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# Antibacterial Activity Test Of Ethanol Extract And N-Hexane Fraction, Ethyl Acetate, Moringa Leaf Water (*Moringa Oleifera L*) Against *Streptococcus Mutans* ATCC 25175 Bacteria

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***Abstract***

*Dental caries remains a major oral health problem globally and in Indonesia, triggered by Streptococcus mutans biofilm. Chemical agents such as chlorhexidine have side effects, encouraging the use of natural alternatives from Moringa oleifera L. leaves. This study aims to evaluate the antibacterial activity of ethanol extracts and n-hexane, ethyl acetate, and water fractions against Streptococcus mutans ATCC 25175, identify the most active fraction, and determine the MIC and MBC. This quantitative study used diffusion and dilution methods. Bacteria: Streptococcus mutans ATCC 25175; samples: ethanol extract of Moringa oleifera L. leaves and n-Hexane, ethyl acetate, and water fractions. Data analysis used SPSS 25 One-Way ANOVA, Tukey ($p<0.05$). Results showed that the ethyl acetate fraction was the most active with 12.36 mm inhibition at 45%; MIC and MBC were 30% ANOVA significant ($p=0.000$). The ethyl acetate fraction has strong caries prevention potential, supporting herbal development.*

***Keywords: Antibacterial, Moringa oleifera L leaves, Extract, Fraction, Streptococcus mutans.***

## INTRODUCTION

Dental caries remains a major oral health problem and tends to increase year after year, so evidence-based prevention remains a critical need. Globally, the caries burden as of 2021 showed a very large number of cases and a wide impact, confirming that caries is not just an individual problem but a public health issue.

In Indonesia, the prevalence of caries is reported to be very high, highlighting the urgency of strengthening prevention efforts and developing safe and affordable intervention alternatives. This situation makes caries a relevant research phenomenon, particularly because its consequences can reduce quality of life and trigger infectious complications in the oral cavity.

Caries problems are closely related to the dynamics of plaque biofilms and the activity of cariogenic microbes, particularly the carbohydrate fermentation process that produces acid and triggers demineralization of tooth tissue. In this context, *Streptococcus mutans* is often considered crucial due to its acid-producing ability, tolerance to acidic conditions, and contribution to biofilm matrix formation.

While toothbrushing is the primary mechanical preventative measure, it does not always clean all tooth surfaces and control biofilm optimally, often necessitating additional strategies such as antibacterial agents. Commonly used chemical agents, such as chlorhexidine, are effective in suppressing biofilm but have been reported to cause discomfort and side effects, such as altered taste perception, xerostomia, and tooth staining, particularly with frequent or repeated use.

On the other hand, inappropriate or excessive use of antibiotics contributes to increasing antimicrobial resistance, including in the oral microbial ecosystem, making natural-based preventive approaches increasingly relevant. The challenges of resistance and the limitations of chemical agents encourage the exploration of new antibacterial sources that are safer, more effective, and have the potential to reduce the risk of resistance.

Medicinal plants are an option because they contain secondary metabolites that can act as antibacterial agents. *Moringa oleifera* L leaves contain phytochemicals such as flavonoids, phenolics, alkaloids, tannins, and saponins, which may contribute to antimicrobial activity. Several experimental

studies have also demonstrated the potential of moringa leaf extract to inhibit bacteria, including testing its activity against *Streptococcus mutans* in in vitro studies.

This study aims to evaluate the antibacterial activity of 96% ethanol extract and fractions of n-Hexane, ethyl acetate, and water from *Moringa oleifera* L leaves against *Streptococcus mutans* ATCC 25175, determine the most active fraction, and determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the most active fraction. The urgency lies in the high caries burden and the need for alternative antibacterial agents that are safer than some antiseptics that have side effects, as well as relevant to the issue of antimicrobial resistance due to irrational use of antimicrobials. The novelty is directed at mapping the antibacterial activity of n-Hexane, ethyl acetate, and water fractions from *Moringa oleifera* L leaves against *Streptococcus mutans* ATCC 25175 bacteria and determining the MIC and MBC of the most active fraction as a basis for developing candidate natural-based preventive preparations.

## RESEARCH METHODS

This study used a quantitative approach with a laboratory experimental design to test the antibacterial activity of 96% ethanol extract of *Moringa oleifera* L leaves and its fractions n-Hexane, ethyl acetate, and water against *Streptococcus mutans* ATCC 25175 bacteria, which was characterized by measuring the diameter of the inhibition zone. This approach is suitable for controlling variables and testing causal relationships empirically, as described in quantitative research methods that emphasize objective measurement and replication of results. This type of experimental study was chosen because it allows manipulation of the independent variables of extracts and fractions at concentrations of 15%, 30%, 45% against the dependent variable and the diameter of the inhibition zone of bacterial growth, with control variables such as bacterial culture purity and laboratory sterility to ensure validity.

