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*Research Article*

# Determination of the Active Chemical Compounds and the Antibacterial Activity of Various Fractions of *Lawsonia inermis* L.

**Sri Mulyaningsih** [1*]

**Febriyati Adji Rachmadani** [2]

[1] Department of Pharmacy, Universitas Ahmad Dahlan, Yogyakarta, Special Region of Yogyakarta, Indonesia

[2] K24 Indonesia, Yogyakarta, Special Region of Yogyakarta, Indonesia

*email: sri.mulyaningsih@pharm.uad.ac.id

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## Abstract

*Lawsonia inermis* L., or henna leaves, are usually used to treat wounds on the skin. *Lawsonia inermis* contain naphthoquinones, flavonoids, tannins, and saponins. The antibacterial activity of *L. inermis* leaf extract in various solvents has been extensively studied. However, which component is responsible for the antibacterial activity is still unknown. This study was intended to investigate the antibacterial effect of *L. inermis* fractions against *Staphylococcus aureus* and to discover the antibacterial chemical class in the most active fraction. The methanol extract was fractionated with *n*-hexane and ethyl acetate subsequently. The antibacterial activity of various fractions was tested using the well diffusion method. TLC-bioautography was used to identify the class of active chemicals as antibacterial agents. Antibacterial activity against *S. aureus* was highest in the ethyl acetate fraction. TLC-bioautography of the ethyl acetate fraction showed inhibition areas at Rf values of 0.25 and 0.53, respectively, indicating the naphthoquinones and phenolic compounds groups. In conclusion, naphthoquinones and phenolic compounds are suggested to contribute to the antibacterial effect of the ethyl acetate fraction of *L. inermis* leaves.

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## INTRODUCTION

*Lawsonia inermis*, or known as *henna* (English name), has local names such as *hennastrauch* (Germany), *hena/mendhi* (Pakistan, India), and *inai/pacar kuku* (Indonesia, Malaysia). It is one of the familiar plants widely found in Asia, including in Indonesia[1]. Generally, the leaves are used by the community as a natural reddish brown dye for coloring nails, hair, and skin. The community often uses *L. inermis* leaves to treat wounds and skin inflammation[2,3].

*Lawsonia inermis* leaves contain large amounts of chemical compounds such as lawsone, flavonoids, tannins, coumarins, sterols, and terpenoids[4]. According to the phytochemical analysis[5], all of the extracts contained naphthoquinones, saponins, flavonoids, and steroids. Lawsone (2-hydroxy-1,4-naphthoquinone), a kind of naphthoquinone, has been identified as the major component in *L. inermis*[6,7].

Many studies have investigated the antimicrobial activity of *L. inermis* leaves extract in various solvents. Usman and Rabiu[5] reported that the aqueous extract of *L. inermis* leaves inhibited *Staphylococcus aureus* and *Epidermophyton floccosum*. The *L. inermis* extract inhibited some microbial isolates at 1000 µg/mL concentrations. The most significant antimicrobial activity of methanol, ethanol, and aqueous *L. inermis* extract against some human pathogenic bacteria, and some fungi were possessed by methanol and ethanolic extracts[8,9]. However, many investigations reported that methanol extract exhibited promising antibacterial activity against some pathogenic bacteria from clinical isolated[10-12]. Leaves extract of *L. inermis* has also been reported to possess good biofilm inhibition and antibacterial activity, which can be explored to develop new drugs for MDR pathogens[13].

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Although *L. inermis* was reported to have acted as an antibacterial agent, the information on which compound is responsible for the antibacterial activity is still unclear. In this study, the methanol extract of *L. inermis* leaves was fractionated with *n*-hexane and ethyl acetate to obtain *n*-hexane, ethyl acetate, and methanol fractions. The purpose of the fractionation is to separate the compounds in the extract into the solvent according to their polarity. Non-polar compounds were screened out with *n*-hexane, semi-polar compounds were sorted out with ethyl acetate, and polar compounds were taken with methanol[14]. The three fractions were then tested against *S. aureus* and continued with TLC-bioautography. This study's objective was to comprehend and determine the antibacterial activity of the active fraction of *L. inermis* leaves and the class of active compounds as an antibacterial agent from the most active fraction against *S. aureus*.

