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Short Communication

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The Potency of *Centella asiatica* Leaf Extract on VEGF Expression and Angiogenesis in Second-Degree Burn Wound in Mice

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ABSTRACT

Burn injuries present a significant global health challenge, with the highest incidence rates reported in Southeast Asia, including Indonesia. Healing burn wounds is a complex and dynamic process involving various cellular and molecular mechanisms, prominently featuring the role of Vascular Endothelial Growth Factor (VEGF) in tissue regeneration and wound repair. VEGF is crucial for inducing and regulating angiogenesis and supplying oxygen and nutrients to the healing tissue. This study aims to evaluate the potential of pegagan (*Centella asiatica*) leaf extract cream 1%, 3%, and 5% daily for 14 days in enhancing VEGF expression and angiogenesis in second-degree burn wounds in mice (*Mus musculus*). This study investigates the application of *C. asiatica* extract cream on second-degree burn wounds in mice, comparing its effects on VEGF protein expression and angiogenesis to those of base cream and silver sulfadiazine cream, with outcomes evaluated using immunohistochemistry (IHC) and hematoxylin and eosin (HE) staining methods. Our findings suggest that *C. asiatica* extract cream promotes reduced burn wound size, significant upregulated VEGF expression, and enhanced angiogenesis in treating burn wounds compared to positive control, with a 5% dose having the best result. The study concludes that *C. asiatica* extract cream may effectively treat burn wound healing through enhancing VEGF expression and angiogenesis.

1. Introduction

The healing of burn wounds involves various cellular and molecular mechanisms, with the Vascular Endothelial Growth Factor (VEGF) being a key protein in regulating blood vessel growth. VEGF plays a pivotal role in inducing and regulating angiogenesis, the formation of new blood vessels, which is crucial for supplying oxygen and nutrients to the damaged tissue (Infanger *et al.* 2004). This process accelerates re-epithelialization, as the newly formed blood vessels facilitate the delivery of essential nutrients and oxygen to the injured tissue, thereby promoting more efficient healing (Ngo *et al.* 2014).

Centella asiatica, commonly known as "Pegagan," has a long history in traditional medicine, reputed for its wound-healing and anti-inflammatory properties. This plant contains various bioactive compounds, including triterpenoids and asiaticoside, which have been extensively studied for their potential to stimulate tissue regeneration and reduce inflammatory responses (Sun *et al.* 2020).

Burn injuries represent a significant global health issue, with the World Health Organization (WHO) reporting approximately 180,000 deaths annually, predominantly in low- and middle-income countries, including Indonesia (Riskesdas 2018; Herlianita *et al.* 2021). Due to the significant impact of burn injuries on quality of life, it is beneficial to explore effective treatments utilizing natural resources. This study focuses on the potential of *C. asiatica* extract cream in enhancing VEGF expression and promoting angiogenesis in second-degree burn wounds in mice (*Mus musculus*). Cream is selected as the vehicle in this study due to its ability to be applied topically on a

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large scale, preferable for extensive burn wounds, and can be compared with the control of this study, silver sulfadiazine cream due to the same topical vehicle type (Yasti *et al.* 2015). While several studies have investigated the effects of *C. asiatica* on angiogenesis, this research uniquely examines its efficacy in a topical cream formulation specifically for second-degree burn wounds, using different doses, compared with controls and standard treatment such as Silver Sulfadiazine cream. By exploring the efficacy of *C. asiatica* cream in burn wound healing, this research aims to expand the knowledge on natural treatments for burns and contribute to the development of effective burn management strategies.

2. Materials and Methods

2.1. Sample Preparation

The preparation of *C. asiatica* extract (CAE) used harvested leaves from a local source and dried at room temperature (25-30°C). CAE was processed by PT Borobudur based on Good Manufacturing Practices with CoA (batch number 056PW01.1.). The *C. asiatica* leaves were dried at dryer machine and then extracted using 70% ethanol, then maltodextrin was added (Widowati *et al.* 2023). The *C. asiatica* extract was then used to make creams with concentrations of 1%, 3%, and 5%, each amounting to 50 g, formulated as per Table 1 (Resti *et al.* 2020).

