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Antibacterial Activity of Maja Fruit Extract Against *Escherichia coli* and Its Potential as Urease Inhibitor for Reducing Ammonia Emission in Poultry Excreta

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Abstract. Essential oil is one of the natural compounds from plant extract that has the potential as a natural antibacterial for inhibiting the hydrolysis of urea compound into ammonia gas in livestock wastes. This research aims to measure antibacterial effects of Maja Fruits Extract (MFE) on *Escherichia coli* and its potential as a urease inhibitor in excreta medium. MFE was extracted using Maceration method. The results indicated that antibacterial activity of MFE was low or resistant, with the biggest diameter of inhibition zone occurred on agar medium was 14.26 ± 0.75 mm. The antibacterial ability of MFE with an addition of 3% (v/v) in liquid medium gave the lowest graph of bacterial growth. MFE has the potential as an antibacterial and urease inhibitor compound, which could be applied to inhibit the hydrolysis of urea by microorganisms so that it can reduce the emission of ammonia gas from livestock wastes, especially chicken excreta.
Key Word: urease inhibitor, antibacterial, essential oils, maja fruit extract, poultry excreta

1. Introduction

Ammonia accumulation produces adverse effects on livestock production activity and potentially causes social problems. Ammonia is formed as a final product from the urea hydrolysis process performed by bacterial urease in feces or excreta, such as *Escherichia coli*. *E. coli* is a type of gram-negative pathogenic bacteria found in feces and has the ability to hydrolyze urea compound into ammonia [1].

Furthermore, *E. Coli* has been observed as a very fast-growing bacteria. At an optimum condition, *E. coli* can grow with doubling time at around 20 minutes [2]. The high growth of bacterial urease will result in a higher rate of urea hydrolysis, which increases ammonia gas emission. After being excreted into the environment, urea is then converted into ammonia by bacterial urease, which is found in feces and excreta. However, urease producing bacteria have not been observed in the urine [3]. Inhibiting the growth rate of urease producing bacteria and the urease activity could be an alternative solution to mitigate the ammonia emission.

Essential oil is one of the natural compounds which has the potential market due to its natural antibacterial activity. The application of essential oil is highly recommended due to the environmentally friendly orientation. It can be obtained from parts of the plant through the extraction process. Its phytochemical content like phenol serves as an antimicrobial. Essential oil could interact with the cell wall and inhibit bacterial activity by changing the permeability of the cell wall for the ion exchange process. The dissipation of the ion gradients process will result in the loss of turgor pressure, inhibition of DNA synthesis, inhibition of enzyme activity, and overall metabolic activities [4]. Marmelosin is one of the types of essential oil that has antimicrobial properties [5]. In the previous study, the marmelosin can be obtained from the extraction of Maja fruit, which is easily found in Indonesia [6]. Based on the aforementioned description, it is necessary to conduct a research to reveal the potential of a natural antimicrobial compound in MFE to inhibit the growth of bacterial urease.

2. Materials and Methods

2.1. Preparation of Maja Fruit Extract (MFE)

Maja fruit in this study was extracted using the maceration method [7] which was conducted at the Laboratory of Leather, Waste, and By-Products Technology at the Faculty of Animal Science. The research was also partly performed at the Integrated Research and Testing Laboratory (LPPT) Universitas Gadjah Mada, Yogyakarta. The Maja fruit was obtained from the local area in the Yogyakarta region. The maceration method commenced by making a powder of Maja fruit and followed by the extracting process. The Maja fruit was dried in an oven for 48 hours. Furthermore, Maja fruit powder was weighed and dissolved with methanol. Stirring was performed using Ultraturai for 30 min, left for 24 hours, and continued with filtration. The filtrate was evaporated with Rotary Vacuum Evaporator and water bath heater at 60ºC and then poured into a porcelain dish and heated with a water bath to obtain a concentrated extract. The concentrated extract of the fruit was packaged in a closed container and stored in the refrigerator. Some of the concentrated extract were mixed with aquadest and made into a solution with different concentrations of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%.

