

In vitro Wound Healing Potential and Antimicrobial Activity of *Clerodendrum Inerme* Leave Extracts

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ABSTRACT

Background: *Clerodendrum inerme* is a medicinal plant which exhibited many pharmacological effects. However, wound healing property of this plant has not been investigated. **Objective:** The present study was designed to evaluate wound healing and antimicrobial activities of *C. inerme* leaves using the *in vitro* model with Human keratinocytes (HaCaT). **Methods:** Cytotoxicity and wound healing effects were determined by MTT and scratch wound healing assay. Antimicrobial activities against cutaneous flora and clinical isolates of bacteria were investigated by broth microdilution assay. **Results:** The results showed that the water extract of *C. inerme* leaves did not exhibit cytotoxic effects on HaCaT cells, while ethanol extracts at higher concentrations significantly decreased cell growth with an IC₅₀ value of 386.8 ± 87.1 µg/mL. The water extract and the lowest concentration (6.25 µg/mL) of the ethanol extract significantly increased percentage of wound closure compared with the untreated group. The water and ethanol extracts of *C. inerme* displayed a broad spectrum of antibacterial activity, inhibiting growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The water extract displayed remarkable activity against methicillin-resistant *S. aureus* with MIC and MBC values ranging from 0.39 to 1.56 µg/mL. Notably, it provided stronger antibacterial activity than vancomycin and also showed antifungal activity against *C. albicans*. **Conclusion:** This study confirms the potential of *C. inerme* leaves for wound healing and antimicrobial therapy and supports the continued utilization of *C. inerme* leaves in traditional medicine. Further studies are needed to clarify the molecular mechanisms through which it exerts such biological effects.

Key words: Antimicrobial activity, *Clerodendrum inerme*, Keratinocytes, Scratch assay, Wound healing.

INTRODUCTION

Wound healing is a physiological process which regenerates and replaces injured cells and involves three different phases including inflammation, new tissue formation, and remodeling.¹ In this process, keratinocytes and fibroblasts, growth factors, cytokines, and their receptors as well as matrix molecules are required.² The critical step for the success of the wound closure is the proliferation and migration of fibroblasts and keratinocytes. Keratinocytes are an important cell component of the outermost layer of the skin. They multiply and migrate into the epidermis' top layers, where they are differentiated and altered as they progress through the cell layers to their final maturity state.³ The wound healing process is adversely affected by oxidants, inflammation, nutritional condition, medications, and hormones. Furthermore, the type and location of the wound, conditions such as diabetes, and microbial infections can modulate effective wound healing.⁴ An infected wound is less likely to heal, thus prevention or cure of infections are required for effective wound healing. Plant compounds were widely reported as potential agents with antimicrobial activity and are preferred because of their general availability, absence of adverse effects, and efficacy in crude preparations.⁵

Clerodendrum inerme L. Gaertn is a mangrove plant that is broadly distributed along the beach forests in South-east Asia, Australia and Pacific islands.⁶ *C. inerme* is a medicinal plant used in the treatment of rheumatism, cough, scrofula, venereal infection, skin diseases, beri-beri, tumors and

wound healing.⁷⁻⁹ The leaves of *C. inerme* are used in conjunction with other plant leaves for the treatment of fever, cough, skin rashes, chronic pyrexia and boils.¹⁰ *C. inerme* exhibits hepatoprotective,⁷ anti-inflammatory,¹¹ anti-bacterial,¹²⁻¹³ anti-oxidant¹⁴ and anticancer activities.¹⁵⁻¹⁷ However, thus far effects of *C. inerme* leave extracts on wound healing have not been reported. The present study aimed to evaluate the pharmacological effects of water and ethanol extracts of *C. inerme* leaves on wound healing.

MATERIALS AND METHODS

Chemical reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trypan blue, dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Phosphate buffered saline and fetal bovine serum (FBS) were purchased from Merck Millipore Corp (Darmstadt, Germany). Powdered Dulbecco's modified Eagle's medium (DMEM), penicillin-streptomycin and 0.25% trypsin-EDTA were purchased from Gibco®, (Grand Island, NY, USA).

Collection of plant material and extracts preparation

C. inerme leave parts were collected in the Pak-Poon Community, Nakhon Si Thammarat, Thailand, in May 2020. The voucher specimen was deposited at the herbarium in the Botanic Garden of Walailak University, Nakhon Si Thammarat, Thailand, under number 01521. Fresh leaves were washed under running tap water and then dried for 36 hours using

a hot air oven at 60 °C. Dried leaves were crushed and subjected to extraction using the maceration process in distilled water and ethyl alcohol (95%) for 7 days at room temperature. Extracts were collected and concentrated at 40 °C under reduced pressure using a rotary evaporator. The dried extracts were stored at 4 °C until further analysis.