Table 1. *C. asiatica* extract cream formula used for this study

Ingredients	Base cream Formula (g)	CAE cream 1% Formula (g)	CAE cream 3% Formula (g)	CAE cream 5% Formula (g)
C. asiatica extract (PT. Borobudur, 056PW01.1)	-	0.5	1.5	2.5
Carboxylmethyl cellulose sodium (planet kimia)	0.5	0.5	0.5	0.5
Stearic acid (kimia market, 07472206)	5	5	5	5
Paraffin oil (kimia market)	4	4	4	4
White petrolatum (kimia market)	3	3	3	3
Trietanolamin (kimia market)	0.5	0.5	0.5	0.5
Sorbitan monostearate (kimia market)	1	1	1	1
Methylparaben (golden era)	Qs	Qs	Qs	Qs
Distilled water	Ad 50	Ad 50	Ad 50	Ad 50

2.2. Induction of Second-Degree Burn Wound in Mice

The burn wound research protocols were approved by the Maranatha Christian University Ethical Committee, Bandung, Indonesia (023/KEP/III/2024). The study, conducted at Aretha Medika Utama, Biomolecular and Biomedical Research Centre, Bandung, from March 2024 to June 2024, involved the procurement and treatment of mice, preparation of cream, and evaluation of VEGF protein expression and angiogenesis in second-degree burn-induced mice.

A total of 35 healthy mice were randomly selected, with each experimental group including 5 mice and an additional mouse per group to prevent dropout. The mice were provided with clean experimental cage, beddings, standard feed, and water. The inclusion criteria were male mice, normal body weight (20-30 g), aged 2 months before the adaptation period, visually healthy, active, and without anatomical abnormalities. Exclusion criteria included skin abnormalities, significant weight loss (>10%) after the adaptation period, and mortality during the treatment period. The mice were divided into seven groups including I: Negative Control (NC: no burn induction and no treatment), II: Positive Control (PC: burn induction and no treatment), III: Vehicle Control (CC : PC + Base Cream as per table 1), IV: Comparative Control (VC: PC + Silver Sulfadiazine Cream), V: PC + CAE cream 1%, VI: PC + CAE cream 3%, VI I: PC + CAE cream 5%. Treatments are applied daily for 14 days (Liu *et al.* 2023).

2.2.1. Burn Wound Induction Methods

The process starts by preparing the designated area on the animal's back, shaving the fur, and cleaning the skin within a 2 cm^2 of the intended burn site. A protective pad is placed under the animal. After ensuring sterile conditions with gloves and alcohol disinfection, anesthesia is administered using ketamine-xylazine, intraperitoneally for sedation. An iron bar was heated in heating plate, and then gently applied to the shaved back skin of mice for 5 seconds without pressure causing 2^{nd} degree burn injury. The area is then compressed with a saline solution for 1 minute to prevent the burn from spreading. Standard wound care protocols are followed afterward to monitor and manage the burn wound effectively (Mahboub *et al.* 2022).

2.2.2. Post-Induction Treatment Protocol

After induction of second-degree burn wounds, each mice was treated according to their groups. The burn wound size reduction of each mice was observed at day 3, 6, 9, 12, and 15. Then, after the 14-day treatment period, all mice were euthanized by cervical dislocation, and tissue samples from the burn wounds were collected for histological examination to evaluate the expression of VEGF and angiogenesis. This examination aimed to assess the effects of each treatment on wound healing processes at a cellular and molecular level.

2.3. Histological Preparation

Histopathological examination was performed using Hematoxylin and Eosin (HE) staining. Skin samples were placed in a formalin solution and fixed with 10% formalin for 2-3 days. This was followed by dehydration with a series of increasing alcohol concentrations for 2 hours, and then cleaned with graded xylol while being continuously shaken. The samples were then embedded in liquid paraffin in stages, reaching a 100% paraffin concentration at 60°C. The samples were stored until paraffin blocks formed at room temperature. The paraffin blocks were sliced using a microtome (Leica RM 2135 BioCut Rotary Microtome) to a thickness of 5 µm. The tissue sections were mounted on glass slides and stained with HE. The slides were then analyzed with a light microscope and ImageJ software to quantify inflammation scores and collagen density (Widowati *et al.* 2024).

2.4. Angiogenesis Measurement Based on Blood Vessels Count

To assess angiogenesis, new blood vessels were counted in each wound tissue sample across 5 high-power fields (400X magnification). The average number of blood vessels from these fields was calculated (Kusmayani *et al.* 2022).

