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Original Article

ANTIBACTERIAL ACTIVITY AND BIOAUTOGRAPHIC EVALUATION OF EXTRACT AND FRACTION FROM TAMOENJU (*HIBISCUS SURATTENSIS* L.) LEAVES

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ABSTRACT

Objective: The tamoenju (*Hibiscus surattensis* L.) is one of the plants as traditional medicines to treat infections. Tamoenju leaves contain alkaloids, flavonoids, saponins, tannins, and steroids, a potential antibacterial agent. This study aimed to determine the antibacterial activity of tamoenju leaves extract and fraction against *Staphylococcus aureus* (ATCC 25923) and *Salmonella typhi* (ATCC 14028), and detect the active compounds using Thin Layer Chromatography (TLC) Bioautography techniques.

Methods: The sample was extracted using maceration method with 96% ethanol as solvent. Fractionation of ethanol extract using the liquid-liquid extraction method using *n*-hexane and ethyl acetate. The agar well diffusion method was used to evaluate the antibacterial activity with various concentrations of 2.5%, 5%, 10%, and 20%, followed by TLC bioautography using *n*-butanol: acetic acid: aquadest (4:1:1) as the mobile phase and silica gel GF 254 as the stationary phase on the most active fraction. Zones of inhibition showed the sensitivity of the tested microorganisms.

Results: The results showed the extract, *n*-hexane, and water fractions were more sensitive to *S. typhi,* while the ethyl acetate fraction was more sensitive to both bacteria. The zone of inhibition increased with the increasing extract and fractions concentration. The bioautography TLC showed that the compounds that had the potential as antibacterial in the most active fraction (ethyl acetate fraction) were flavonoids.

Conclusion: The extract and fraction of tamoenju leaves have antibacterial activity. Ethyl acetate fraction had the highest antibacterial activity. The compounds predicted to have antibacterial activity against the two tested bacteria were flavonoids.

Keywords: Antibacterial activity, Bioautography, *Hibiscus surattensis* L. *Staphylococcus aureus*, *Salmonella typhi*, Zone of inhibition

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INTRODUCTION

Tamoenju (*Hibiscus surattensis* L.) is one of the Malvaceae family plants with the potential as an antibacterial agent. *H. surattensis* L. is an annual plant that lives as herbs or shrubs and uses as a potherb. Ethnopharmacological studies show the efficacy of tamoenju to treat wound infections, abscesses, urethritis and venereal diseases [1, 2]. The results of previous studies showed that the tamoenju leaves could inhibit the growth of bacteria [3-5]. Previous studies reported the presence of alkaloids, flavones, flavanones, glycosides, tannins, saponins, and steroids in the ethanol extract of tamoenju leaves [6-9] that could inhibit bacterial growth [10, 11].

The tamoenju leaves, which are the subject of this research, are commonly used to treat infections related to the respiratory, skin, urinary tract, intestinal tract, and other infectious diseases Antibacterial is one the potentials that are good enough to be developed in the health sector to treat various diseases caused by bacteria. The disease is caused by bacteria that are pathogenic for humans, such as *Staphylococcus aureus* and *Salmonella typhi*. *S. aureus* is a gram-positive bacterium that is commonly the causative agent of multiple human infections, including skin and soft tissue infections, pulmonary infections, gastroenteritis, and urinary tract infections [12, 13]. While the bacterium *S. typhi* is one of the gram-negative bacterium that causes typhoid fever which is transmitted through food and drink contaminated by feces. Typhoid fever is a disease that often occurs in developing countries, including Indonesia and is one of the endemic diseases [14]. Based on this, it is necessary to separate the antibacterial compounds contained in the tamoenju extract using several solvents with different polarity levels (*n*-hexane, ethyl acetate, and water fractions). In addition, it is necessary to research the antibacterial potential in the fraction and the class of chemical compounds that give the activity to the fraction with high activity.

Thin layer chromatography (TLC) bioautography is a simple method that is specific for detecting spots on TLC chromatograms that have antibacterial activity. This method is a relatively inexpensive and simple technique that has been utilized alternative for detecting active substances, looking for antibacterial and detecting groups of compounds. The basis of this method is the agar diffusion technique, in which antibacterial compounds are transferred from the TLC plate to an agar medium containing the bacterium. Antibacterial activity was indicated by the formation of a clear zone on the bioautogram [15].