Cell culture

Human keratinocytes (HaCaT) were provided by Professor Ramida Watanapokasin from Department of Biochemistry, Faculty of Medicine, Srinakharinwirot University, Thailand. HaCaT cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 mg/mL streptomycin. Cultures were incubated in a humidified atmosphere with 5% CO₂ at 37 °C. Cultured cells were dispersed/distributed every 72 h. The confluent culture was subcultured using 0.25% of trypsin-EDTA.

Cytotoxic assay

An MTT test was used to determine the viability of HaCaT cells. Cells (1×10^4 cells/well) were seeded in a 96-well plate in DMEM culture media (Gibco, Grand Island, NY, USA) supplemented with 10% FBS and incubated for 24 hours at 37 °C in a 5% CO₂ atmosphere. Water and ethanol extracts at various concentrations (0-400 g/mL) were applied to cells for 24 hours. After treatment, a 0.5 mg/mL MTT solution was added to each well and incubated at 37°C in a humidified atmosphere with 5% CO₂ for 2 hours. The culture medium was removed and cells were suspended in 100 µL DMSO. Absorbance at 570 nm was determined by a Microplate spectrophotometer and the percentage of cell viability was calculated relative to the control. The IC₅₀ value was calculated using the software GraphPad Prism 3.03 (GraphPad Software Inc, CA). Data were recorded for three independent experiments and are shown as mean ± SEM.

In Vitro wound healing scratch assay

The scratch wound-healing assay was used with modifications as previously described.¹⁸ Briefly, HaCaT cells (5×10^4) were seeded into 12-well cell culture plates and allowed to grow to 90% confluence as a monolayer. Cells were starved with serum-free medium for 24 hours. The monolayer was gently scratched with a sterile 200 µL pipette tip. After scratching, the culture medium was removed, and cellular debris was removed by washing with PBS solution. Serum-free medium with non-cytotoxic concentrations of the extract of *C. inerme* leaves was added and incubated for 24 hours at 37 °C in a 5% CO₂ atmosphere. Cells without treatment and cells treated with allantoin (Sigma-Aldrich, Germany) (50 µg/mL) were used as negative and positive control, respectively. Images of the scratched areas were photographed at 0 and 24 hours post-treatment using an inverted microscope (Carl Zeiss Microscopy, NY, USA) and analyzed using NIH ImageJ software (Bethesda, Maryland, USA). Wound healing was estimated by the percentage of scratch closure at each dose point relative to the control. Three independent experiments were performed for each data point and the percentage of wound closure was calculated using the following formula:

$$\frac{[(\text{Wound area } t_0 - \text{Wound area } t_{24}) / \text{Wound area } t_0] \times 100}{}$$

Bacterial strains and culture conditions

Antimicrobial activity of water and ethanol extracts was tested against Gram-positive bacteria including *Staphylococcus aureus* ATCC25923, *S. epidermidis* ATCC12228 and methicillin-resistant *S. aureus* (MRSA) and Gram-negative bacteria including, *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853, was tested by a modified broth microdilution method according to the Guidelines of the Clinical and Laboratory Standards Institute Guidelines.¹⁹ The bacteria were

grown in trypticase soy medium (Merck, Germany) at 37°C. *Candida albicans* was grown on Sabouraud dextrose agar (BD Difco, MD, USA). A single colony of bacteria was isolated by cross streaking on medium and incubated at 37 °C for 18-20 hours.

Antimicrobial activities

Minimum inhibitory concentration (MIC) was the lowest concentration of samples that did not result in bacterial growth. Water and ethanol extracts of *C. inerme* were dissolved in 10% DMSO and two-fold dilutions were made. Microorganisms obtained from the overnight broth culture were suspended in Muller-Hinton broth (Merck KGaA, Darmstadt, Germany). The bacterial cells suspension was mixed with the diluted test agents in a microtiter plate (*Thermo Fisher Scientific*, MA, USA). The final concentration was 0.5×10^5 CFU/mL. The final concentration of the extracts ranged from 0.39 to 100 µg/mL and microtiter plates were incubated at 37 °C for 18-20 hours. The MIC was observed. Aliquots were plated onto Muller-Hinton agar plates and incubated at 37 °C for 18-20 hours. The minimum bactericidal concentration (MBC) was recorded as the lowest concentration of the extracts preventing bacterial growth completely.