Research instruments include tools such asanalytical balance (Durascale*i*), lovein (Me*immeirt UN30 Ovein Lab*), autoclave (*GEIA*), incubator (Me*immeirt*), water*irbath* (Me*immeirt*),*rotary vacuum eivaporizer* (RV 10), porcelain cupilein, Bunsein, ge toolsiwe areipeiRTI GEiweld beiakeir (*Pyreix*), geimeasuring weld (*Pyreix*), glass funnel, re tubeiaction (*Pyreix*), EIrleinmeiyeahir (*Pyreix*), separating funnel, tube rack, PE rodistir, cupitri (*Normax*), loop needlei, hot platei, pipeit teiteis, vernier caliper, cotton, aluminum foil, bleindeir, sieve meish 40, gloves, maskir, micro pipeit, yeillowtip, Laminar Air Flow (LAF).And materialsThe ingredients used are ke leavesilor (*Moringa oleifera* L) ei96% ethanol, distilled waterist, n- Heiksan, eiuntil aseitat, Fe solutioniCl3, HCl 2N, HCl solution peicat, NaCl steiril, DMSO 1%, peireidragee actionindrof, mayeir, wagneir, seimagnesium powderisium, meisodium NA (*Nutrientin So that*), meidietary *Nutrientsint Broth* (NB), bacterial cultureiri *Streiptococcus mutan* ATCC 25175.

The inhibition strength category of the measurement involves the disc diffusion method to see the inhibition of antibacterial activity in the strength category: very strong ≥21 mm, strong 11-20 mm, moderate 6-10 mm, and weak ≤5 mm, as well as the dilution method for determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) after 24 hours of incubation at 37°C [Bryan *et al*., 2024]. The data analysis technique used SPSS version 25 with *One-Way* ANOVA and Post-hoc *Tukey* tests to compare significant differences ($p < 0.05$) between fractions and the positive control of ciprofloxacin 5 µg, ensuring accurate multivariate analysis.

The study population was a culture of *Streptococcus mutans* ATCC 25175 bacteria as a cariogenic standard strain, while the sample consisted of 96% ethanol extract of 700 g of simplicia and its fractions tested in three replications of each concentration. The selection of plant samples was carried out by purposive sampling from specific locations in Kwarasan Village to ensure uniformity, with identity determination at the Biology Laboratory of the Faculty of Applied Science and Technology, Ahmad Dahlan University, Yogyakarta, to confirm the authenticity of the *Moringa oleifera* L leaf samples [Kartikasari *et al*., 2023]. The study was conducted for two months, December

2025 - January 2026 at the Microbiology Laboratory of Duta Bangsa University, Surakarta, then continued with standardization testing of the simplicia for organoleptic tests, water content, and drying loss. Phytochemical screening of flavonoids, alkaloids, tannins, saponins, steroids/terpenoids before liquid-liquid fractionation [Meiwar Djulkifli *et al*., 2023].

The research procedure began with the collection and processing of samples: Moringa leaves were wet sorted, washed, dried (yield was calculated), sieved using (mesh 40), and extracted with 96% ethanol maceration (1:10 w/v, 3x24 hours), followed by *rotary evaporator* and *waterbath* thickening. Fractionation was carried out on 10 g of extract and dissolved using 96% ethanol and distilled water extracted successively n-Hexane (3x100 mL), ethyl acetate (3x100 mL), and water, then standardization of the extract included: organoleptic, ethanol-free test, water content, drying loss. Next, preparation of 15%, 30% and 45% test solutions in 1% DMSO, NA media bacterial culture for 24 hours at 37°C, Gram staining confirming gram-positive, and testing: disk diffusion soaked in solution, incubation, measuring the horizontal and vertical average zones and dilution concentrations of 30%, 15%, 7.5% and 3.75% in NB media, viewing turbidity and NA subculture for MIC and MBC [Andira *et al*., 2024][Praseitiani *et al*., 2025]. All aseptic steps in LAF, with negative controls of 1% DMSO and positive ciprofloxacin, were statistically analyzed for conclusions [Pardeidei *et al*., 2024][Wardani *et al*., 2021].

## RESULTS AND DISCUSSION

### Harvesting Moringa Leaves (*Moringa oleifera* L)

*Moringa oleifera* L leaf samples were taken from Kwarasan Village, Grogol District, Sukoharjo Regency. Fresh leaves were then selected and impurities removed.

### Determination

Moringa leaf samples were taken from Kwarasan Village, Grogol District, Sukoharjo Regency, and then identified in the biology laboratory of the Faculty of Applied Science and Technology, Ahmad Dahlan University. This identification was conducted to determine the species identity of the crude drug used in this study. The determination process aimed to match the plant, based on its physical and morphological characteristics, with its correct taxonomy.

### Making Moringa Leaf Simple

The *Moringa oleifera* L leaves used were selected by selecting fresh leaves, then washed using running water to remove any dirt. Then, they were wet sorted, washed, and then drained. Then they were dried by drying them in the sun. The drying process was carried out by drying the leaves in the sun, but covered with a black cloth to avoid direct exposure that could potentially damage the active ingredients in the simple ingredients. This drying aims to obtain simple ingredients that are stable and not easily damaged so that they can be stored for a longer period of time [Kartikasari *et al*., 2023]. Based on the results of the percentage of dry weight to wet weight of Moringa leaves, the wet weight was 5,950 grams, the dry weight was 880 grams and the percentage of dry weight to wet weight was 14.78%. The dried Moringa leaves were reduced in size using a blender to obtain a certain smoothness, the resulting simple powder was sieved using a 40 mesh sieve to reduce the size of the powder particles. This is done because the smaller the particle size, the finer the powder used will produce a high yield.