2.2. Preparation of Bacterial Cultivation

The initial bacterial suspension was performed by equivalent to the turbidity of 0.5 Mc Farland (turbidity of a mixture of barium sulphate and HCl) or comparable to the number of bacteria 1,108 CFU/g or 250 to 300 colonies in solid media [8]. Some colonies of bacteria were taken and diluted to the concentration level in accordance with the concentration of 0.5 Mc Farland.

2.3. Bacterial inhibition Test of MFE in Solid Medium

The bacterial inhibition was observed using the disk-diffusion method with paper discs. [8]. The medium was prepared with a composition of 1 g of meat extract, 1 g of peptone, 1 g of agar powder, 0.5M NaCl, and 100 ml of aquadest. All the components of nutrient media and tools were sterilized in an autoclave at a temperature of 121°C for 15-17 min. One ose of pure cultures of *Escherichia coli* was then cultured in 10 ml of sterile aquadest and homogenized as an active culture. Sterile Petri dishes were filled with nutrient medium. Furthermore, 100 µl of the active culture of *Escherichia coli* was added after solidification. The paper disc (6 mm diameter) was applied in extract and controlled by dripping the extract solution. The paper Discs were waited until they dried and then placed on the surface of bacterial media using sterile tweezers and slightly pressed. The bacterial media that had been installed with paper discs were then incubated at a temperature of 30°C for 18-24 hours. The inhibition zone was formed, whose diameter was measured using callipers to determine the effectiveness of the antibacterial.

2.4. Bacterial Inhibition Test of MFE in Liquid Medium

The inhibition of *Escherichia coli* in liquid medium commenced by culturing of 1 ose of bacterial culture into 20 ml of NB medium and incubated for 24 hours. One ml of the incubated bacterial suspension was then taken and transferred to 10 ml of NB for each tube in each bacterium and then measured its Optical Density (OD600). After the OD was measured, Maja fruit was extracted with a concentration of 1%, 2%, and 3% and added to NB liquid medium. Meanwhile, the control would be compared using an aquadest. After the treatments were performed, the NB liquid medium was placed in a water bath and measured its OD every 1 hour. Three replications were conducted to test the inhibitory activity on bacterial growth.

2.5. The potent urease inhibitor test of Maja fruit extract in chicken excreta

The ammonia reduction was tested using the nesslerization method, beginning by making a standard solution with different concentrations of 0 ppm (blank), 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm. The standard solution was then read using a spectrophotometer at a wavelength of 425 nm. The absorbance value read on the spectrophotometer was used as a standard curve that could be used as a reference for determining the concentration of samples. The sample concentration could be determined by observing the relationship between the concentration of the standard solution and the absorbance value of the sample.

The measurement of the additional effect of Maja fruit extract on chicken excreta for reducing ammonia emission was determined by the total ammonia trapped in boric acid [9]. The media of excreta were made by mixing the fresh excreta (obtained from a local layer poultry farm in Yogyakarta, Indonesia). The excreta were collected using plastic for covering the ground under the cages. The excreta were mixed with aquadest with a ratio of 1:10 (i.e. 20 g of excreta and 200 ml of aquadest), stirred to homogeneous, put into the 500 ml Erlenmeyer flask and placed in a rotary shaker (120 rpm). The excreta medium was treated with the addition of Maja fruit extract at 0%, 5%, 7.5% and 10% (v/v). The concentration of ammonia was measured by taking 1 ml of the acid sample. Samples were taken once every 3 hours and added with 0.2 ml of Nessler solution. The samples were left for 10 minutes and read on a spectrophotometer with a wavelength of 425 nm.

2.6. Data Analysis

The data of antibacterial assay design were prepared in Completely Randomized Design Method and the results were analysed by using ANOVA and Duncan's Multiple Range Test for the significant test.

