

Antibacterial Activity of *Euphorbia tirucalli* against *Lactobacillus acidophilus*: An In Vitro Study

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ABSTRACT

Dental caries is a common oral disease that occurs due to excessive interaction between tooth structure, bacterial biofilm, and glucose. *Lactobacillus acidophilus* is the main cause of dentin dental caries, which can be prevented using anticaries agents. However, current anticaries agents may cause some side effects. Therefore, there is a need for natural anticaries agents with minimal side effects. *Euphorbia tirucalli* contains flavonoids, tannins, and saponins that contribute to its antibacterial activity. This study aimed to determine the antibacterial activity of *E. tirucalli* against *L. acidophilus* bacterial growth. This was an in-vitro study with a post-test-only control group design, divided into three groups. The positive control group received 0.2% chlorhexidine digluconate, the negative control group received aquadest, and the intervention groups received extracts with concentrations of 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, and 100 mg/mL. Minimum Inhibitory Concentration (MIC) was examined post-treatment using the agar dilution method. The differences in colony growth were analyzed using Cramer's V and Fisher's Exact Test, with $p < 0.05$ considered significant. *E. tirucalli* extracts showed antibacterial effects against *L. acidophilus* with a concentration of 40 mg/mL as the MIC. Extract groups with concentrations of 40 mg/mL and 100 mg/mL showed significant differences in *L. acidophilus* growth compared to the negative control group ($p < 0.001$). The study concluded that *E. tirucalli* extract has antibacterial activity against the growth of *L. acidophilus*, with MIC of 40 mg/mL.

Keywords: Antibacterial activity; Anticaries; Caries; *Euphorbia tirucalli*; *Lactobacillus acidophilus*

INTRODUCTION

Caries is a multifactorial disease mediated by biofilms. Dental caries arises from excessive interactions between tooth structure, microbial biofilms that form on tooth surfaces, and glucose. The dynamic process of caries consists of rapid alternation between demineralization and remineralization. If demineralization adequately removes hydroxyapatite crystal mineral ions, it can initiate carious lesions in the tooth structure (Pitts et al., 2017).

Dental caries is the most common oral disease in the world (Holm-Pedersen et al., 2015). Half of the world's population suffers from caries and their quality of life can be directly affected, as caries can cause pain, loss of tooth structure, and even loss of teeth, which can cause damage to the aesthetic appearance and function of the mouth (Liao et al., 2021; WHO, 2017). According to 2018 Riskesdas, Indonesia has a caries prevalence of 88.8% (Riset Kesehatan Dasar (Riskedas) 2018).

The process of dental caries begins with the presence of oral microorganisms

(such as *Streptococcus sanguinis* and *Actinomyces sp.*) that gather on the teeth surfaces, allowing cariogenic microorganisms (such as *S.mutans* and *Lactobacillus sp.*) to attach and form a sticky biofilm called dental plaque (Singh et al., 2019). *Lactobacillus* in dental plaque will hydrolyze glucose from foods and drinks to produce acid which can cause a decrease in pH to less than 4.5. The acid produced can diffuse into the pores of the enamel and dentin. If it encounters a mineral that can absorb acid, the mineral will immediately be broken down and produce a cavity in the teeth after several months (Pitts et al., 2017; Samaranyake 2018; Singh et al., 2019).

Lactobacillus can be found in the oral cavity and saliva in individuals with active caries (Singh et al., 2019). The most common *Lactobacillus* that causes dental caries is *L. acidophilus* (Wijaya et al., 2017). Furthermore, *L. acidophilus* is the most common bacteria found in dental caries that have reached the dentin (Widjiastuti et al., 2017).

Caries can be suppressed using anticaries agents. In general, the anticaries effect can be found in several chemicals such as chlorhexidine, but there are side effects from the use of these chemicals (Liao et al., 2021). Side effects that are

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commonly discovered include dry mouth (xerostomia), discolored tongue, and changes in taste sensations, especially salty and bitter (Brookes et al., 2020). These side effects have led to many innovations of natural anticaries agents from plant extracts, which are added to oral health products such as toothpaste and mouthwash to increase their antibacterial properties (Chen et al., 2020).

E. tirucalli is a species belonging to the *Euphorbiaceae* family. *E. tirucalli* twigs extract has activity as an anthelmintic, antirheumatic, analgesic, antioxidant, antiviral, antifungal, and antibacterial due to the diversity of bioactive constituents it contains (de Lima et al., 2021; Mali & Panchal 2017; Mohamad 2017).