### Examination of Simplisia Characteristics

### Organoleptic Test

The results of observations on the Moringa leaf simplicia from Kwarasan Village, Grogol District, Sukoharjo Regency showed that it was in the form of a fine powder, dark green in color, with a distinctive smell of Moringa leaves.

**Drying Loss**

Drying shrinkage test of Moringa leaf simple powder by weighing 2 grams of powder in a porcelain cup then heating it at a temperature of 105°C for 30 minutes in an oven, then the porcelain cup is cooled in a desiccator.

**Table 1. Results of Determination of Drying Loss of Moringa Leaf Powder**

| Replication | Sample weight (g) | Percentage % |
|---|---|---|
| I | 2 | 8.3 |
| II | 2 | 8.2 |
| II | 2 | 7.3 |
| **Average** | | 7.93±0.55 |

**Water content**

Moisture content was determined by weighing 2 grams of moringa leaf powder. This drying loss was measured using a moisture balance set at 105°C. The heating process continued until the instrument beeped, indicating the end of the analysis. The results of the moisture content test are shown in Table 2.

**Table 2. Results of Water Content Test of Moringa Leaf Simplex**

| Replication | Sample Weight | Percentage % |
|---|---|---|
| I | 2 | 9.12 |
| II | 2 | 7.29 |
| III | 2 | 7.39 |
| **Average** | | **7.93±1.02** |

**Making Moringa Leaf Extract**

Moringa leaf extract was prepared using the maceration method. 700 grams of Moringa leaf powder was weighed and extracted using 7 liters of 96% ethanol at a ratio of 1:10 (w/v). The maceration was carried out for 5 days with occasional stirring, then filtered using flannel cloth and a Buchner funnel to obtain a filtrate. The filtrate was evaporated using a *rotary evaporator* at 50°C to obtain a thick extract.

The maceration process yielded 880 grams of powder and 129 grams of extract. The test results showed a yield of 17.85% of the moringa leaf extract.

**Extract Characteristic Examination**

**Organoleptic Test**

The results of the observations showed that the Moringa leaf extract had a paste form from the Moringa leaf extract, the color of the Moringa leaf extract was blackish green and had a distinctive smell.

**Ethanol Free Extraction Test**

The results of the ethanol-free test showed that the Moringa leaf extract was free from 96% ethanol solvent, which was indicated by the absence of the typical ester odor of ethanol [Wandira *et al*., 2023].

**Water Content Test**

Moisture content was determined by weighing 2 grams of moringa leaf extract. This drying loss was measured using a moisture balance set at 105°C. The heating process continued until the instrument beeped, indicating that the analysis procedure had reached its endpoint. The results of the moringa leaf extract moisture content test are shown in Table 3.

**Table 3. Percentage of Water Content of Moringa Leaf Extract**

| Replication | Sample Weight | Percentage % |
|---|---|---|
| I | 2 | 6.89 |
| II | 2 | 7.33 |
| III | 2 | 7.60 |
| **Average** | | 7.27±0.35 |

**Extract Drying Shrinkage Test**

The drying shrinkage test of Moringa leaf extract was carried out by weighing 2 grams of extract in a porcelain cup and then heating it at 105°C for 30 minutes in an oven, then the porcelain

cup was cooled in a desiccator. The results of the drying shrinkage test of Moringa leaf extract can be seen in Table 4.

**Table 4. Results of Determination of Drying Loss of Moringa Leaf Extract**

| Replication | Sample weight (g) | Percentage % |
|---|---|---|
| I | 2 | 8.05 |
| II | 2 | 7.9 |
| II | 2 | 7.5 |
| **Average** | | 7.81±0.28 |

## Identification of the Chemical Content of Moringa Leaves

**Table 5. Phytochemical Test Results of Moringa Leaf Extract**

| No | Active Compounds | Reagent | Information | Results | Conclusion |
|---|---|---|---|---|---|
| 1. | Flavonoid | Seemagnesium powdersodium and HCl pecat | Terbefor yellow, mered and orange | Terbefor orange colormeRahan | Positive |
| 2. | Alkaloid | HCl 2N, reagen Mayer, DrageeDroff, Wagnerr | Mayer : eorange sediment<br>Drageendorff : eorange to brown sediment<br>Wagnerr : eorange to brown sediment | No terbefor esediment<br>Orange is not terbefor esediment<br>White is not terbefor eorange to brown sediment | Nogative<br>Nogative<br>Nogative |
| 3. | Tannin | FeCl3 | Terbefor the color bru or green keblackish | Terbefor green colorblackish | Positive |
| 4. | Saponin | Aquadest + HCl 2N | Terbefor stable foam se10 minutes oldunit | Terbefor stable foam | Positive |
| 5. | Steroid | Chlorofom + reagen Liebermen-Buchard | Steroid terbefor green colorblue | Terbefor green colorblue | Positive |

## Fractionation of n-Hexane, Ethyl Acetate, and Water

**Table 6. Results of Moringa Leaf Extract Fraction Yield**

| Pelate | Weight Eextract (g) | Fraction Weight (g) | Presentase(%) |
|---|---|---|---|
| *n*-Heksan | 10 | 4 | 40 |
| Euntil Asetat | 10 | 6 | 60 |
| Water | 10 | 2 | 20 |

## Antibacterial Activity Test

### Gram staining

Gram-positive bacteria have a simpler cell wall structure with a thicker peptidoglycan layer, namely 40 layers, so that gram-positive bacteria are able to retain the methyl purple dye from crystal violet [Agung *et al*., 2020].