2.5. VEGF Expression Measured Using Immunohistochemistry

The Immunohistochemistry (IHC) method was used to detect antigen-antibody binding and observe target protein expression in treated tissue cells. This technique involves immunological reactions (antigen-antibody interactions) and chemical reactions (enzyme-substrate interactions). Antibodies were conjugated with peroxidase enzymes to visualize the antigen-antibody complexes. The IHC procedure included sectioning, deparaffinization, dehydration, antigen retrieval, blocking steps, incubation with primary antibodies (Anti-VEGF, Elabscience, E-AB-81493) and secondary antibodies (Polyperoxidase-anti-Mouse/Rabbit IgG, Elabscience, E-IR-R217B), application of DAB chromogen, counterstaining with HE, dehydration, clearing, and slide mounting (Widowati *et al.* 2022).

2.6. Statistical Analysis

Data analysis was conducted using SPSS. Levene's and Shapiro-Wilk tests assessed data homogeneity and distribution. Normally distributed data were analyzed with One-Way ANOVA, followed by post hoc tests if significant differences ($p<0.05$) were found. For non-normally distributed data, the Kruskal-Wallis test was used, followed by Mann-Whitney test.

3. Results

3.1. Burn Wound Size Reduction

Burn wound size reduction are defined as percentage of wound reduction compared to wound's original size. CAE cream (1, 3, 5%) were also showed reductions the burn injury significantly ($p<0.05$), CAE 5% was the most effective compared to CAE cream 1, 3%. CAE 5% was comparable with SS cream (Table 2). Statistical analysis confirm that these reductions are significant, indicating the effectiveness of the treatments applied.

3.2. Angiogenesis

Group I (Negative Control or NC) group showed the lowest angiogenesis score at 2.80±0.75, indicating minimal blood vessel formation. Group II (Positive Control or PC) and Group III (Vehicle Control or VC) had similar angiogenesis scores at 7.08±1.06 and 7.36±1.03, respectively, suggesting moderate blood vessel formation. Group IV (Comparative Control or CC) exhibited significantly higher angiogenesis at 14.12±2.06 compared to the other groups, indicating substantial blood vessel formation. Group V, Group VI, and Group VII showed increasing angiogenesis scores of 9.76±1.42, 11.88±1.53, and 13.20±1.42, respectively, suggesting a dose-dependent increase in blood vessel formation with higher concentrations of CAE cream (Figure 1).

These results indicate that treatment with CAE cream at different concentrations (1%, 3%, and 5%) led to increased angiogenesis compared to NC significantly ($p<0.05$), with higher concentrations showing greater efficacy in promoting blood vessel formation in the burn wound model.

Table 2. Effect of CAE cream toward burn wound reduction

Group	Days of observation				
	Day 3	Day 6	Day 9	Day 12	Day 15
I	100.00±0.00^{dA}	100.00±0.00^{eA}	100.00±0.00^{dA}	100.00±0.00^{dA}	100.00±0.00^{dA}
II	1.60±1.95^{aA}	8.90±3.52^{aB}	24.90±2.95^{aC}	38.90±2.53^{bD}	47.10±2.77^{aE}
III	2.20±2.02^{aA}	5.20±4.31^{aA}	18.90±8.71^{aB}	37.40±3.19^{bC}	49.10±4.92^{aD}
IV	14.19±5.76^{cA}	24.30±3.33^{cB}	40.90±7.24^{cC}	53.30±4.92^{cD}	74.10±3.25^{cE}
V	6.00±2.15^{bA}	15.47±1.95^{bB}	21.40±1.74^{aC}	30.67±3.97^{aD}	60.90±1.13^{bE}
VI	8.30±1.82^{bA}	25.30±3.33^{cB}	32.10±1.47^{bC}	42.00±2.55^{bD}	63.60±2.72^{bE}
VII	14.30±3.21^{cA}	34.60±2.53^{dB}	41.20±1.79^{cC}	51.30±2.31^{cD}	71.60±2.70^{cE}

*Groups were designated as follows: I: negative control (normal rats), II: positive control, III: vehicle control (cream base), IV: comparative control (silver sulfadiazine cream), V: CAE cream 1%, VI: CAE cream 3%, VII: CAE cream 5%. Data are presented as mean ± standard deviation. Lowercase superscript letters within the same column (a, b, c, d, and e) indicate significant differences between treatments on the same day. Uppercase superscript letters within the same row (A, B, C, D, E) indicate significant differences between observation days within the same treatment group