The composition of the ethanol extract from *E. tirucalli* contains gallic acid, gallic acid derivatives, and flavonoids (de Lima et al., 2021). The extract of *E. tirucalli* also contains tannins, polyphenols, and saponins which have antimicrobial properties that can inhibit the growth of bacteria, including those that cause caries (Goldberg 2016; Mohamad 2017; Sugumar et al., 2010). The mechanism of bacterial inhibition by each active component is different: tannins can have a toxic effect on bacteria by increasing the bacterial hydroxylation process (Cushnie et al., 2014), while polyphenols inhibit bacterial growth by inhibiting c-di-AMP which controls various functions in bacteria (Opoku-Temeng & Sintim 2016).

Previous study has shown that *E. tirucalli* twig extract can suppress the growth of caries-causing bacteria, such as *S. mutans* and *S. sobrinus* (Mohamad 2017). However, previous research has not proven that *E. tirucalli* can suppress the growth of *L. acidophilus*. Therefore, further studies on antibacterial mechanisms from plants are still needed (Chen et al., 2020). In this study, concentrations of 10, 20, 30, 40, and 100 mg/mL of *E. tirucalli* twigs extract were chosen based on previous studies (Mohamad 2017).

MATERIALS AND METHODS

Instruments and Materials

Materials used for this study are *E. tirucalli* twigs, *L. acidophilus*, aquadest, 0.2% chlorhexidine digluconate (*Minosep*), MRS agar media powder (*Merck*), 96% ethanol, filter paper (*Whatman*), NaCl solvent, sterile cotton swabs, labels, and candle. The tools used for this study are Petri dishes (*Pyrex*), autoclave (*GEA*), stirrers, hotplate (*Boeco*), erlenmeyer flasks (*Pyrex*), micropipettes (*Socorex*), sterile yellow tips, sterile containers, measuring cups (*Herma*), test tubes (*Pyrex*), sterile ose, bunsen lamp, vortex mixer (*Boeco*), incubator

(*Memmert*), grinder (*L69D1AA101096*), vacuum rotary evaporator (*IKA*), McFarland 0.5 solvent, water bath (*Memmert*), lighter, glass jar, marker, and scales (*Mettler Toledo*).

Preparation of *E. tirucalli* Twigs Extract.

The *E. tirucalli* plant used originated from Semarang, Central Java, Indonesia, and aged approximately 12 years. The twigs were collected from a single brood weighing approximately 2 kilograms, and then dried in an oven at 60°C for approximately 48 hours or until fully dry. The dried twigs were crushed using a grinder to form simplicia. About 208 grams of simplicia were placed into the maceration vessel and 1000 mL of 96% ethanol solvent was added. The mixture was stirred several times and left to macerate for 3x24 hours. After maceration, the extract was filtered using Whatman paper to separate the filtrate and residue. The filtrate was evaporated using a vacuum rotary evaporator at a temperature of 45 to 50°C with a pressure of 0.08 MPa until a thick extract was obtained.

Preparation of Agar Media Solution

Sterile containers are labeled for each group. Approximately 18.6 grams of MRS agar powder were dissolved in 300 mL of aquadest. The mixture was stirred using a stirrer while heating on a hotplate until the powder was completely dissolved, and the solution came to a boil. The media was then sterilized using an autoclave for 15 minutes at a temperature of 121°C and a pressure of 15 psi, then was cooled to a temperature of 50°C.

Preparation of *E. tirucalli* Twigs Extract

Group P1 (10 mg/mL) by mixing 250 mg of extract with 25 mL of media solution; Group P2 (20 mg/mL) by mixing 500 mg of extract with 25 mL of media solution; Group P3 (30 mg/mL) by mixing 750 mg of extract with 25 mL of media solution; Group P4 (40 mg/mL) by mixing 1000 mg of extract with 25 mL of media solution; Group P5 (100 mg/mL) by mixing 2500 mg of extract with 25 mL of media solution.

Preparation of Agar Media

The media mixed with the extract and control solution was transferred to a petri dish, dried, and stored in the refrigerator for one day before growing the bacteria.

Preparation of Bacteria Suspension

L. acidophilus was grown on MRS agar and incubated at 37°C for 48 hours. The bacterial culture of *L. acidophilus* was transferred to a sterile ose and suspended in sterile distilled water. The

suspension was adjusted to a turbidity of 0.5 McFarland, and 1 mL of the suspension was mixed with 9 mL of aquadest to obtain a bacterial concentration of 10^7 CFU/mL.

