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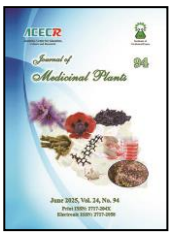

Research Article

Wound healing promoting activities of *Dimetia capitellata* (Wall. ex G.Don) Neupane & N.Wikstr. leaves

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ABSTRACT

Background: The leaves of *Dimetia capitellata* (Wall. ex G.Don) Neupane & N.Wikstr. have been used as a traditional medicine to treat skin wounds in some human communities. **Objective:** This study aimed to evaluate the wound healing activities of the 70% ethanolic extract of this plant's leaves (DcEE) and its ethyl acetate (DcEAF), petroleum ether (DcPEF), and water (DcWF) fractions. **Methods:** The nitric oxide levels in LPS-activated RAW 26.7 cells were measured by Griss assay. The proliferation and migration of cells were assessed by MTT and scratchings methods, respectively. In an in vivo assay, skin wounds were created in mice and then daily treated with DcEE, and the wound contraction percentages were determined every day. The mRNA levels of inflammation-related genes were evaluated using qRT-PCR. **Results:** We found that the DcEE contained 74.7 ± 2.9 mgGAE/gE polyphenols and 66.5 ± 2.8 mgQE/gE flavonoids, and showed a weak antioxidant activity. The DcEE treatment also reduced the *in vitro* nitric oxide level released from LPS-stimulated RAW 26.7 macrophages by 89.8±8.2% and decreased the mRNA levels of inflammation-related genes *NF-κB1* and *TNF-α* in wound tissues of the examined mice, suggesting its anti-inflammatory activity. Besides, DcEE stimulated fibroblast and keratinocyte proliferation and induced keratinocyte migration. Importantly, in the *in vivo* assay, the wound closure time in DcEE-treated mice was reduced approximately 2 days in comparison with that in the control mice. **Conclusions:** These findings provided some evidence to prove the traditional utilization of *D. capitellata* leaves in skin wound treatment.

1. Introduction

Skin is the largest organ of our bodies that plays as a mutifuntional barrier to protect the inside of the body from the environment and thus, an injury in the skin may promote the infection of pathogens and seriously affect the human health. Therefore, although skin wounds can naturally heal by themselves, many efforts have been made to promote the injury recovery. Among them,

Abbreviations: qRT-PCR, quantitative reverse transcription PCR; LPS, lipopolysaccharides; TPC, total (poly)phenol content; TFC, total flavonoid content; DPPH, 2,2-diphenyl-1-picrylhydrazyl; RSA, radical scavenging activity; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-Diphenyltetrazolium Bromide; NO, nitric oxide

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herbal plants have been widely used as wound healing agents by many people in the world for ages. As an example, the plant *Achillea millefolium* has a long history of being used as a medicine for skin wound treatment since the Trojan War when the Greek hero Achilles used it to treat the wounds of his soldiers [1]. The longstanding use of herbal plants, as in case of *A. millefolium*, makes them acceptable and trusted by some communities in the world. However, most of traditional herbal medicines are relied on belief rather than scientific evidence, leading to controversy in using them. Therefore, a lot of studies have been carried out to understand the action mechanisms as well as to prove the efficacy and safety of traditional medicines, in order to integrate them into the mainstream healthcare.

Dimetia capitellata (Wall. ex G.Don) Neupane & N.Wikstr. is a herbal plant which is widely used to treat wounds, inflammation, and skin ulcers by some ethnic minorities in Vietnam, as well as to treat fractures, wounds and snake-bites in some other countries [2, 3]. This plant was formerly classified as *Hedyotis capitellata var. mollis* (Pierre ex Pit.) T.N. Ninh but reclassified as *Dimetia capitellata* (Wall. ex G.Don) Neupane & N.Wikstr. in 2015. The later name has been accepted since 2024 [4]. Accordingly, *D. capitellata* has been previously reported to possess antibacterial and anti-inflammatory activities [3, 5], and contain β-Carboline alkaloids and anthraquinones functioning as anti-inflammatory agents [6, 7]. Though these findings suggested the potentiality of this plant in wound healing, the action mechanisms remain unclear. Therefore, this study was performed with the aim to examine the effects and mechanisms of *D. capitellata* in promoting wound recovery, with the hope of providing some scientific evidence for the ethnomedicinal use of this plant in treating injury.