3. Results and Discussion

3.1. Antibacterial activity of Maja fruit extract

The results of antibacterial activity from Maja fruit extract using the disk diffusion assay method at different concentrations indicated the presence of an inhibitory zone (Figure 1.). The diameter of the inhibition zone could be determined around the hole with a clear zone. The higher the concentration of Maja fruit extract resulted, the more visible the zona area of inhibition formed. The results of the measurement area of inhibitory zones are shown in Figure 2.

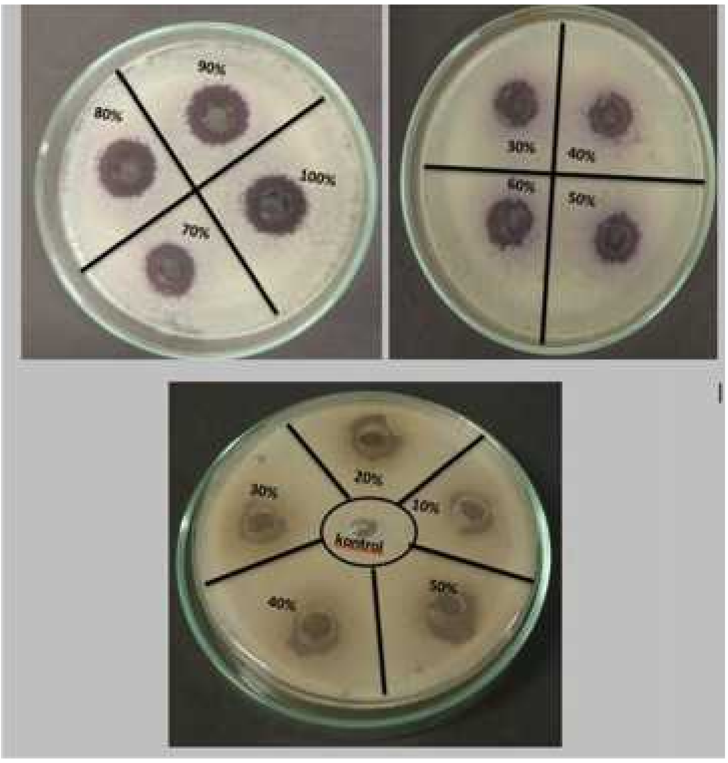

Figure 1. Inhibitory effect of antibacterial activity of Maja Fruit Extract on *E. coli*

