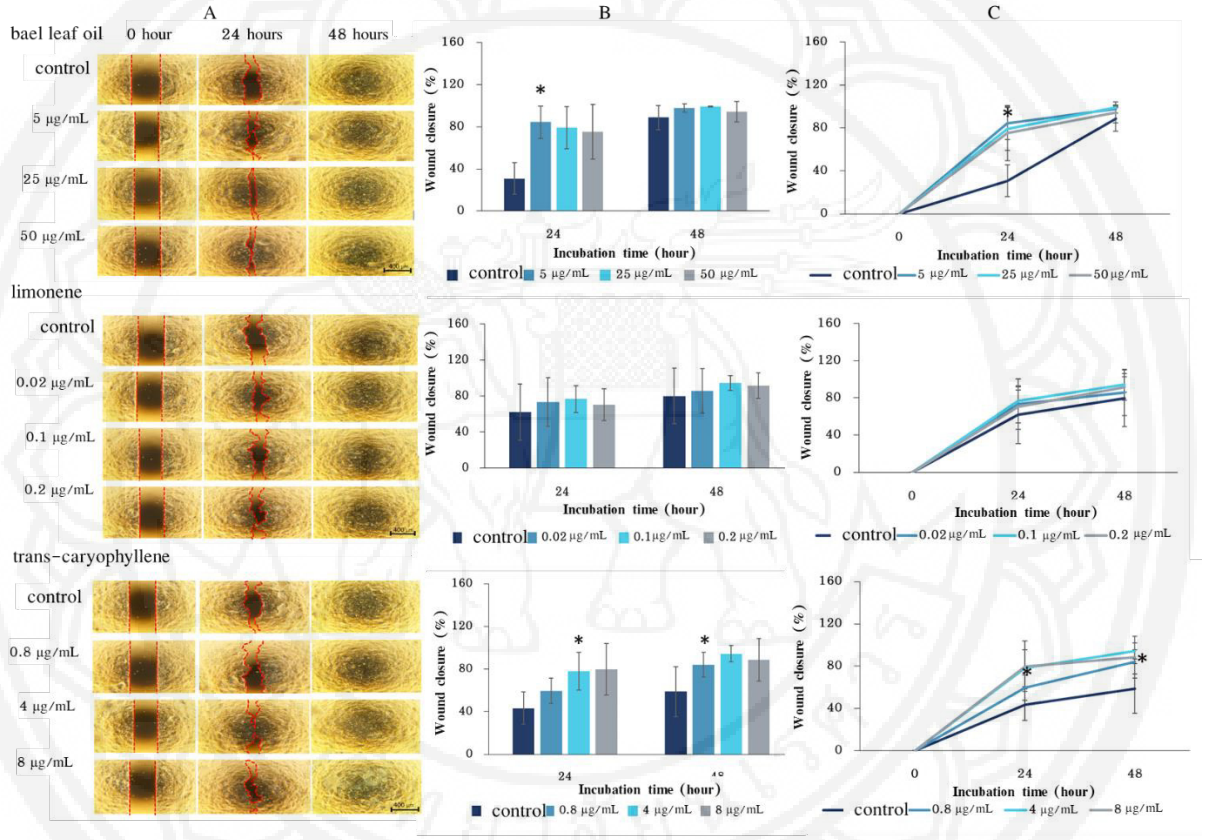

Figure 2 Wound healing effect of bael leaf oil, limonene, and trans-caryophyllene in HaCat cells. Bael leaf oil (5, 25, 50 µg/mL), limonene (0.02, 0.1, 0.2 µg/mL), and trans-caryophyllene (0.8, 4, 8 µg/mL) were applied to HaCat cells after the cell gap was made. The wound area was measured at 24 and 48 hours. (mean±SD, n = 3, *: p<0.05 vs. vehicle group). (A) Representative microscopic images of wounds and the scale bar is 400 µm long. (B) Percentage of wound closure. (C) Graphical representation of wound closure

The significant wound closure was observed when the cells were treated with 5 µg/mL of the oil for 24 hr with 84.51% wound closure compared to 30.81% in the control group. Additionally, the significant wound closure was observed following the administration of trans-caryophyllene at a dose of 4 µg/mL for 24 hr and 0.8 µg/mL for 48 hours; with 77.99% wound closure compared to 53.45% in the control group and 83.99% compared to 58.82% in the control group. There was no significant wound closure when the cells were treated with limonene.

The concentrations used in the wound healing investigation were once more exposed to the cell viability assay at 24 hr to confirm cell viability in the 2%FBS media and to ascertain if the wound was closed by cell migration or in conjunction with proliferation. The results suggested that trans-caryophyllene at 4 µg/mL improves wound closure by cell migration and in association with proliferation. In contrast, the oil at 5 µg/mL showed considerably improved wound closure without promoting cell proliferation (Figure 3, Table 2).