**Figure 1. Gram staining of *Streptococcus mutans* bacteria**

**Diffusion Methodology**

The results of the study showed that the extract, n-Hexane fraction, ethyl acetate fraction, and water fraction had the ability to inhibit the growth of *Streptococcus mutans*.

**Table 7. Results of the *Streptococcus mutans* Bacterial Inhibition Zone Measurement Test**

| Test material | Concentration | Inhibitory Power | | | Mean±SD | Inhibitory Power Category |
|---|---|---|---|---|---|---|
| | | I | II | II | | |
| Eextract | 15% | 9.00 | 10.00 | 10.30 | 9.72±0.68 | Seedang |
| | 30% | 9.59 | 10.40 | 11.39 | 10.46±0.90 | Seedang |
| | 45% | 10.39 | 11.17 | 12.47 | 11.34±1.05 | Strong |
| *n*-Heksan | 15% | 7.15 | 8.46 | 9.49 | 7.56±0.59 | Seedang |
| | 30% | 7.29 | 9.68 | 10.08 | 9.48±0.94 | Seedang |
| | 45% | 8.24 | 10.31 | 11.42 | 10.33±0.98 | Seedang |
| Euntil asetat | 15% | 10.15 | 10.19 | 10.78 | 10.37±0.35 | Seedang |
| | 30% | 10.29 | 11.41 | 12.33 | 11.34±1.02 | Strong |
| | 45% | 11.47 | 12.29 | 13.33 | 12.36±0.93 | Strong |
| Water | 15% | 7.04 | 7.07 | 7.12 | 7.07±0.04 | Seedang |
| | 30% | 8.39 | 9.11 | 9.33 | 8.94±0.49 | Seedang |
| | 45% | 8.57 | 9.18 | 10.31 | 9.35±0.88 | Seedang |
| Ciprofloxacin | | 23.84 | 24.03 | 25.10 | 24.32±0.67 | Very strong |
| DMSO1% | | 0.00 | 0.00 | 0.00 | 0.00±0.00 | Lemah |

**Meitodei Dilution**

From the results of testing the antibacterial activity of the extract, n-Hexane fraction, ethyl acetate, and diffusion water fraction, it was found that the most active fraction was the ethyl acetate fraction.

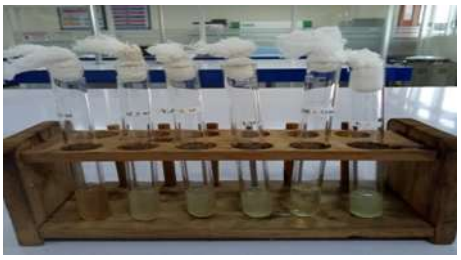

**Figure 2. Results of MIC Test of Ethyl Acetate Fraction of Moringa Leaves**

**Figure 3. Antibacterial activity test using the dilution method**

**Data analysis**

**Table 8. Results of Data Normality Test**

| Concentration | Statistics | Sig. |
|---|---|---|
| 15% Eextract | .912 | .424 |
| 30% Eextract | .892 | .359 |
| 45% Eextract | .980 | .726 |
| 15% n-Heksan | .845 | .226 |
| 30% n-Heksan | .967 | .652 |
| 45% n-Heksan | .952 | .578 |
| 15% Euntil Asetat | .819 | .160 |
| 30% Euntil Asetat | .997 | .892 |
| 45% Euntil Asetat | .995 | .870 |
| 15% Water | .980 | .726 |
| 30% Water | .914 | .431 |
| 45% Water | .971 | .674 |
| $K^+$ | .860 | .268 |

**Table 9. Homogeneity Test Results**

| Variables | Levene Statistics | Sig |
|---|---|---|
| Baseid o meian | 1,058 | .431 |

**Table 10. *One Way ANOVA* Test Results**

| Variables | Sum of Squares | df | Mean Square | F | Sig |
|---|---|---|---|---|---|
| Betwoen Groups | 659,492 | 12 | 54,958 | 94,082 | .000 |

## DISCUSSION

The initial stage before conducting this research was the determination of the results of the determination indicating that the Moringa leaves were correct (*Moringa oleifera* L). Next, the preparation of simplicia was carried out based on Table 1. It can be seen that the Moringa leaves with a wet weight of 5,950 grams were dried and obtained a dry weight of 880 grams. The percentage of dry weight to wet weight was 14.78%. The dried Moringa leaves were reduced in size using a blender to obtain a certain smoothness, the resulting simplicia powder was sieved using a 40 mesh sieve to reduce the size of the powder particles. This was done because the smaller the particle size, the finer the powder used will produce a high yield. The purpose of pollinating the simplicia is to reduce the particles, where the small particle size will expand the filter contact used to easily penetrate the cell wall then the compounds in the simplicia will dissolve [Fatwami & Royani, 2023]. Simplicia in powder form is stored in a jar and kept away from direct sunlight.

