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Antibacterial Activity of Ethanol Extract of Thorn Spinach Leaves (*Amaranthus spinosus* L.) against *Streptococcus mutans*

Aktivitas Antibakteri Ekstrak Etanol Daun Bayam Duri (Amaranthus spinosus L.) terhadap Streptococcus mutans

Ria Dwi Setiani*, Guntur Trimulyono
Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya
*e-mail: ria.20017@mhs.unesa.ac.id

Abstract. Dental caries is a disease that attacks oral health that many people suffer from. Dental caries is caused by the activity of the bacteria *Streptococcus mutans*. Thorn spinach (*Amaranthus spinosus* L.) is a medicinal plant that has secondary metabolite compounds that can have antibacterial properties. This research aims to determine the antibacterial activity and optimal concentration in inhibiting the growth of *S. mutans*. The stages of this research include preparation, extraction, making sample solutions, phytochemical screening, rejuvenation and making bacterial suspensions, as well as antibacterial activity tests. The antibacterial activity test used extract concentrations of 20%, 40%, 60% and 80%. These extract concentrations produced an average diameter of the inhibition zone 0.25 ± 0.50 mm; 1.50 ± 2.38 mm; 5.13 ± 0.75 mm; and 6.38 ± 0.25 mm. Data were analyzed using the *Kolmogorov-Smirnov* test, *Duncan* test, and *Anova* test. Based on the analysis data, it was concluded that there was antibacterial activity at each extract concentration, the concentration that produced the highest average diameter was the 80% concentration, but statistically it was not significantly different from the 60% concentration. This research proves the ability of thorn spinach leaves to inhibit the growth of *S. mutans* which causes dental caries.
Keywords: antibacterial activity; inhibitory properties; *Amaranthus spinosus* L. extract; dental caries; *Streptococcus mutans*; crops.

Abstrak. *Karies gigi merupakan penyakit yang menyerang kesehatan mulut yang banyak diderita oleh masyarakat. Karies gigi disebabkan oleh adanya aktivitas bakteri Streptococcus mutans. Bayam duri (Amaranthus spinosus L.) merupakan salah satu tanaman obat yang memiliki senyawa metabolit sekunder yang dapat bersifat antibakteri. Penelitian ini bertujuan untuk mengetahui adanya aktivitas antibakteri dan konsentrasi optimal dalam menghambat pertumbuhan S. mutans. Tahapan pada penelitian ini meliputi preparasi, ekstraksi, pembuatan larutan sampel, skrining fitokimia, peremajaan dan pembuatan suspensi bakteri, serta uji aktivitas antibakteri. Uji aktivitas antibakteri menggunakan konsentrasi ekstrak 20%, 40%, 60%, dan 80%. Konsentrasi ekstrak tersebut menghasilkan rata-rata diameter zona hambat berturut-turut sebesar 0,25 ± 0,50 mm; 1,50 ± 2,38 mm; 5,13 ± 0,75 mm; dan 6,38 ± 0,25 mm. Data dianalisis dengan uji Kolmogorov-Smirnov, uji Duncan, dan uji Anova. Berdasarkan hasil analisis, disimpulkan bahwa terdapat aktivitas antibakteri pada tiap konsentrasi ekstrak. Konsentrasi yang menghasilkan rata-rata diameter tertinggi adalah konsentrasi 80%, namun secara statistik tidak berbeda nyata dengan konsentrasi 60%. Penelitian ini membuktikan adanya kemampuan daun bayam duri dalam menghambat pertumbuhan S. mutans penyebab karies gigi.*
Kata kunci: *aktivitas antibakteri; daya hambat; ekstrak Amaranthus spinosus L.; karies gigi; Streptococcus mutans; tanaman.*

INTRODUCTION

Dental caries is a major problem that attacks oral health, especially the teeth. The results of the 2018 Basic Health Research stated that the largest proportion of dental problems in Indonesia was dental caries, with a percentage of 45.3% (RI, 2018). Dental caries is caused by cariogenic foods (Afiati *et al.*, 2017). If treatment is not immediately carried out, this disease can cause pain, tooth damage and infection (Afrinis *et al.*, 2020). Dental caries is usually caused by the activity of the bacteria *Streptococcus mutans* (Nurani and Zakiyah, 2022).

