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Melaksanakan penulisan artikel : *Antibacterial Activity, Cytotoxicity, and Phytochemicals Screenings of Binahong (Anredera cordifolia (Ten.) Steenis) Leaf Extract* di *Journal of Advanced Pharmacy Education & Research (JAPER)* pada bulan Januari 2023.

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Bandung, 17 Januari 2023
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Table of Contents

Volume 14 | Issue 1 - 2024

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Antibacterial activity, cytotoxicity, and phytochemicals screenings of binahong (*Anredera cordifolia* (Ten.) p. 1-7 steenis) leaf extract (<https://japer.in/article/antibacterial-activity-cytotoxicity-and-phytochemicals-screenings-of-binahong-anredera-cordifoli-hnuhiud2aemiydb>)

Vinna Kurniawati Sugiaman

 Views: 61 Downloads: 23


Can liver enzyme profile be a predictor of NAFLD in type-2 diabetes mellitus (T2DM) patients? p. 8-12 (<https://japer.in/article/can-liver-enzyme-profile-be-a-predictor-of-nafld-in-type-2-diabetes-mellitus-t2dm-patients-4wsmaraeuhppze2>)

Jacobus Albertus Auwyang

 Views: 23 Downloads: 13


Assessment of parenteral dosage forms course objectives including objective structured practical examination by E-learning method (<https://japer.in/article/assessment-of-parenteral-dosage-forms-course-objectives-including-objective-structured-practical-exa-iz706ccygh9nboy>)

Faten Hamed

 Views: 21 Downloads: 8


Does on-line learning affect to achieve the minimal competencies of entry level pharmacists in Indonesia? p. 21-25 (<https://japer.in/article/does-on-line-learning-affect-to-achieve-the-minimal-competencies-of-entry-level-pharmacists-in-indon-hiqys3un9o1oycu>)

Agung Endro Nugroho

 Views: 29 Downloads: 11


Evaluation of the characteristics of iron metabolism markers in patients with CHF-related anemia in Jordan p. 26-33 (<https://japer.in/article/evaluation-of-the-characteristics-of-iron-metabolism-markers-in-patients-with-chf-related-anemia-in-eppneusksh6vm7y>)

Derar H. Abdel-Qader

 Views: 17 Downloads: 6


Enhancement of Icaria aphrodisiac effect by self nano emulsifying drug delivery system (SNEDDS) method p. 34-39 (<https://japer.in/article/enhancement-of-licaria-aphrodisiac-effect-by-self-nano-emulsifying-drug-delivery-system-snedds-met-lxwnijmai8kzhab>)

Septiana Indratmoko

 Views: 29 Downloads: 13


Comparison of caffeine content in different types of coffee in Saudi Arabia (<https://japer.in/article/comparison-of-caffeine-content-in-different-types-of-coffee-in-saudi-arabia-lrm-oc7jv8j5qadbvpy>) p. 40-44

Lina Abdulkareem Baz

 Views: 35 Downloads: 13

Chromatogram profile of andrographolide-glucuronate and andrographolide glycine in rabbit feces extract p. 45-51 (<https://japer.in/article/chromatogram-profile-of-andrographolide-glucuronate-and-andrographolide-glycine-in-rabbit-feces-extr-p8qq1vxlbyayog>)

Theresia Ratnadevi

 Views: 23 Downloads: 8

CHATGPT4 (AI) shaping the future of medical laboratory sciences by improving teaching, learning, and assessment p. 52-55
([https://japer.in/article/chatgpt4-ai-shaping-the-future-of-medical-laboratory-sciences-by-](https://japer.in/article/chatgpt4-ai-shaping-the-future-of-medical-laboratory-sciences-by-improving-teaching-learning-and-ovbnemmh5upiczn)

[improving-teaching-learning-and-ovbnemmh5upiczn](https://japer.in/article/chatgpt4-ai-shaping-the-future-of-medical-laboratory-sciences-by-improving-teaching-learning-and-ovbnemmh5upiczn))