Time-kill assay

Time-kill assay was used to study the killing activity of the water extract on MRSA at hourly intervals for 6 hours and 24 hours. The bacterial cells inoculum approximately 0.5×10^6 CFU/mL was added to Muller-Hinton broth (1:10), supplemented with water extract at concentrations of 0.5 MIC, MIC, 1 MIC, and 2 MIC and incubated at 37°C. Viable counts were performed by a drop plate method. The test was carried out in duplicate and results are shown as the mean of the log numbers of bacterial cells.

Statistical analysis

All data were subjected to statistical analysis using the SPSS 23.0 software package (IBM Software Inc, Chicago, IL, USA). Data are reported as mean ± standard error of the mean (±SEM) of three independent experiments. Statistical significance was analyzed by the one-way ANOVA test. Differences between treated group and untreated group were determined statistically significant at $p < 0.05$.

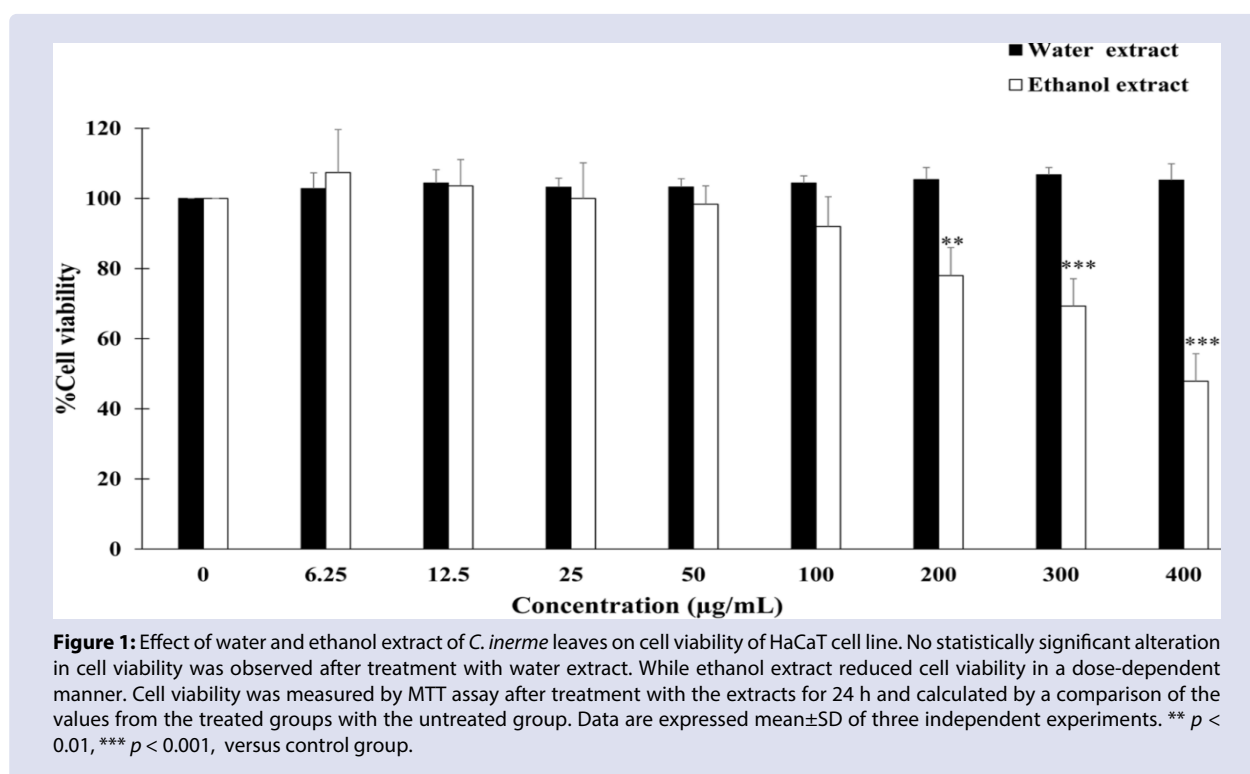
RESULTS

Cytotoxic effect of *C. inerme* leaf extracts

The cytotoxic effect of water and ethanol extracts from *C. inerme* leaves on HaCaT cells were evaluated by a viability assay using MTT. As shown in Figure 1, there was no cytotoxic effect and an increase in cell growth was observed at extract concentrations of 6.25 to 400 µg/mL for 24 h. The IC₅₀ value was determined to >200 µg/mL. The ethanol extract did not exhibit a cytotoxic effect at lower concentrations. However, a cytotoxic effect was observed at a concentration of 200 µg/mL and an IC₅₀ value of 386.8 ± 87.1 µg/mL. We found that the ethanol extract at concentrations from 6.25 to 25 µg/mL increased cell viability and 50-100 µg/mL of ethanol extract produced a cell viability of more than 90%. The data obtained in this study indicate better viability of HaCaT cells at lower concentrations.