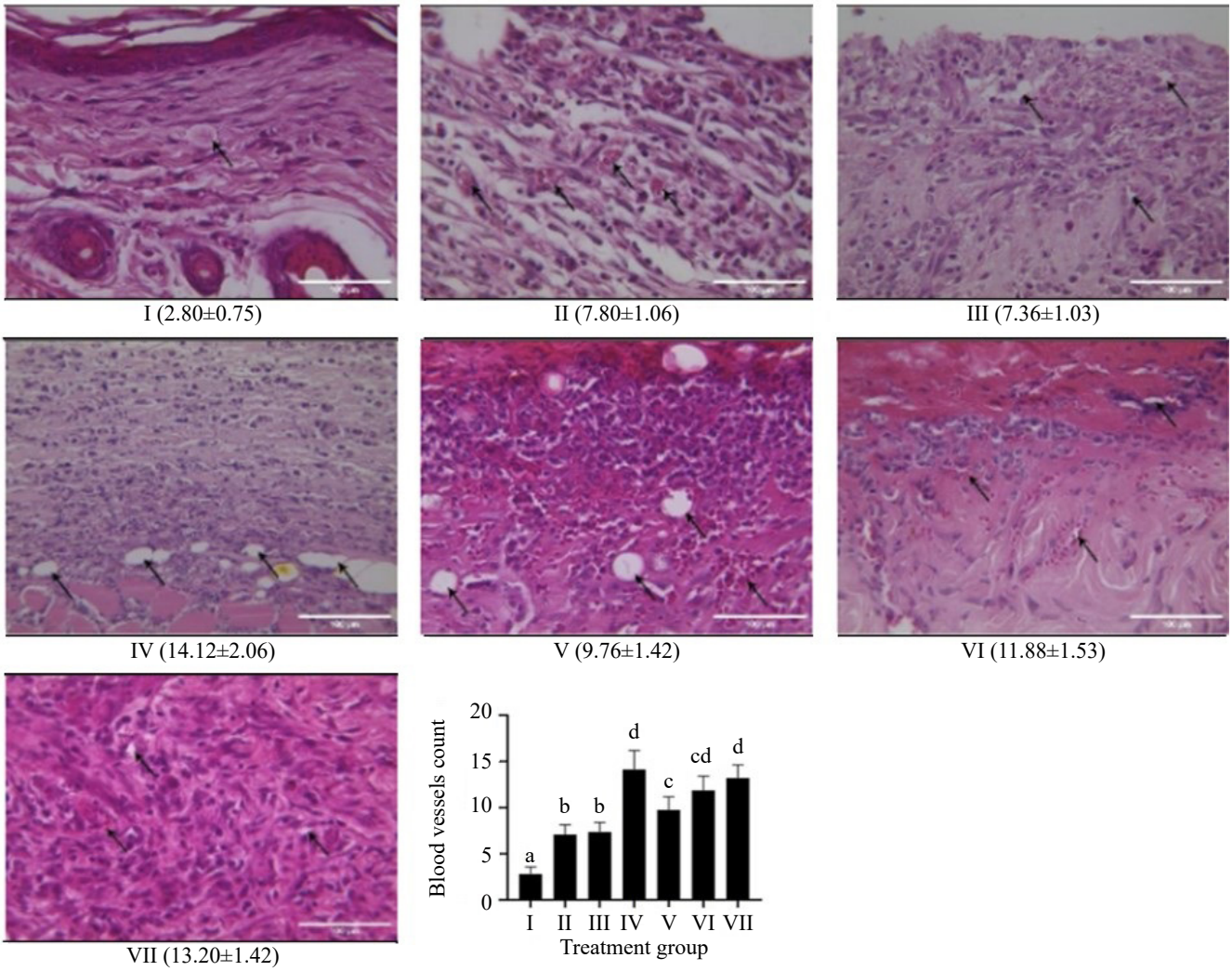

Figure 1. Effect of CAE cream toward angiogenesis in burn wound mice. Angiogenesis was observed at 400x magnification with a 100 µm scale bar, black arrow sign was angiogenesis. The groups were as follows: I: negative control (normal rats), II: positive control, III: vehicle control (cream base), IV: comparative control (silver sulfadiazine cream), V: CAE cream 1%, VI: CAE cream 3%, VII: CAE cream 5%. Data presented as mean ± standard deviation. Different superscript letters (a, b, c, cd and d) indicate significant differences between treatments

