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Antibacterial Activity Test of *Staphylococcus aureus* from Henna Leaf Extract (*Lawsonia inermis* Linn)

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Abstract

An antibacterial activity test against *Staphylococcus aureus* using henna leaf extract (*Lawsonia inermis* Linn.) has been conducted. This study aimed to determine the secondary metabolite components and the major compounds present in the henna leaf extract, as well as its inhibitory activity against *Staphylococcus aureus*. The secondary metabolite components were identified through phytochemical screening, while the major compounds were determined using GC-MS. The antibacterial activity was tested at concentration variations of 75%, 50%, 25%, and 12.5%. The resulting inhibition zones were then categorized according to standard criteria. The significance of the effect of concentration on the inhibition zones was analyzed using a one-way ANOVA test, followed by LSD test at $\alpha = 0.05$. The results showed that the henna leaf extract tested positive for flavonoids, alkaloids, tannins, and quinones and GC-MS analysis shows compounds included 1,5-heptadiene-3,4-diol, 2-methyl-; 2-undecene, 2-methyl-; cyclotrisiloxane, hexamethyl-; 2,6-dihydroxyacetophenone; and 2,3-dihydroxybenzoic acid. The antibacterial activity test results showed that different concentrations (12.5%, 25%, 50%, and 75%) produced different inhibition zones; however, all were still classified within the same category, namely moderate. Statistical analysis indicates a significant effect of concentration on the inhibitory activity against the test bacteria, where F test = 4.771 > F crit = 4.066 at $\alpha = 0.05$. Further analysis using the LSD test revealed two groups with significantly different inhibitory effects: the 75% and 50% concentration groups showed significantly higher inhibitory activity compared to the 25% and 12.5% groups.

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INTRODUCTION

Indonesia is known as one of the countries with the greatest biodiversity in the world. There are approximately 30,000 species of flowering plants spread throughout the archipelago (Cahyaningsih et al., 2021). Indonesia's diverse natural ingredients can be utilized as traditional medicine. One such plant is henna leaves, also known as inai leaves (Muharni et al., 2025), which have long been used as nail dye and in bridal traditions (Anggraeni & Mariana, 2023). In addition to its medicinal properties, henna leaves possess various biological activities, including antioxidant, antibacterial, antimicrobial, anti-inflammatory, analgesic, and anticancer properties (Joyroy et al., 2025).

Several studies have shown that secondary metabolites from henna leaves can be used as medicine, including antibacterial, anti-inflammatory, antifungal, analgesic, antipyretic, and antioxidant properties (Zubardiah et al., 2008). Henna leaves contain secondary metabolites such as flavonoids, carbohydrates, quinones, steroids, and phenols, which have antibacterial properties (Sharma & Anjana, 2018). Antibacteri-

als offer significant potential for development in the health sector, treating various bacterial diseases, such as food poisoning and typhoid fever.

Staphylococcus aureus is a bacteria that causes major infections in humans, found in the nasal cavity and skin of most of the human population. This bacteria is classified as a pathogenic bacteria that can cause poisoning in humans, especially through food that comes into contact with humans during handling. Poisoning caused by this bacteria is classified as an intoxication case, namely the ingestion of staphylococcal enterotoxin (Puspadewi et al., 2014). Research conducted by Sharma and Anjana (2018) has demonstrated the potential of henna plants, including their leaves, as antibacterial agents. Dita et al. (2021) reported that the inhibitory activity of ethanol extract from henna leaves dried for 3–4 days against S. aureus falls into the "strong" category. This finding indicates that it is necessary to examine the inhibitory activity of ethanol extract from fresh henna leaves in order to obtain more comprehensive data. At the same time, the use of samples from different habitats may provide comparative information regarding inhibitory activity. Lingga et al. (2025) reported that the growth location influences the secondary metabolite content of plants. Based on these considerations, a study entitled "Antibacterial Activity Test of S. aureus from Henna Leaf Extract (Lawsonia inermis L.)" was conducted.

METHOD

Tools

The tools used in this study were 500 mL Pyrex beakers, vials, stirring rods, spatulas, Pyrex funnels, porcelain dishes, containers, scales, a set of GC-MS equipment: columns, ovens, detectors, inlets, and MS scanners, scissors, loop wires, Petri dishes, laminar air flow apparatus, vortex tubes, alcohol, test tubes, test tube racks, tweezers, Erlenmeyer flasks, colony counters, incubators, refrigerators, matches, Bunsen burners, rulers, vernier calipers, evaporators, and autoclaves.