## MATERIALS AND METHODS

### *Materials*

Plant materials were obtained from Merapi Farma Herbal Yogyakarta, and the identification of the plant was carried out by the Biology Laboratory, Faculty of Science and Applied Technology, Universitas Ahmad Dahlan, Yogyakarta, with the voucher specimen number 087/Lab.bio/B/VI/2019. *Staphylococcus aureus* ATCC 25923, Mueller Hinton Agar (Oxoid), Brain Heart Infusion medium (Oxoid), 1% $BaCl_2$ (Merck), 1% $H_2SO_4$ (Merck), NaCl 0.9% (Merck), 1% Dimethylsulfoxide (Merck), Vancomycin 1% (Vancep), and silica plate $GF_{254}$ (Merck). The instruments used were digital balance (Ohaus), oven (Binder), micropipettes (Soccorex), biosafety cabinet (Monmouth Scientific), incubator (Binder), autoclave (Shenan), TLC chamber (Camag), UV lamps, and glassware (Pyrex).

### *Methods*

*Preparation of methanol extract*

A total of 2 kg of *L. inermis* leaves were washed and dried in the oven. The dried *L. inermis* leaves were then ground with a blender and sieved with a 50-mesh sieve. An amount of 250 g of *L. inermis* leaves powder was macerated with 1000 mL of methanol. The maceration process was carried out at room temperature for the first six hours while shaking, then allowed to stand for 18 hours. Remaceration was done in the same manner. Afterward, the macerate was filtrated using a Buchner funnel. The filtrate was then evaporated with a rotary evaporator until a thick extract was obtained[15].

*Fractionation of methanol extract of L. inermis*

A total of 15 g of *L. inermis* extract was subsequently dissolved in methanol and fractionated with *n*-hexane and ethyl acetate. Each *n*-hexane fraction and ethyl acetate fraction were evaporated with a rotary evaporator to get the *n*-hexane, ethyl acetate, and methanol fraction. Every fraction was weighed to give the yield of fractionation.

*Antibacterial activity test against S. aureus*

The well diffusion method was used to conduct the antibacterial activity test. A sterile cotton swab was used to apply the *S. aureus* bacterium suspension with a 1 x $10^8$ CFU/mL density to the Mueller Hinton agar surface. Then the surface of the agar is perforated and dripped with test and control samples. Next, the plate was incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones was measured.

*Phytochemical compound testing of the ethyl acetate fraction by reaction test*[16]

The test solution was made in a concentration of 1% w/v by dissolving 250 mg of the ethyl acetate fraction of L. inermis in 25 mL of distilled water. The test was carried out to determine the presence of naphthoquinone, flavonoid, tannin, and saponin compounds.

*Naphthoquinone test*

As much as 1 mL of the test solution was added to a few drops of 1 N NaOH, a positive solution containing naphthoquinone will show a red color.

*Flavonoid test*

The test solution was dropped on filter paper, then treated with ammonia vapor. If it causes a yellow color, it indicates the presence of flavonoids.

*Tannin test*

A positive solution containing naphthoquinone will show a red color when one mL of the test solution is added to a few drops of 1 N NaOH.

*Saponin test*

A total of 1 mL of the test solution was shaken vigorously for 10 seconds. If the foam is formed for not less than 10 minutes as high as 3-10 cm and by the addition of 2 N HCl the foam does not disappear, it is positive for saponins.

*TLC-Bioautography*

As the mobile phase, the ethyl acetate fraction of *L. inermis* leaves was separated using TLC with chloroform : acetone : formic acid (6 : 1.5 : 0.5). The surface medium of MH agar was sprayed with a bacterial suspension evenly. After that, the silica gel plate was placed on the surface of the MHA agar medium in an inverted position and left for 30 minutes to allow diffusion based on the reference with minor modifications[17]. Then the plate was removed, and the petri dish was incubated at 37°C for 24 hours. After incubation, the inhibition zone was observed. The inhibition zone that appears was measured by the Rf value and compared with the chromatogram detected with spraying reagents to determine the group of active compounds.

