Analysis of the best secondary metabolite compound of binahong leaves (*Anredera cordifolia (Ten.) Steenis*) against *Staphylococcus epidermidis*

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Abstract. Subclinical mastitis is a disease that causes inoptimal milk production and milk quality. Subclinical mastitis can be treated with the help of herbal plants. Binahong leaf (*Anredera cordifolia (Ten.) Steenis*) is one of the herbals that contains several antibacterial secondary metabolite compounds. This study aims to identify the secondary metabolite of binahong leaf extract that has the best antibacterial activity against bacteria that cause subclinical mastitis. The herbal ingredient used was binahong leaf extract, and the bacterium that causes mastitis was *S. epidermidis*. The analyses used were TLC bioautography. The data were analyzed descriptively. The results showed that the growth of *S. epidermidis* could be inhibited by binahong leaf extract with an inhibition zone of 11 mm identified in the fraction with an *Rf* value of 0.95. The fraction was a triterpenoid compound marked purple after being sprayed with Lieberman burchard. It can be concluded that the best secondary metabolite compound in binahong leaf extract is triterpenoid. Triterpenoid in binahong leaf extract at *Rf* 0.95 showed the best activity in inhibiting the growth of *S. epidermidis* as one of the bacteria that causes subclinical mastitis with an inhibitory zone of 11 mm.

1. Introduction

Livestock farming is one of the agricultural subsectors that plays a vital role in providing animal protein for the community. Currently, the demand for animal protein continues to increase annually. One of the animal proteins experiencing a significant rise in demand is milk. This trend is driven by economic prosperity, increased population, and extended lifespan [1]. Furthermore, the quality of domestic milk, including its protein content, remains below standard. This is concerning, as milk protein is an essential nutrient and milk serves as a critical source of animal protein, necessitating efforts to optimize its availability. Besides being caused by feed [2], the suboptimal milk production and protein content in Indonesia

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maybe due to the high incidence of mastitis [3]. Subclinical mastitis may result in decreased milk protein levels and overall milk production [4].

Subclinical mastitis is caused by bacterial infections, with *Staphylococcus epidermidis* being one of the causative agents. This bacterium belongs to the *Staphylococcus* species, which is a primary cause of subclinical mastitis in ruminants [5]. Subclinical mastitis has adverse effects on milk production and milk quality [4]. Therefore, specific measures are necessary to prevent or treat subclinical mastitis in dairy cattle.

One approach to managing subclinical mastitis is the use of antibiotics. However, the use of antibiotics has been prohibited due to their negative effects, such as residues in animal products [6]. As an alternative, herbal plants with potential therapeutic properties have been explored for treating mastitis. Herbal plants are rich in active compounds, specifically secondary metabolites, which have antibacterial properties against mastitis-causing pathogens. Additionally, herbal remedies are considered safer for long-term use. One herbal plant with potential for treating subclinical mastitis is *Anredera cordifolia* (binahong leaves) [3], because it contains a variety of bioactive chemicals or secondary metabolites that can fight off subclinical mastitis bacteria. These metabolites commonly exhibit activities such as antibacterial, antioxidant, and anti-inflammatory properties [7]. The secondary metabolites found in binahong leaves, such as phenol, flavonoid, saponin, and tanin [3].

Each secondary metabolite compound found in plants have different effects on bacteria [8]. Therefore, it is important to identify which compounds in binahong leaves exhibit the best antibacterial activity against *S. epidermidis*, a bacterium responsible for subclinical mastitis, as part of a broader exploration of binahong's potential in combating mastitis-causing bacteria. A previous study conducted by [3] successfully identified the types of secondary metabolite compounds in binahong leaf extract and fractionated them using Thin Layer Chromatography (TLC). The current research was carried out to carry on the work of [3] by using TLC bioautography, a detection method that combines TLC and biological activity testing [9]. TLC Bioautography allows for the identification of compounds responsible for antibacterial activity, indicated by the appearance of inhibition zones [10]. This research aims to analyze the best secondary metabolite compound from binahong leaf extract with the most effective antibacterial activity against *Staphylococcus epidermidis*, the bacterium responsible for subclinical mastitis, as an effort to enhance milk production and milk protein.