Streptococcus mutans is a bacteria that can stick to the tooth surface and form a biofilm on the tooth surface (Rai *et al.*, 2020). *Streptococcus mutans* is able to form colonies that will stick to the tooth surface and result in tooth mineralization (Suryani *et al.*, 2019). These bacteria will attach to the tooth

surface with hydrophobic bonds which will then ferment sucrose and will result in acid production and cavity formation (Kayalvizhi *et al*., 2016). *Streptococcus mutans* causes the lining of the teeth to become destroyed (Zelnicek, 2014).

Dental caries is usually treated with antibiotics. Continuous administration of antibiotics causes antibiotic resistance (Seko *et al*., 2021). Shami *et al*., (2019) stated that *S. mutans* bacteria have the ability to resist several antibiotics such as *erythromycin, lincomycin,* and *penicillin*. Jubair (2015) also stated that *S. mutans* bacteria are resistant to the antibiotic *erythromycin* and susceptible to *cefotaxime* and *ciprofloxacin*. Bacteria that are resistant to antibiotics will have difficulty dying and will multiply (Fatisa, 2013), so there is a need for other alternatives in treating dental caries.

Plants are often used as an alternative for treatment and health maintenance which have been used for a long time (Harefa, 2020). According to Nisyapuri *et al*., (2018), people already know the procedures for using plants as alternative medicine based on the results of intergenerational inheritance and personal experience. Traditional medicines can be sourced from plants or what are commonly known as medicinal plants (Puspitasari *et al*., 2021). Medicinal plants are often used because they have several advantages, namely relatively small side effects and are more suitable for treating metabolic and degenerative disorders (Harefa, 2020). Medicinal plants also have relatively cheap prices because when using them you don't have to buy them like modern medicine but you can take them from the surrounding environment (Utami *et al*., 2019).

Thorn spinach (*Amaranthus spinosus* L.) is a medicinal plant that is widely distributed in India, Sri Lanka and many other tropical countries (Mondal *et al*., 2016). This plant is classified as a wild plant with habitats in bushes, roads, rubbish dumps and in the yard where it grows to a height of 50-100 cm (Mondal *et al*., 2016). By the Talang Tribe, Riau, thorn spinach is used as an alternative medicine to treat various health problems (Almurdani *et al*., 2017). The Dayak people also use thorn spinach leaves to treat dry eyes, increase blood pressure, urinary problems, eczema, boils and fever (Sari *et al*., 2015). Thorn spinach leaves, known as "Purundawa Bunga" by the people of Amesiu Village, Southeast Sulawesi, are usually used by boiling them to treat several diseases that many people in the village suffer from, such as asthma, heart disease, leukemia, typhus, ulcers, hypertension, vomiting blood, diabetes, kidney stones, colon cancer, gout, and rheumatism (Alkawi *et al*., 2021). All parts of the thorn spinach plant can be used as medicine, but people more often use the leaves by boiling, rubbing, or compressing the injured part (Jafar and Djollong, 2018). Thorn spinach (*A. spinosus* L.) has several active compounds that can inhibit the growth of microorganisms (antimicrobial compounds) (Almurdani *et al*., 2017). Research that has been conducted shows that the spinach plant is traditionally used to treat digestive diseases, as a diuretic, as an antipyretic, to increase appetite, and to treat gallbladder disease (Prajitha and Thoppil, 2017). Methanol extract of *A. spinosus* L. roots has anti-fungal activity against *Dermatophyte* sp. which produces an inhibitory zone of 21-32 mm with a minimum inhibitory concentration of 2.5-10 mg/mL (Das *et al*., 2012). Research conducted by Djindadi *et al*., (2020), found that spinach leaf extract has the ability to stop the growth of *Staphylococcus aureus* bacteria. *Amaranthus spinosus* L. can also be used as an antidiabetic and antidepressant (Mondal *et al*., 2015).