## RESULTS AND DISCUSSION

*Yield of extraction and fractionation of L. inermis leaves*

The maceration of *L. inermis* leaves obtained 47.96 g of methanol extract with a yield of 19.18%. In the study by Sharma and Goel[18], the yield of methanol extract of *L. inermis* nails was 17%. The extraction yield we obtained is greater than that of the previous study due to several factors, such as geographical conditions, sampling time, or other factors.

The fractionation using *n*-hexane obtained a *n*-hexane fraction of 1.4610 g with a yield of 9.74%. The *n*-hexane fraction had a greenish color due to the presence of chlorophyll. With a yield of 25.55%, the ethyl acetate fraction displayed a reddish-brown color of 3.8333 g. The residue in the form of methanol fraction is 8.0585 g with a yield of 53.72%.

*Antibacterial activity of the fractions of L. inermis against S. aureus*

The antibacterial activity test was performed on MHA media seeded with *S. aureus* using the well diffusion method. **Table I** reveals that the inhibitory zone diameter of the methanol extract from *L. inermis* leaves is 11.33 mm. The *n*-hexane, ethyl acetate, and methanol fractions result in 8.33 mm, 9.50 mm, and 0 mm, respectively. Remarkably, the methanol extract had the most significant inhibitory zone, though the methanol fraction had none. According to Nwodo *et al*.[19], fractionation occasionally led to increased activity but occasionally led to decreased activity. This represents a situation where fractionation leads to loss of activity, suggesting that components of the extract may have acted synergistically or additively to produce the activity observed in the extract. Another study found that some fractions of *Tamarindus indica* showed no activity against *type P. aeruginosa* and *E. coli* strains, unlike the extract.

The antibacterial activity of the methanol extract was greater compared to each fraction. The highest antibacterial activity was confirmed in crude methanol extract, possibly due to all the antibacterial compounds in its fractions[20]. These results indicate that the active chemical compounds as antibacterial agents were spread into these fractions and were not collected in one certain fraction. Previous studies showed that the chemical compounds contained in plants provide a synergistic or additive effect in causing pharmacological effects[21]. If these compounds are separated, it will cause a decrease in their pharmacological activity. However, the ethyl acetate fraction had the greatest antibacterial activity compared to the other fractions. The positive control in the antibacterial activity test was vancomycin because it is sensitive to *S. aureus*. As a

negative control, 1% DMSO was utilized to dissolve practically all polar and non-polar substances. There was no bactericidal action in 1% DMSO. **Table I** shows the diameter of the inhibitory zones of the methanol extract and the fractions.

**Table I.** Diameter of inhibition zones of methanol extract and various fractions against *S. aureus* using well diffusion method

| Sample tested (w/v) | Diameter of inhibition zone (mean± SD in mm) |
|---|---|
| Methanol extract 10% | 11.33 ± 0.29 |
| *n*-hexane 10% | 8.33 ± 0.52 |
| Ethyl acetate 10% | 9.50 ± 0.87 |
| Methanol fraction 10% | 0.00 ± 0.00 |
| Vancomycin 1% | 21.00 ± 0.00 |
| DMSO 1% | 0.00 ± 0.00 |

Furthermore, the antibacterial activity of the ethyl acetate fraction of *L. inermis* leaves was tested at different concentrations to determine the concentration that could inhibit the growth of bacteria. The concentrations of ethyl acetate fraction tested were 5, 15, and 20 %w/v. **Table II** shows that the diameter of the inhibition zone increased as the concentration of the ethyl acetate fraction was raised. The 20% ethyl acetate fraction produced the largest inhibition diameter of 10.67 mm. Statistical analysis with the Kruskal-Wallis test showed that there were differences in the antibacterial activity of each concentration tested.

**Table II.** Diameter of inhibition zones of the ethyl acetate fraction against *S. aureus* using well diffusion method

| Concentration of ethyl acetate fraction (w/v) | Diameter of inhibition zone (mean ± SD in mm) |
|---|---|
| Vancomycin 1% | 21.00 ± 0.00 |
| DMSO 1% | 0.00 ± 0.00 |
| 5% | 8.25 ± 0.43 |
| 15% | 9.67 ± 0.72 |
| 20% | 10.67 ± 0.80 |

*The phytochemical content of ethyl acetate fraction of L. inermis leaves*

The results of the phytochemical screening test (**Table III**) show that the ethyl acetate fraction of *L. inermis* leaves contains naphthoquinones, flavonoids, and tannins. In the naphthoquinone test, when the ethyl acetate fraction of *L. inermis* leaves was dripped with 1 N NaOH solution, the color changed to brownish red due to the presence of a chromophore group in the ethyl acetate fraction of *L. inermis* leaves so that the addition of a hydroxyl group from NaOH will give a red color[6,15]. When testing for flavonoids, a more intense yellow color appears on filter paper that has been treated with ammonia vapor, indicating the presence of flavonoid[22].