3.3. VEGF Expression

VEGF expression is indicated by the appearance of brown coloration in the tissue samples, which is a positive indicator of angiogenesis activity and healing processes. Based on the research findings, it can be concluded that the use of CAE cream at various concentrations (1%, 3%, and 5%) enhanced VEGF expression significantly ($p<0.05$) during the burn wound healing process in mice (Figure 2).

This result suggests that CAE cream, at concentrations of 1%, 3%, and 5%, had the potential to significantly enhance VEGF expression ($p<0.05$) compared to PC, and VC in burn wound model. These results support the use of *C. asiatica* as a wound healing agent by enhancing angiogenesis through stimulation of VEGF expression.

4. Discussion

C. asiatica is rich in bioactive components such as triterpenoid saponins, triterpenoid genins, essential oils, flavonoids, and phytosterols, with asiatic

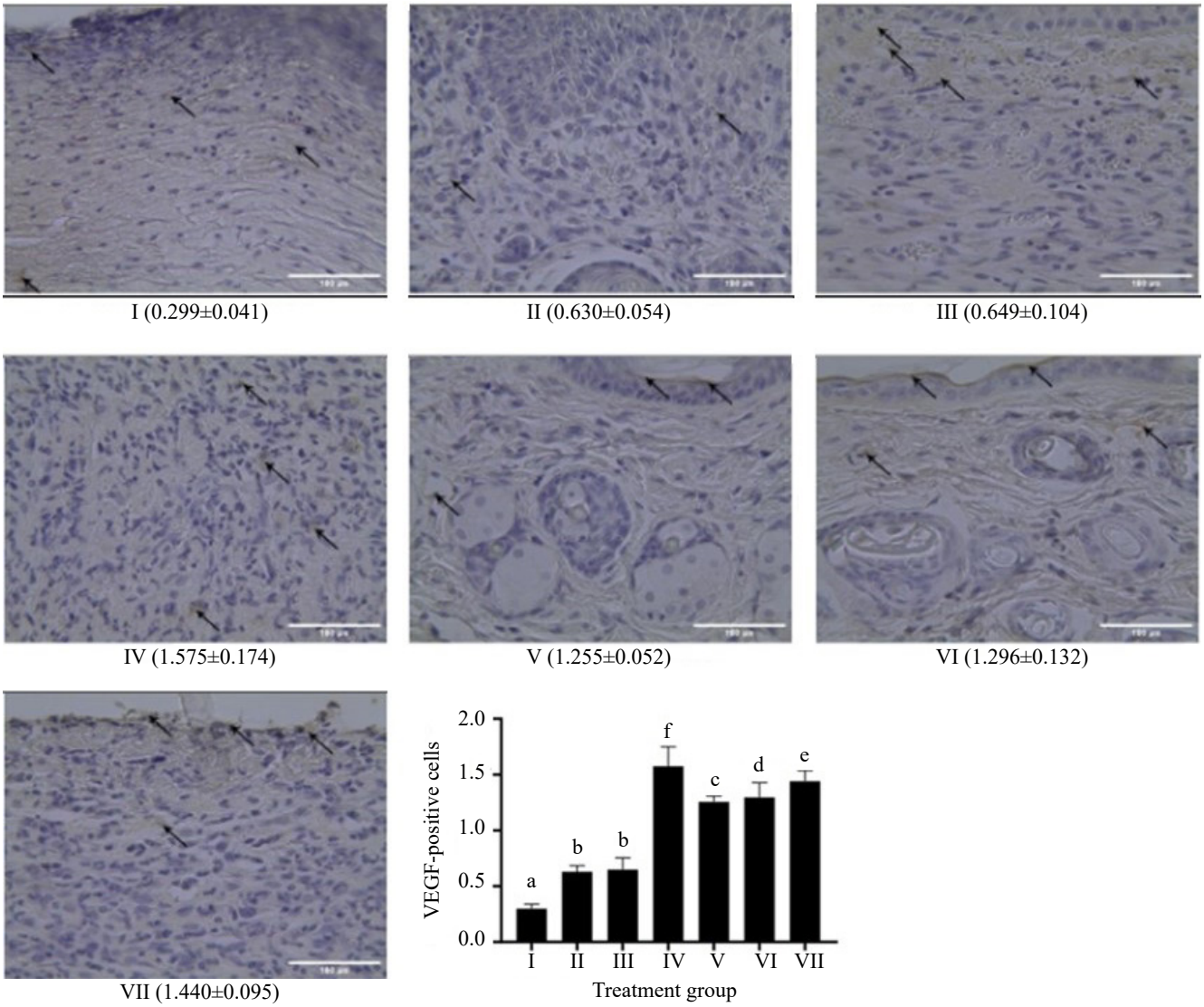

Figure 2. Effect CAE cream toward VEGF expression in burn wound mice. I: negative control (normal rats), II: positive control, III: vehicle control (cream base), IV: comparative control (silver sulfadiazine cream), V: CAE cream 1%, VI: CAE cream 3%, VII: CAE cream 5%. VEGF-positive cells count (black arrows). Data presented as mean ± standard deviation. Different superscript letters (a, b, ab, c, and d) indicate significant differences between treatments

acid being a primary metabolite. Previous studies, including Diniz *et al.* (2023), have demonstrated that *C. asiatica* extract, particularly asiatic acid, accelerates wound healing by enhancing collagen synthesis, cell proliferation, fibroblast proliferation, and epithelial cell re-epithelialization during the proliferative and remodelling phases of wound repair. Additionally, Asiatic acid modulates the inflammatory phase by inhibiting immune cell recruitment, reducing the synthesis of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β), and growth factors (TGF-β, PDGF, VEGF). It also inhibits the increase in serum IL-17/IL-23 levels, contributing to its anti-inflammatory effects (Diniz *et al.* 2023).

The present study demonstrated that the application of *C. asiatica* extract cream significantly enhances the reduction of burn wound size. The findings revealed that CAE 5% was comparable with the Silver Sulfadiazine cream on day 15. However, the group treated with 5% *C. asiatica* cream also exhibited remarkable wound size reduction. The significant reduction in wound size observed with CAE cream can be attributed to its rich composition of bioactive compounds, such as triterpenoid saponins, flavonoids, and phytosterols. These components are known to enhance collagen synthesis, promote fibroblast proliferation, and facilitate re-epithelialization, all crucial processes in wound healing (Diniz *et al.