Agar Dilution Method for Determining MIC

The bacterial suspension was taken using a micropipette with a 1 μ L tip and placed onto the treated MRS agar medium. Each group was given 10 spots of bacterial suspension with a bacterial concentration of 10^4 CFU at each spot. The bacteria were left to dry and then incubated using facultative anaerobic technique (candle jar technique) at 37°C for 48 hours. Bacterial growth in each group was observed.

Phytochemical Test

Qualitative Phytochemical Test

Flavonoid Test

Initially, 50 mg of the sample was placed into a flask, to which 10 mL of 4N hydrochloric acid was added. Hydrolysis was then carried out using a rotary evaporator for 30 minutes. The sample was subsequently cooled and extracted using 5 mL of diethyl ether. The diethyl ether phase was collected and evaporated using nitrogen gas. A sample spot of 20 μ L was applied to a cellulose plate, along with the comparator. The plate was placed into a chamber, saturated with a mobile phase of butanol-acetic acid-water in a ratio of 3:1:1 and developed to the edge. The plate was allowed to dry and then examined under UV light, followed by the application of an aluminum chloride reagent.

Tannin Test

The sample was weighed, then extracted using 10 mL of diethyl-ether for 20 hours. The sample underwent drying using filter paper. The obtained residue was subsequently boiled in 5 mL of distilled water for 2 hours, cooled and filtered. A 10 μ L sample spot was applied to a silica gel plate. The plate was then positioned within a chamber saturated with a mobile phase composed of ethyl acetate, acetic acid, formic acid, and water in a ratio of 100:5:5:13. It was allowed to develop until reaching the plate's edge. After the development process, the plate was dried and examined under UV light, and then it was treated with a ferric chloride reagent.

Saponin Test

A quantity of 50 mg of the sample was weighed and placed into a flask, then 10 mL of 2N sulfuric acid was subsequently added. Hydrolysis was then initiated by using a rotary evaporator for 30 minutes. The sample was subsequently cooled

and extracted using 5 mL of chloroform. The chloroform phase was collected, evaporated with nitrogen gas, and reconstituted to 500 μ L. Then 10 μ L of it was spotted onto a silica gel 60 F254 plate. The plate was put into a chamber saturated with a mobile phase of chloroform and methanol in a ratio of 95:5 and developed to the edge. The plate was taken out, dried, and then examined under UV light. It was further treated by spraying the sample with anisaldehyde sulfuric acid reagent and heated to a maximum spot at a temperature of 110°C.

Quantitative Phytochemical Test

Flavonoid Test

A total of 50 mg of extract was mixed with 0.3 mL of 5% sodium nitrite. After a 5-minute interval, 0.6 mL of 10% aluminum nitrate was added, allowed to sit for another 5 minutes, and then 2 mL of 1 M sodium hydroxide was added. The next step involved adding distilled water until the total volume reached 10 mL in a volumetric flask. Standard dilutions were prepared at concentrations of 0.5, 1, 2, 5, 10, 25, 50, 75, and 100 ppm, and their absorbances were measured at a wavelength of 510 nm. Primary data was obtained from the absorbance of the quercetin reference solution, a calibration curve was constructed, and a linear regression equation ($y = bx + a$) was derived from the reference calibration curve. The results were expressed in milligrams per gram.

Tannin Test

A total of 50 mg of extract underwent a 20-hour extraction with diethyl ether. It was then filtered, and any remaining diethyl ether was evaporated. Subsequently, distilled water was added to the sample to achieve a total volume of 10 mL. A mL sample solution was then extracted and combined with 0.1 mL of Folin Ciocalteu reagent, followed by vortexing. After a 5-minute interval, 2 mL of 20% sodium carbonate was added, vortexed once more, and left to sit for an additional 5 minutes. Distilled water was subsequently added until it reached the 10 mL volume. Dilutions were prepared, starting at concentrations of 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 ppm. These dilutions were incubated for 30 minutes at room temperature, and the absorbance was measured at a wavelength of 760 nm.

