

Wound Healing Activity of Binahong (*Anredera cordifolia* (Ten.) Steenis) Leaves Extract towards NIH-3T3 Fibroblast Cells

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Abstract

Anredera cordifolia (Ten.) Steenis, also known as Binahong, is a family of Basellaceae, a medicinal plant that had been growing well for many years. Binahong leaf extract can stimulate fibroblast and collagen formation which will accelerate the process of wound healing. This study was carried out to observe the proliferation kinetics and migratory activity of 3T3 fibroblast cell lines induced by *Anredera cordifolia* (Ten.) Steenis ethanol extract. *Anredera cordifolia* (Ten.) Steenis powder was extracted by maceration with 70% ethanol. Proliferation activity was assessed by using the MTT assay. Cell migration activity was determined using scratch assay. The data obtained were analyzed statistically with data analysis, descriptive analysis, and continued with the bivariate analysis of correlation between case groups. The results of phytochemical analysis test on the content of Binahong leaf extract's secondary metabolites confirmed the presence of saponins, tannins, alkaloids, triterpenoid, flavonoid, phenolics, steroids, and glycosides. Fibroblast cell proliferation was increased from 0 h until 72 h with the highest proliferation rate at the concentration of 62.5 µg/mL (127.89 ± 16.12). The results showed that there was a significant difference in the mean proliferation rate of fibroblast cells between the Binahong leaf extract, Aloclair[®] and the control group (p value = 0.0001). *Anredera cordifolia* (Ten.) Steenis leaf extract has chemical compounds, such as saponins, tannins, alkaloids, triterpenoid, flavonoid, phenolics, steroids, and glycosides. It is potentially effective as a wound healing agent which stimulates proliferation of fibroblast cells.

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Introduction

The use of alternative medicine to treat a wound is in practice as many studies had proven that poly-molecular traditional medicine provided more beneficial effects than the single molecule based allopathic medicine in many cases.¹ The entire wound healing process involves a complex series of events that begins at the moment of injury and can continue for months to years. The paradigm shift from allopathic medicine to the integration of herbal plants in modern medicine to

accelerate wound healing has been the prime goal of several researches.²

Anredera cordifolia (Ten.) Steenis, also known as Binahong, is a family of Basellaceae, a medicinal plant that had been growing very well for many years. In Indonesia, the Binahong plant is still uncommon but in Vietnam, this plant is high in demand and often used as a vegetable in Taiwan. In China and Taiwan, this plant is known to have tremendous benefits and is consumed for more than a millennium. Almost all parts of the Binahong plant like tubers, stems and leaves could be used in herbal therapy.³ Binahong leaf extract can stimulate fibroblast and collagen formation which will accelerate the process of wound healing.^{4,5}

The ingredients of Binahong leaf extract that affects wound healing are saponins, tannins, terpenoids, alkaloids, and flavonoids.^{6,7} Saponin is known to have an influence in stimulating

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proliferation of fibroblasts and collagen formation, which in turn plays a role in the process of wound healing.^{4,5} Saponins play a role in the synthesis of TGF- β 1 and modifications of the TGF- β 1 and TGF- β 2 receptors in fibroblasts. This will stimulate the synthesis of fibronectin. Moreover, saponins have the ability to act as a cleanser and possesses antiseptic properties which serve to kill and prevent the growth of microorganisms that arise in the wound, thus preventing severe infections in the wound.⁸

Tannins and alkaloids have antimicrobial properties and antioxidants that aid the wound healing process by preventing and keeping the wound area from being damaged by free radicals and inhibit the growth of pathogenic bacteria in the wound.⁹

Flavonoids act as a potent antioxidant, anti-bacterial, anti-aging, anti-leukemic and as a potent vasodilator. The antimicrobial effect of flavonoids is by inhibiting the synthesis of DNA, proteins and lipids of bacteria. Flavonoids inhibit the function of the cytoplasmic membrane by forming complex compounds against extracellular proteins that interfere with the integrity of bacterial cell membrane. Flavonoids also have a tendency to bind to bacterial proteins and are capable of inhibiting the enzyme activity involved in the metabolic processes of bacteria.^{10,11}

Topical application of Binahong (*Anredera cordifolia* (Ten.) Steenis) leaf paste showed higher percentage of wound contraction in the wound healing process of *Mus musculus* mice. Binahong leaf paste can be used widely as an alternative for wound care after further studies were conducted.¹² The Binahong leaf extract is beneficial in wound healing, where flavonoid acted in anti-inflammatory mechanisms, inhibition of free radical activity, and enhanced epithelialization.¹³

The purpose of this research is to observe the proliferation kinetics and migratory activity of 3T3 fibroblast cell lines induced by *Anredera cordifolia* (Ten.) Steenis ethanol extract.