Makhoahle Pakiso Moses



Views: 22 Downloads: 7

Analysis of online health information seeking among HIV patients in Indonesia p. 56-62

([https://japer.in/article/analysis-of-online-health-information-seeking-among-hiv-patients-in-indonesia-](https://japer.in/article/analysis-of-online-health-information-seeking-among-hiv-patients-in-indonesia-vndgywchvmegwq)

[vndgywchvmegwq](https://japer.in/article/analysis-of-online-health-information-seeking-among-hiv-patients-in-indonesia-vndgywchvmegwq))

Hesty Utami Ramadaniati



Views: 26 Downloads: 9

Knowledge, attitude, and practice of menstrual hygiene at a medical and health sciences university p. 63-72

([https://japer.in/article/knowledge-attitude-and-practice-of-menstrual-hygiene-at-a-medical-and-health-](https://japer.in/article/knowledge-attitude-and-practice-of-menstrual-hygiene-at-a-medical-and-health-sciences-university-lq1towkefazke3z)

[sciences-university-lq1towkefazke3z](https://japer.in/article/knowledge-attitude-and-practice-of-menstrual-hygiene-at-a-medical-and-health-sciences-university-lq1towkefazke3z))

Rajani Dube



Views: 38 Downloads: 13

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(<https://japer.in/journal/archives>)



Current Issue

(<https://japer.in/issue/vol-14-issue-1-2024-5aon>)

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Antibacterial activity, cytotoxicity, and phytochemicals screenings of binahong (*Anredera cordifolia* (Ten.) steenis) leaf extract

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ABSTRACT

Streptococcus mutans can cause caries in human teeth. Currently, people are starting to turn to herbal plants, one of which is the binahong plant known to be used as an antibacterial. Herbal plants must have biocompatibility properties with the cell in the body. To determine the antibacterial effectiveness of the ethanol extract of binahong leaves against *S. mutans* and to determine its toxic effect on fibroblast cells. The antibacterial activity of binahong leaf extract was evaluated by the agar diffusion method (Kirby-Bauer). The method used for the cytotoxicity test in this study is the MTS Assay method. The phytochemical screening test of the sample ethanol extract of binahong leaves contained secondary metabolites of tannins, flavonoids, saponins, and triterpenoids. The results showed that binahong can be used as an antibacterial with an average inhibition zone diameter of 14.513mm at a concentration of 100%. There was no cytotoxicity effect on fibroblast cells in vitro. The IC₅₀ result of 983.88g/mL is safe to use as a limit value. Binahong plant which contains active biological components has significant antibacterial activity against *S. mutans* and a very low toxic effect on fibroblast cells, so it can be used and developed as an ingredient of natural antibacterial.

Keywords: Antibacterial, Binahong leaf, Caries, Cytotoxicity, *Streptococcus mutans*

Introduction

The oral cavity is an integral part of the body, which is an ideal place for the growth of bacteria that have an important role in the body. However, if this environment is not balanced, then bacteria that were originally not pathogenic can become pathogenic and cause disease in the oral cavity [1, 2]. One of the diseases that can occur in the oral cavity is caries. Caries is a

multifactorial infectious disease caused by three main factors, namely agents or microorganisms, host factors, substrates or diet, and coupled time factors [3]. In the occurrence of dental caries, microorganisms have a very important role. One of the pioneer microorganisms that can cause dental caries is *Streptococcus mutans*, which is the most cariogenic bacteria [4]. These bacteria will ferment sucrose into lactic acid which will cause the pH in the oral cavity to be low, thus causing a demineralization process [5].