* 2023). The dose-dependent efficacy is observed in this research, where the higher the dose, the higher the efficacy (1% vs 3% vs 5%), bioactive compounds are effective in promoting tissue repair and regeneration. This could mean that if more different doses are studied, the results may reveal the peak effectiveness and possibly pinpoint a more effective dose for burn wound treatment. Comparing the results across different treatment groups, it is evident that both the 3% and 5% CAE cream significantly outperformed the Vehicle Control (cream base) and Positive Control (untreated burn). The statistical analysis confirmed that these reductions were substantial and significant, underscoring the therapeutic potential of *C. asiatica* in burn wound management. (Arribas-Lopez *et al.* 2022).

According to a systematic review by Arribas-Lopez *et al. C. asiatica* accelerates wound healing through increased angiogenesis, possibly mediated by the stimulation of collagen I, Fibroblast Growth Factor (FGF), and VEGF production. This occurs through the activation of the TβR2 kinase-independent pathway. During early angiogenesis, FGF enhances endothelial cell proliferation, while VEGF contributes to new capillary formation by regulating endothelial cell proliferation, differentiation, and migration. VEGF also stimulates vasodilation and extracellular matrix formation (Arribas-Lopez *et al.* 2022). These findings are consistent with our study, where CAE cream at various concentrations (1%, 3%, and 5%) enhanced VEGF expression and angiogenesis during mice's burn wound healing process. The 5% dose of CAE cream is considered the best result compared to 1% and 3% because of more effective burn wound size reduction and the best VEGF and angiogenesis count, contributing to the proliferative phase of burn wound healing.

Further research is necessary to explore additional mechanisms through which *C. asiatica* enhances wound healing, optimize its formulation and concentration, and clarify its comparative efficacy with standard treatments. Understanding these aspects will facilitate the development of effective *C. asiatica*-based therapies for improving clinical outcomes in burn wound care.

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