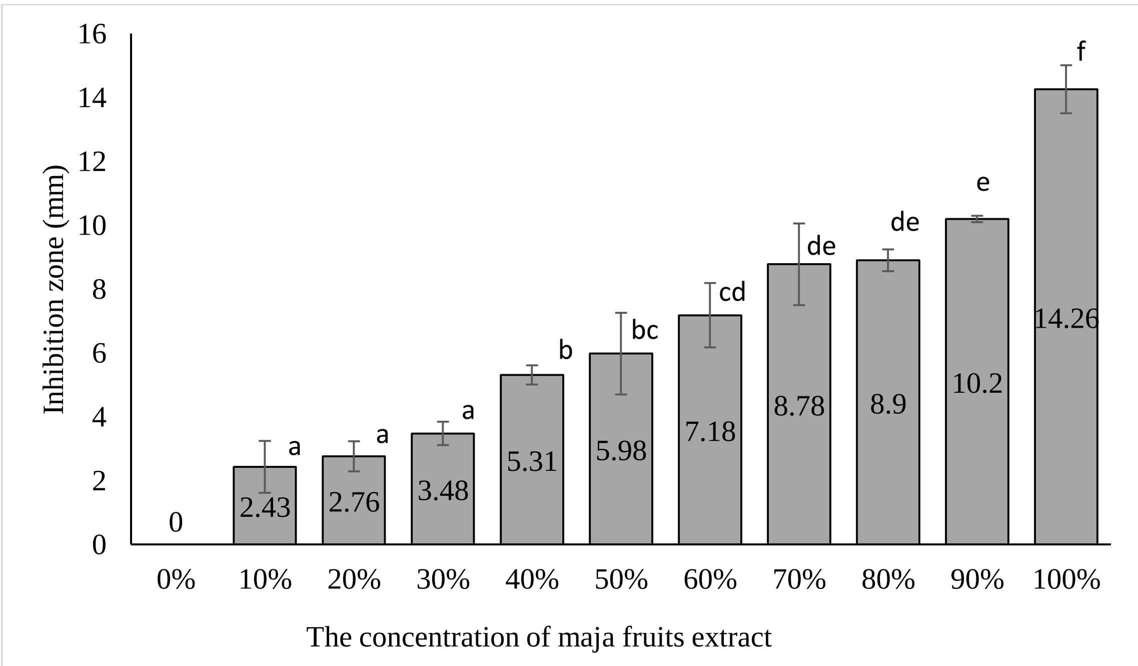

Figure 2. The average of inhibition zone from the addition of Maja fruit extract at different concentration

The antibacterial activity of Maja fruit extract can be seen from the bacterial inhibitory zone (Figures 1 and 2), and the graph of bacterial growth reduction in the liquid medium (Figure 3). The treatment of higher concentrations of Maja fruit extract resulted in an increasingly larger diameter of the inhibition zone, and the graph of cell density absorbance was significantly lower than the control. The antibacterial activity zone from Maja fruit extract was observed at 2.3 - 14.26 mm from

Escherichia coli (Gram-negative bacteria). The antibacterial strength can be described as follows: strong if the inhibition zone diameter is 20 mm or more, moderate the diameter is 16 to 20 mm, and low or resistant if the diameter is less than 16 mm [10]. The antibacterial activity in Maja fruit extract was suspected because it has phytochemical contents of essential oils including marmelosin [6], alkaloids, phenols, saponins, and tannins [11]. Those compounds were proven to have an antibacterial function [12]. The content of marmelosin in Maja fruit at different conditions was measured before [6], in immature fruit was observed by 108.65 µg/g, while in mature condition was by 65.82 µg/g, and fermented condition by 23.01 µg/g. Essential oils have the ability to change and penetrate the bacterial lipid membranes, making them more permeable and causing ion and cytoplasmic leaked. Damage to the cell wall and cytoplasmic membrane causes lysis of cell and death to bacteria [13]. Some essential oils from plant extracts have antibacterial abilities with weak effects on or resistance to Gram-negative bacteria. [14] Gram-negative bacteria have a more complex cell wall arrangement and contain lipopolysaccharides on the outer membrane of cells that can limit the diffusion of hydrophobic molecules from essential oils into bacterial cells [15].