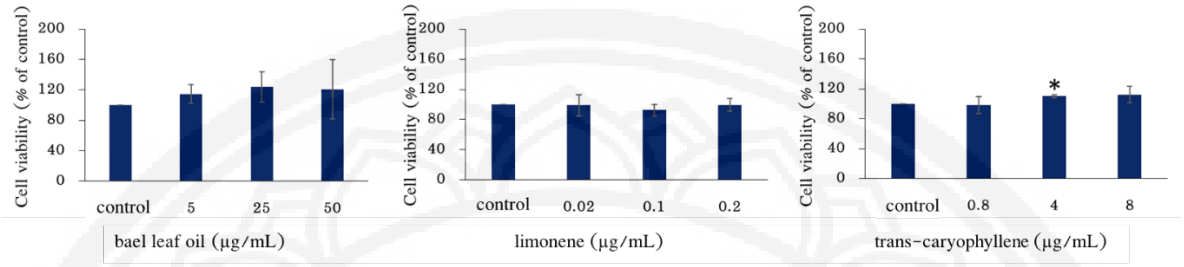

Figure 3 Effect of bael leaf oil, limonene, and trans-caryophyllene on HaCaT cell viability in 2% FBS-DMEM at 24 hours (mean±SD, n = 3, *: p<0.05 vs. vehicle group)

Table 2 Summary of the results for wound closure and cell viability in 2% FBS-DMEM at 24 hours

Sample	Concentration (µg/mL)	% Wound closure	% Cell viability
Bael leaf oil	0	30.81	100
	5	84.51 (↑)	114.78 (↔)
	25	79.05 (↔)	123.95 (↔)
	50	75.17 (↔)	120.76 (↔)
Limonene	0	62.03	100
	0.02	73.29 (↔)	98.92 (↔)
	0.1	76.70 (↔)	92.44 (↔)
	0.2	70.67 (↔)	99.58 (↔)
Trans-caryophyllene	0	43.45	100
	0.8	59.65 (↔)	98.19 (↔)
	4	77.99 (↑)	100.09 (↑)
	8	79.77 (↔)	112.38 (↔)

(↔ = not significantly different compare to control, ↑ = significantly different)

Effect on the expression of AQP3

AQP3 is a membrane protein that is essential for skin hydration. It functions as a channel for water transfer across the plasma membrane. As a result, qPCR was applied to measure the expression of AQP3 following exposure to the oil (5 µg/mL) and trans-caryophyllene (4 µg/mL), which has been proven to significantly improve wound closure. AQP3 was significantly enhanced after exposure to the oil and trans-caryophyllene (figure 4).

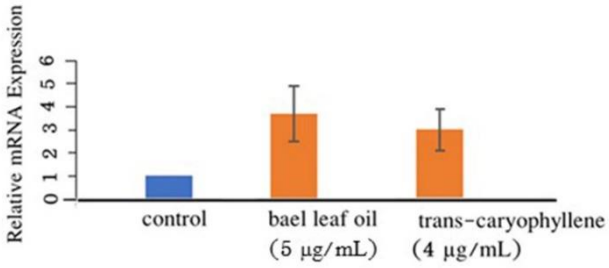

Figure 4 AQP3 expression level in HaCat cells. HaCat cells were treated with either bael leaf oil (5 µg/mL) or trans-caryophyllene (4 µg/mL) for 24 hours, and the mRNA expression level of AQP3 was analyzed by real-time RT-PCR (mean±SD, $n = 3$)

Discussion

In this investigation, wound gaps were produced by scratching or placing a Culture-Insert. These techniques appear to be equivalent ways to make a gap devoid of cells. Yet, a closer examination reveals significant differences between these two approaches that might have an impact on the assays' conclusions. The Culture-Insert approach creates physical cell exclusion by inserting an insert on the culture surface before cell seeding. The cells are intact, and the wound gap is clearly defined. Scratching, on the other hand, may cause damage to the cells and alter the gap size (Jonkman et al., 2014). However, the results of scratching and inserting a Culture-Insert exhibited the same pattern. Therefore, a scratch assay and Culture-Insert were combined as the outcome of the wound healing.

In this work, trans-caryophyllene and limonene concentrations have been established to be equivalent to those observed in bael leaf oil. The wound-healing activity of bael leaf oil has been found to associate with trans-caryophyllene. The findings agree with the previous report that trans-caryophyllene accelerated the growth of new tissues and epithelium in wounds on the skin of animals (Gushiken et al., 2022; Koyama et al., 2019). Trans-caryophyllene is a sesquiterpenoid presented in a variety of plants that produce essential oils. It is used as a flavor enhancer and in cosmetics and has received approval from the Food and Drug Administration (FDA) and the European Food Safety Authority (EFSA) (Francomano et al., 2019).

Limonene, though, has been found to rebalance the wound environment in diabetic mice by modulating various pro-inflammatory and anti-inflammatory cytokines and therefore promote wound healing (Ahmad, Khan, Ansari, & Ahmad, 2014). The results of this study demonstrated that the amount of limonene in bael leaf oil was insufficient to cause wound healing, implying that the wound-healing action of the oils is attributable to trans-caryophyllene rather than limonene.

AQP3 is the most prevalent skin aquaglyceroporin. It is thought that the AQP3 plays a role in the healing of wounds by acting as a water channel promoting cell migration and as a glycerol transporter romoting keratinocyte proliferation and differentiation (Boury-Jamot et al., 2009) The AQP3 gene expression has been demonstrated to exert in HaCat cells, in the migration assay, which revealed that propolis stimulated keratinocytes to heal the wound (Martinotti et al., 2019). Bael leaf oil and trans-caryophyllene have the potential to induce wound closure but had not previously been tested on wound mechanisms. An RT-realtime PCR was, therefore, carried out to better understand the effects of bael leaf oil and trans-caryophyllene on wound healing. The results

indicated that skin aquaglyceroporin appears to support the wound healing function of bael leaf oil and trans-caryophyllene.

Conclusion and Suggestions

This study demonstrated the wound-healing stimulating property of the essential oils from bael leaves, which are associated more with trans-caryophyllene activity than limonene activity. The skin aquaglyceroporin proved to facilitate the healing property. These findings could be a valuable source for an active ingredient that could be employed as a molecule for the manufacture of wound healing medication. The findings also provide scientific evidence supporting the use of bael leaf oil for wound treatment. This would be achievable, however, following extensive *in vivo* therapeutic and toxicological studies.

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