Effect of *C. inerme* leaf extracts on cell migration

The effect of water and ethanol extracts of *C. inerme* leaves on cell migration was determined by a scratch wound healing assay. Cells with and without treatment by *C. inerme* leaf extracts were allowed to migrate into the denuded area for 24 h at 37 °C and wound closure was determined by using a microscope with Image J software. Allantoin was used as a positive control drug at a concentration of 50 µg/mL. The water extract of *C. inerme* leaves (6.25-100 µg/mL) significantly



enhanced the wound closure rate when compared to the untreated group after 24 h (Figure 2A). Cells treated with water extracts at 6.25, 12.5, 25 and 50 µg/mL showed a dramatic enhancement in wound closure. The water extract at 100 µg/mL also showed a potent wound healing effect on HaCaT by $54.1 \pm 9.9\%$ ($p < 0.01$) when compared to the control group where only $30.6 \pm 7.8\%$ were observed (Figure 2B).

In contrast, the ethanol extract of *C. inerme* leaves displayed a significant effect on wound closure when compared to the control group ($p < 0.05$). However, at higher concentrations it did not affect the wound closure rate of HaCaT cells (Figure 3). The results indicate that water and ethanol extracts at low concentrations are suitable like traditional remedies to improve wound healing whereby the water extract had a marked effect on cell proliferation and migration.

Antimicrobial activities of *C. inerme* leave extracts

Antimicrobial activity of water and ethanol extracts of *C. inerme* on both reference and clinical isolated strains of bacteria are shown in Table 1. The water extract demonstrated antimicrobial activity against *S. aureus*, *S. epidermidis* and *C. albicans* with MIC values of 0.78, 1.56, and 6.25 µg/mL, respectively. The ethanol extract displayed activity with the MIC values of 1.56, 3.13 and 12.50 µg/mL against *S. aureus*, *S. epidermidis*, and *C. albicans*, respectively.

Interestingly, the water extract of *C. inerme* displayed remarkable activity against MRSA with MIC and MBC values ranging from 0.39 to 1.56 µg/mL and thus provided stronger antibacterial activity than vancomycin, which displayed 3 times higher MIC values and 3 times higher MBC values Table 1. It also had antifungal activity against a clinical isolate of *C. albicans*. MIC of the water and ethanol extracts tested with *E. coli* and *P. aeruginosa* were higher than 100 mg/mL, the highest concentration tested.

Time-kill assay

Methicillin-resistant strains of *S. aureus* were incubated with the water extract at amounts of 0.5, 1 and 2 MIC and samples were analyzed at

time intervals for bactericidal activity. At all test concentrations, the water extract showed a significant bactericidal activity on all clinical isolated MRSA. Bactericidal activity was defined as a decrease of ≥ 3 log units in CFU/mL. From the results, 99% killing was observed at 6, 5, and 4 h when treated with 0.5MIC, MIC, and 2MIC, respectively. Moreover, the most significant bactericidal activity ($\geq 99.9\%$ killing) was observed at 24, 6, and 5 h at 0.5MIC, MIC, and 2MIC, respectively.

DISCUSSION

Plants and their extracts have been used for the treatment of wounds since ancient times. Treatments using plant-extracts are widespread, available, easily accessible and lack side effects. Often their use is based on tradition without any scientific evidence of efficacy and with little knowledge about active compounds or their modes of action. In this study we first demonstrate that water and ethanol extracts of *C. inerme* leaves are conducive to an improvement of wound repair. We have performed a cytotoxicity test first to determine a range of crude extract concentrations that have no cytotoxic effects on HaCaT cells. We found that extract concentrations ranging from 6.25 to 400 µg/mL of water extracts had no significant effect on cell viability and even enhanced the cell viability of HaCaT cells. Ethanol extracts at lower concentrations (6.25-25 µg/mL) increased cell viability whereas at higher concentrations cell growth was significantly inhibited. Water and ethanol extracts were selected at their non-toxic concentrations to determine the effect on a possible stimulation of HaCaT cell migration. We found that both water and ethanol extract are suitable as traditional remedies to improve wound healing. The *C. inerme* water extract exhibited a better-wound healing capacity than the ethanol extract. Several phytochemical constituents have been isolated from *C. inerme* leaves such as alkaloids, steroids, tannins, phenolic compounds, flavonoids, and terpenoids which possess various properties such as antioxidation and antimicrobial activities, promote wound healing and skin regeneration.^{4,20-23} A previous study showed that extracts of leaves, stem bark and roots of *K. africana* and *S. hispidus* contain alkaloids, saponins, tannins, flavonoids, carbohydrates, and saponin glycosides