Materials

The materials used in this study were henna leaves (*Lawsonia inermis* L.), 95% ethanol (C_2H_5OH), Staphylococcus aureus ATCC 25923, penicillin, agar, NaCl, distilled water, 1M NaOH, HCl, magnesium, Meyer's reagent, gelatin, Whatman filter paper, plastic wrap, cotton swabs, label paper, sterile cotton, and aluminum foil.

Preparation of Henna Leaf Samples

The henna plants used were obtained from the Puunggapu Indah area, Andoolo District, South Konawe Regency, Kendari City, Southeast Sulawesi. Fresh henna plant samples were used.

Extraction of Henna Leaf (*Lawsonia inermis* L.)

200 g of henna leaf powder was weighed and macerated for 3 x 24 hours in 95% ethanol. The maceration was carried out using ethanol until completely submerged, then filtered using filter paper to obtain a liquid macerate. The resulting macerate was then evaporated to remove the solvent, resulting in a thick macerate (Thantry et al., 2026)

Phytochemical Test of Henna Leaf Extract (Hayat et al., 2020)

Flavonoid Test

Five drops of henna leaf extract were placed in a test tube and heated. Next, 0.05 g of magnesium powder and 5 drops of concentrated HCl were added. A color change indicates the presence of flavonoids (Moges et al., 2025).

Alkaloid Test

5 drops of henna leaf extract were placed in a test tube, followed by 3 mL of chloroform and 3 drops of Meyer's reagent. A precipitate formed, indicating the presence of alkaloids (Moges et al., 2025).

Tannin Test

5 drops of henna leaf extract were placed in a test tube and heated. Next, 5 drops of gelatin were added. A white precipitate formed, indicating the presence of tannins (Kulshreshtha & Saxena, 2022).

Quinone Test

5 drops of henna leaf extract were placed in a test tube, followed by 5 drops of 1 M NaOH. A color change indicated the presence of quinones (Rahimah et al., 2019).

Identification compounds using GC-MS

The components in cajuputi oil were determined using GC-MS. The operating conditions of GC- MS were programmed from 60 to 250 °C at 10 °C /min and injection temperature was from 70 to 250 °C. The results obtained were in the form of chromatogram and mass spectrum. The data obtained were further interpreted by comparing with NIST MS and WILLEY library.

Antibacterial Activity Testing (Nursin et al., 2019and Musta, 2022)

Sterilization of Equipment and Materials

All equipment was thoroughly washed and dried. Vials, test tubes, Erlenmeyer flasks, and Petri dishes were wrapped in paper. They were then sterilized using an autoclave at 121°C for 15 minutes. Aseptic processing was carried out in a previously cleaned Laminar Air Flow apparatus, then sterilized with a UV lamp for approximately 1 hour before use in the antibacterial test.

Media Preparation and Sterilization

This medium was prepared by dissolving 22.1 g of Nutrient Agar in 260 mL of distilled water in Erlenmeyer flasks. Nutrient Agar (NA) media was sterilized in an autoclave at 121°C.

Microorganism Rejuvenation

The microorganism used in this study was *S. aureus* ATCC 25923. This bacteria was rejuvenated by taking one or two loops of the bacteria, then placing them in a test tube containing 10 mL of sterile liquid medium and incubating them for 24 hours.

Antibacterial Activity Testing

A 20 mL NA medium was pipetted into an Eppendorf tube, and 10 µL of *S. aureus* ATCC 25923 bacterial inoculum was added and shaken until homogeneous. Once homogeneous, the medium was poured into a Petri dish using circular motions until the medium adhered to the surface of the dish. Then, it was allowed to stand for several minutes until solid. Then, paper discs soaked in the test solution (12.5% henna leaves) were placed on the surface of the solid medium. Streptomycin served as a positive control, and distilled water served as a negative control. The Petri dish was then tightly closed and wrapped in plastic wrap. It was then refrigerated for 2 hours. After that, it was incubated for 5 hours at 37°C and the clear zone formed was measured.