Based on this description, there are several previous studies that examined the ethanol extract of spinach leaves and the presence of several active compounds that have antibacterial activity. Therefore, further research is needed to examine the ethanol extract of spinach leaves regarding its antibacterial activity against *S. mutans* as an alternative to the use of antibiotics.

MATERIALS AND METHODS

This research is experimental research and is included in the quantitative research category. This research was carried out in three laboratories, namely the Microbiology Laboratory, Basic Biology Laboratory, and Genetics and Molecular Biology Laboratory, FMIPA, Universitas Negeri Surabaya. The *S. mutans* bacterial reculture process and antibacterial activity testing were carried out in the Microbiology Laboratory, the extract maceration and evaporation process was carried out in the Basic Biology Laboratory, and the bacterial suspension turbidity measurement process was carried out in the Genetics and Molecular Biology Laboratory. Sampling of thorn spinach leaves was carried out in several areas in Taman District, Sidoarjo Regency.

The tools used were test tubes, petri dishes, 250 mL Erlenmeyer, glass beaker, ossicle needle, blender, analytical balance, filter, 6 mm cork borer, spirit lamp, oven, hot plate, 200 µL micropipette, 1000 µL micropipette, rotary vaccum evaporator (BUCHI V-850), incubator (Imperial-III Model 302), Laminar Air Flow (LAF) ESCO, UV-VIS spectrophotometer, autoclave (Tomy ES-215). The materials

used include the bacterial suspension *S. mutans* ATCC 25175 obtained from Brawijaya University, thorn spinach leaves, sterile distilled water, Nutrient Agar (NA) media (Merck KgaA number: VM966650), antibiotics *amoxicillin*, H2SO4 1%, BaCl2 1%, NaCl 0.9%, hydrochloric acid (HCl) 1%, *Dragendroff's* reagent, chloroform, anhydrous acetic acid, sulfuric acid (H2SO4), Mg (Magnesium), FeCl3 1%, blue tip (*Eppendorf*), yellow tip (*Eppendorf*), aluminum foil, PP plastic, plastic wrap, paper, filter paper, and cotton.

The spinach leaf samples used were obtained from various areas in Taman District, Sidoarjo Regency. The thorn spinach leaves (*A. spinosus* L.) chosen were leaves that were characterized by being mature green (dark), thick, and somewhat stiff (Yulia and Ranova, 2019), and still fresh (Hadisoebroto *et al.*, 2016). Thorn spinach leaves are washed using running water until clean and then air-dried without using heat from the sun for ± 7 days (Handoyo and Pranoto, 2020). After that, the dried thorn spinach leaves are crushed and sifted to produce simplicia powder (Taurina and Andrie, 2023).

The extraction method at the maceration stage was carried out by adding simplicia powder using 96% ethanol then soaking for 3 days and carried out in stages with a ratio of simplicia powder and solvent of 1:3, 1:2, and 1:2 (Puspitasari and Proyogo, 2016). Each maceration process was carried out for 24 hours and was stirred occasionally (Dewatisari, 2020). To separate the extract from the solvent, the filtrate is filtered and evaporated at a temperature of 40°C (Hadisoebroto *et al.*, 2016).

Phytochemical screening was carried out by adding the extract with several reagents. The alkaloid test was carried out by adding 0.5 g of the extracted sample with 0.5 mL of 1% HCl and 1 to 2 drops of *Dragendroff's* reagent. If the color changes to orange, the sample is declared positive for the alkaloid test (Jati *et al.*, 2019). For the steroid test, 1 g of sample was added to 2 mL chloroform and shaken. Next, two drops each of anhydrous acetic acid and sulfuric acid were added. If the color changes to green or blue, then the sample is positive for a steroid test (Rumagit, 2015). The flavonoid test was carried out by taking 2 g of sample and heating it for 5 minutes. After going through the heating process, the sample was given 0.1 g of magnesium (Mg) and 5 drops of concentrated hydrochloric acid. If the color changes to orange or red, the sample contains flavonoids (Ergina *et al.*, 2014). The terpenoid test was carried out by adding 2 g of sample with 3 drops of concentrated hydrochloric acid and 1 drop of concentrated sulfuric acid then homogenizing. If the color changes to red or purple, the sample is declared positive for the terpenoid test (Ergina *et al.*, 2014). Tannin testing is carried out by adding 1 g of sample with 2-3 drops of 1% Iron (III) chloride (FeCl3). If the color change that occurs is blackish green or inky blue, the sample is declared positive for containing tannin (Noviyanty *et al.*, 2020).