The test on tannin compounds, when added to the gelatin solution in the ethyl acetate fraction of *L. inermis* leaves, forms a precipitate due to the nature of the tannins, which can precipitate protein so that the tannin test with the addition of gelatin solution, which is a protein will be precipitated by the tannins[23]. In the saponin test, our finding showed that within 10 minutes, the foam slowly disappeared when HCl was added, indicating that the ethyl acetate fraction of *L. inermis* leaves did not contain saponin[16].

**Table III.** The phytochemical screening of ethyl acetate fraction of *L. inermis*

| Reaction test | Result | Presence |
|---|---|---|
| Naphthoquinone | Brownish red | Present |
| Flavonoid | Yellow | Present |
| Tannin | Brown precipitate | Present |
| Saponin | No foam | Absent |

*TLC-Bioautograaby of ethyl acetate fraction of L. inermis*

The results of the TLC-bioautography of the ethyl acetate fraction of *L. inermis* leaves can be seen in **Figures 1** and **2**. **Figure 1** shows two inhibition zones formed on MHA inoculated *S. aureus* with Rf values of 0.25 and 0.53. After the TLC plate was sprayed with 10% KOH, a spot with the Rf value of 0.25 appeared to be a positive red-brown color, indicating the presence of naphthoquinone compounds (**Figure 2** and **Table IV**). The appearance of a reddish-brown color is due to the addition of

a hydroxyl group from KOH[15]. After the plate was sprayed with $FeCl_3$, a spot with the Rf value of 0.53 (**Figure 2** and **Table IV**) showed a blue-black color, indicating the presence of phenolic compounds[16]. The reaction forms a blue-black color due to the formation of complex compounds between metal atoms of iron (Fe) and non-metal atoms. The presence of phenolic compounds is in line with previous research, which stated that *L. inermis* contains phenolics[11,24]. Husni *et al*.[25] reported that the ethanol extract of *L. inermis* leaves has a total phenolic content of 16.02 g/100 g.

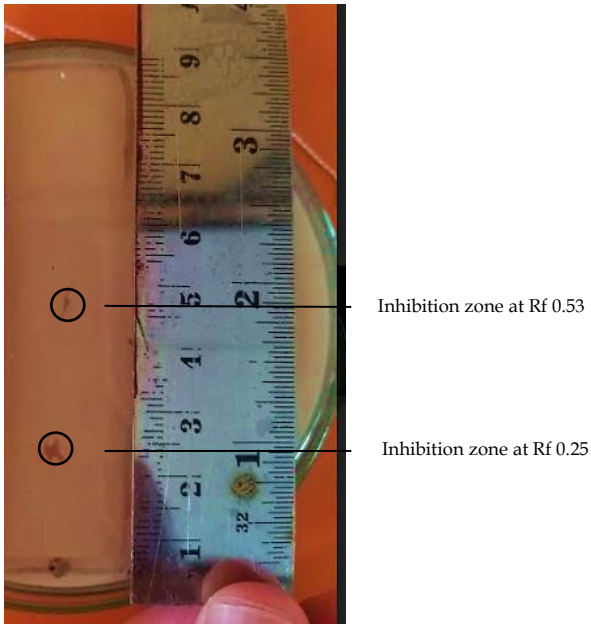


**Figure 1.** The bioautography result of the ethyl acetate fraction of *L. inermis* leaves on Mueller Hinton Agar inoculated by *S. aureus*. The inhibition zones are depicted with black circles.