Data Analysis

Inhibitor zone of anti that are resulted was categorized according Davis and Stout (1971) in Ouchari et al., (2019) as shown in Table 1.

Table 1. Inhibitor Zone Category

Inhibitor Zone (mm)	Category
> 20	Very Strong
11-20	Strong
6-10	Medium
≤ 5	Weak

Inhibition zones at each concentration were analyzed by one-way ANOVA to test concentration effects on antibacterial activity. H_0 was accepted if F test ≤ F criteria; otherwise, H_1 was accepted. All decisions used a significance level of $\alpha = 0.05$. If H_1 was accepted, a post hoc LSD test was run at $\alpha = 0.05$ to evaluate inhibition zone differences among concentrations, using criteria similar to the F-test.

RESULTS AND DISCUSSION

Sampling and Processing of Henna Leaf

A total of 200 g of henna leaf powder was extracted using a maceration method with ethanol for 3 x 24 hours at room temperature. This maceration process works through a soaking mechanism that creates a pressure difference between the inside and outside of leaf cells, causing the cell walls and membranes to break down and release secondary metabolites from the cytoplasm into the organic solvent (Yulianingtyas & Bambang, 2016). Ethanol was chosen as a solvent based on its ability to dissolve both polar and nonpolar compounds, thanks to the presence of hydroxyl groups (-OH) that interact with polar compounds and methyl groups (-CH3) that bind to nonpolar compounds (Verdiana et al., 2018).

Several important factors influencing extraction results include the type of solvent, stirring speed, particle size, and the volume and type of solvent used. This maceration method has several advantages, including simple equipment, low operational costs, and the ability to maintain the stability of thermolabile compounds that are susceptible to heat. The yield obtained from henna leaves is 3.55% (w/w) in 200 g of henna leaf powder. According to Komala et al., 2019, the yield of henna leaves is 22.6% in 1.68 kg of henna leaf powder. This difference depends on the number of henna leaf samples. The Buton people usually use henna leaves as medicine and as a natural nail dye. However, fresh leaves have a short shelf life.

Pawarti et al. (2023) reported that extraction yield is significantly influenced by sample mass, solvent polarity, and extraction time, where optimization of these parameters can significantly increase metabolite yield. Thus, the maceration method using ethanol has been proven to be an effective technique for extracting bioactive compounds from henna leaves, while

proper post-harvest handling and processing are crucial to maintain the quality and extend the usefulness of henna leaves for traditional and pharmaceutical applications.

Phytochemical Screening of Henna Leaf Extract

Phytochemical screening, a qualitative analysis test, was performed on sample extracts to determine the presence of active compounds such as flavonoids, tannins, alkaloids, and quinones in henna leaf extract. The results of the phytochemical screening of henna leaf extract are shown in Table 1.

Table 1. Results of Phytochemical Screening of Henna Leaf Extract

Secondary Metabolite Compounds	Phytochemical Screening Results	Note
Flavonoids	Color change observed	+
Alkaloids	Precipitate observed	+
Tannins	White precipitate observed	+
Quinones	Yellowish-brown solution observed	+

Based on the results of phytochemical screening of henna leaf extract, it was found that the extract contains alkaloids, flavonoids, tannins, and quinones. This is consistent with Mustapha et al. (2024), who reported that henna leaf extract contains secondary metabolites, and is further supported by Altemimi et al. (2017), who stated that various medicinal plants generally contain major groups of phytochemical compounds such as flavonoids, alkaloids, and tannins with significant biological activities. In the flavonoid test, flavonoids are soluble in polar solvents and therefore soluble in water.

The appearance of color in the extract layer after the addition of HCl and magnesium powder indicates the presence of flavonoids. Flavonoids possess anti-inflammatory, antioxidant, anticarcinogenic, antiallergic, and antibacterial properties. They exhibit antibacterial activity by interacting with bacterial DNA. This interaction leads to disruption of cell wall permeability as well as damage to bacterial microsomes and lysosomes (Widyaningrum et al., 2021). Overall, these data demonstrate that henna leaf extract has great potential to be developed as a natural source of bioactive compounds for pharmaceutical applications as well as traditional medicine.