Pure cultures of *S. mutans* were taken using a loop needle then inoculated on slanted NA media and incubated at 37°C for 24 hours (Yulvizar, 2013). The bacterial culture was then inoculated in 10 mL of liquid 0.9% NaCl and its turbidity was measured (Afni *et al.*, 2015). The turbidity of the bacterial suspension was measured using a UV-Vis Spectrophotometer at a wavelength of 625 nm until the absorbance value was around 0.08-0.10, which is equivalent to a bacterial count of 1.5 x 108 CFU/mL, where the blank used was NaCl solution (Septiani *et al.*, 2017).

The extract solution was made by weighing the thick extract of spinach leaves, 0.2 g each; 0.4g; 0.6 g and 0.8 g were then diluted using 10% DMSO solvent until the volume reached 1 mL (Angelina *et al.*, 2015), resulting in extract concentrations of 20%, 40%, 60% and 80%. The antibacterial activity test uses a well technique, namely by making holes in the media that has been added with microbes (Putra and Rahayu, 2017). 1 mL of the test bacterial suspension was put into a petri dish then added with 15 mL of NA medium after which it was homogenized. After the media solidifies, holes are made in the media with a cork borer with a diameter of 6 mm. Each petri dish had 3 holes filled with extract solution, amoxicillin antibiotic solution (positive control), and 10% DMSO solution (negative control). 50 µL of each test solution was added into the well using a micropipette (Hadisoebroto *et al.*, 2016). Each treatment was repeated 4 times. Next, the petri dish was incubated at 37°C for 24 hours. The inhibition zone formed was measured using a ruler in millimeters (mm) (Faidiban *et al.*, 2020). The average of the measurement results is calculated using the following formula.

$$Inhibition\ Zone\ Diameter = \frac{(\mathrm{Vd}-\mathrm{Dd})+(Hd-Dd)}{2} \quad (1)$$

Information:

Vd: Vertical Diameter
Hd: Horizontal Diameter
Dd: Disc/Well Diameter

The data was then analyzed using the *Kolmogorov-Smirnov* test for normality test, then an ANOVA test was carried out to determine the effect of the treatment given. Next, the *Duncan* test was carried out to compare the results of each treatment. All stages of data analysis were carried out using *SPSS 23.0 for Windows 10*.

RESULTS

Based on the extraction process that has been carried out, from 700 g of thorn spinach leaf simplicia, 16.8 g of thick extract is produced. In the phytochemical screening test, the results showed that the ethanol extract of thorn spinach leaves contained secondary metabolite compounds such as alkaloids, terpenoids, steroids, flavonoids and tannins (Figure 1). Data on the results of phytochemical screening can be seen in Table 1.

Table 1. Results of phytochemical screening test of ethanol extract of spinach leaves

Phytochemical test	Results
Alkaloid	Positive
Steroid	Positive
Flavonoid	Positive
Terpenoid	Positive
Tanin	Positive