Saponin Test

A total of 50 mg of the extract was mixed with 2 mL of 25% H_2SO_4 (sulfuric acid), then autoclaved for 120 minutes at 110°C. After autoclaving, the mixture was extracted with ether, filtered, and the resulting filtrate was left to dry. A mL of water was added, followed by vortex

extraction for 5 minutes. Then, 50 μ L of anisaldehyde was added, agitated, and allowed to stand for 10 minutes. The solution was then heated in a water bath at 60°C for 10 minutes, and water was added to achieve a final volume of 10 mL in a volumetric flask. Standard dilutions were prepared, starting from 12.5, 25, 50, 100, 200, and 400 ppm, and the absorbance was assessed at a wavelength of 435 nm.

RESULTS

The yield of *E. tirucalli* twigs extract obtained from the maceration of 208 grams of simplicia using 96% ethanol was 11.51 grams. The results of the phytochemical test showed that the *E. tirucalli* twigs extract positively contained flavonoids, tannins, and saponins. The highest content of the three compounds in *E. tirucalli* twigs extract is tannin with a percentage of 13.58% w/w (Table I).

The agar dilution test showed that extract concentrations of 40 mg/mL (group P4) and 100 mg/mL (group P5) could inhibit 100% growth of *L. acidophilus* at 10 spots each containing 10^4 CFU of bacteria. The results on the petri dishes in the P4 and P5 groups showed a significant difference when compared to the negative control group. In the negative control group, there was high-density growth of *L. acidophilus*, while in the P4 and P5 groups, there was no growth of *L. acidophilus*, similar to the positive control group. Petri dishes containing *E. tirucalli* twigs extract at a concentration of 10 mg/mL (group P1) showed bacterial growth at a lower density compared to the negative control group. The petri dish containing the extract at a concentration of 20 mg/mL (group P2) showed a lower density of bacterial growth compared to group P1.

The petri dish containing the extract at a concentration of 30 mg/mL (group P3) showed bacterial growth with a lower density than group P2 and was barely visible. The results showed that the minimum concentration of *E. tirucalli* twigs extract that could inhibit the growth of *L. acidophilus* bacteria was 40 mg/mL (Table II).

The material collection process produces 2000 grams weight of *E. tirucalli* twigs. The research continued with the drying process of *E. tirucalli* twigs using an oven at 60°C for 48 hours and the result was 208 grams weight of dried *E. tirucalli* twigs. These results indicated that the weight of the dried twigs is about 10% of the weight of fresh *E. tirucalli* twigs. Dried and finely ground *E. tirucalli* twigs were then extracted using 96% ethanol solvent, and the final result was a viscous extract weighing 11.59 grams. These

results indicated that the extract was about 6% of the dried *E. tirucalli* twigs.

E. tirucalli twigs extract was used as a sample for phytochemical tests. The results of the qualitative phytochemical test of this study showed that the ethanol extract of *E. tirucalli* twigs contained flavonoids, tannins, and saponins. These results are similar to previous studies (Manongko et al., 2020; Mohamad 2017), but previous studies did not mention the percentage of these contents.

The results of the quantitative phytochemical test in this study showed that the ethanol extract of *E. tirucalli* twigs contained 13.58%w/w of tannins, 2.66%w/w of flavonoids, and 1.88%w/w of saponins. The content of flavonoids in this study (2.66% w/w or equivalent to 26,600 ppm) was greater than the content of flavonoids in previous studies (824.15 ± 25.55 ppm) (Le et al., 2021). Differences in the method of quantifying the flavonoid can lead to different interpretations of results, as well as differences in the origin and growth environment of *E. tirucalli* plants, which may contribute to the occurrence of such discrepancies.

In this study, tannins showed the highest amount of content, which can be attributed to their polarity, causing them to dissolve maximally in ethanol solvents (Halimu et al., 2017). Flavonoids and saponins exhibited a smaller amount than tannins, which is also influenced by polarity. Flavonoids are categorized into two types of polarity, namely flavonoid glycosides and aglycones are flavonoids with polar properties, while isoflavones, flavones alcohols, and flavonols have non-polar properties (Hendryani et al., 2015). Saponins, which are polar compounds that can dissolve in ethanol solvents, also have aglycone or sapogenin, a hydrophobic group that makes them non-polar (Agustina et al., 2017). Flavonoids and saponins are not entirely polar, allowing these two ingredients to not dissolve optimally in ethanol and show lower yields than tannins.