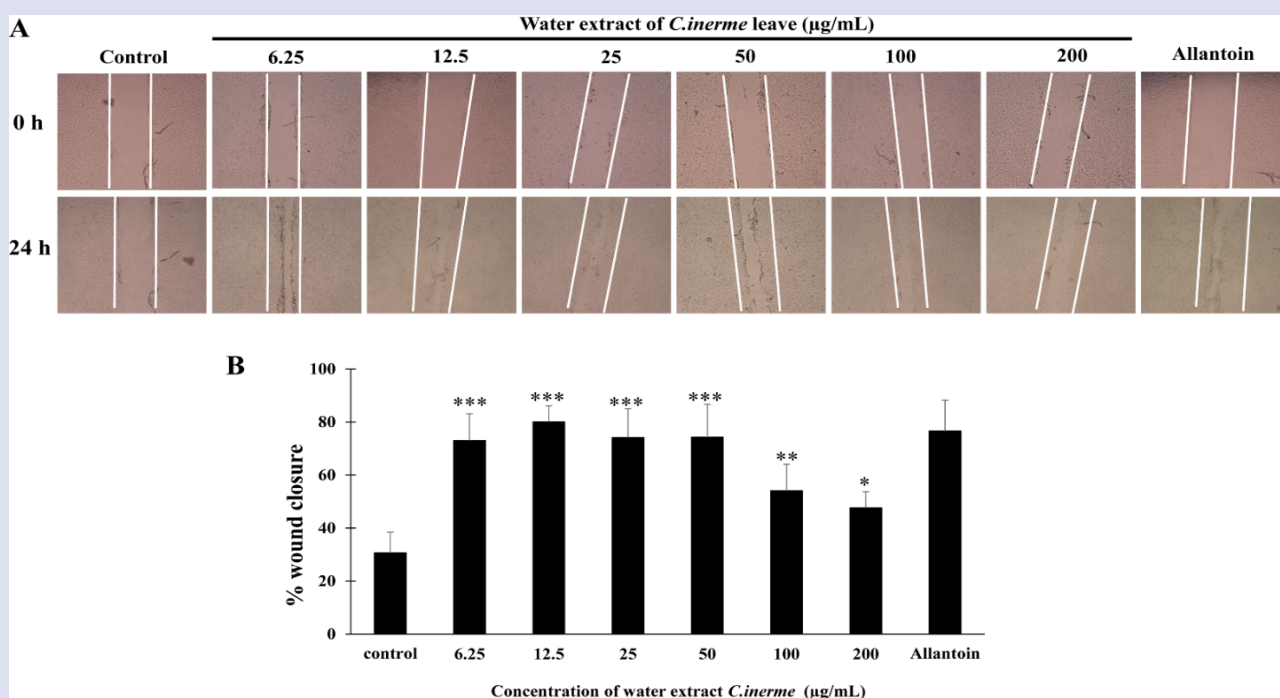


Figure 2: Effect of water extract of *C. inerme* leaves on cell migration was determined by scratch wound healing assay. **(A)** Digital images captured at 5x magnification using an inverted microscope at 0 and 24 after treatment. **(B)** The wound closure rate in percentage of HaCaT cell after treatment with water extract of *C. inerme* leaves for 24 h. Quantitative analysis of the migration rate was analyzed with the use of ImageJ software. Data are expressed as mean ± standard deviation from three individual experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, versus control group.