The presence of tannins is detected by the appearance of a precipitate after the extract is added to gelatin. Tannins are water-soluble, so when dissolved in hot water, their solubility increases (Puspitasari and Desrita 2019). Tannins are a group of compounds that can function as a treatment for diarrhea, stop bleeding, treat hemorrhoids, and act as antibacterials (Andriyani et al., 2010). Tannins are plasmolytic, which can shrink cell walls or membranes, disrupting their permeability. Due to impaired permeability, cells cannot carry out vital activities, resulting in stunted growth or death (Wulandari, 2020).

Siamtuti et al. (2017) reported that tannins have antibacterial activity by precipitating proteins and inhibiting the activity of microbial enzymes, thereby disrupting bacterial growth and cell function. Thus, it is clear that tannins play an important role as natural bioactive compounds with strong pharmacological potential. Therefore, plant extracts containing tannins can be considered promising candidates for further development in pharmaceutical and therapeutic applications.

Alkaloid compounds can be dissolved in polar solvents. The presence of alkaloid compounds is detected using Mayer's reagent, which causes a precipitate to form. Alkaloid compounds have antibacterial activity by inhibiting cell wall synthesis, causing bacterial disfigurement and lysis. The use of alkaloids has physiological activity that can be widely applied in medicine (Marselia et al., 2015). Further studies by Sarjono et al. (2025) reported that alkaloid compounds derived from natural sources demonstrate strong antibacterial activity through disruption of bacterial metabolic processes and inhibition of microbial growth.

The presence of quinone compounds is detected by the appearance of a brownish color after the addition of NaOH. Quinones form irreversible complexes with nucleophilic amino acids in proteins, causing the protein to lose its function and have the ability to generate stable free radicals (Fatmawati, et al., 2022). Thus, alkaloid compounds play an important role as bioactives in plant extracts and have great potential to be developed as natural antibacterial agents in pharmaceutical applications.

Characterization of Compounds in Henna Leaf Extract

The GC-MS analysis method combines chromatography and mass spectroscopy. GC-MS analysis can identify the chemical compounds predicted to be present in a natural dye. The results of GC-MS testing of henna leaf extract are shown in Figure 1.

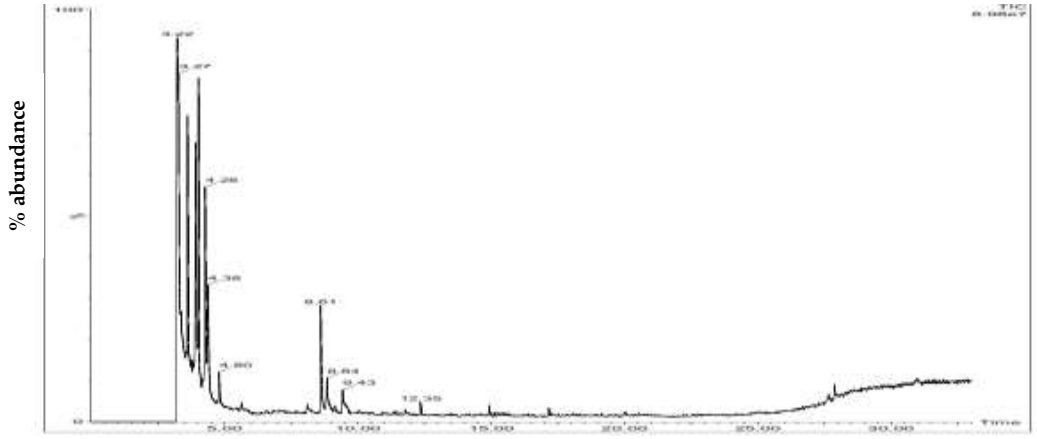

Figure 1. Chromatogram of Henna Leaf Extract

Table 2. Compounds Identified as Secondary Metabolites

Retention Time	Molecular Mass	% Area	Molecular formula	Compound
3,914	142	1697301,25	$C_8H_{14}O_2$	1,5-Heptadiene-3,4-diol, 2-methyl-
4,383	168	763356,88	$C_{12}H_{24}$	2-Undecene, 2-methyl
4,802	222	230201,23	$C_6H_{18}O_3Si_3$	Cyclotrisiloxane, hexamethyl
9,429	296	174677,11	$C_{14}H_{24}O_3Si_2$	2,6-Dihydroxyacetophenone, TMS derivative
12,359	370	68567,00	$C_{16}H_{30}O_4Si_3$	2,3-Dihydroxybenzoic acid, 3 TMS derivative