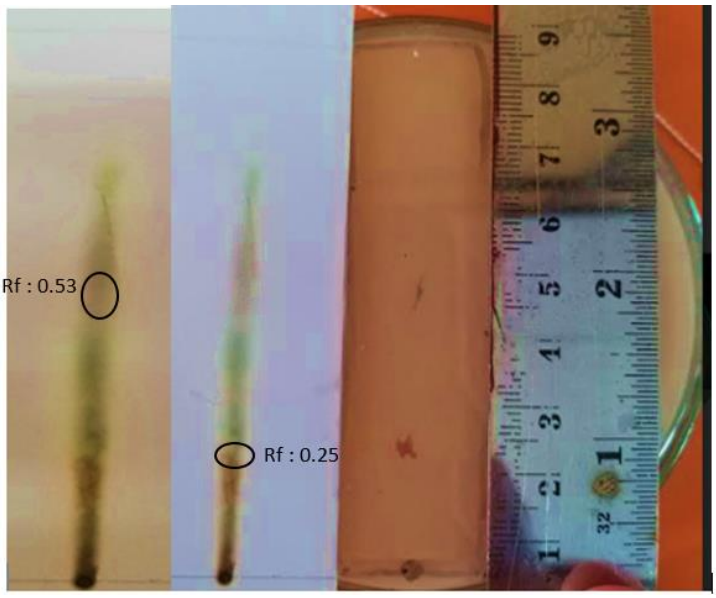


**Figure 2.** The chomatogram of ethyl acetate fraction of *L. inermis* leaves after sprayed with $FeCl_3$ (**a**), and with KOH 10% (**b**).

**Table IV.** Result of TLC-bioautography of the ethyl acetate fraction of *L. inermis* leaves

| Rf | Detection | Color | Chemical group |
|---|---|---|---|
| 0.25 | KOH 10% | Brownish red | Naphthoquinone |
| 0.53 | $FeCl_3$ | Blue black | Phenolic compounds |

The mechanism of phenolic compounds in inhibiting bacterial growth is to irreversibly bind to nucleophilic amino acids from proteins, causing protein inactivation and becoming non-functional while also inactivating adhesins and enzymes on microbial membranes. The presence of phenolic groups with a high protein binding affinity can inhibit microbial enzymes while also boosting membrane affinity, resulting in increased antibacterial action[26]. Luis *et al*.[27] investigated the mechanism of action of a phenolic compound and hypothesized that it was linked to polyphenol-membrane contact. The presence of a phenolic compound was connected to increased permeability and depolarization of the cell layer, as well as a decrease in respiratory action in the *S. aureus* ATCC 25923 strain. The component of activity of a phenolic compound is connected to cell layer damage and changes in the vigorous metabolism of *S. aureus* cells[8,27]. A phenolic compound suppresses a hemolysin secretion in *S. aureus*; a membrane-dependent activity further supports the initial findings[28]. Simple phenols' activities are thought to be mediated through contact with sulfhydryl groups in microbial enzymes, inhibiting those enzymes or nonspecific protein interactions[29].

Naphthoquinones are found naturally in various plants and are considered promising antibacterial agents. Increased ROS production, followed by apoptotic cell death, is the mechanism of action for this antibacterial agent. Various naphthoquinone compounds have pharmacological effects, including antibacterial, anticancer, antitubercular, antimalarial, and trypanocidal properties. Naphthoquinone analogs are highly lethal to infected cells due to their capacity to create reactive oxygen species (ROS) and can inhibit cellular enzymes involved in apoptosis and cell proliferation. Consequently, these compounds serve as models for developing clinical antibacterial drugs[30]. Another study confirmed 2-hydroxy-1,4-naphthoquinone found in *L. inermis* as the main compound that may be an antibacterial agent[31]. However, its specific mechanism of action needs further research. Although the class of compounds with antibacterial action was identified in this investigation, the actual name of the active chemical cannot be determined. Therefore, additional investigation is required to identify and isolate the active substance.

## CONCLUSION

These results indicate that the ethyl acetate fraction of *L. inermis* leaves contains naphthoquinones, flavonoids, and phenolic compounds that can inhibit bacterial growth. The results also suggest that other phytochemical compounds may contribute to the antibacterial activity of *L. inermis* leaves, and further study needs to be done to explore them.

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## AUTHORS' CONTRIBUTION

**Sri Mulyaningsih**: designed, directed, and managed the study; drafted manuscript preparation; edited and reviewed article. **Febriyati Adji Rachmadani**: collected data.

## DATA AVAILABILITY

None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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