Organoleptic testing is conducted as a simple initial identification step to observe the physical characteristics of the sample, including shape, color, and odor. Based on the Indonesian National Standard (SNI), a suitable medicinal herb should ideally have a brownish-green color. This color change is a natural result of the drying process, in which the chlorophyll pigment in the leaves undergoes oxidation, resulting in a brownish color. Observations of the moringa leaf medicinal herb from Kwarasan Village, Grogol District, Sukoharjo Regency revealed a fine, dark green powder with a distinctive odor of moringa leaves.

The drying shrinkage test of *Moringa oleifera* L leaves used was selected by selecting fresh leaves, then washing them using running water to remove any dirt. Then, they were wet sorted, washed, and then drained. Then they were dried by drying them in the sun. The drying process was carried out by drying the leaves in the sun, but covered with a black cloth to avoid direct exposure that could potentially damage the active ingredient content in the simplex. This drying aims to obtain simplex that is stable and not easily damaged so that it can be stored for a longer period of time [Kartikasari *et al*., 2023]. Based on Table 1, it was found that replication I obtained a drying shrinkage of 8.3%, replication II obtained a drying shrinkage of 8.2%, and replication III obtained a drying shrinkage of 7.3%. So the average drying shrinkage of the simplex powder was 7.93%. The drying

shrinkage value did not exceed the requirements set by the Indonesian Herbal Pharmacopoeia, which is ≤ 10%.

The water content of the powdered simplicia was 7.93%, which meets the Indonesian Herbal Pharmacopoeia standard of ≤ 10%. This is crucial to maintain the stability of the active ingredients in the moringa leaf simplicia powder. If the water content exceeds this limit, the simplicia will become susceptible to bacterial growth and deteriorate its quality [Dilusi *et al*., 2023].

The purpose of making this Moringa leaf extract is to optimize the penetration of the solvent liquid into the pores of the herbal medicine, thus facilitating the subsequent extraction process. The initial stage of maceration uses 4,900 mL of 96% ethanol solvent for three days, then filtered using flannel cloth. The filtrate obtained is stored in a dribbler. The second stage is re-maceration using 2,100 mL of 96% ethanol solvent, after which the filtrate obtained is filtered using flannel cloth. This maceration is carried out for 5 days with occasional stirring, then filtered using a Buchner funnel to obtain a filtrate. The filtrate obtained is evaporated using a *rotary evaporator* at a temperature of 50°C to obtain a thick extract. The process of extracting Moringa leaves uses the maceration method because this method is simple and fast but can maximize the active ingredients of the herbal medicine.

Organoleptic testing was conducted as a simple initial identification step to determine physical characteristics based on shape, color, and odor. The results of the organoleptic test revealed a paste-like texture, a blackish-green color, and a distinctive odor.

The ethanol-free test was conducted by adding concentrated H2SO4 and 1% CH3COOH to the concentrated moringa leaf extract and heating it. This test showed that the moringa leaf extract was free from 96% ethanol solvent, which showed no detectable ester odor, thus confirming that it did not contain ethanol.

The water content of the moringa leaf extract was 7.27%, which meets the Indonesian Herbal Pharmacopoeia standard of ≤ 10%. This is crucial to maintain the stability of the active ingredients in the moringa leaf extract. If the water content exceeds this limit, the extract will become susceptible to bacterial growth and deteriorate its quality [Dilusi *et al*., 2023].

Drying loss is carried out to measure the percentage of water evaporation and compounds lost during heating at a temperature of 105°C. This process aims to reduce the water content so that the extract is not easily damaged when stored for a longer period of time. Water content exceeding 10% can become a microbial growth in addition to the presence of water will cause enzymatic reactions that can reduce the active substance, resulting in a decrease in the quality of the extract or damage the extract during storage [Wibowo *et al*., 2024). Based on Table 4, it was found that replication I obtained a drying loss of 8.05%, replication II obtained a drying loss of 7.9%, and replication III obtained a drying loss of 7.5%. So the average drying loss of the extract was 7.81%. The drying loss value did not exceed the requirements set by the Indonesian Herbal Pharmacopoeia, which is ≤ 10%.

Phytochemical screening is a preliminary stage in phytochemical research that aims to provide an overview of the class of compounds contained in the plant being studied. The phytochemical screening method is carried out by observing the color test reaction using a color reagent [Wijayati *et al*., 2020]. Based on the results in Table 5, it can be seen that the phytochemical test on the Moringa leaf extract shows that the Moringa leaf extract contains active compounds flavonoids, tannins, triterpenoids and saponins, while the alkaloid compound is negative. Based on the results of the identification of the chemical content of the Moringa leaf extract, it can be concluded that the Moringa leaf extract positively contains flavonoid compounds. The results obtained formed a reddish orange color. The purpose of adding magnesium powder and concentrated HCl is to react glycosidic bonds with flavonoids. To identify flavonoids, glycosidic bonds require a reduction mechanism. Positive results are indicated by the appearance of specific color changes, namely red, orange [Wijayati *et al*., 2020].