2. Materials and methods

2.1 Materials

The equipment used includes envelope paper, a 60°C oven, blender, sieve, plastic, magnetic stirrer, rotary evaporator, dark glass bottles, TLC plates (Thin Layer Chromatography), TLC chamber, pencil, ruler, UV lamp (254 nm and 366 nm), scissors, micropipette, 100°C and 110°C ovens, petri dishes, digital scale, beaker, hotplate, glass stirrer, test tubes, Erlenmeyer flask, cotton, inoculation loop, Bunsen burner, autoclave, aluminum foil, incubator, and tissue.

The materials used include binahong leaves, 95% ethanol, chloroform, ethyl acetate, methanol, distilled water, *S. epidermidis* bacteria, and media such as Nutrient Agar (NA), Nutrient Broth (NB), and Mueller Hinton Agar (MHA).

2.2 Methods

2.2.1 Extraction of binahong leaves

The extraction of binahong leaves followed the method described by [3]. After being dried and milled into a powder, the binahong leaves were extracted using the maceration process with a 1:5 (w/v) solvent ratio of 95% ethanol. In particular, 1000 milliliters of 95% ethanol were combined with 200 grams of the powdered leaves. A rotary evaporator was used to evaporate the solvent, producing a thick extract that was used to make the binahong leaf extract.

2.2.2 Thin Layer Cromatography (TLC)

The TLC process was conducted following the method described by [3]. A TLC plate measuring 8 x 1.5 cm was prepared, with the upper margin set at 0.5 cm and the lower margin at 1.5 cm, allowing for a solvent movement distance of 6 cm. The extract was spotted onto the lower margin of the plate and allowed to dry. The eluent used was a mixture of chloroform, ethyl acetate, methanol, and water in a ratio of 5:2:1:1. The TLC plate was then developed using the selected eluent, which had been saturated for 10 minutes, until the eluent reached the upper margin. The fractions on the plate were observed under UV light at 254 nm and 366 nm. The Liebermann-Burchard reagent was sprayed to detect triterpenoid/steroid saponins, while FeCl3 was sprayed to detect polyphenols/phenols. Plates sprayed with FeCl3 were heated at 110°C for 10 minutes, whereas those sprayed with Liebermann-Burchard were heated at 100°C for 10 minutes. The Rf values for each fraction were observed and calculated using the following equation:

$$Rf = \text{Distrance traveled by the compound : Distance traveled by the solvent} \qquad (1)$$

2.2.3 TLC bioautography

The bioautography method employed in this study was the overlay bioautography method described by [9]. The extract was spotted onto a TLC plate and then eluted using the eluent selected during the TLC experiment. The TLC plate was placed on a Petri dish and overlaid with MHA media mixed with a suspension of *S. epidermidis* bacteria, followed by solidification. The plate was incubated at 37°C for 24 hours, then the inhibition zones were observed. Then, the zone was measured and categorized as follows: (1) weak: inhibition zones less than 5 mm (+), (2) moderate: zones between 5-10 mm (++), (3) strong: zones between 10-20 mm (+++), and (4) very strong: zones exceeding 20 mm (++++). The widest inhibition zone in a specific fraction indicated the compound with the strongest antibacterial activity within that fraction. To identify the compound at the spot with the best antibacterial activity, the results were compared with the chromatogram from the TLC experiment that had been sprayed with reagents to determine the compound in the fraction.

2.3 Data analysis

Data were analyzed descriptively and presented in the form of figure.

3. Results and discussion

In the initial stage, the TLC results showed a pattern similar to the previous study by [3]. The TLC results, consistent with the previous study, provide a foundation for continuing the research with the bioautography TLC method aimed at exploring the compounds most responsible for antibacterial activity against S. epidermidis.