2. Materials and methods

2.1. Extract preparation

Dimetia capitellata (Wall. ex G. Don) Neupane & N.Wikstr. leaves were collected in months of July to August from Bidoup – Nui Ba National Park, Lam Dong province, Vietnam. The whole plants were also collected for the scientific name identification, which was performed at the Laboratory of Plants, VNUHCM-University of Science, Vietnam (voucher specimen: PHH0004894).

The leaf extract was prepared using the method reported previously [8]. The leaves were washed and dried at temperature below 40˚C to a moisture content of below 10%, then ground into fine powder. The leaf powder was soaked in 70% ethanol (1:10, w/v) for 24 hours (three times) and then filtered through Whatman No. 1 filter paper to prepare the crude ethanolic extract (DcEE). A portion of EcEE was evaporated using a rotary evaporater to 10% of the initial volume and then the same volume of water was added to prepare the condensed crude extract. The condensed crude extract was partitioned with an equal volume of petroleum ether (four times) to get aqueous fraction and petroleum ether fraction (DcPEF). The aqueous fraction was further partitioned with an equal volume of ethyl acetate (four times) to yield fractions of ethyl acetate (DcEAF) and water residues (DcWF). All extracts including DcEE, DcPEF, DcEAF, and DcWF were finally evaporated to stable masses and dissolved in DMSO for further experiments.

2.2. Total (poly) phenolic content determination

The total (poly)phenolic content (TPC) was measured using Folin - Ciocalteu assay, following the method described by Do et al., with minor modifications [9]. Briefly, 80 µL sample was mixed with 800 µL of 0.2 N Foline-

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Ciocalteu reagent (Sigma) in a glass tube. After that, 800 µl of 7.5% Na_2CO_3 solution and 320 µl distilled water were added. Then, the reaction was mixed thoroughly and incubated for 30 minutes in dark and the absorbance at wavelength 760 nm was measured. TPC of the extract was determined using a gallic acid standard curve (25-400 µg/ml) and reported as gallic acid equivalent per gram dry extract (mg GAE/gE) or per gram dried leaf weight (mg GAE/gdw).

2.3. Total flavonoids content determination

The total flavonoid content (TFC) was measured following the method previously described by Do et al. with minor modification [9]. Briefly, 200 µl sample was mixed well with 120 µl of 5% $NaNO_2$ solution in a glass tube by vortexing for 3 minutes. Then 800 µl of 1 M NaOH solution, 120 µl of 10% $AlCl_3$ solution, and 760 µl distilled water were added and the absorbance at 415 nm wavelength was read. TPC of the extract was determined using a quercetin standard curve (31.25-1000 µg/ml) and reported as quercetin equivalent per gram dry extract (mg QE/gE) or per gram dried leaf weight (mg QE/gdw).

2.4. Antioxidant activity evaluation using DPPH scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity of DcEE was measured according to Yakaew et al. [10] with modifications. Briefly, 2 ml of 0.2 mM DPPH reagent (Sigma, USA) was mixed with 0.5 ml extract at tested concentrations (12.5-200 µg/ml extract in 70% ethanol solvent). The mixture was kept for 30 minutes at room temperature and the result was read at 517 nm wavelength. The radical scavenging activity percentage (%RSA) was determined using the formula: $\%RSA = (1-A_S/A_B)x100$, where A_S refers to the absorbance of the sample being tested, and A_B refers to the absorbance of the blank solution, which consists of 500 µl of 70% ethanol mixed with 2000 µl of DPPH solution. The antioxidant activity of DcEE was measured using a galic acid standard curve (50-200 µg/ml) and shown as gallic acid equivalent antioxidant capacity per gram extract (GAEAC/gE). The IC_{50} value was determined by log-probit analysis of the extract at different concentrations.

2.5. Griess assay for nitric oxide inhibitory activity

Griess assay was used to measure nitric oxide (NO) concentration as reported by Ahmad et al. [3]. Accordingly, 10^4 RAW 264.7 mouse macrophage cells (ATCC TIB-71) in 100 µl of DMEM-F12 medium containing 10% fetal bovine serum (FBS) were added into each well of 96-well plates and then cultured at 37˚C with 5% CO_2. After 12 hours of cultivation, the medium was substituted by 100 µl extract, which had been serially diluted in fresh medium. Cells were then stimulated by 0.5 µg/ml lipopolysaccharides (LPS) for 24 hours. After that, 50 µl of the culture medium was taken out and 50 µl of Griess reagent (0.1% N-(1-naphtyl) ethylenediamine dihydrochloride and 1% sulfonamide in 2.5% H_3PO_4) was provided into wells, followed by spectrophotometric measurement at 550 nm. Dexamethasone (50 µg/ml) was added instead of the extract into the positive control well.