MATERIALS AND METHODS

Chemicals and reagents

The materials used were a maceration container, blender (Signora), hotplate (Vendille), oven (Oxone), incubator (Eyela), autoclave (Eyela), UV lamp 254 and 366 nm (CAMAG), vacuum rotary evaporator (EYELA N-1 200 B), petri dish (Pyrex Iwaki), analytical balance (Ohaus), vortex (Labnex VX 200), UV-Vis spectrophotometer (CECIL 7000 series), micropipette (Dragonlab), caliper (NANKAI), chamber, shaker water bath (Grant), Laminary Air Flow (Stream Line), and spare steel (well). The materials used were tamoenju leaves obtained from Alindau village, Donggala district, Central Sulawesi. *Staphylococcus aureus* (ATCC 25923) and *Salmonella typi* (ATCC 14028) bacteria were obtained from the Pharmacy Microbiology Laboratory, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Tadulako University. The ingredients used included distilled water, 96% ethanol, *n*-hexane, ethyl acetate (Bratachem, Indonesia), *n*-butanol (Merck, Germany), DMSO (Merck, Germany), GF254 TLC plate (Merck, Germany), 10% sulfuric acid (H_2SO_4) (Merck, Germany), 1% $FeCl_3$ (Merck, Germany), 10% $AlCl_3$, anisaldehyde-sulfuric acid reagent, Lieberman Burchard reagent, 0.5 McFarland solution, 0.9% NaCl solution, Nutrient Agar (NA) medium (OXOID, UK), Nutrient Broth (NB) medium (OXOID, UK).

Sample extraction and fractionation

Extraction was carried out by maceration method using 96% ethanol for 5x24 h to obtain a thick extract. Furthermore, fractionation was carried out by partitioning method using solvents of different polarity with n-hexane and ethyl acetate [9]. Calculated the yield of the extract and the fraction obtained.

Bacteria suspension culture

The inoculated bacteria, *S. aureus* and *S. typhi*, were each taken one ose and then suspended in a tube containing 10 ml of 0.9% NaCl solution. Then compared with the standard 0.5 Mc Farland solution. Observation of turbidity using a spectrophotometer which is more accurate in determining turbidity.

Antibacterial activity test

Modified methods [16] were used for the test of antibacterial activity. Testing the antibacterial activity of the extract and fraction of tamoenju leaves against *S. aureus* and *S. typhi* was carried out using the agar diffusion method. The test media was made with 2 layers of agar media. The base layer was made by pouring 15 ml of Nutrient Agar (NA) into each of the Petri dishes and then allowed to solidify. After solidification, the surface of the base layer is made into 6 holes using a tube whose diameter is adjusted like a disc, and the distance is adjusted so that the observation area does not overlap. The 0.1 ml bacterial suspension was mixed into the NA culture medium. Then 15 ml of NA was poured into each petri dish which was placed as a second layer as a backup. After the second layer has solidified, the backing is removed aseptically using tweezers from each petri dish to form a well that will be used in the bacterial test. Test solution concentration 2.5; 5; 10; and 20% w/v (loading dose 0.025; 0.05; 0.1 and 0.2 mg/µl), control negative (DMSO) and positive control (chloramphenicol) as much as 50 µl put into wells and incubated at 37 °C for 24 h. The clear zone formed was measured using a calliper. The test was carried out in 3 repetitions.

Bioautography TLC test

The bioautography TLC test was carried out on the extract or fraction with the highest activity. A total of 0.1 ml of bacterial suspension was inoculated into a sterile petri dish and then added 20 ml of NA medium. Then the fraction with the highest inhibitory activity was spotted on the TLC plate and eluted using the eluent *n*-butanol: acetic acid: water (4:1:1). Then removed and aerated using a hair dryer. The TLC plate was then affixed to the surface of the medium for 3 h and then removed. Then it was incubated at 37 °C for 1x24 h under aerobic conditions. The clear zone formed on the medium was observed. The zone of inhibition seen on the agar medium was compared with the results of the TLC plate observations in visible light and below UV lamp (254 and 366 nm) and stain detection reagent [17].

Identification of compounds

The TLC plate as the result of the bioautography TLC test was then identified the class of antibacterial compounds by spraying stain-seeking reagents on the plate, such as 1% $FeCl_3$ to detect phenolic compounds (bluish black), 10% H_2SO_4 to detect saponin compounds (purple), 10% $AlCl_3$ to detect flavonoid compounds (yellow), Dragendorff reagent to detect orange alkaloid compounds on a yellow background, Lieberman-Burchard reagent to detect steroid compounds (brown) and triterpenoids (purple-blue) [18].

RESULTS AND DISCUSSION

Sample extraction and fractionation

The percentage yield of the ethanol extract of tamoenju leaves obtained was 20.35%. Percentage yield is the ratio between the amount of viscous extract obtained from the extraction process and the initial weight of the dried plant. The yield calculation aims to estimate how much dry plant material will be needed to produce the specified extract. The fraction was then evaporated to obtain dry fractions of *n*-hexane (FH), ethyl acetate (FEA), and water fraction (FA) with 3.36%, 6.78%, and 47.08% yields, respectively. The purpose of fractionation is to separate compounds based on their polarity. Polar compounds will enter polar solvents, and non-polar compounds will enter non-polar solvents. The percentage of yield obtained from each fraction is different. This is due to differences ability to attract compounds from each solvent used in the fractionation process. The percentage yield of the water fraction is greater than that of ethyl acetate and n-hexane. Based on this, it can be assumed that the percentage of polar compounds in tamoenju leaves is higher compared to nonpolar and semipolar compounds.