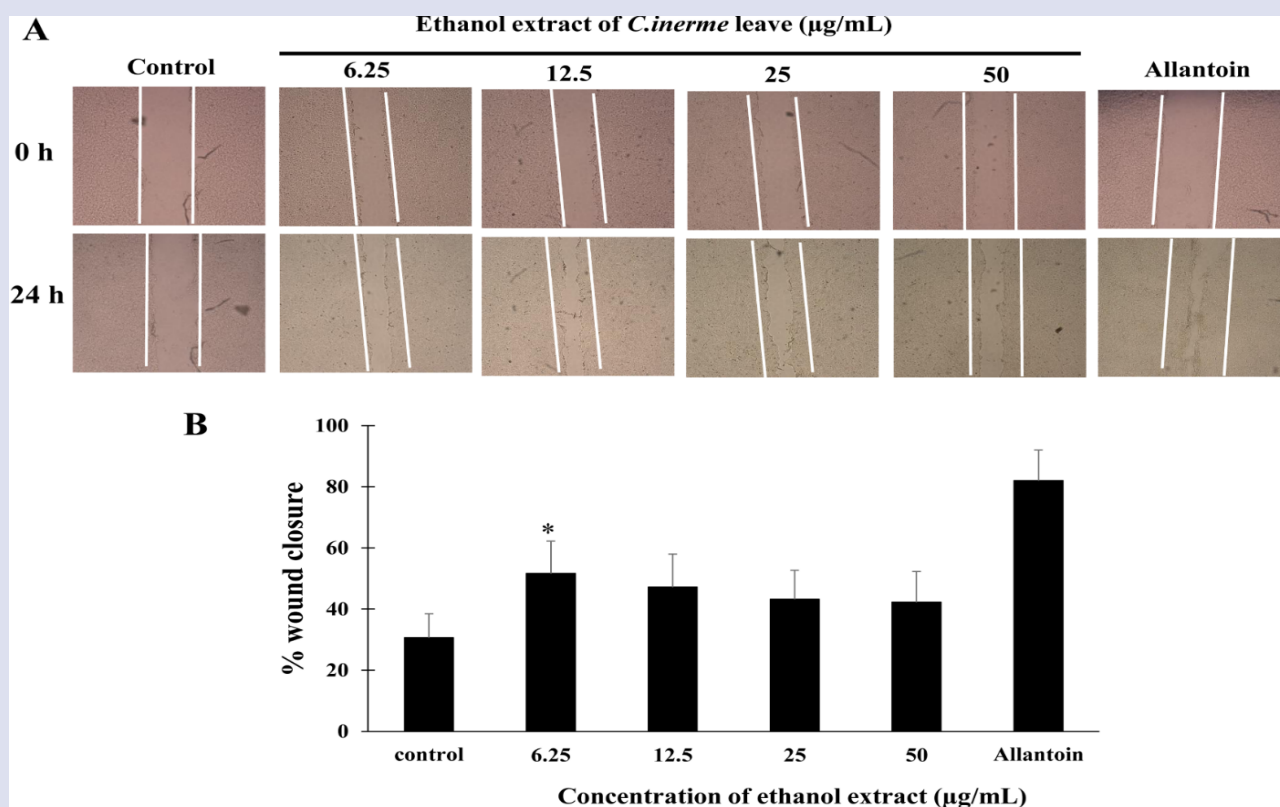


Figure 3: Effect of ethanol extract of *C. inerme* leaves on cell migration was determined by scratch wound healing assay. **(A)** Digital images captured at 5x magnification using an inverted microscope at 0 and 24 after treatment. **(B)** The wound closure rate in percentage of HaCaT cell after treatment with water extract of *C. inerme* leaves for 24 h. Quantitative analysis of the migration rate was analyzed with the use of ImageJ software. Data are expressed as mean ± standard deviation from three individual experiments. * $p < 0.05$ versus control group.

Table 1: The minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *C. inerme* leaf extracts against skin microorganisms.

Microorganisms	MIC/MBC ($\mu\text{g/mL}$)		Antibiotics ($\mu\text{g/mL}$)
	Water extract	Ethanol extract	
<i>Staphylococcus aureus</i> ATCC25923	0.78/1.56	1.56/1.56	0.62/1.25 ^a
Methicillin-resistant <i>Staphylococcus aureus</i> 1	0.39/0.78	0.78/1.56	1.25/125 ^a
Methicillin-resistant <i>Staphylococcus aureus</i> 2	0.39/0.78	0.78/1.56	1.25/125 ^a
Methicillin-resistant <i>Staphylococcus aureus</i> 3	0.39/0.78	0.78/1.56	1.25/125 ^a
Methicillin-resistant <i>Staphylococcus aureus</i> 4	0.39/1.56	0.78/1.56	1.25/125 ^a
Methicillin-resistant <i>Staphylococcus aureus</i> 5	0.39/1.56	0.78/1.56	1.25/125 ^a
<i>Staphylococcus epidermidis</i> ATCC12228	1.56/1.56	3.13/3.13	0.78/1.56 ^a
<i>Candida albicans</i> (clinical isolated)	6.25/6.25	12.50/12.50	0.25/0.25 ^b
<i>Escherichia coli</i> ATCC25922	>100/>100	>100/>100	0.50/0.50 ^c
<i>Pseudomonas aeruginosa</i> ATCC27853	>100/>100	>100/>100	1.50/3.00 ^c