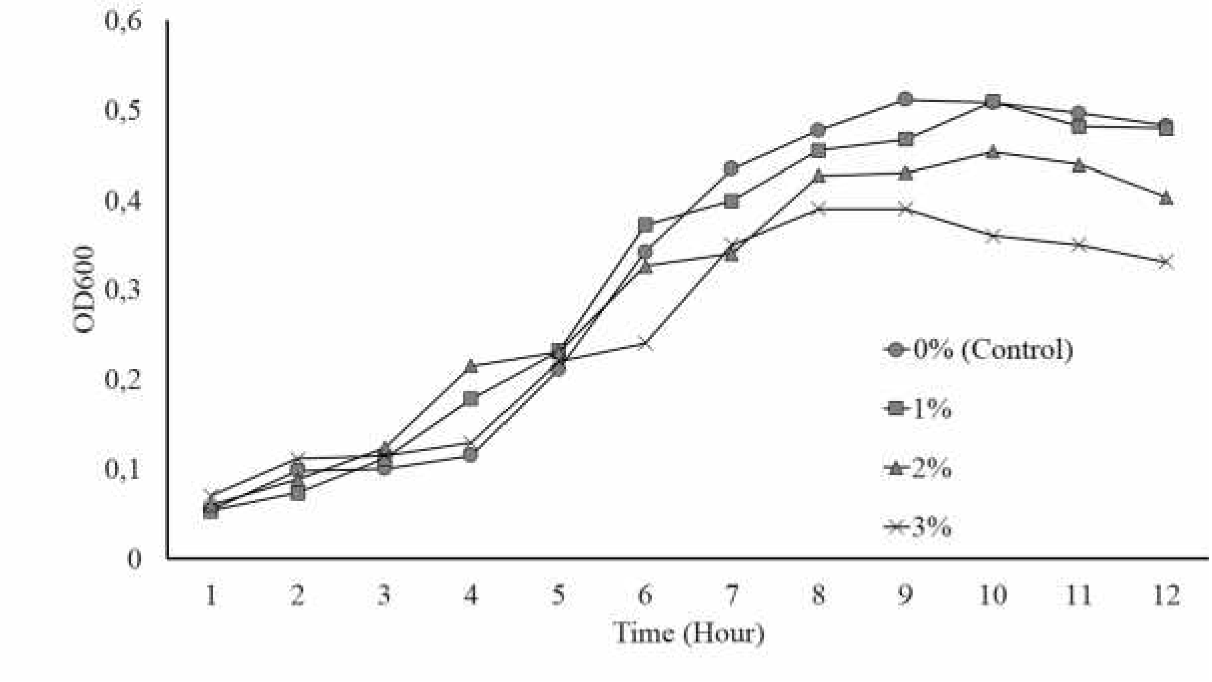

Figure 3. The growth graph of *Escherichia coli* on liquid nutrient medium with the addition of maja fruit extract

The low antibacterial effect of Maja fruit extract on *E. coli* in this study is consistent with previous research [16] that the antibacterial results from the leaves extract of Maja plant also gave low effect on Gram-negative bacteria (*Salmonella typhi, Salmonella paratyphi-A, Escherichia coli,* and *Sarcina lutea*). However, it had a strong effect on groups of Gram-positive bacteria (*Shigella dysenteriae, Bacillus cereus, Bacillus subtilis, Bacillus megaterium, and Staphylococcus aureus*). Accordingly, it is possible that Maja fruit extract can also have a strong antibacterial effect on a group of gram-positive bacteria. This requires further research because the mechanism of antibacterial activity of essential oils from plant extracts depends on the type of bacteria. Some essential oils are more active against Gram-positive bacteria, but the other essential oils are more active against Gram-negative bacteria or more active against both of them as antibacterial [17].

3.2. Potential of Maja Fruit Extract (MFE) as Urease inhibitor

The effects of MFE antibacterial activity added with different concentrations in the layer hen excreta on the accumulation of ammonia emission trapped in the boric acid solution are shown in Figure 4. The addition of 5%, 7.5% and 10% MFE in the excreta medium indicated that the amount of ammonia accumulated in boric acid was lower than the control. In this study, the addition of 10% MFE gave the lowest amount of ammonia emission accumulation after a 28-hour period of cultivation. These results indicate that MFE has the potential as a urease inhibitor by inhibiting the activity of microorganisms contained in the chicken excreta medium. The emission of ammonia odour in livestock wastes can be reduced by using essential oils from plant extracts such as thymol as a natural antibacterial [4]. Essential oils from plant extracts contain natural antibacterial compounds like phenol and its derivate, which can be used to inhibit the hydrolysis of urea into ammonia gas (urease inhibitor), so that it can reduce the formation of odours from livestock waste.