2.6. Cell proliferation assay

One hundred milliliters of HaCaT (Fisher Scientific) or NIH/3T3 (ATCC CRL-1658) cell culture at the density of 10^5 cells/ml was dispensed into individual well of a 96-well microplate and the plate was kept at 37˚C with

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5% CO_2. After 24 hours of incubation, the medium was removed and 100 µl of the extract diluted in DMEM-F12 medium containing 1% FBS was added. Cells continued being cultured for 48 hours at 37˚C with 5% CO_2. Subsequently, each well received 10 µl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) (Sigma, USA) solution. After 3 hours of incubation, 100 µl of 0.1 N HCl in isopropanol was added into wells following the medium removal. The cell density was determined through absorbance at 550 nm.

2.7. Migration assay

A volume of 500 µl of human keratinocytes HaCaT culture at the density of 10^4 cells/ml was seeded into each well of a 24-well plate, which was then incubated at 37˚C with 5% (v/v) CO_2 for 24 hours. Scratches were created using a 200 µl pipet tip, followed by the replacement of the culture medium with 500 µl of DcEE diluted in fresh DMEM-F12 medium containing 1% FBS. Cells cultured in DMEM-F12 medium containing 10% FBS and in DMEM-F12 medium containing 1% FBS and 0.25% DMSO were used as the positive and negative controls, respectively. Cells were observed under an inverted microscope. Three different areas in the scratch were pictured and marked to monitor the cell migration. After incubated at 37˚C with 5% CO_2 for 18 hours, cells were fixed with 4% paraformaldehyde and dyed with crystal violet. The marked areas were pictured again. ImageJ software was employed for image analysis.

2.8. Experimental animals

Healthy Swiss albino mice (18-20 g) were provided by Pasteur institute in Ho Chi Minh city, Vietnam. Mice were housed at room temperature in separate cages and fed ad libitum for two weeks until they reached 25 ± 3 g. The mice were then allocated to four groups of six mice. Group I were treated with solvent (35% ethanol) and considered as a negative control. Groups II, III and IV were treated with DcEE at the doses of 50, 25, and 10 mg/kg/day, respectively. To create wounds, mice were anaesthetized by intraperitoneal injection of 15 mg/kg Zoletyl 50 and 5 mg/kg Xylazine. The dorsal surface of mice was depilated and disinfected with 70% ethanol. After that, a 10 mm-diameter circular area was cropped inside the shaved region using a biopsy punch and the skin was removed. The DcEE was applied to the wound every day. Wound contraction percentage was daily evaluated. All procedures were conducted with the utmost care to reduce animal suffering, and the experiment received ethical approval from the HCMUS Animal Ethics Committee on December 19, 2023 (Approval No. 1256/KHTN-ACUCUS).

2.9. RNA extraction and quantitative reverse transcriptase-PCR

On the forth day following surgery and treatment, wound tissue samples (30 mg) were collected for the total RNA isolation using TRIzol reagent (Invitrogen, USA). After that, a 20 µl reverse transcription reaction containing 1 µg of total RNA was performed using the PrimeScript RT reagent kit (Takara, Japan), followed by the realtime-PCR with primer sets for reference gene *GADPH* and inflammation-related genes TNF-α and NF-κB1, which were described previously [11]. Gene expression fold changes were determined using the $2^{-\Delta\Delta CT}$ method.

2.10. Statistical analysis of the data

Data were shown as mean ± SD of at least three independent experiments. Statistically analysis was performed using one-way analysis of variance (ANOVA) by GraphPad Prism 8 software.

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3. Results

3.1. The TPC, TFC and radical scavenging activity of DcEE

Since some (poly)phenolic and flavonoid compounds were reported to have wound healing activities [12, 13], we first checked the contents of these compounds in DcEE. We found that DcEE contained 74.7 ± 2.9 mgGAE/gE (10.2 ± 0.3 mgGAE/gdw) polyphenols and 66.5 ± 2.8 mgQE/gE flavonoids (~9.1 ± 0.3 mgQE/gdw). In addition, we also analyzed the antioxidant activity of DcEE. The result from DPPH assay showed that this extract exhibited the radical scavenging activity with IC_{50} of 204.5 ± 3.7 µg/ml, while that of acid gallic was 2.9 ± 0.1 µg/ml (Table 1).