To prevent caries, many antibacterial agents have been developed recently. However, many of these antibacterial agents can cause cell death in the oral mucosa which in turn can lead to a decrease in the body's defense mechanisms. Therefore, caries prevention agents need to be developed namely, agents that have an antibacterial effect but their effect on oral tissues must be evaluated so that they are safe for cells in oral tissues [1]. One of the dominant cells in connective tissue in the oral mucosa is

Access this article online

Website: www.japer.in

E-ISSN: 2249-3379

How to cite this article: Sugiawan VK, Pranata BMD, Susila RA, Pranata N, Rahmawati DY. Antibacterial activity, cytotoxicity, and phytochemicals screenings of binahong (*Anredera cordifolia* (Ten.) steenis) leaf extract. J Adv Pharm Educ Res. 2024;14(1):1-7. <https://doi.org/10.51847/BXxQtsSI1s>

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fibroblasts which function to produce an extra matrix of connective tissue cells. Fibroblasts are also the largest cell component that composes dental pulp, periodontal ligament, and gingiva [6, 7]. Therefore, one of the efforts that can be developed is by utilizing medicinal plants that contain various biologically active components that can act as antibacterial, not antibacterial. toxic to cells, and can regenerate cells with minimal side effects [4].

The use of medicinal plants is growing rapidly among the people of Indonesia because these plants are efficacious for curing various kinds of diseases [8]. In Indonesia, the drug industry has succeeded in utilizing about 300 types of plants from around 9,600 types of plants that have the potential to be developed into medicinal plants [9]. One of the plants that can be developed into medicinal plants is the binahong leaf (*Anredera cordifolia* (Ten.) Steenis) [8]. Binahong is a medicinal plant from mainland China since the 14th century which is known by the original name *dheng san chi*, all parts of the plant can be used, starting from the roots (tubers), stems, and leaves. Binahong leaf was chosen in this study because this leaf is an herbal plant that contains several active compounds, including tannins, flavonoids, saponins, and triterpenoids, and is widely found in Southeast Asia and the tropical region of the United States [10, 11].

The active compound acts directly as an antibacterial against *Streptococcus mutans* by interfering with its function so that the growth of the bacteria that causes dental caries will be inhibited [12, 13]. Where the active compound derived from the binahong leaves must have good biocompatibility properties, both locally and systemically [14].

The purpose of this study was to determine the antibacterial effect of binahong leaf extract against *S. mutans* and to determine its toxic effect on fibroblast cells so that the use of binahong leaf extract in preventing caries would be safe for tissues in the oral cavity.

Materials and Methods

Binahong leaf ethanol extract (BLE) production

Binahong leaves used in this study were obtained from the Agricultural Technology Research and Development Installation Manoko, Lembang, West Java, Indonesia. The selected plants were planted at an altitude of 1.200 above sea level, with an age of about 6 months. Plant determination was carried out in the Botanical Division of the BRIN Biological Research Center, Cibinong, Bogor City, West Java, Indonesia. BLE was made by the maceration method using 70% ethanol solution and carried out a phytochemical test.

Making streptococcus mutans growing media

The growing media used in this study were Mueller Hinton Agar (MHA) (Himedia, M173) and Mueller Hinton Broth (MHB) (Himedia, M391).

BLE preparation for antibacterial test

The stock solution of BLE used was prepared by dissolving 3000mg of extract paste in 3 mL of 10% DMSO. Preparation of Working Solution (WS) Series: The stock dilution of the BLE was carried out using 10% DMSO to make a concentration series are 100%, 75%, 50%, 25%, 12.5%, 6.25%, and 3.125%.

Preparation of streptococcus mutans inoculum (ATCC 25175®TM)

The inoculum was obtained by inoculating *Streptococcus mutans* colonies that had been cultured for 24 hours in MHA medium into MHB medium. The turbidity of the solution was then adjusted to the turbidity of the standard McFarland 0.5.

Agar disk diffusion

Dip a sterile cotton swab into the bacterial suspension and wipe it on the surface of the MHA. Place a 6mm paper disc on the plate, and drop 10µL of each extract concentration, 0.1% Clindamycin, and 10% DMSO, doing 3 repetitions. Then incubate (Thermo IH3543) the agar plate at 37°C for 24 hours. Measure the diameter of the inhibition zone formed using a caliper (Modern, Sigma 6”).