To test their activity against the growth of *L. acidophilus*, the flavonoids, tannins, and saponins content was determined. The agar dilution method was employed in this study, not only to determine the effect of *E. tirucalli* twigs extract on the growth of *L. acidophilus* but also to establish the lowest concentration of *E. tirucalli* twigs extract that could inhibit *L. acidophilus*' growth. The agar dilution method was carried out by instilling the test bacteria (*L. acidophilus*) with a concentration of 10^4 CFU per 1 μ l as much as 10 spots on MRS agar media, which had been mixed with *E. tirucalli* twigs extract at concentrations of 10 mg/mL, 20 mg/mL, 30 mg/mL, 40 mg/mL, 100 mg/mL (Golus et al., 2016; Weinstein 2018).

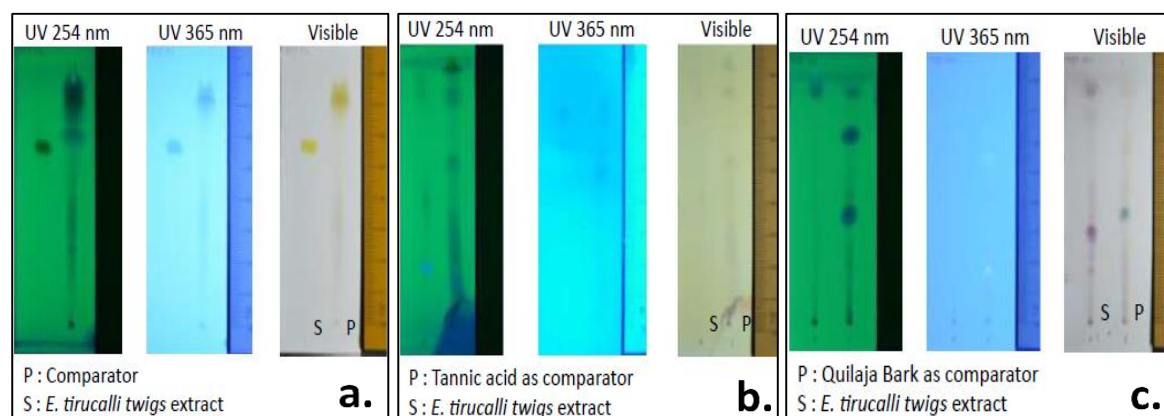


Figure 1. Results of the qualitative phytochemical test of *E. tirucalli* twigs extract

(a) Flavonoids. Flavonoid spots are visible in yellow, with a detected value of 0.85; (b) Tannins. Tannin spots are visible in grayish blue, with detected values of 0.33, 0.62, 0.88; (c) Saponins. Saponin spots are visible in bluish-green, with a detected value of 0.42.

Table I. Phytochemical Test Results of *E. tirucalli* Twigs Extract

Tested content	Existence	Percentage (%b/b)
Flavonoids (quercetin equivalent)	Positive	2.66
Tannins (tannin acid equivalents)	Positive	13.58
Saponins (from Quillaja bark)	Positive	1.88

%w/w is % weight/weight. %w/w = 1000 ppm.

Table II. Agar Dilution MIC Method Test Results

Group	Bacterial Growth (percentage)
K- (aquadest)	10/10 (100%)
K+ (0.2% chlorhexidine digluconate)	0/10 (0%)
P1 (10 mg/mL)	10/10 (100%)
P2 (20 mg/mL)	10/10 (100%)
P3 (30 mg/mL)	10/10 (100%)
P4 (40 mg/mL)	0/10 (0%)
P5 (100 mg/mL)	0/10 (0%)

The agar dilution method was preferred over the broth dilution method as the latter encountered several difficulties in antibacterial activity tests made from plant extracts using the liquid dilution test method. This can lead to misinterpretation of the results, with the main problem often being the difficulty of differentiating media turbidity and determining changes in turbidity due to bacterial growth, especially when the extract used has strong turbidity as in this study (Golus et al., 2016).

The agar dilution method was chosen for the antibacterial activity test with the raw plant extract ingredients because this method allows for a more even mixing of the extracts in the agar media. Additionally, this method enables the observation

of bacterial growth without the need for additional tools, as bacteria naturally form visible growth. Therefore, the detection of bacterial growth is not influenced by any color changes in the agar media. The agar dilution method provides a simpler and clearer determination of bacterial growth on the surface of agar media compared to the broth dilution method (Golus et al., 2016).

The results of this study demonstrated that the P4 group (extract concentration of 40 mg/mL) and P5 group (extract concentration of 100 mg/mL) in the petri dishes inhibited 100% of the growth of *L. acidophilus*. The results of the study in the P1 group (extract concentration of 10 mg/mL), P2 group (extract concentration of 20 mg/mL), and P3 group (extract concentration of 30 mg/mL)

exhibited a decrease in *L. acidophilus* density. The higher the concentration of the extract used, the lower the growth density of *L. acidophilus*.