Table 1. DPPH-scavenging activity of *D. capitellata* ethanol extract

Unit	Activity	
	DcEE	**Galic acid**
IC_{50} (µg/ml)	204.5 ± 3.7	2.9 ± 0.1
mg GAEAC/gE	14.3 ± 0.3	-
mg GAEAC/gdw	4.7 ± 0.1	-

3.2. In vitro anti-inflammatory activities of DcEE and its fractions

Inflammation is the second phase of wound healing process, occurs within 24 hours after injury and usually lasts for 2-5 days. In this phase, the neutrophils, monocytes, and macrophages are attracted to the wound area to eliminate infections and debris from the wound, while also releasing cytokines and growth factors, which are important for the next phase in healing. However, excessive or prolonged inflammation and high level of cytokines can delay the healing process or lead to chronic wounds [14]. Since a reduction of NO is considered as an important signal of inflammatory inhibition, the reduction of NO production in LPS-induced RAW 264.7 macrophage was utilized as an *in vitro* assay to evaluate anti-inflammatory activity in previous studies. We here found that the NO level was remarkably reduced in DcEE or DcPEF-treated cells. DcEE at the concentration of 62.5 µg/ml reduced NO level by 89.8±8.2%, while DcPEF (20 µg/ml) reduced NO level by 76.2 ± 6.2% (Table 2). The other fractions DcEAF and DcWF showed little or no effect on NO level.

Table 2. Reduced NO production from LPS-stimuated RAW 264.7 cells upon *D. capitellata* extract treatments (n = 3)

Extract	Concentration (µg/ml)	Percentage of NO inhibition (%)
Ethanol	62.5	89.8 ± 8.2
	15.625	57.1 ± 3.9
Petroleum ether	20	76.2 ± 6.2
	5	29.9 ± 3.0
Ethyl acetate	20	2.7 ± 1.4
	5	9.0 ± 5.5
Water	62.5	8.16 ± 3.7
	15.625	5.31 ± 1.5
Dexamethasone	50	83.33 ± 7.2

3.3. DcEE and its fractions induced fibroblast and keratinocyte proliferation

The proliferation of fibroblasts and keratinocytes plays a crucial role in injury recovery. Fibroblasts contribute to wound closure by producing components of connective tissue and secreting some growth factors while keratinocytes restore epithelial function and secret cytokines to support other cells [15, 16]. We here demonstrated that 62.5 µg/ml DcEE

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slightly stimulated the proliferation of HaCaT keratynocytes and NIH/3T3 fibroblasts (Figures 1A, E). Besides, DcEE at lower concentrations (15.625 µg/ml and 31.25 µg/ml) also showed positive effects on NIH/3T3 proliferation (Figure 1E). Regarding extract fractions, DcEAF (15.625 µg/ml) and DcPEF (7.8175 µg/ml) significantly enhanced the proliferation of HaCaT and NIH/3T3 since the cell densities in these samples were approximately 1.5 to 2 times higher than those in the control sample (Figures 1B, C, F, G). In contrast, DcWF had no effect on the proliferation of these two cell lines (Figures 1D, H).