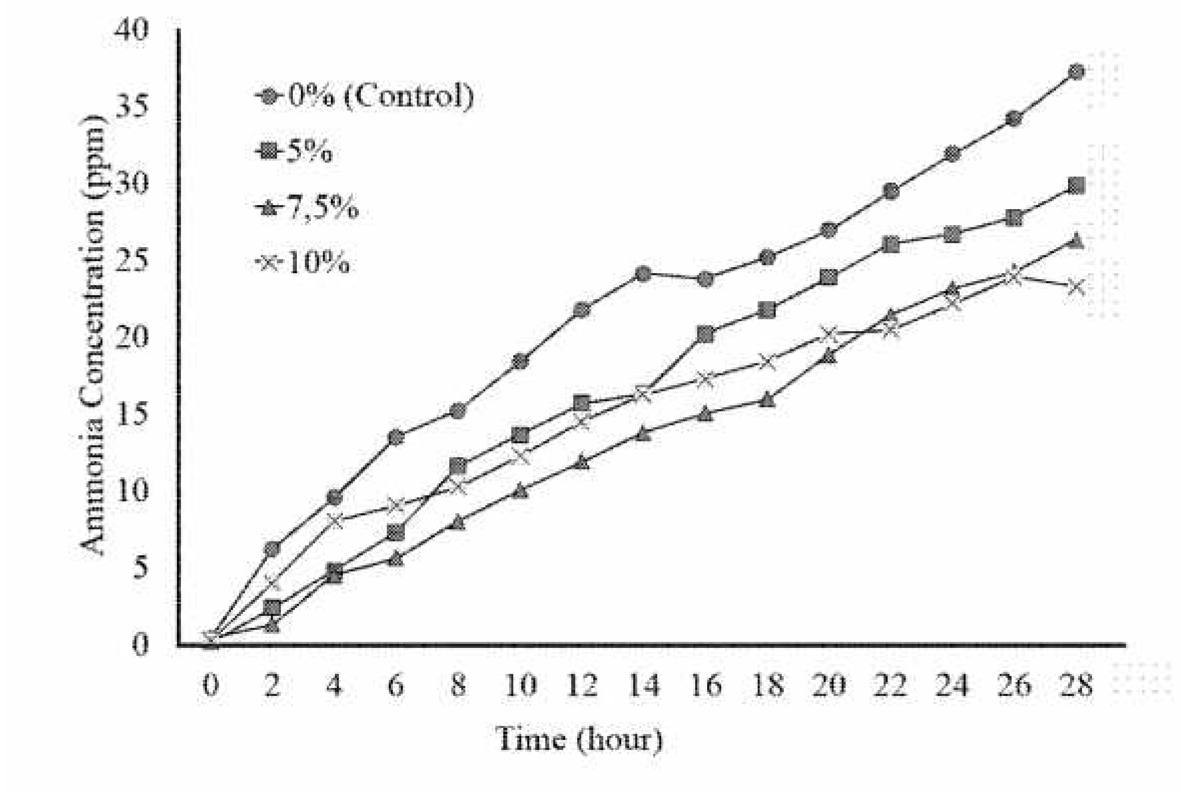

Figure 4. The graph of ammonia concentration trapped in boric acid solution

MFE has the potential as a urease inhibitor compound in chicken excreta to reduce odour formation from the emission of ammonia. Chicken excreta contains nutrition from indigestible feeds as well as a large and diverse population of microorganisms [18]. Various microorganisms, including Gram-negative and Gram-positive bacteria, are identified living in excreta with the largest population predominated by *Bacillus* and *Streptococcus* bacteria [19]. Chicken excreta contains approximately 70% uric acid, of which component is easily hydrolysed into ammonia gas by bacterial urease such as *Helicobacter pylori*, *Pseudomonas* spp., *Proteus* spp., *Escherichia coli, Staphylococcus* spp. and *Canavalia ensiformis* [20];[21]. Although the antibacterial effect of Maja fruit extract was low on the growth *of E. coli*, the addition of Maja fruit extract has indicated to inhibit the rate of emission and ammonia concentration. It is suspected that the essential antibacterial compounds in the extract of Maja fruit can have an effect on the bacterial urease group other than *E. Coli* contained in chicken excreta. The contents of antibacterial from phytochemical compound, including marmelosin, saponins, alkaloids, tannins and phenols in MFE can interfere with the microbial metabolic process in the medium.