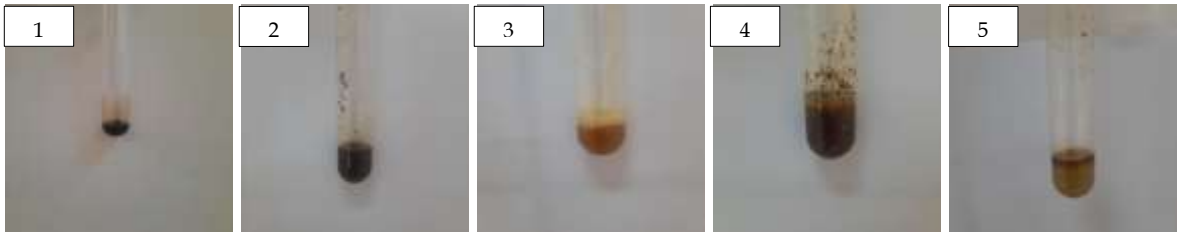

Figure 1. Phytochemical screening test results; (1) Tannin Test; (2) Terpenoid Test; (3) Alkaloid Test; (4) Flavonoid Test; (5) Streioid Test

Based on the antibacterial activity test, it was found that each extract concentration had an effect on the growth of *S. mutans* bacteria, which was characterized by the presence of an inhibition zone that was formed (Figure 2). Data on the average diameter of the inhibition zone formed is in Table 2.

Table 2. Data on the average diameter of the inhibition zone formed in the antibacterial activity test of ethanol extract of thorn spinach leaves against *S. mutans* bacteria.

Treatment	Mean Inhibition Zone Diameter (mm)*
Negative Control (DMSO 10%)	0,00 ± 0,00^{a}
Concentration 20%	0,25 ± 0,50^{a}
Concentration 40%	1,50 ± 2,38^{a}
Concentration 60%	5,13 ± 0,75^{b}
Concentration 80%	6,38 ± 0,25^{b}
Positive Control (*Amoxicillin*)	10,88 ± 1,25^{c}

Note: *) Different letters indicate that significantly different result based on Duncan test ($p< 0.05$)

In the *Kolmogorov-Smirnov* test, data on the average diameter of the inhibition zone from various treatments was normally distributed with a sig value (0.120) > α value (0.05). Next, the data were analyzed using a *one-way ANOVA* test and obtained a significance value (0.00) < α value (0.05). This shows that there are real differences between treatments. The data was then analyzed using the *Duncan* test and it was found that in each treatment the concentration, positive control and negative control were significantly different from each other as indicated by different notations (Table 2). The negative control treatment, 20% concentration treatment, and 40% concentration treatment were not significantly different and are expressed with the notation a. However, the negative control treatment, 20% concentration treatment, and 40% concentration treatment were significantly different from the 60% concentration treatment, 80% concentration treatment, and positive control. The 60% concentration treatment was not significantly different from the 80% concentration and is shown with the notation b. The positive control, negative control and all concentration treatments were

significantly different from each other and are shown with the notation c. The analysis results showed that each extract concentration had an effect on *S. mutans*.