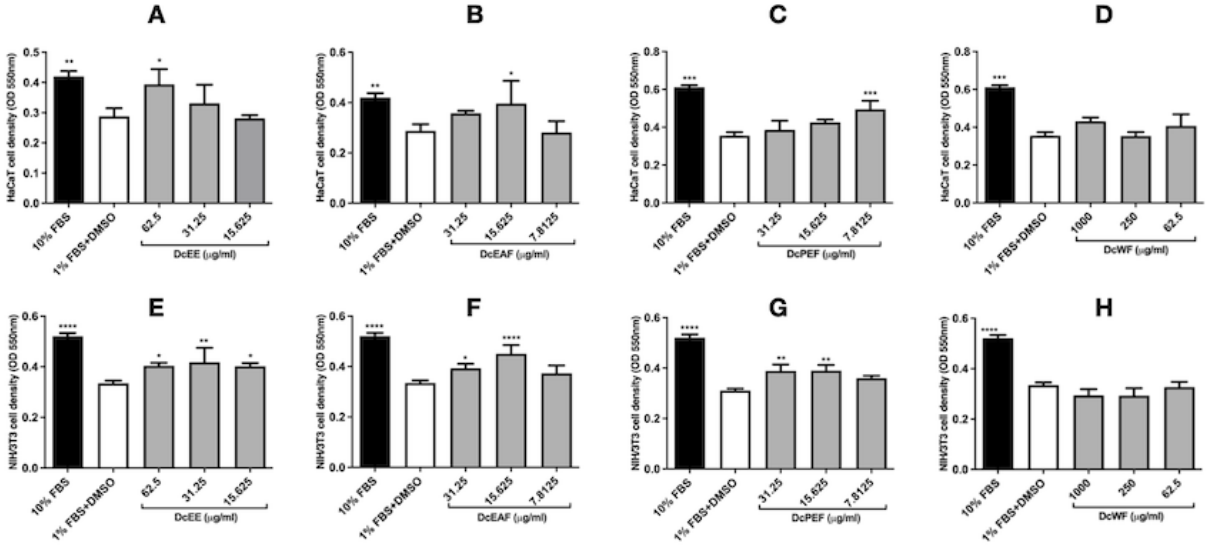

Fig. 1. The effect of *D. capitellata* extract on HaCaT keratinocytes (A-D) and NIH/3T3 fibroblasts (E-H) proliferation. Cells were treated with DcEE (A, E), DcEAF (B, F), DcPEF (C, G), DcWF (D, H) at different concentrations, or grown in DMEM medium containing 1% FBS and 0.25% DMSO (negative control) or in DMEM medium containing10% FBS (possitive control). The cell densities in the extract-treated samples were compared to that of the negative control (*P ≤ 0.05; ** P ≤ 0.01, *** P ≤ 0.001, **** P < 0.0001).

3.4. DcEE induced keratinocyte HaCaT migration

To examine the ability of *D. capitellata* leaves in inducing keratinocyte migration, we treated HaCaT cells with DcEE or its fractions for 24 hours. We found that DcEE significantly stimulated HaCaT migration since the gap closure rate was doubled by the treatment with 15.625 µg/ml DcEE (Figure 2). However, the differences between the effects of the extract fractions DcAEF, DcPEF, and DcWF on keratinocyte migration and that on the control were not statistical (data not shown).

3.5. DcEE accelerated wound closure process and reduced the expression of inflammation-related genes in mice

Wound areas of different mice groups were examined after being treated with DcEE. The daily treated mice with DcEE enhanced wound closure significantly. After 13 days, wounds on mice treated with 50 mg/kg/day DcEE recovered 99.19 ± 0.56% while those on control mice recovered 61.06 ± 3.62%. And importantly, the median time to complete wound closure in these treated mice was approximate 2 days shorter than that in the control mice (Table 3).

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In addition, we also evaluated the impact of DcEE on the transcriptional expression of two inflammation-related genes *TNF-α* and *NF-κB1*, which were previously reported to be elevated during inflammation [17-20]. We here found that the treatments of DcEE at all tested doses significantly reduced the *TNF-α* and *NF-κB1* mRNA levels in the wound sites (Figure 3), suggesting the *in vivo* anti-inflammation activity of this extract.