Methods

Cell line

Swiss 3T3 albino mouse fibroblasts were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100

g/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO₂ (all Gibco-BRL, Netherlands).

Preparation of extract

Leaves of fully grown Binahong plants were plucked and used as sample for this study. Initially, before pulverizing the Binahong leaves, a few steps were taken. The leaves were washed under running water to remove sand and dirt, drained, weighed, and its wet weight was noted. Binahong leaves were collected and weighed as much as 10 kg, then washed and dried under 40 °C and later, weighed in as dry weight of 400 grams. Furthermore, the leaves were crushed, and pulverized Binahong leaves were obtained. A total of 400 grams of pulverized Binahong leaves were placed in a closed vessel and soaked with 70% ethanol that had been distilled for 5 days at room temperature. After 5 days, the liquid is removed and filtered with filter paper, then the powder was soaked again with 70% ethanol that had been distilled for 2 days and a final filtering process was conducted. Then, vacuum rotary evaporator and water bath was used to evaporate the solvent until the extract became desiccate.¹³

Examinations of secondary metabolites in Binahong

Testing of flavonoids and tannins was done using spectrophotometry and saponins were tested using a thin-layer chromatographic scanner.

Cell proliferation

Ethanol extract was submitted for cytotoxicity test. 3T3 cell line (5×10^3 cells/mL) was grown in DMEM complete medium. After 24; 48 and 72 h treatment, MTT assay was performed and cell viability was counted to calculate the antiproliferative activity.¹⁴

In vitro wound healing assay

The migration assay was carried out with NIH-3T3 cells that were seeded at 5×10^4 cells/well in 24-well plates and incubated for 24 h at 37°C. Cultured cells were washed with PBS. Scratch was done at the bottom center of the well within the cell layer using a yellow tip. Cell residues in the plate were washed with PBS and treated with Binahong extract and Aloclair®

Plus Gel (Sinclair Pharma Srl, Milan, Italy), then incubated for 24 h at 37°C and documented under an inverted microscope to measure rate of cell migration after 0, 6 and 24 h. The space from the scratch treatment between control and treatment groups were quantified using the ImageJ software (National Institute of Mental Health, Maryland, USA) and defined as the cell migration area.^{8,14,15}

Statistical analysis

The results were presented as means ± SD. The data obtained were analyzed statistically with data analysis, descriptive analysis, and continued with bivariate analysis with one-way Anova to analyze the correlation between case groups. The statistical analysis was carried out by using the Statistical Package for the Social Sciences version 21 (IBM® Inc, New York, USA).

Results

No	The active ingredients of secondary metabolites of Binahong	Test result
1	Saponin	+
2	Tannin	+
3	Triterpenoid	+
4	Alkaloid	+
5	Flavonoid	+
6	Phenolic	+
7	Steroid	+
8	Glycoside	+

Table 1. Tally Sheet Containing Secondary Metabolites of Binahong Leaf Extract.

The active Ingredient of Secondary Metabolites of Binahong	Test			Σ Test	Testing Methods
	Test 1	Test 2	Test 3		
Saponins	1.08	1.07	1.09	1.08	TLC scanner
Tannins	1.56	1.61	1.58	1.58	Spectrophotometry
Flavonoids	0.41	0.39	0.37	0.39	Spectrophotometry

Table 2. Quantitative Analysis of the Secondary Metabolites in Binahong Leaf Extract.

The results of the phytochemical analysis test on the content of Binahong leaf extract's secondary metabolites [done in Laboratory of the Research Institute for Spices and Medicinal

Plants (Balitro)] and quantitative analysis of the differences in the content of Binahong leaf extract's secondary metabolites, such as saponins, tannins, and flavonoids are shown in Table 1 and 2.