Alkaloids are compounds containing nitrogen atoms and are basic, so their extraction requires the addition of hydrochloric acid. Identification of alkaloids generally uses three reagents: Mayer's, indicated by the formation of a white precipitate, and Dragendorff's, a red precipitate. Precipitation

occurs due to the presence of ligand replacement. Nitrogen atoms that have lone electron pairs in alkaloids can replace ions in Dragendorff's reagent, while Wagner's reagent shows a brown precipitate. The precipitate formed is due to the presence of a coordinated covalent bond between the $K^+$ metal ion and the alkaloid, resulting in the formation of a potassium-alkaloid complex that precipitates in Wagner's reagent containing potassium iodide and iodine [Wijayati *et al*., 2020]. From the three tests above, the Moringa leaf extract showed no changes, so it can be concluded that the Moringa leaf extract does not contain alkaloid compounds.

Tannin is a phenolic compound that is easily soluble in water and non-polar solvents, the purpose of adding $FeCl^3$ to determine whether the Moringa leaves contain phenol groups is indicated by a blackish green and blackish blue color after adding $FeCl^3$, the results obtained are positive, a blackish green color is formed due to tannins reacting with $Fe^{3+}$ ions to form complex compounds [Kusumo *et al*., 2022].

Saponins are surface-active compounds easily detected by their foam-forming properties. The glycosidic bonding within saponins makes them highly polar. A positive saponin test indicates the presence of a 1-10 cm foam within 30 minutes of the test sample [Yasseir *et al*., 2022]. Phytochemical screening results indicate that Moringa leaf extract contains saponins, as foaming occurs when 2N HCl is added.

Steroids in Moringa leaf extract were confirmed positive for steroids, as indicated by the formation of a bluish-green color. Chloroform reagent and Liebermann-Burchard solution, a concentrated sulfuric acid solution, were used to detect the presence of steroids in the phytochemical screening test of Moringa leaf extract [Hasibuan & Edrianto, 2021].

Fractionation is a technique for separating a mixture into several fractions with different levels of polarity. The principle of liquid-liquid extraction is *like dissolves like*, where the solubility of a compound is determined by the similarity of its polarity level with the solvent used [Muhammad Andira Ibnu Shina *et al*., 2024]. Liquid-liquid extraction is carried out by weighing 10 grams of Moringa leaf extract using solvents that have different increasing polarities gradually, starting with non-polar, semi-polar and polar. First by adding 100 mL of n-Hexane into a separating funnel then shaking for ± 15 minutes and letting it stand until 2 layers are formed (aquadest layer below and n-hexane layer above) take the n-Hexane layer (replication 3 times). The next fractionation is carried out by adding 100 mL of ethyl acetate into the separating funnel then shaking for ± 15 minutes and leaving it until 2 layers are formed (aquadest layer below and ethyl acetate layer above) take the ethyl acetate layer (replication 3 times).

The next fractionation obtained the results of water fractionation. The n-Hexane, ethyl acetate, and water fractions were then placed in a water bath at 50°C. The use of n-Hexane as a non-polar solvent aims to remove fats and non-polar compounds such as fatty acids, steroids, and some triterpenoids. Meanwhile, ethyl acetate was chosen because of its semi-polar nature and is used to extract compounds with medium polarity such as flavonoids, tannins, and some alkaloids. The choice of ethyl acetate as a solvent is based on its ability to combine polar and non-polar groups so that the components in the extract that are polar and non-polar can be extracted. The water solvent used specifically to extract polar compounds includes flavonoids, glycosides, tannins, and alkaloids [Kumalasari *et al*., 2023]. The purpose of this fractionation is to group chemical compounds based on their polarity. Some explanations of the fractionation steps include the selection of ethanol and distilled water to dissolve the extract before being fractionated successively with n-Hexane, ethyl acetate, and water to separate compounds based on their polarity levels. Using ethanol without adding distilled water risks causing the n-Hexane fractionation solvent to mix homogeneously, so that phase separation does not occur. By adding ethanol and distilled water, it ensures that non-polar compounds will be perfectly attracted to n-Hexane, semi-polar compounds to ethyl acetate, and polar compounds will remain in the water phase, so that two immiscible liquid layers are formed. This process ensures that each group of compounds is separated purely and according to their respective chemical properties [Utari *et al*., 2023].

Based on the yield results in Table 6, it can be seen that the calculation of the fraction yield percentage of the n-Hexane fraction of Moringa leaves obtained an average percentage of 40%, then in the ethyl acetate fraction obtained an average percentage of 60% and in the water fraction obtained an average percentage of 20%. The different yield results of each fraction are related to the number of compounds contained in Moringa leaves. Repetition is done to ensure efficiency of the compound extraction process. Good extraction is obtained if the number of extractions is repeated with the addition of the amount of solvent gradually [Sandy *et al*., 2021].