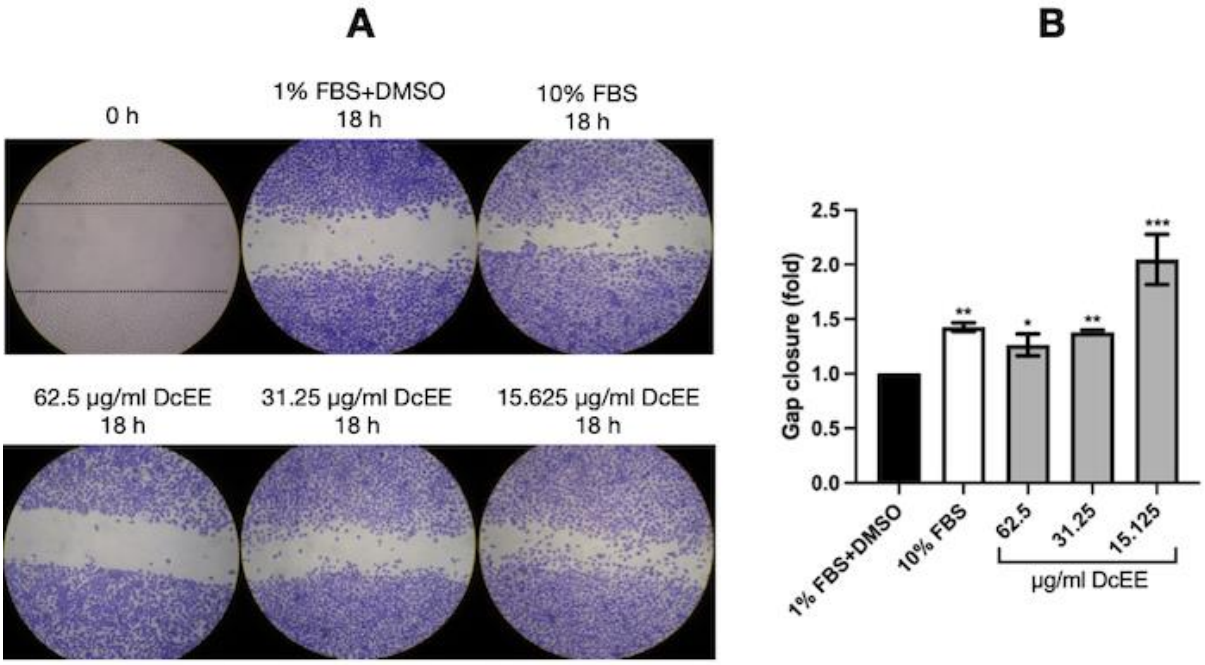

Fig. 2. The effect of DcEE on keratinocytes HaCaT migration. Cells were exposed to DcEE at different concentrations or 0.25% DMSO (negative control) in DMEM medium supplemented with 1% FBS. Representive images of cell migration (A) and gap closure (B) after 18-hour treatment with DcEE were shown. The closure rates of the extract-treated samples were compared to that of the negative control (* $P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.001$).

Table 3. Effect of DcEE on percentage of wound contraction (%) and wound closure time (day)

Treatment	Percentage of wound contraction (%)			Time to complete wound closure (days)
	Day 7	Day 10	Day 13	
Ethanol 35%	22.18 ± 4.14	41.03 ± 2.13	61.06 ± 3.62	16.06 ± 0.5
DcEE (100mg/kg/day)	29.57 ± 3.04	64.75 ± 2.59	97.84 ± 0.37*	13.8 ± 0.7
DcEE (50mg/kg/day)	32.24 ± 3.87	68.15 ± 4.37	99.19 ± 0.56*	13.15 ± 0.4*
DcEE (25mg/kg/day)	32.19 ± 3.92	63.34 ± 3.55	97.12 ± 1.33*	14.1 ± 0.6
DcEE (10mg/kg/day)	20.50 ± 5.49	45.18 ± 4.73	70.51 ± 9.16	15.1 ± 1.2

(*) Statistical difference (p-value ≤ 0.05)

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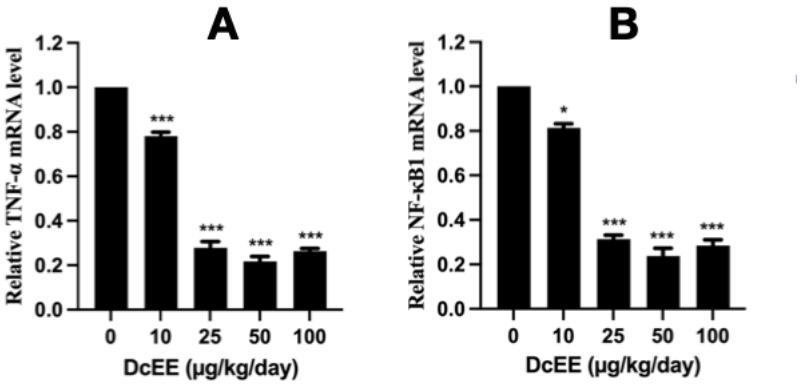

Fig. 3. DcEE reduced the TNF-α (A) and NFκB-1 (B) mRNA levels in treated wounds. For comparison of mRNA expression levels, each gene's mRNA level was normalized against that of *GADPH*. The mRNA level in 35% ethanol solvent treated sample (0 µg/kg/day) was considered as 1. The differences between the mRNA levels in DcEE-treated wounds and that in the non treated-wounds were analyzed by two-tailed unpaired Student's t-tests (*P ≤ 0.05; ***P ≤ 0.001).