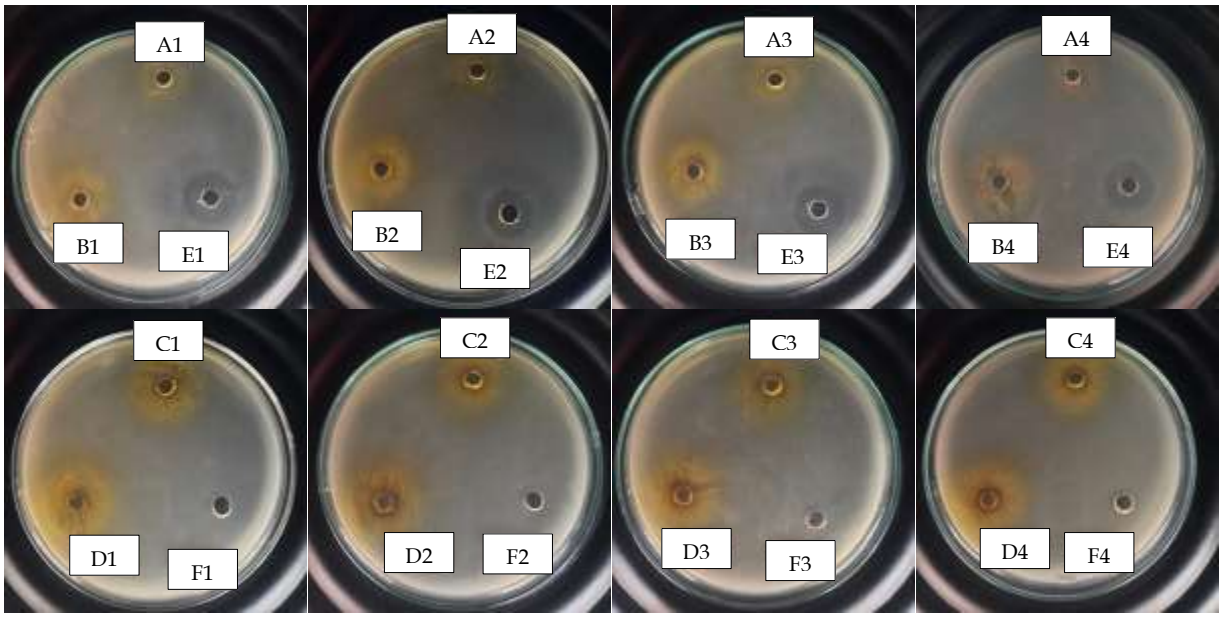

Figure 2. Antibacterial test results; (A1-4) Extract concentration 20%; (B1-4) Extract concentration 40%; (C1-4) Extract concentration 60%; (D1-4) Extract concentration 80%; (E1-4) Positive control (*Amoxicillin*); (F1-4) Negative control (DMSO 10%)

DISCUSSION

Based on the data obtained, it is known that various concentrations of thorn spinach leaf ethanol extract can inhibit the growth of *S. mutans*. This is characterized by the formation of an inhibition zone. The use of a negative control in the form of 10% DMSO did not produce antibacterial activity. DMSO is a compound that can separate special compounds and is not toxic (Kusumawati *et al.*, 2015). DMSO solvent is a solvent that is capable of separating special polar or non-polar compounds found in plants (Fatonah *et al.*, 2021). DMSO can extract alkaloid, flavonoid and tannin compounds because these three compounds are polar, DMSO also has the ability to extract steroid and terpenoid compounds because both are non-polar compounds (Vifta and Advistasari, 2018). The antibiotic *amoxicillin* was used as a positive control, this is because is classified as a penicillin antibiotic which has a broad spectrum and is bacteriostatic (Handayani *et al.*, 2017).

The positive control using the antibiotic *amoxicillin* was able to suppress the growth of *S. mutans* with an average inhibition zone formed of 10.88 ± 1.25 mm. In the negative control using 10% DMSO there was no zone of inhibition because 10% DMSO could not suppress the growth of *S. mutans*. The antibiotic *amoxicillin* as an antibacterial inhibits the combination of the cell wall peptidoglycan structure in three phases (Supari, 2016). The first and second phases inhibit amino acid synthesis in the cytoplasm, the third phase occurs outside the cell by completing the cross-linking of new subunits (Soares *et al.*, 2012).

At each concentration of 20%, 40%, 60%, and 80% the average inhibition zone produced was 0.25 ± 0.50 mm; 1.50 ± 2.38 mm; 5.13 ± 0.75 mm; and 6.38 ± 0.25 mm. From the observation data, it was found that the concentration that produced the highest average diameter was at a concentration of 80%, however based on statistical tests the 80% concentration did not show any significant difference to the 60% concentration. The antimicrobial ability of a substance is known from the formation of an inhibition zone (Zulkarnain *et al.*, 2021). The higher the concentration, the more active ingredients it contains, this causes different inhibitory zones to form at different extract concentration levels (Afifi, 2018).

The cell wall structure of the *S. mutans* bacteria is relatively simple, which makes it susceptible to the activity of antimicrobial compounds such as phenolics and penicillin (Yumas, 2017). The compounds contained in the ethanol extract of spinach leaves (**Table 1**) have mechanisms for inhibiting the growth of different *S. mutans* bacteria. Antimicrobial compounds that inhibit microbial

growth have different mechanisms, including inhibiting cell wall formation, disruption of cell membranes, and inactivation of metabolic enzymes (Nurhamidin *et al.*, 2022).