Bacterial growth from each group was compared with the negative and positive control groups. In the negative control group (aquadest), *L. acidophilus* was observed, while in the positive control group (0.2% chlorhexidine digluconate), no growth of *L. acidophilus* bacteria was detected.

The results revealed that the *E. tirucalli* twigs extract had the highest tannin content compared to flavonoids and saponins, with tannins comprising 13.58%w/w, flavonoids comprising 2.66%w/w, and saponins comprising 1.88%w/w. This indicates that the growth of *L. acidophilus* is inhibited by tannins, which possess broad-spectrum antibacterial properties against both gram-positive and gram-negative bacteria. *L. acidophilus* belongs to the gram-positive bacteria group characterized by a cell wall consisting of a thick peptidoglycan layer. Tannins function by penetrating the cell wall and damaging the internal cell membrane of the bacteria (Kaczmarek 2020).

Tannins can disrupt bacterial attachment, leading to a decreased ability of bacteria to adhere to surfaces and eventually causing bacterial cell death. Tannins also hinder the absorption of glucose and amino acids necessary for bacterial growth, thus inhibiting bacterial growth and metabolism (Farha et al., 2020; Kaczmarek 2020). The effectiveness of tannins in inhibiting bacterial growth is higher in gram-positive bacteria compared to gram-negative bacteria, due to differences in the structure of the bacterial cell wall (Farha et al., 2020).

Flavonoids, which are secondary metabolites present in the *E. tirucalli* twigs extract, demonstrate effective inhibition of bacterial growth by forming complexes with extracellular cell wall proteins and disrupting bacterial membranes (Mohamad 2017). Additionally, the *E. tirucalli* twigs extract used in this study contains saponins. Saponins act as an antibacterial by causing lysis of the bacterial wall and leakage of the intracellular components from the bacterial cell (Khan et al., 2018).

The minimum inhibitory concentration (MIC) determined in this study was 40 mg/mL. The results demonstrated a decreasing trend in the growth density of *L. acidophilus* as the concentration of *E. tirucalli* twig extract increased. This indicates that the extract of *E. tirucalli* twigs has a dose-dependent effect on the growth of *L. acidophilus*.

DISCUSSION

In previous research, the effect of *E. tirucalli* twig extract on *S. mutans* and *S. sobrinus* was tested using the agar diffusion method. Previous researchers observed that *E. tirucalli* twig extract could inhibit the growth of *S. mutans* and *S. sobrinus* through the agar diffusion method by measuring the diameter of the inhibition zone caused by the extract. The study reported that concentrations of 20 and 30 mg/mL of *E. tirucalli* twigs extract exhibited an inhibitory effect on *S. mutans* and *S. sobrinus*, as indicated by an increase in the diameter of the bacterial inhibition zone with increasing concentration (Mohamad 2017).

The agar diffusion method is less precise for testing the antibacterial effect of the extract because the extract components tend to have reduced diffusion into the media. Additionally, volatile extract components can evaporate during incubation (Golus et al., 2016). Furthermore, the sensitivity assessment of the extract has not been standardized by the Clinical and Laboratory Standards Institute (CLSI) in the guide to Antimicrobial Susceptibility Testing.

This research has advantages and limitations. The advantages include the utilization of plant extract materials that are rarely studied, as well as the significant variation in bacterial growth density observed among each group. The study describes the extraction method based on the raw weight of the plant material and the concentration of the extract used in terms of weight per volume (mg/mL), which enhances reproducibility. Moreover, the researcher employed the agar dilution method which is, highly recommended for studies involving plant extracts to ensure accurate assessment of the MIC and to avoid confusion caused by high turbidity resulting from plant extracts.

However, this study has certain limitations. The minimum bactericidal concentration (MBC) of *E. tirucalli* twigs extract against *L. acidophilus* was not calculated. The treatment groups were limited to the extract form, and other antibacterial compounds beyond flavonoids, tannins, and saponins were not measured. Additionally, this study used crude extract. It is recommended for further research to use pure extracts of substances from *E. tirucalli*.

CONCLUSION

The extract of *E. tirucalli* twigs exhibits antibacterial activity against *L. acidophilus*, with a MIC of 40 mg/mL.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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