According to Davis and Stout, the criteria for antibacterial strength based on the diameter of the inhibition zone are divided into several categories [15];

Weak: 5 mm or less.

Medium: 5-10 mm.

Strong: 10-20 mm.

Very Strong: more than 20 mm.

BALB/3T3 thawing cell procedure (ATCC®CCL-163)

Take the cell from the liquid nitrogen tank (-196°C) and a 3T3/BALB cell culture medium was prepared by mixing 10% FBS, 1% Antibiotic-Antimycotic, 1% Amphotericin B, 1% MEM Vitamins, 1% L-Glutamine, 0.2% Nanomycopolitine, 0.1% Gentamicin and DMEM High Glucose basal medium up to 100% of the total volume in a 50mL tube. Cells were centrifuged (MW260r) at 1600rpm for 5 minutes, and then the pellet was resuspended with 4mL of culture medium. The cell suspension was placed in flask T25 and was incubated (Thermo IH3543) at 5% CO₂, at 37°C, and observed under an inverted microscope (Olympus CKX-41-F32FL) until the confluent was about 70-80%.

BALB/3T3 cell subculture procedure

The culture medium was removed and then rinsed with PBS. Add 2mL of Trypsin-EDTA, and then incubate for 3 minutes, CO₂ 5% at 37°C. Cells were centrifuged at 1600rpm for 5 minutes. Use 1 mL of culture medium to resuspend the pellets. then divided into 2 T25 flasks and incubated at 5% CO₂, 37°C. Every 2-3 days, during cell treatment, the growth medium is replaced.

Preparation of ble sample solution for cytotoxicity test

The stock solution of BLE used was prepared by dissolving 50mg of extract paste in 1 mL of 100% DMSO. Preparation of WS Series: The stock dilution of the BLE was carried out using 10% DMSO to make a concentration series, which are 5.000µg/mL, 2500µg/mL, 1250µg/mL, 625µg/mL, 312.5µg/mL, 156.25µg/mL, 78.13µg/mL, and 39.06µg/mL.

Cytotoxicity test procedure

Hemocytometer (Neubauer) was used to count cells and cells were grown at a density of 5x10³cells/well on a 96-well plate, then incubated (Thermo IH3543) for 24 hours at 37°C, 5%CO₂. The sample was added to each well plate, then incubated for 24 hours at 37°C, 5% CO₂, and added to each well plate 20µl of MTS, incubated for 3 hours at 37°C, 5% CO₂. The absorbance was measured using spectrophotometry (Multiskan GO Thermo Scientific 51119300) and read at a wavelength of 490nm.

IC₅₀ calculation

IC₅₀ calculation in this study uses probit regression analysis using IBM SPSS Statistics version 26.

Statistics

The test to prove the antibacterial effectiveness of the ethanol extract of binahong leaves against Streptococcus mutans used the Kruskal Wallis non-parametric test, while to prove its toxic effect, the One Way Anova test was used with the Shapiro-Wilk normality test (p>0.05), which was then followed by Dunnett T3 Post Hoc. Test.

Results and Discussion

Determination test results

The results obtained from the determination test are:

Scientific Name: *Anredera cordifolia* (Ten.) Steenis

Local Name: Binahong

Tribe/Family: Basellaceae

Phytochemical screening test results

The results of the phytochemical screening test of the sample showed that the ethanolic extract of binahong leaves contained

secondary metabolites of tannins, flavonoids, saponins, and triterpenoids.