Based on the results of GC-MS analysis of henna leaf extract, a number of secondary metabolite compounds were identified, namely 1,5-Heptadiene-3,4-diol, 2-methyl- at a retention time of 3.914 with a % area of 1697301; 2-Undecene, 2-methyl at a retention time of 4.383 with a % area of 763356.88; Cyclotrisiloxane, hexamethyl at a retention time of 4.802 with a % area of 230201.23; 2,6-Dihydroxyacetophenone, 2 TMS derivative at a retention time of 9.429 with a % area of 174677.11; and 2,3-Dihydroxybenzoic acid, 3 TMS derivative at a retention time of 12.359 with a % area of 68567.00. The identified compounds indicate the presence of phenolic compounds that potentially contribute to the antimicrobial activity of henna leaves. Wanjare and Surve (2021) also showed GCMS results that Lawsonia plants can be used as anti-inflammatory, anti-allergic, absorbent, demulcent, anti-itch, and antidermatic agents.

Antibacterial Activity of Henna Leaves

The antibacterial activity of henna leaves was tested using the sterile paper disc diffusion method. The henna leaves used as an antibacterial were controlled with streptomycin as a positive control and distilled water as a negative control.

Streptomycin was used as a positive control due to its ability to treat various bacterial infections. This antibacterial works by inhibiting bacterial cell wall synthesis by binding to one or more streptomycin-protein bonds, thus inhibiting the final transpeptidase step in peptidoglycan synthesis in the bacterial cell wall. This results in cell wall biosynthesis being inhibited and the bacterial cell breaking down (lysis). This mechanism is similar to that used by penicillin to inhibit the growth of *S. aureus* bacteria. Aqua, as a negative control, acts as an emulsifier between the henna leaf extract and the nutrient agar (NA) medium.

This mechanism also is supported by studies showing that antibiotics, such as penicillin, act by inhibiting peptidoglycan synthesis through interaction with penicillin-binding proteins (PBPs), thereby disrupting cell wall formation and causing bacterial cell lysis (Ghooi & Thatte, 1995).

Antibacterial activity can be seen by the formation of a zone of inhibition around the paper disc, a clear area or zone around the paper disc that is free of bacteria. Antibacterial strength or classification is determined by measuring the diameter of the inhibition zone formed from the tested extract. Research on the activity test of henna leaf extract on the growth of *S. aureus* bacteria showed that henna leaf extract has inhibitory power against *S. aureus* bacteria. This is evidenced by the presence of a clear zone diameter around the disc containing henna leaf extract after incubation for 24 hours. Zannat, et al., (2023) also reported similar findings and this study adds evidence of henna leaves potential as a natural antibacterial agent.

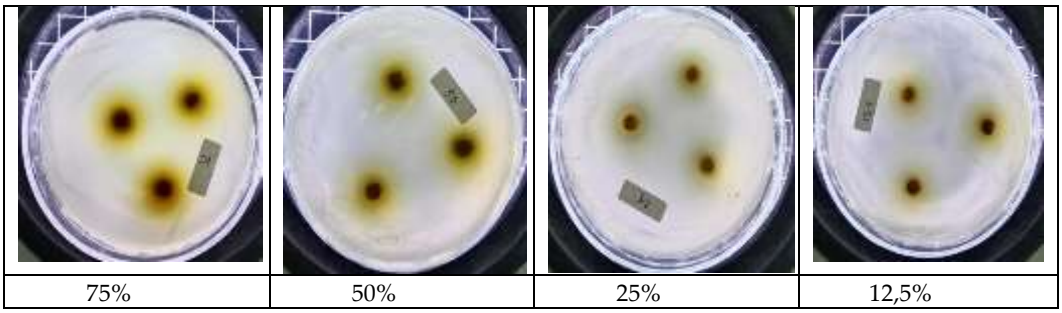

Figure 2. The Result of Antibacterial Test of Henna Leaf Extract

Figure 2. shows that each concentration of henna leaf extract exhibits varying activity. The data above shows that the average diameter of the clear zone of henna leaf extract against *S. aureus* bacteria falls within the moderate to strong category. This is consistent with the fact that the higher the concentration of an antimicrobial, the larger the clear zone formed. The higher the concentration of an antimicrobial, the greater the active ingredient, thus increasing its effectiveness in inhibiting bacteria and forming a wider clear zone. Conversely, at lower concentrations, the antimicrobial substance contained in an antimicrobial is reduced, resulting in decreased activity. Meanwhile, when categorized, all tested concentrations produce inhibition zones that fall within the same category. That is the medium category