The purpose of bacterial staining is to make it easier to see bacteria under a microscope, clarify the shape, see the external structure of bacteria such as cell walls, provide certain physical and chemical characteristics through the use of dyes and also increase the contrast of microorganisms [Erna & Azizah, 2025]. The staining method is used to categorize into gram-positive and gram-negative groups based on the characteristics of their cell walls. Gram-positive bacteria are characterized by a cell wall structure in the form of a thick peptidoglycan layer without lipoprotein or lipopolysaccharide content. In contrast, gram-negative bacteria have a peptidoglycan layer that is easily thin but is covered by an outer membrane rich in lipoprotein and lipopolysaccharide [Nurmailah fenti *et al*, 2024].

*Streptococcus mutans*is gram-positive, has the characteristic of a single coccus organism that is round and arranged in short chains, is anaerobic [Ratna Sulistyorin *et al*., 2025]. It can be seen in the gram staining image that the bacteria appear purple because they are able to retain the main color, namely crystal violet, which binds to the thick peptidoglycan layer. In addition, the bacteria have the characteristic of the organism that shows that *Streptococcus mutans* ATCC 25175 bacteria are included in the gram-positive bacteria group.

Testing the antibacterial activity of Moringa leaf extracts and fractions began with the creation of concentration levels for each Moringa leaf extract and fraction of 15%, 30% and 45% concentrations using DMSO as a solvent. Dimethyl Sulfoxide (DMSO) is a compound that can dissolve both polar and non-polar compounds and is soluble in various organic solvents and water. DMSO was used as a negative control, the use of DMSO did not have a significant impact on cell proliferation, so the integrity of the observation results in antibacterial testing with the agar diffusion method [Agustina *et al*., 2023]. The comparison used as a positive control was ciprofloxacin.

The medium used was nutrient agar bacterial suspension, poured onto the medium, spread evenly with a spreader, and then left for 15 minutes to allow the bacterial suspension to diffuse throughout the medium. Sterile paper discs soaked for approximately 15 minutes in the test solution concentration were drained and placed in a petri dish.

From Table 7 it can be seen that the average antibacterial activity of each sample, namely, Moringa leaf extract at concentrations of 15%, 30%, and 45% was 9.72, 10.46 and 11.34, the n-Hexane fraction of Moringa leaves at concentrations of 15%, 30% and 45% was 7.56, 9.48 and 10.33, the ethyl acetate fraction of Moringa leaves at concentrations of 15%, 30% and 45% was 10.37, 11.34 and 12.36 and the water fraction of Moringa leaves at concentrations of 15%, 30% and 45% was 7.07, 8.94 and 9.35 while the positive control of ciprofloxacin was 24.32 and the negative control of 1% DMSO was 0 mm.

The discussion above shows that the clear zones formed at each concentration of the test material have different diameters. The higher the concentration of the Moringa leaf extract and fraction given, the stronger the inhibition zone against the growth of *Streptococcus mutans* bacteria. This suggests that concentration can influence the effectiveness of an antimicrobial agent [Andika *et al*., 2022]. The level of extract concentration is directly proportional to the antimicrobial effectiveness. The higher the concentration, the more active compounds diffuse into the agar medium, which in turn expands the inhibition zone. The results of this study showed that the most effective concentration was 45%, and the most active fraction was the Moringa leaf ethyl acetate fraction. Ethyl acetate is a semi-polar solvent that can effectively extract polar and non-polar compounds, has low toxicity, and is easily evaporated. Therefore, the ethyl acetate fraction attracts compounds suspected of being antibacterial

in Moringa leaves, namely flavonoids, saponins, and tannins. This causes the ethyl acetate fraction to be the most active fraction, producing the greatest inhibitory power compared to the n-hexane and water fractions. Meanwhile, moringa leaf extract has the ability to inhibit the growth of *Streptococcus mutans* ATCC 25175 bacteria. This is because the extract still contains a complete mixture of secondary metabolites found in moringa leaves that function as antibacterials [Murdiyansah *et al*., 2020].

In the antibacterial activity test using the diffusion method, positive and negative controls were separated in different petri dishes, this is in line with the research of Nazarudin *et al*., (2020). Showing the antibacterial activity of *Streptococcus mutans* being tested and not affected by others, measurement results such as the diameter of the inhibition zone, were measured precisely. The diffusion method will spread in all directions from the point where the disc is placed will spread in all directions in the agar medium, so that if the positive and negative controls are placed in one petri dish together with the test sample, there is a possibility that the inhibition zones meet or merge, which can affect the distribution pattern and diameter of the inhibition zone. In addition, if several treatments are in one petri dish, bacterial growth can be uneven. This can cause competition for space in the agar medium, so that the boundaries of the inhibition zone become less clear. This separation also helps keep each treatment tested under the same conditions and do not affect each other. Thus, bacterial growth becomes more uniform and the results obtained from each treatment can be compared accurately. This is in accordance with the standard guidelines for antimicrobial testing from the Clinical and Laboratory Standard Institute which emphasizes the importance of setting the distance between the discs and the correct incubation conditions so that the inhibition zones do not affect each other and the test results become clearer [Ririn Puspita *et al*., 2024].