Table 1. Results of Phytochemical Screening of Binahong Leaf Ethanol Extract

No	Secondary Metabolites	Test Method	Test Results
1	Tanin	Reagent FeCl ₃ 1%	++
2	Flavonoid	a. Reagent concentrated HCl+Mg	-
		b. Reagent H ₂ SO ₄ 2N	+
		c. Reagent NaOH 10%	+
3	Saponin	Heated	++
4	Triterpenoid	Reagent-concentrated H ₂ SO ₄ +anhydrous CH ₃ COOH	+

(+): A little; (++) : Medium; (+++) : Many; (-) : There isn't any

The antibacterial Effectiveness of BLE against Streptococcus mutans with the average (mean) and standard deviation (stdev) is presented in **Table 2**.

Table 2. Inhibitory Zone Diameter of BLE

Group	Inhibition Zone Diameter		Inhibition Zone Category (Davis-Stout)
	mean	stdev	
Positive Control	14.570	0.233	Strong
Negative Control	0.000	0.000	Weak
BLE 3,125%	0.000	0.000	Weak
BLE 6,25%	0.000	0.000	Weak
BLE 12,5%	7.133	0.252	Medium
BLE 25%	8.753	0.197	Medium
BLE 50%	9.500	0.737	Medium
BLE 75%	11.970	0.070	Strong
BLE 100%	14.513	0.096	Strong

Antibacterial Effectiveness Test Results The antibacterial effectiveness test of the ethanol extract of binahong leaves (BLE) against Streptococcus mutans with the average (mean) is presented in **Figure 1**.

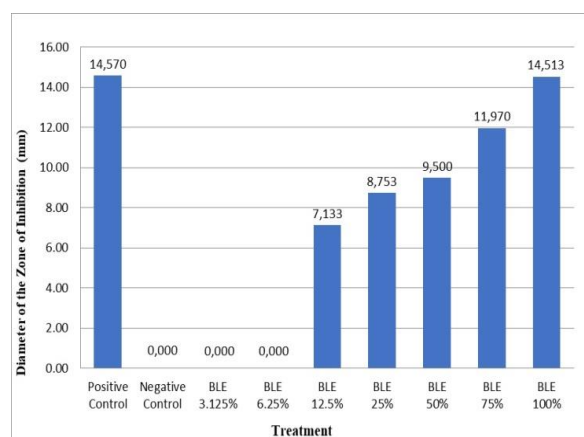


Figure 1. Average Inhibition Zone Diameter of Binahong Leaf Ethanol Extract (BLE)

Based on **Table 2** and **Figure 1**, it can be seen that the average diameter of the inhibition zone of the ethanol extract of binahong leaves in the positive control group was 14.57mm, and the average inhibitory power in the 100% BLE group was 14.513mm, the 75% BLE group was 11.970mm, the average of the BLE 50% group was 9.5mm, the average in the 25% BLE group was 8.753mm and the average in the 12.5% BLE group was 7.133mm. This indicates that the 100% BLE group is closest to the average diameter of the positive control group (**Figure 2**).

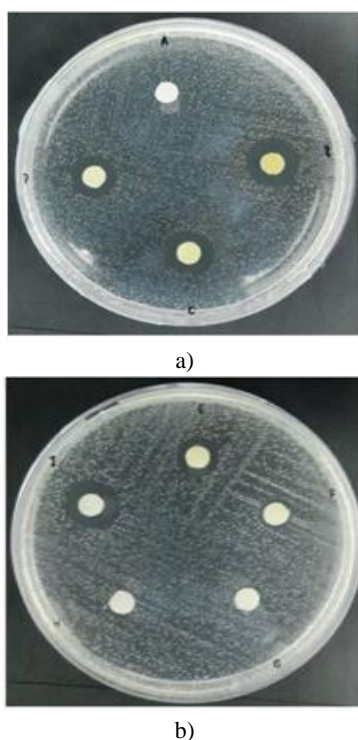


Figure 2. Observation of the zone of inhibition against *Streptococcus mutans*
Description: A: Negative Control (DMSO 10%), B: BLE 100%, C: BLE 75%, D: BLE 50%, E: BLE 25%, F: BLE 12.5%, G: BLE 6.25%, H: BLE 3.125%, I: Clindamycin 0.1%

From the results that have been obtained and processed statistically, it can be seen that the Chi-Square obtained is 25.451 > Chi-table 15.507, so it can be concluded that there are significant differences in antibacterial effectiveness between the concentration groups of binahong leaf ethanol extract. So, the higher the concentration of ethanol extract of binahong leaves, the higher the inhibitory power in inhibiting the growth of *Streptococcus mutans* bacteria.