Alkaloids are compounds that have the potential to be antibacterial agents (Kurniawan and Aryana, 2015). Alkaloids are compounds that can be obtained by extracting plant materials using organic solvents (Maisarah and Chatri, 2023). Alkaloids are better at suppressing the growth of gram-positive bacteria (Yumas, 2017). Alkaloids have an antibacterial effect, this is because alkaloids can damage cells by destroying the peptidoglycan part of the cell and disabling the formation of the cell wall layer (Sepriani *et al.*, 2020). The peptidoglycan components in bacteria are disrupted due to destruction by alkaloid compounds (Yumas, 2017).

Steroids are antibacterial compounds that are associated with cell lipid membranes. Steroids can cause liposomes to rupture and reduce membrane integrity, causing cell brittleness or lysis (Sari *et al.*, 2017). Steroids in plants are in the form of sterols (Suryelita *et al.*, 2017). The cell wall will interact with the surface of sterol molecules and cause changes in the primary structure of the cell wall, these changes lead to the formation of pores or holes and degradation of cell components (Sadiah *et al.*, 2022).

Flavonoids are metabolite compounds that have anti-inflammatory, anti-diabetic and antibacterial properties (Alfaridz, 2018). The antibacterial activity contained in this compound has been widely tested and shows positive results against many bacteria (Kumar and Pandey, 2013). High amounts of flavonoids can be bactericidal on bacteria (Christabel *et al.*, 2018). The mechanism of flavonoids in antibacterial action is by destroying the cytoplasmic membrane and cell walls of bacteria (Christabel *et al.*, 2018). Flavonoids will attack the phospholipids that make up the cytoplasmic membrane of bacteria. This will cause leaks in the membrane and loss of substances that help cells process food, thereby causing cell death. (Lutpiatina, 2015).

Terpenoids are compounds that are easily soluble in all types of solvents (Wulansari *et al.*, 2020). The antibacterial mechanism of terpenoids depends on interactions with porins, by means of which terpenoids form strong polymer bonds and can damage the porins (Wahdaningsih *et al.*, 2014). Damaged porins will reduce cell wall permeability and cause nutritional deficiencies in cells, resulting in inhibited cell development (Wulansari *et al.*, 2020).

Tannins are classified as secondary metabolite compounds. Tannins contain polyphenol cores which can bind to proteins and inhibit protein synthesis (Hadi *et al.*, 2015). Tannins have antidiarrheal, antibacterial, antioxidant and astringent properties (Malangngi *et al.*, 2012). The antibacterial power contained in tannins includes enzyme inactivation, reactions in cell membranes, and inhibition of the genetic material of bacterial cells (Fratiwi, 2015). Apart from that, tannins also have the ability to precipitate proteins or interfere with cell membrane protein transport (Suryani *et al.*, 2019). Tannin acts as an antibacterial by inhibiting DNA topoisomerase activity thereby inhibiting bacterial cell formation (Nor *et al.*, 2018).

Based on the test results, it was concluded that the ethanol extract from spinach leaves showed antibacterial activity against the growth of *S. mutans* bacteria. Therefore, it is hoped that this research can become a basis for further research in the future as an effort to develop antibacterial products based on ethanol extract from spinach leaves.

CONCLUSION

In this study, the ethanol extract of thorn spinach leaves had antibacterial activity against *S. mutans*, a concentration of 80% was the concentration of ethanol extract of thorn spinach leaves which produced the highest average diameter of the inhibition zone, namely 6.38 ± 0.25 mm, but based on statistical tests. There was no significant difference between 80% concentration and 60% concentration.

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Authors:
Ria Dwi Setiani, Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Jalan Ketintang Gedung C14 Surabaya 60231, Indonesia, e-mail: ria.20017@mhs.unesa.ac.id
Guntur Trimulyono, Biology Study Program, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Jalan Ketintang Gedung C14 Surabaya 60231, Indonesia, e-mail: gunturtrimulyono@unesa.ac.id

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