Figure 1 shows that the Binahong leaf extract had increased the proliferation rate of 3T3 fibroblast cell line (*in vitro*). Proliferation of 3T3 fibroblast cells were increased from 0 h until 72 h with the highest proliferation rate noted at the concentration of 62.5 ppm (127.89 ± 16.12).

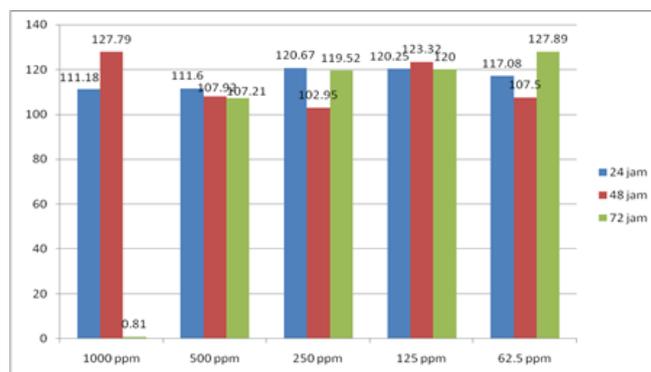


Figure 1. Proliferation Kinetics of Fibroblast Cell Activity Towards 3T3 Cell Lines of Anredera Cordifolia (Ten.) Steenis.

Table 3 shows the statistical analysis of wound healing assay between Binahong leaf extract, Aloclair® and the control group. The highest fibroblast proliferation rate after 6 hours of exposure was found in the Binahong extract group (33,54 ± 1,30). Application of Aloclair® 250 ppm gel obtained the average value of 23.17 ± 1.34, while the control group obtained the mean value of 16.04 ± 1.22. The results showed that there was a significant difference in the mean proliferation rate of fibroblast cells (p value = 0.0001).

Treatment	6 hrs	24 hrs
Aloclair 250 ppm	23,17 ± 1,34	48,29 ± 0,85
Binahong 62,5 ppm	33,54 ± 1,30	55,08 ± 1,04
Control	16,04 ± 1,22	38,32 ± 2,08

Table 1. Statistical Analysis of Wound Healing Assay Between Binahong Leaf Extract and Aloclair.

The highest fibroblast proliferation rate after 24 hours of exposure was found in the

Binahong leaf extract group (55.08 ± 1.04). Application of Aloclair® 250 ppm gel obtained the mean value of $48,29 \pm 0,85$, while the control group obtained the mean value of $38,32 \pm 2,08$. The results showed that there was a significant difference in the mean proliferation rate of fibroblast cells (p value = 0.0001).

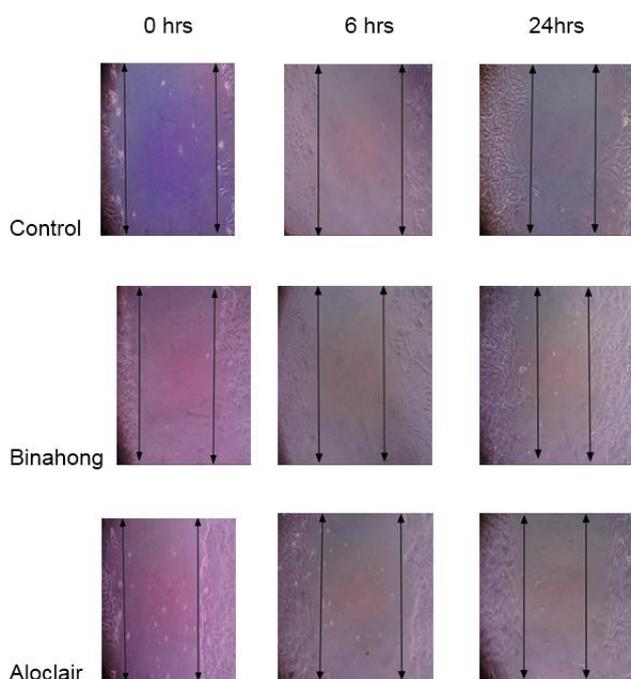


Figure 2. Comparison of Wound Distance in 3T3 Fibroblast Cell Line Between Binahong Leaf Extract, Aloclair® and the Control Group.