Cytotoxicity test results

The results of measuring the viability of fibroblast cells that have been treated with BLE can be seen in **Table 3**.

Table 3. Cytotoxicity Test Results of BLE on BALB/3T3 cells

Treatment	Cell Viability (%)	Cell Inhibition (%)
Negative Control	100.00 ± 1.21 ^g	0.00 ± 1.21 ^a
Positive Control DMSO 1%	93.48 ± 1.32 ^{hcd}	6.52 ± 1.32 ^{de}

BLE 500µg/mL	84.07 ± 0.73 ^{ab}	15.93 ± 0.93 ^f
BLE 250µg/mL	87.92 ± 0.87 ^{abc}	12.08 ± 0.87 ^{ef}
BLE 125µg/mL	92.51 ± 0.14 ^{bc}	7.49 ± 0.14 ^{de}
BLE 62.5µg/mL	94.91 ± 0.26 ^{de}	5.09 ± 0.26 ^{cd}
BLE 31.25µg/mL	96.31 ± 0.29 ^f	3.69 ± 0.29 ^b
BLE 15.63µg/mL	99.05 ± 0.32 ^g	0.95 ± 0.32 ^a
BLE 7.81µg/mL	100.95 ± 0.74 ^g	-0.95 ± 0.74 ^a
BLE 3.91µg/mL	106.84 ± 1.99 ^g	-6.84 ± 1.99 ^a

Data are presented in mean ±SD. Different superscript marks indicate a significant difference (p < 0.05) Dunnett T3 post hoc test.

In this study, it can be seen in **Figure 3**, that the number of living cells is inversely proportional to the increase in concentration. The concentration group of 3.91g/mL had the highest number of living cells with a viability of 106.84%, indicating that this concentration could trigger the growth and proliferation of fibroblast cells. This is supported by the percentage of inhibition in **Figure 4**, the ethanol extract of binahong leaves at a concentration of 7.81g/mL was -6.84%.

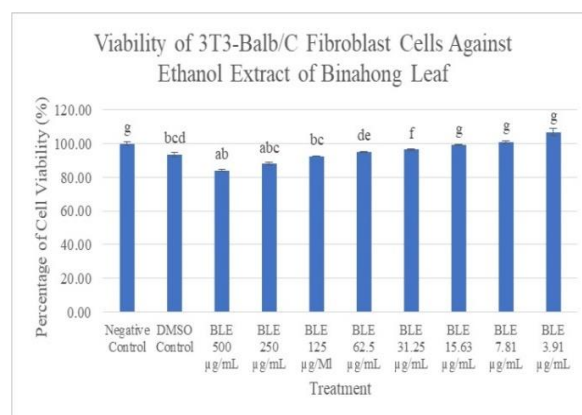


Figure 3. Viability of BALB/3T3 cells after being given the Binahong Leaf Ethanol Extract with various concentrations. The data are presented in the mean ±SD. Different superscript signs (a, ab,b,c,d) showed a significant difference (p < 0.05) Dunnett T3 post hoc test