^a = vancomycin, ^b = amphotericin B, and ^c = gentamicin

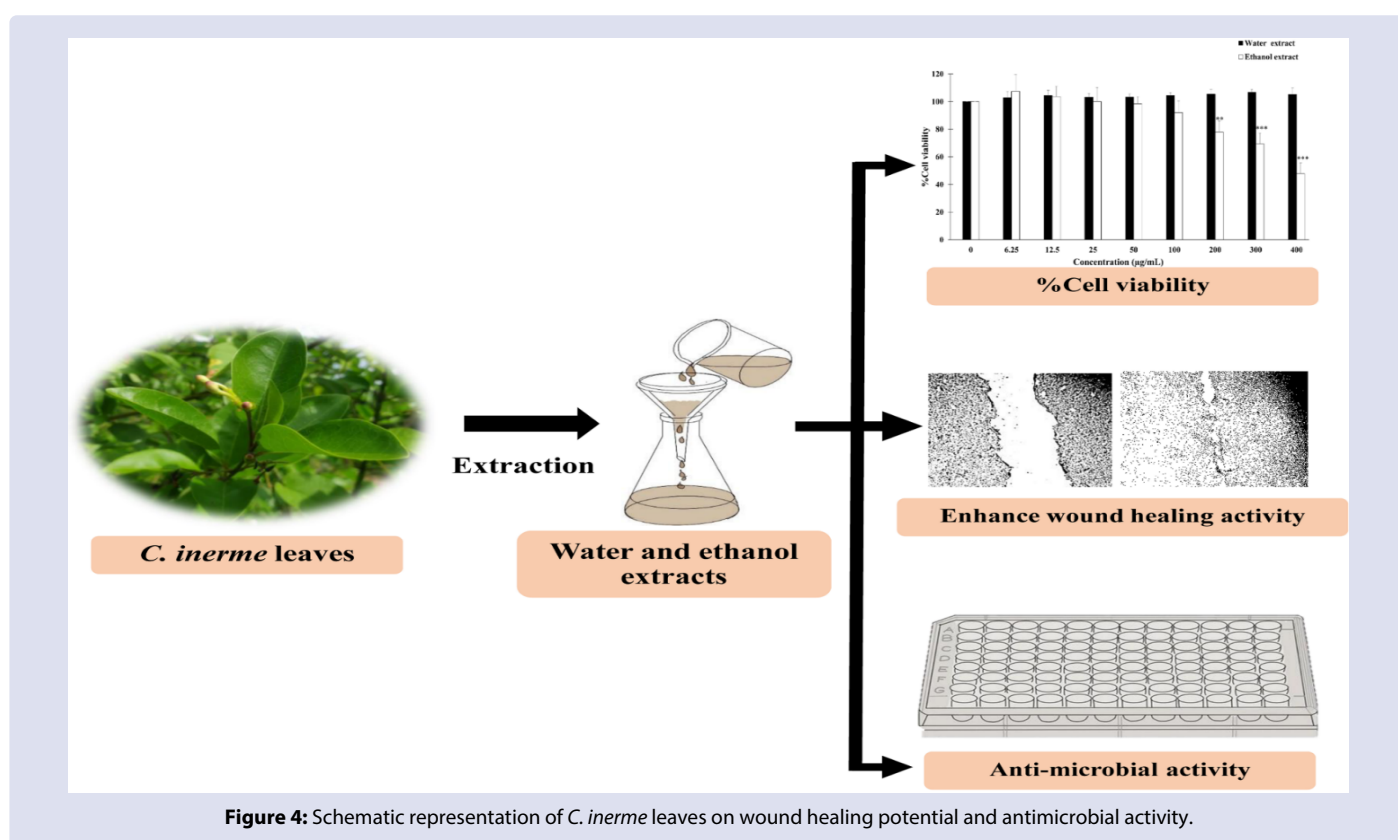


Figure 4: Schematic representation of *C. inerme* leaves on wound healing potential and antimicrobial activity.

which exhibit antimicrobial, antioxidant and enhanced wound healing properties.²⁴ Likewise, Agyare et al. reported the presence of tannins, flavonoids, alkaloids, and glycosides in *J. flava* and *L. welwitschii* extracts and their antimicrobial and wound healing properties.²⁵ Some studies have reported that high concentrations of flavonoid contents of *A. bracteolata* inhibit cell proliferation of human fibroblast and HaCaT cell.^{26,27} Flavonoids contained in a hydro-ethanol extract of *C. officinalis* stimulate potentiated wound healing by stimulating *in vitro* and *in vivo* proliferation of dermal fibroblasts.²⁸ Recently the potential of other species such as *Aloe muth-muth* and *Digitaria ciliaris* in wound healing of the immortalized human keratinocyte (HaCaT) cell line was reported.^{29,30}

We have also evaluated *in vitro* antimicrobial activity of the water and ethanol extracts of *C. inerme* leaves. The water extract had demonstrated substantial activity against methicillin-resistant *S. aureus* and *S. Aureus*. The activity of the water extract with MIC values ranged from 0.39 to 0.78 $\mu\text{g/mL}$, respectively. The MBC was 2- to 4-fold greater than the

MIC, suggesting that the water extract is highly bactericidal against these pathogenic species. Moreover, extracts of *C. inerme* demonstrated antifungal activity against *C. albicans*, whereas it no significant activity against 2 tested strains of Gram-negative bacteria.