Antibacterial activity test

The extract and fractions of tamoenju leaves (*H. surattensis* L.) were then tested for antibacterial against *Staphylococcus aureus* and *Salmonella typhi* bacteria using the well method with a concentration series of 2.5%, 5%, 10%, and 20% which was dissolved using 10% DMSO solvent. The well or hole cup method has advantages over other methods because this method is easier to measure the inhibition zone formed and is more sensitive. In the well method, a more thorough and more homogeneous osmolarity process occurs so that it is stronger to inhibit bacterial growth [19]. The resulting clear area is an indication of the sensitivity of bacteria to antibiotics or other antibacterial materials used as test material. This study used a comparison of chloramphenicol as a positive control because chloramphenicol is a broad-spectrum antibiotic, where its broad spectrum of action includes both gram-positive and gram-negative bacteria. Chloramphenicol is basically bacteriostatic. The negative control used is DMSO. This negative control serves to prove that the solvent has no effect on the antibacterial activity caused by the test solution [20].

The results of the antibacterial activity test showed that the extract and fraction of tamoenju leaves could inhibit the growth of the test bacteria. The results of this study succeeded in proving the activity of extracts and fractions of tamoenju leaves as antibacterial so that they can support their traditional use for treating wounds, urethritis, venereal disease, and abscesses. The results of the inhibition zone measurements can be seen in fig. 1.

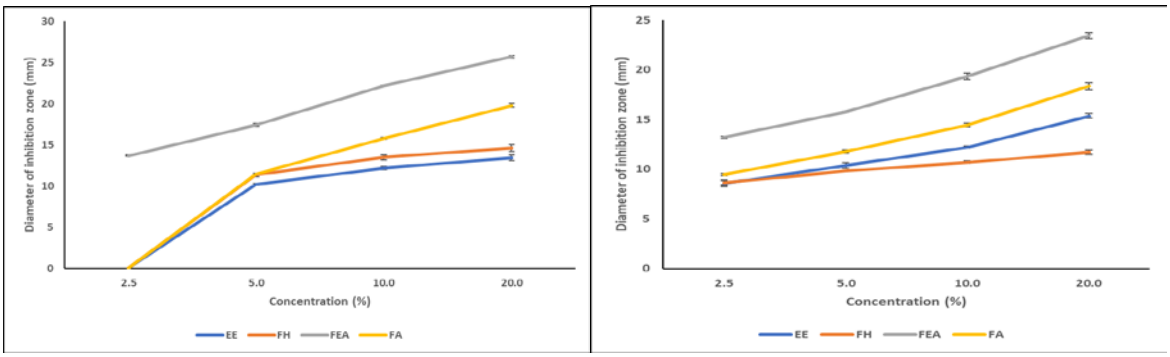

Fig. 1: The results of the antibacterial activity of tamoenju leaf extract and fraction, (a) Antibacterial activity test results against *S. aureus* (b) Antibacterial activity test results against *S. typhi*

The EE extract has strong activity as an antibacterial against *S. aureus* and *S. typi* bacteria. The FH and FA had a moderate-strong inhibitory ability on *S. aureus* and *S. typhi.* The FEA has very stronger antibacterial potential against *S. aureus* and *S. typhi* compared to

extract and other fraction. This indicates that *S. aureus* (gram-positive bacteria) and *S. typhi* (gram-negative bacteria) are more susceptible to compounds in the ethyl acetate fraction. The FEA has the strongest activity because in previous studies the levels of secondary FEA was identified as chemical components that provide activity by the TLC bioautography method.

The results of the inhibition zone measurements in fig. 2 show that the higher the concentration of extract or fractions of tamoenju leaves, the higher the concentration of tamoenju leaves extract the larger the zone of inhibition given. *S. aureus* was successfully inhibited by EE, FH dan FA of tamoenju leaves at a concentration of 5% while the FEA started at a concentration of 2.5%. The growth of *S. typhi*-tested bacteria was inhibited by ethanol extract and fractions at a concentration of 2.5%. This shows that the extract and fractions of tamoenju leaves are more sensitive to *S. typhi*. The lowest concentration difference in inhibiting the two bacteria was due to the sensitivity and response of the test bacterial cells to antibacterial compounds in the extract or fractions of tamoenju leaves [21]. Generally, gram-positive bacteria are more sensitive to compounds with antibacterial activity than gram-negative bacteria. The difference in sensitivity between gram-positive and gram-negative bacteria is caused by differences in the structure of the cell walls of each bacterium. Gram-positive bacteria have a more straightforward bacterial cell wall structure than gram-positive bacteria, making it easier for antibacterial compounds to enter the cell. The cell wall structure of gram-negative bacteria is more complex and has three layers. The outer layer is a lipoprotein, the middle layer is peptidoglycan, and the inner layer is lipopolysaccharide.