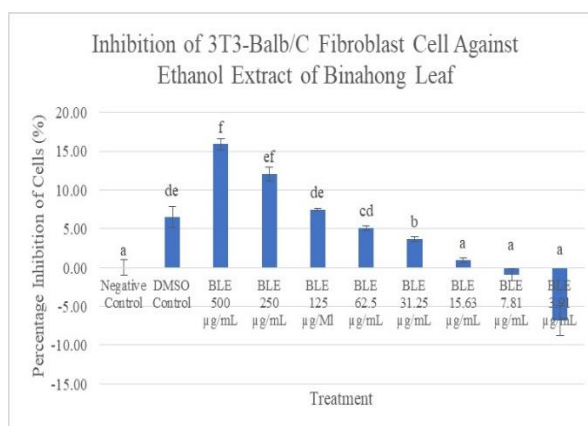


Figure 4. Percentage of inhibition of BALB/3T3 cells after being given Binahong Leaf Ethanol Extract with various concentrations. Data are presented in mean ±SD. Signs of different superscripts (a, ab,b,c, cd,d) showed significant differences (p < 0.05) Dunnett T3 post hoc test

The criteria for showing cytotoxicity can be seen in the classification according to ISO 10993-5: Biological Evaluation of Medical Devices, which states that if the relative cell viability for the extract concentration of a sample is less than 70%, then a substance or compound is considered to have toxic properties. So it can be concluded that the cell viability of the ethanol extract of binahong leaves which is still above 90% is considered a safe dose [16].

Calculation of IC_{50} value

The IC_{50} value is the potential of a substance in inhibiting biological functions up to 50%, the value is taken from the results of probit statistics with a probability of 0.5 (which means 50%), which means that the ethanolic extract of binahong leaves can inhibit 3T3-Balb/C fibroblast cells by 50% at a concentration 983.88g/mL.

The results of laboratory experimental research on the antibacterial effectiveness of the ethanolic extract of binahong leaves with various concentrations showed that the ethanolic extract of binahong leaves had an antibacterial effect which was indicated by its effectiveness in inhibiting the growth of *Streptococcus mutans* bacteria as presented in **Table 1**. Based on the results of qualitative phytochemical tests, ethanol extracts of Binahong leaves contain active compounds such as tannins, saponins, flavonoids, and triterpenoids. It is the content of the active compounds in the binahong leaves that can play an important role as an antibacterial and can inhibit the growth of *Streptococcus mutans* bacteria which is the bacteria that causes dental caries [12, 13].

The binahong plant used in this study was found to contain different amounts of flavonoid, triterpenoid, saponin, and tannin compounds. This can happen because it is influenced by internal factors and external factors, including biotic and abiotic conditions. Internal factors include genetic factors and physiological factors such as the level of development of a plant. Meanwhile, external factors are influenced by abiotic environmental factors, namely climate and biotic environment such as interactions with other organisms, and are influenced by geographical conditions of environmental stress that induce secondary metabolite biosynthesis or defensive responses [17, 18]. Climate change such as temperature, drought, CO_2 , light, salinity, UV light, ozone, and other factors can cause changes in plant metabolism which will affect the secondary metabolites they contain. Therefore it can be said that each plant species requires special environmental conditions for its growth [19, 20]. Tannin compounds are one type of compound that is included in the polyphenol group and has properties that are soluble in water and organic solvents that are often found in plants. This compound has a phenol group that is antiseptic and can form complex compounds with proteins in bacteria through hydrogen bonds. The formation of hydrogen bonds between tannins and proteins is what allows bacterial cell proteins to be denatured so that bacterial metabolism is disrupted. Tannins also have toxic properties, so these compounds can damage bacterial cell

membranes and are thought to cause shrinkage of the cell wall or bacterial cell membrane, thereby disrupting the permeability of the cell itself, and ultimately the cell cannot carry out its activities [21, 22].

As an antibacterial, saponin compounds will be associated with lipopolysaccharides in the bacterial cell wall. This will decrease the surface tension and increase the permeability of the cell wall so that antibacterial substances can easily enter the cell. This will cause an interaction that causes the cell wall to undergo lysis and result in bacterial death [23].