Water was the most effective solvent for extracting antibacterial constituents from *C. inerme* which indicates that the antibacterial constituents of *C. inerme* are highly polar. The susceptibility pattern we have observed correlates well with those obtained for other extracts of medicinal plant.³¹ Gram-negative bacteria are generally less susceptible to antibacterial agents than Gram-positive organisms due to the low permeability of their cell walls.³²

CONCLUSION

The results presented in our study indicate that *C. inerme* leaves can exhibit prominent antimicrobial and wound healing activity (Figure 4). The findings further support traditional applications of *C. inerme* leaves

for the cure of infections and as wound healing agent. Future studies are needed to identify and isolate major components responsible for wound healing. The effects of *C. inerme* extracts on signaling pathways underlying proliferation and migration of keratinocytes warrant further investigation as well as the development of hydrogel bandages containing *C. inerme* leaf extracts.

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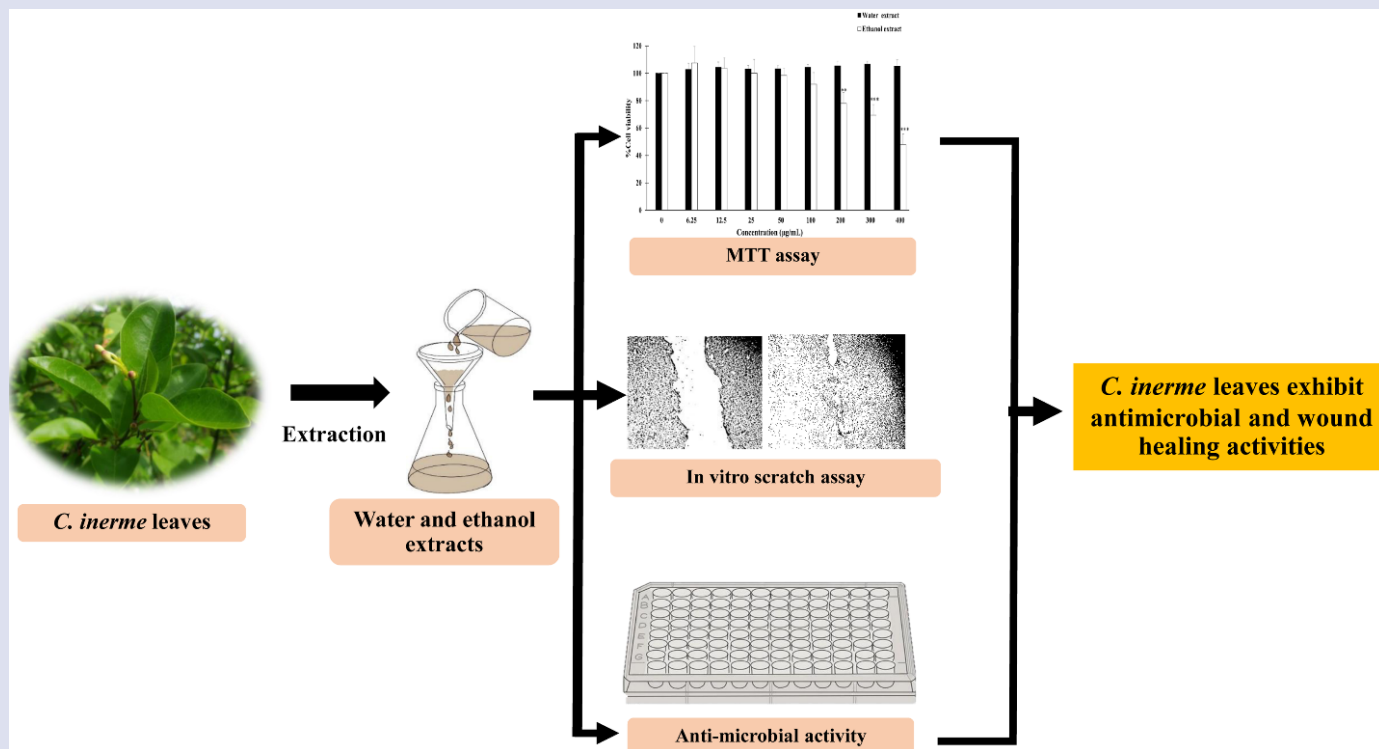
CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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GRAPHICAL ABSTRACT



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