Phytochemicals exert their antimicrobial activity through different mechanisms, such as tannins. Tannin's action has been reported to be antibacterial through the action of iron deprivation, hydrogen bonding, or non-specific interactions with vital proteins including various enzymes in cells metabolizing process of the microorganisms [22];[23]. The antibacterial function of tannin compounds is reported to be effective against *Helicobacter pylori* [24], *Staphylococcus aureus* [25], *Proteus vulgaris* [26], *Shigella boydii, Shigella flexneri, Escherichia* coli and *Pseudomonas aeruginosa* [27].

The marmelosin compound is a type of furanocoumarin from the phenol compound derivative. The present report, the mechanism of phenol as an antibacterial has been reported that its presence toxic and may poisoning the protoplasm, damage and penetrate cell walls and precipitate proteins, inhibiting activation of essential enzymes, inhibiting the ATP synthesis and DNA cell synthesis which results in disruption of the metabolism process in bacterial life and bacterial cells become ill or even be killed furthermore [28].

Marmelosin is a bioactive compound in the extract of Bael plant (*Aegle marmelos*) which has been reported to function as an antihelmintic and antibacterial [29]and proven to be able inhibition Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and Gram-Negative (*Klebsiella pneumoniae, Proteus mirabilis, Escherichia coli, Salmonella paratyphi A and Salmonella paratyphi B*) [30];[31]. Saponins have been reported to function as antifungal and antibacterial, which have been reported to be effective against *Aspergillus ochraceous, Curvularia lunata, Bacillus subtilis, Pseudomonas aeruginosa, and Salmonella typhimurium* [32]. Furthermore, the research proved that saponin gave significant antifungal in soilborne pathogens (*Fusarium oxysporum, Fusarium oxysporum f. sp. lycopersici, Fusarium solani, Phytium ultimum and Rhizoctonia solani*), water-borne pathogens *(Botrytis cinerea, Alternaria alternata, and Alternaria porri*) and the biocontrol fungus *Trichoderma harzianum* [33]. Saponin could affect the catabolic enzymes in antimicrobial mechanisms qhich indicates an activity not only in the electron transport chain, but also in other sites of energy metabolism of bacteria and fungi [32].

Although the antibacterial mechanism of alkaloids is still unclear, the antibacterial abilities of these compounds have been proven effective on *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Staphylococcus aureus, Streptococcus faecalis, Vibrio cholera, Enterococcus faecalis, Shigella dysenteriae* [34];[35]. The previous study reported that the alkaloid causes lysis and morphological changes in bacterial cell [36], and this compound is known as a DNA intercalator and an inhibitor of DNA synthesis through topoisomerase inhibition [37].

The bioactive compound of Maja fruits extracts has the potential as an alternative solution for further studies for reducing ammonia formation in livestock wastes. The use of natural antibacterial compounds such as Maja fruit extract as a urease inhibitor is considered appropriate because it does not cause residues to the environment. This is in consistent with the opinion of [4] that the use of essential oils from plant extracts as urease inhibitors is an environmentally friendly solution to reduce the emergence of odours from livestock wastes. Potential advantages of using these natural antimicrobial chemicals are that microbial fermentation of waste is inhibited, which then reduce the rate of ammonia and odour emissions, reduce the production of global warming gases, and conserve nutrients in the waste for increased fertilizer value. Pathogenic faecal coliforms should be minimized, and these oils can be used as natural pesticides, which can play a significant role in controlling insects in livestock wastes.

4. Conclusion

It can be concluded that the antibacterial ability of the Maja Fruit Extract is low or resistant. The most significant inhibition zone occurs in the addition of a 100% concentration of Maja fruit extract on agar medium at 14.26 ± 0.75 mm. The antibacterial ability of Maja fruit extract with an addition of 3% in liquid medium gives the lowest graph of bacterial growth. Maja fruit extract has the potential as an antibacterial and urease inhibitor compound, which can be applied to inhibit the hydrolysis of urea by microorganisms, so that it can reduce the emission of gaseous ammonia from livestock wastes, especially chicken excreta.

5. Acknowledgments

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