Antibacterial effects can also occur due to the presence of flavonoid compounds, where these compounds will inhibit cytoplasmic membrane function, nucleic acid synthesis, and the energy metabolism of bacteria [24]. Flavonoids can form complexes with bacterial cell walls, and extracellular proteins, and can damage bacterial cell membranes because flavonoid is lipophilic [25]. Flavonoids in some plants are known to have antibacterial properties by releasing transduction energy to the bacterial cytoplasmic membrane and inhibiting bacterial motility, that the hydroxyl group in the flavonoid structure causes changes in organic components and nutrient transport which can eventually lead to the toxic effect on bacteria [23].

Triterpenoid as an antibacterial has a mechanical action is on the outer membrane of the bacterial cell wall by reacting with the porin (transmembrane protein), forming a strong polymer bond, resulting in the destruction of the porin. Damage to the porin will reduce the permeability of the bacterial cell wall which is the entrance and exit of the compound and cause the bacterial cell to lack nutrients so that bacterial growth is inhibited or dead [26].

In addition to knowing the role of the biologically active content as an antibacterial of this medicinal plant, the toxic properties of cells and tissues also need to be considered before this plant will be used in humans [27]. Therefore, a cytotoxicity test on fibroblast cells is needed to directly determine the toxic effect of a substance on tissue culture and observe cell growth, reproduction, and morphological effects. The principle of this test is to measure the ability of cell viability in a material [28, 29]. The mechanism of cytotoxicity of a substance to cell culture occurs through the destruction of cell membranes, prevention of protein synthesis, binding to cell receptors, inhibiting polydeoxynucleotide elongation, and enzymatic reactions. The parameter of this test is the value of inhibition concentration (IC_{50}). This value indicates the concentration of extracts or ingredients that can inhibit cell proliferation by 50% in the total population [29].

The results of this study indicate that the average dose used in this test is still within a safe dose, based on the classification according to ISO 10993-5: Biological Evaluation of Medical Devices, which states that the relative cell viability for the extract concentration of a sample is more than 70% then a substance or compound is considered not to have toxic properties [16]. The determination of IC_{50} is a measure of drug efficacy that indicates the amount of concentration of a chemotherapeutic agent required to inhibit biological processes up to 50%. IC_{50} obtained in this study can be

used as a reference for further research on the potency of the extract. binahong leaf ethanol.

In this study, the IC₅₀ value was 983.88 g/mL, thus the IC₅₀ result of the ethanolic extract of binahong leaves was safe to use as a limit value because it did not interfere with cell proliferation. If the dose used exceeds the IC₅₀ value, it can disrupt the cell proliferation phase, because fibroblast migration does not occur or even fibroblasts die. So it can be said that the concentration of ethanol extract from binahong leaves used in this study was relatively safe because the cell viability value was still above 90%. In addition to having an antimicrobial effect, the active compound content of binahong leaves can also play a role in the tissue healing process because when used in certain doses it is not toxic and has a role as an antioxidant, anti-inflammatory, and antibacterial so that it can be used in the wound healing process [30]. Saponin compounds can increase monocyte proliferation thereby helping to increase the number of macrophages that will secrete growth factor TGF-β1 which will play a role in various phases of wound healing [31, 32].

The content of tannins and triterpenoids which act as antibacterial also acts as an astringent in wounds and helps in the differentiation process of myofibroblasts [33, 34]. The content of flavonoids can inhibit the formation of prostaglandins formed by arachidonic acid and other inflammatory mediators such as histamine and serotonin. The high content of flavonoids in binahong leaves plays a role in the cell proliferation phase during the wound healing process by increasing the process of mitogenesis, cell interactions, and molecular adhesion [31].

Conclusion

Binahong plant (*Anredera cordifolia* (Ten.) Steenis) which contains active biological components of tannins, flavonoids, saponins, and triterpenoids has significant antibacterial activity against *S. mutans* and a very low toxic effect on fibroblast cells, so it can be used and developed as an ingredient. natural antibacterial and helps in the process of repair and regeneration.

Acknowledgments: The author would like to thank Maranatha Christian University, Indonesia

Conflict of interest: None

Financial support: The present study was supported by Maranatha Christian University Indonesia.

Ethics statement: None

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