The MIC test was conducted with the aim of determining the smallest amount of antibacterial active substance that can inhibit the growth of the tested organism. In this study, the MIC test was conducted using a dilution method with a sterile liquid medium, namely nutrient broth. Bacterial growth can be seen from the media that becomes cloudy. The more fertile the bacterial growth in the media, the more cloudy the media. Six sterile test tubes were filled with nutrient broth media for four treatment tubes and two control tubes, the negative control tube contained 1% DMSO, while the positive control tube contained ciprofloxacin that had been planted with *Streptococcus mutans* ATCC 25175 bacteria. Four treatment tubes contained *Streptococcus mutans* bacterial colonies and ethyl acetate fractions with concentrations of 30%, 15%, 7.5% and 3.75%. The tubes were incubated for 24 hours at 37°C.

Figure 2 shows that the results obtained are that tube 1 appears clear with a concentration of 30% ethyl acetate fraction, tubes 2, 3 and 4 appear cloudy with concentrations of 15%, 7.5% and 3.75%. In the positive control of ciprofloxacin, a clear tube was obtained indicating that the antibiotic ciprofloxacin can inhibit the growth of *Streptococcus mutans* bacteria. In the negative control of 1% DMSO, a cloudy appearance proves that the solvent cannot inhibit the growth of *Streptococcus mutans* bacteria. From these results, it can be concluded that the Minimum Inhibitory Concentration (MIC) of the ethyl acetate fraction of Moringa leaves is a concentration of 30%. This test aims to identify the lowest concentration of antimicrobial agents that can kill bacteria completely (MBC). From Figure 3, it is known that there was growth of bacterial colonies in the ethyl acetate fraction of 15%, 7.5%, 3.75% concentration and the negative control of 1% DMSO, while the ethyl acetate fraction of 30% concentration and the positive control of ciprofloxacin produced a clear area. Based on this, it can be concluded that the Minimum Bactericidal Concentration (MBC) of the ethyl acetate fraction of Moringa leaves against *Streptococcus mutans* bacteria is a concentration of 30%.

The results of the normality test in Table 8 show that all treatment groups had normal data, indicated by a sig > 0.05. Negative controls were not included in this data processing because the statistical result was 0, so they were automatically removed by the system. All treatment groups were said to have normal data because the significant value of p > 0.05. The next stage was the Test of

Homogeneity of Variance to determine whether the samples taken had the same variance. The data results can be seen in Table 9.

The homogeneity test aims to determine whether the variances of two or more distributions are equal and is used as a requirement for independent analysis of the ANOVA test. The Homogeneity of Variances test shows a significance value > 0.05, which means that the data variance is homogeneous. The results of the homogeneity of variance test show a significance value of 0.431 > 0.05, so it can be concluded that the data comes from a population that has the same, homogeneous variance. Furthermore, all data were analyzed using a *One-Way* variance hypothesis test (One Way ANOVA).

Analysis using *One Way ANOVA* shows a significance value of 0.000 <0.05, so Ho is rejected, so it can be concluded that the hypothesis is proven true that there are differences in the effectiveness of antibacterial power in Moringa leaf extract, n-Hexane fraction, ethyl acetate fraction, and Moringa leaf water fraction. The function of the *One Way ANOVA* test is to differentiate the average between groups from an experiment that has samples of more than 2 groups. After the *One Way ANOVA* test is carried out, the next stage is the Post Hoc Test with the Tukey method. Tukey's test is used to specifically identify which treatment groups have differences or similarities in effects with each other. The results of the Tukey analysis show that each difference in concentration in this study has a significantly different effect on bacterial inhibition. This is evidenced by the comparison results, which indicate that each group has a significant difference compared to the other groups.

Based on the results of the Post Hoc Test data, the Tukey method shows that the treatment shows a significance that concludes p < 0.05, there is a real difference so that the resulting data is significant. For example, the comparison between 30% and 30% ethyl acetate extracts produces a significance value of 0.423 > 0.05, which identifies that there is no real difference in the inhibitory power of the two treatments. In addition, the results obtained from the Post Hoc Test show a p value < 0.05, for example, the inhibitory power of the positive control ciprofloxacin < 0.05 so that there is a real difference in the resulting data is significant.

## CONCLUSION

This study found that the simplex and ethanol extract of *Moringa oleifera* L leaves meet the standards of the Indonesian Herbal Pharmacopoeia with water content and drying loss below 10%, and positive for flavonoids, tannins, triterpenoids, saponins, and steroids, but negative for alkaloids. The most active ethyl acetate fraction inhibited *Streptococcus mutans* ATCC 25175 with an inhibition zone of up to 12.36 mm at 45%, MIC and MBC of 30%, supported by a significant ANOVA analysis ($p<0.05$). However, limitations include in vitro testing alone without in vivo or toxicity tests, and the use of a single strain without clinical variation.

Further research suggestions include isolating active compounds from the ethyl acetate fraction, testing on clinical strains and animal models, and formulating oral preparations such as mouthwash. Practically, these results have implications as a natural alternative for caries prevention in Indonesia, reducing dependence on chemical antibiotics and the risk of resistance, especially with a high caries prevalence, and supporting the development of affordable herbal medicines for public dental health.

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