Discussion

This study showed that the Binahong leaf extract had chemical compounds, such as saponins, tannins, alkaloids, triterpenoid, flavonoid, phenolics, steroids, and glycosides (Table 1 and 2). The results of this study was similar to the research conducted by Kaur, et al. and Yuliani, et al. A research conducted by Astuti, et al., showed that saponin, triterpenoid, and steroid compounds were only found on the stems and leaves of the plant, whereas alkaloid compounds were mainly found on the leaves, stems, and tubers of plants.^{5,12} The results of this study is also comparable to the study by Lestari D, et al., in Bandung, Indonesia where the phytochemical screening of crude drug, extract and fractions of Binahong leaves had confirmed the presence of saponin, flavonoid, alkaloid and triterpenoid in Binahong leaves.¹⁶

In Figure 1, Binahong leaf extract with the concentration of 1000 ppm had proven to be toxic towards the 3T3 fibroblast cells. Hence, it can be assumed that the Binahong extract with the concentration of 500 ppm is within the safety margin whereas 1000 ppm is considered overdose and capable of inducing necrosis of the 3T3 fibroblast cells. As a rule, the therapeutic effect or toxic action of a drug depends critically on the response of a single organ (or cells) or a limited number of organs. Experimentation on isolated organs or cells offers several disadvantages; (1) Unavoidable tissue injury during dissection, (2) loss of physiological regulation of function in the isolated tissue, and (3) the artificial milieu imposed on the tissue. In this study, as the concentration is raised by a constant factor, the increment in effect diminishes steadily and tends asymptotically toward zero, the closer one comes to the maximally effective concentration.¹⁷ Binahong leaf extract with the concentration of 62,5 ppm showed the greatest ability in stimulating fibroblast cell proliferation (Figure 1). This low dose in comparison to the other extract concentrations (125 ppm, 250 ppm, 500 ppm, 1000 ppm) indicates a greater potency of the Binahong leaf extract as a drug as it is capable of stimulating fibroblast proliferation at a concentration of 62,5 ppm.¹⁸

In Table 3 and Figure 2, the Binahong leaf extract with the concentration of 62,5 ppm showed greater potency of stimulating wound contraction by inducing 3T3 fibroblast proliferation compared to Aloclair® (Aloe vera) gel of 250 ppm and the control group. The *Anredera cordifolia* (Ten.) Steenis contain triterpenoids/ steroids, saponins, tannins, flavanoids, glycosides which has the ability to promote fibroblast proliferation.^{3,5,8} A study by Zhang, et al., found that saponins from *Anredera cordifolia* (Ten.) Steenis stimulated fibroblast proliferation and myofibroblast differentiation in the wound and thus accelerated wound closure.¹⁵ The mechanism of action of saponins in wound healing is to stimulate the production of type I collagen, which has an important role in wound closure and increases rate of epithelialization in tissues. Flavonoids act by inhibiting the lipid peroxidation process and are responsible for scavenging free radicals, thus preventing and retarding cellular necrosis, and increasing vascularization at the wound site. Inhibition of

lipid peroxidation is believed to enhance the viability of collagen fibrils by increasing collagen fibers and vascularization, preventing cellular damage, and promoting DNA synthesis. Flavonoids, glycosides, and tannins are known to act as astringents and antibacterials. Polyphenols, being compounds with antioxidant properties, also play a role in wound healing, through inhibition of lipid peroxidation, similar to flavonoids. The use of antioxidants in wound healing is due to the fact that cell proliferation, suppression of inflammation, and contraction of collagenous tissues are inhibited by the presence of free radicals.¹⁶ It is the presence of saponins, alkaloids, and flavonoids in Binahong leaves that presumably plays a significant role in the proliferation of NIH-3T3 fibroblast cells in the current study.

Conclusion

The *Anredera cordifolia* (Ten.) Steenis leaf extract has chemical compounds, such as saponins, tannins, alkaloids, triterpenoid, flavonoid, phenolics, steroids, and glycosides. It is potentially effective as a wound healing agent which stimulates proliferation of fibroblast cells. Further studies are required to assess the molecular mechanism in stimulating proliferation of fibroblast cells.

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