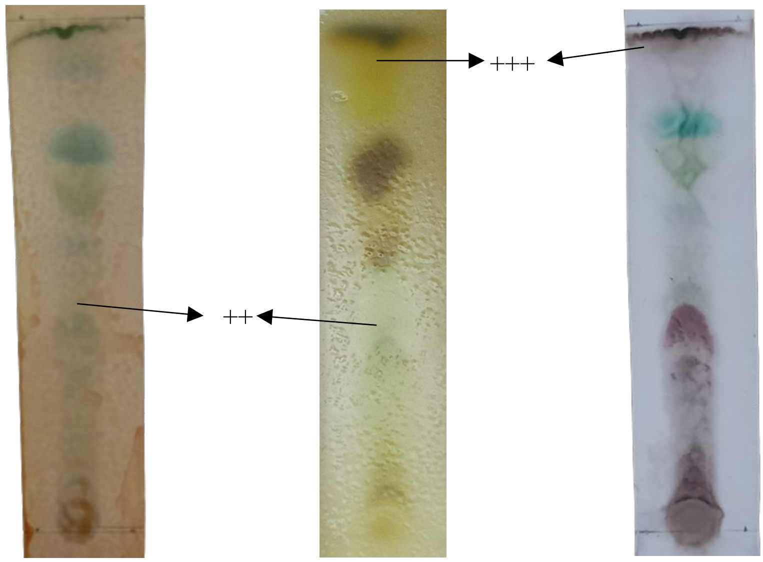

Fig. 1. Identification of fractions with antibacterial activity in binahong leaf extract against *S. epidermidis.* (a) = TLC sprayed with $FeCl_3$; (b) = TLC bioautography; (c) = TLC sprayed with Lieberman burchard

Fig.1 illustrates antibacterial activity zones as distinct spots, indicating active fractions from binahong leaf extract against *S. epidermidis*. Each inhibition zone varies in size, with the widest zone (+++) observed at an *Rf* value of 0.95 and measuring 11 mm. The inhibitory effect on the bioautogram was compared to chromatograms treated with $FeCl_3$ and Liebermann-Burchard reagents. The *Rf* region with the widest inhibition zone exhibited a purple color after Liebermann-Burchard treatment, identifying it as triterpenoid saponins. Liebermann-Burchard is commonly used to detect saponins, where the color change distinguishes the triterpenoid saponins appear purple [11].

Triterpenoids in nature can combine with sugar groups to form triterpenoid saponins. In this structure, the triterpenoid becomes part of the aglycone, while the sugar forms the glycone portion. In triterpenoid saponins, the antibacterial activity is attributed to the aglycone component, specifically the triterpenoid [14]. These saponins appear purple when sprayed with Liebermann Burchard reagent due to the presence of triterpenoid groups [15].

The inhibition effect results from the influence of secondary metabolites present on the chromatogram. These compounds diffuse into the MHA medium mixed with the *S. epidermidis* bacterial suspension. The size of the inhibition zones created indicates the different levels of antibacterial efficacy of each secondary metabolite. The bioautogram's broadest inhibition zone indicates the secondary metabolite with the strongest antibacterial activity against S. epidermidis. This inhibition zone reflects the bactericidal effect of the compound. The inhibition occurs due to the transfer of compounds from the chromatogram into the *S. epidermidis* medium, where they interact with the bacteria. Antibacterial compounds prevent S. epidermidis from growing, which causes a distinct inhibition zone to appear as a result of the limited bacterial growth.

According to the results of the bioautography test, the triterpenoids in the binahong leaf extract exhibits the greatest antibacterial effect against *S. epidermidis*. These compounds play the most dominant role in antibacterial activity. This finding aligns with the study conducted by [12], which also demonstrated that triterpenoids possess antibacterial activity against *S. epidermidis*. Triterpenoids interact with porins, which are transmembrane proteins. Such interactions form strong polymer bonds, leading to porin damage. Porin damage reduces the permeability of the bacterial cell membrane, as porins act as gateways for substances entering and exiting the cell. Reduced membrane permeability results in nutrient deprivation for the bacteria, ultimately inhibiting their growth or causing cell death [13].

The case of subclinical mastitis has been shown to reduce milk production and milk protein. The efforts to prevent mastitis are expected to help optimize milk production and milk protein in livestock. The use of binahong leaves as a natural alternative will ensure the safety of the milk produced by the animals. In addition, the approach using natural ingredients can increase consumer acceptance of livestock products. Healthy animals will provide optimal performance, which can be seen in milk production and quality, including optimal milk protein content. It is hoped that these efforts will create milk with good and sustainable quantity and quality.

4. Conclusion

According to the research findings, the biggest inhibition zone showed that the triterpenoids in the binahong leaf extract at a fraction with an *Rf* value of 0.95 had the highest efficacy in preventing the growth of *Staphylococcus epidermidis*, one of the bacteria that causes subclinical mastitis. This finding confirms that binahong leaves have the potential to address subclinical mastitis cases, thereby optimizing livestock health, which in turn can help maximize milk production and milk protein.

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