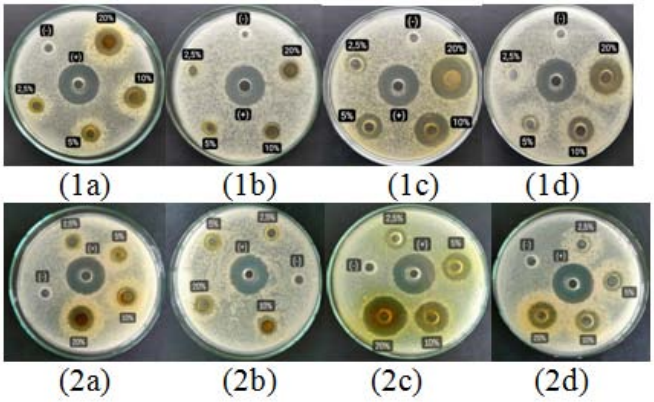

Fig. 2: Antibacterial activity test results against *S. aureus* (1a) EE, (1b) FH, (1c) FEA, (1d) FA and antibacterial activity test results against *S. typhi* (2a) EE, (2b) FH, (2c) FEA, (2d) FA

The test results of the bioautography TLC method showed one spot at Rf 0.8 which indicated a clear zone against *S. aureus* and *S. typhi* (fig. 3). These results indicate that FEA has a broad spectrum for Gram-positive and Gram-negative bacteria.

Based on the identification results using all the reagents used, the stain on the sample (Rf 0.8) gives a color change from a less visible stain to a lighter brownish-yellow color after being sprayed using aluminium trichloride 5% reagent. While detection by spraying with 10% H_2SO_4 (saponins), 1% $FeCl_3$ (phenolic), Dragendorff (alkaloids), and Liebermann-Burchard (steroids/triterpenoids) gave negative results. This shows that the class of compounds that provide this activity is the flavonoid group. Flavonoid compounds will give a yellow color after reacting with 10% aluminium trichloride because flavonoid compounds will form complexes with aluminium [22]. The principle of the reaction aluminum chloride and flavonoid is that aluminium chloride forms stable acid complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols.

The previous research stated that the FEA contained kaempferol, morin, quercetin, rhamnazin and trifolin compounds which belonged to the flavonoid group [8]. These compounds contributed to the antibacterial activity of the FEA. The antibacterial activity of kaempferol, quercetin and morin compounds might be due to their ability to complex with the bacteria cell wall and, therefore, inhibit microbial growth, and coagulates proteins by inactivating enzymes and disrupting cell walls [23-25].

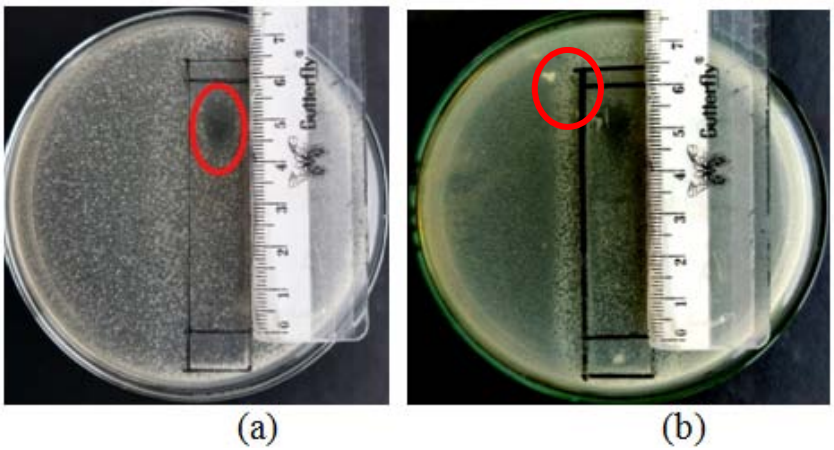

Fig. 3: TLC bioautography, (a) *Staphylococcus aureus* (b) *Salmonella thypi*

CONCLUSION

The results showed that the ethyl acetate fraction tamoenju leaves give the highest activity in inhibiting the growth of *S. aureus* and *S. typhi* bacteria with the identification of flavonoid compounds (Rf 0.8) which provide inhibitory activity.

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Nil

AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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