4. Discussion

Skin wound is a common injury that we frequently experience during our lifetimes. Most wounds are minor and can naturally heal by themselves. But some wounds cannot heal properly on their own and thus, they need to be treated with some medicine to promote the healing process. *D. capitellata* (Wall. ex G.Don) Neupane & N.Wikstr. has been traditionally used for a long time as a wound healing agent in some communities. We here reported several properties of *D. capitellata* leaves which can provide some evidence for the use of this plant in treating skin injury.

Firstly, we found that *D. capitellata* leaves contained significant amounts of polyphenol and flavonoid compounds, in accordance with previous study of Nhung et al. [21]. These compounds were reported to have wound healing activity due to their antioxidant, anti-inflammatory, proliferation, angiogenesis and re-epithelialization effects [22-25]. Besides, since the excess generation of free radical at wound site might cause protein, DNA, and tissue damages that lead to healing delay, we determined the antioxidant activity of DcEE and found that the IC_{50} value of this extract in the DPPH assay was 204.52±3.73µg/ml, very much higher than that of the gallic acid (2.93±0.05 µg/ml) (Table 1), suggesting its weak antioxidant activity. Therefore, the presence of polyphenol and flavonoid compounds, but not the antioxidant activity, might contribute to the wound healing activity of *D. capitellata* leaves.

In addition, NO is a key reactive molecule involved in both normal physiological functions and pathophysiological processes. Excessive production of NO, mainly by macrophages during inflammatory phase, may be toxic to cells due to its capacity to cause DNA and protein damages and mitochondrial respiratory inhibition [26, 27]. Therefore, regulating the production of NO can be considered as an important therapeutic approach for the prevention and treatment of inflammation [28, 29]. DcEE and its fraction DcPEF were found to exhibit an inhibition effect on the production of NO (up to 89.8%) in LPS-stimulated RAW 264.7 cells (Table 2) without cytotoxicity to this cell line (data not shown). This *in vitro* anti-inflammatory capacity correlated to the reduced *TNF-α* and *NF-κB1* gene expression at wound sites of DcEE treated mice (Figure 3). In addition,

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β-Carboline alkaloids and anthraquinones, the main active compounds in *D. capitellata*, have been reported to have strong anti-inflammatory activities, that suggested a reasonable explanation for the anti-inflammatory activity of *D. capitellata* [6, 7, 30, 31].

Lastly, we demonstrated that the extracts of *D. capitellata* stimulated the proliferation of fibroblast NIH/3T3 and keratinocyte HaCaT cell lines (Figure 1). Moreover, the DcEE also improved the *in vitro* migration of HaCaT cell line (Figure 2) but not the fibrolast cell line (data not shown). Keratinocytes and fibroblasts proliferation and migration are the major processes in wound healing [32, 33]. Therefore, the ability to stimulate the growth and migration of these cells is an important parameter for testing wound healing activity *in vitro* [34, 35]. Importantly, our *in vivo* result on mouse excision wound model revealed that DcEE reduced the time of wound closure (Table 3), which might be due to the stimulation of keratinocytes and fibroblast proliferation as well as keratinocyte migration. These findings provided an important piece of evidence in support to the use of this plant in wound treatment.

To explore more information regarding the would healing activity of *D. capitellata* (Wall. ex G.Don) Neupane & N.Wikstr., some experiments including histological analysis and tensile strength measurement during and after wound closure should be performed in the future study. In addition, identification of its active compounds is also requied for the development of effective wound healing medications. On the other hand, it should be noted that the skin structure of mice and human beings is somewhat different, with mouse skin being thinner and containing much more hair follicles [36]. The immune response of mice also differs from that of human [36]. Therefore, it is not guaranteed that the effects of *D. capitellata* on mice could be observed on human beings. However, despite these limitations of using mouse model to access the would healing activity of plant extract, the findings in this study are valuable and could serve as a foundation for the future clinical research.

5. Conclusion

This study strongly demonstrated that *D. capitellata* (Wall. ex G.Don) Neupane & N.Wikstr. leaves had wound healing activity thank to its TPC and TFC, its anti-inflammatory effect, and its capacity to induce fibroblasts and keratinocytes proliferation and migration. Besides, the ethanol extract from the leaves could shorten the wound closure time in examined mice. The results, taken together, provided some scientific evidence in support to the use of *D. capitellata* leaves in treating skin wounds.

Author contribution

NTTN did most of experiments. NMC did several experiments. NTMT wrote the manuscript and had primary responsibility for final content. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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