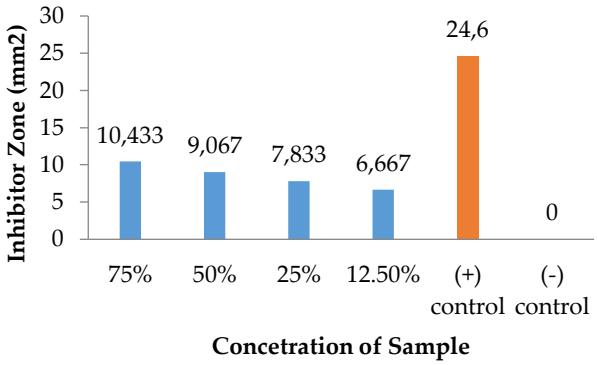

Figure 3. The Diagram of Antibacterial Test Result of Henna Leaf Extract

Table 3. The Result of Anova One Way test

Source of Variation	SS	df	MS	F test	P-value	F crit
Between Groups	23.5933	3	7.8644	4.771	0.0343	4.066
Within Groups	13.1866	8	1.6483			

Moreover, to determine the significance of the effect of concentration variation on the inhibitory activity of henna leaf extract against *S. aureus*, an F test was conducted, and the results are presented in Table 3. Its shows that at $\alpha = 0.05$, F test = 4.771 > F crit= 4.066, indicating that H_1 is accepted. This means that there is a significant effect of differences in concentration on the inhibitory activity against the test bacteria. It also indicates that at least one treatment concentration has a significantly different inhibitory effect compared to the others; therefore, a further analysis using the LSD test was performed. The LSD test result was 2.306, which was then used as a reference to determine the differences in inhibitory activity at each treatment concentration, as shown in Table 4.

Table 4. The Result of LSD Test

Concentration of Sample	Inhibitor Zone (mm^2)	Group	Different Average
75%	10.4333	A	-
50%	9.0666	A	1.3666
25%	7.8333	B	2.6000
12.5%	6.6667	B	1.1667

Table 4 reveals two groups with significantly different inhibitory effects. The inhibitory

activity of the 75% and 50% sample groups is significantly different from that of the 25% and 12.5% sample groups. These findings are in agreement with studies, which reported that *Lawsonia inermis* leaf extracts exhibit significant antibacterial activity against *S. aureus*, with stronger inhibition observed at higher concentrations (Zannat, et al., 2023). This condition demonstrates that henna leaf extract exhibits a clear concentration-dependent antibacterial effect against *S. aureus*, with higher concentrations providing significantly greater inhibition.

CONCLUSION

Based on the research results, it can be concluded that the compounds in henna leaf extract (Lawsonia inermis Linn.) based on GC-MS characterization are 1,5-Heptadiene-3,4-diol, 2-methyl-, 2-Undecene, 2-methyl, Cyclotrisiloxane, hexamethyl, 2,6-Dihydroxyacetophenone, and 2,3-Dihydroxybenzoic acid. The antibacterial activity test results for *Staphylococcus aureus* extract of henna leaf showed different inhibitory power for each concentration variation of 12.5%, 25%, 50%, and 75%, indicating the diameter of the inhibition zone formed was in the medium category. The results of the statistical analysis showed a significant effect of different treatment concentrations on their inhibitory activity against the test bacteria, where F test= 4.771 > F crit = 4.066 at α = 0.05. Further analysis using the LSD test revealed two groups with significantly different inhibitory effects. The inhibitory activity of the 75% and 50% sample groups was significantly different from that of the 25% and 12.5% sample groups.

RECOMMENDATION

In this study, only crude extract of henna leaves was used to evaluate its inhibitory activity against S. aureus. This condition, caused by the absence of separation of active compound components, resulted in the inhibitory potential of the sample not being optimal. Therefore, a further step is required, namely the separation of active compounds for further activity testing using pure secondary metabolites isolated from henna leaf extract, so that their antibacterial activity can be compared.

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