

An In Vitro Antibacterial Activity Test of Meniran Herbs' (Phyllanthus Niruri L.) Ethanol Extract Against Mycoplasma gallisepticum causes Chronic Respiratory Disease (CRD) in Broiler Chickens

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An In Vitro Antibacterial Activity Test of Meniran Herbs' (*Phyllanthus Niruri L.*) Ethanol Extract Against *Mycoplasma gallisepticum* causes Chronic Respiratory Disease (CRD) in Broiler Chickens

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Abstract

Chronic Respiratory Disease (CRD) is a chicken respiratory disease that attacks both broilers and layers. Chronic Respiratory Disease (CRD) has important economic significance in the intensification of chicken farms because this disease can cause huge economic losses. *Meniran* plant (*Phyllanthus niruri Linn*) is one of the plants that can be used as prevention and alternative treatment as a substitute of antibiotic caused by *Mycoplasma galisepticum* causes Chronic Respiratory Disease (CRD) in broiler chickens. The chemicals contained in *meniran* include *tannins*, *saponins*, *alkaloids* as antibacterials. The purpose of this study is to determine the activity of *meniran herbs'* (*Phyllanthus Niruri Linn*) as antibacterial to eradicate *Mycoplasma galisepticum*. The method of this study is dilution method which included *Minimum Inhibitory Concentration* [MIC] and *Minimum Bactericidal Concentration* (MBC). *Minimum Inhibitory Concentration* [MIC] was taken by making the concentration of *meniran* extract as much 65%, 62,5%; 60%; 55%; 50%; 45%; 40%. It was then added *Mycoplasma gallisepticum* bacteria. The result of this study is *Meniran's* activation test on *Mycoplasma galisepticum* obtained a dose of 62,5% could eradicate *Mycoplasma galisepticum* causes Chronic Respiratory Disease (CRD) in broiler chickens. *Meniran herbs'* (*Phyllanthus niruri linn*) is effective as antibacterial at concentrations of 30% against *Mycoplasma gallisepticum* causes Chronic Respiratory Disease (CRD) in broiler chickens.

Keywords: *Meniran herbs'* (*Phyllanthus Niruri Linn*), *Mycoplasma Galisepticum*, Chronic Respiratory Disease (CRD).

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1. Introduction

Mycoplasma gallisepticum was a bacteria that causes respiratory disease called *Chronic Respiratory Disease* (CRD). This bacteria had no cell wall, therefore often called *prokaryotic eubacteria* [4]. *Mycoplasma gallisepticum* infected by destroying the host cell then producing clinical symptoms and causing an immune reaction from host and inflammatory response [7]. This bacteria attacked the surface of epithelial host cells to damage the mucous membranes and decrease the motility of cilia in the respiratory tract. *Mycoplasma gallisepticum* was actually a non-invasive bacteria [10] but spread through cell gaps and capillaries due to its very small cell size and then hemadsorped and formed colonies [4]. *Chronic Respiratory Disease* [CRD] has significance economic meaning, because it can cause big economic losses and high number of morbidity. The morbidity caused by CRD was quite high to reach more than 25% [7].

In this decade, the use of antibiotics to eradicate *Mycoplasma gallisepticum* began to decline its potential because it has caused resistance, thus difficult to handle. Treatment using herbs has several advantages, like relatively low toxicity, safe, no residue left [21]. One of the treatment for bacterial diseases that safe is to use herbs. Indonesia as a tropical country has a wealth of potential herbs. Many plants contain antimicrobial compounds because it contains bactericidal compounds [bacteria killer] and bacteriostatics [bacteria growth inhibitors] and also as immunomodulators.

Meniran plants [*Phyllanthus niruri* Linn] is one of the plants that can be used as an alternative prevention and treatment caused by *Mycoplasma galisepticum* disease. *Meniran* contains several active substances that contribute to minimize bleeding [18]. The chemicals contained in *meniran* include flavonoids, alkaloids, saponins, and tannins [2]. The efficacy of this plants come from the content of various chemical compounds namely lignans, tannins, polyphenols, alkaloids, flavonoids, terpenoids and steroids [19]. Based on the above background, it conducted a study in vitro antibacterial activity test of *meniran* herbs' ethanol extract [*Phyllanthus Niruri* L.] against *Mycoplasma gallisepticum* causes Chronic Respiratory Disease [CRD] in broiler chickens.

2. MATERIAL AND METHODS

This study used laboratory exploration method through below steps: 1) Screening *Mycoplasma* on broiler farms in Probolinggo, Blitar, Lamongan and Mojokerto, 2) Isolation and Identification of *Mycoplasma* in broilers farmsin Probolinggo, Blitar,

Lamongan and Mojokerto and activation Test and Potential Extract of *Meniran* against *Mycoplasma gallisepticum*.

2.1. Diagnosis Procedures

This study was conducted by screening *Mycoplasma gallisepticum* followed by identification isolation test. The tests were performed by prediction test and ratification test, and isolation-identification through biochemical tests namely *Indole*, *Methyl Red*, *Voges-Proskauer*, and *Citrate* (IMViC) (BSN, 2008).

2.2. Screening Test

2.2.1. Rapid Plate Agglutination Test (RPAT)

The main principle of *RPAT* is the binding of standard (modified) antigens with appropriate serum (antibody) samples resulting in agglutination. This test is widely used to detect *Mycoplasma gallisepticum*. The test is quick, easy, no special treatments and tools so it can be done anywhere. It conducted by mixing one drop of serum with one drop of antigen above the plate. It continued with stirring it for 5 seconds, 1 minute and 2 minutes and then see the result. Positive result if there is precipitation/agglutination formed and negative result if no agglutination occurs. The precipitate is an antibody-antigen bond [17].

2.3. Isolation identification

Trachea and lungs organ samples of live chickens suspected of having *CRD* infection, were then cultured in a liquid medium and incubated at 37°C. Culture media that changed color from red to clear yellowish red then reproduced on solid media. If the culture on the liquid medium turned yellowish but cloudy, it was then filtered using a "Millipore" filter of 300 nm. After being filtered then as much as 0.2 ml reproduced back into liquid medium and solid medium. The liquid medium was incubated directly in the incubator at 37°C, while the solid medium culture was incubated in an incubator at 37°C for 5 to 14 days.

Observation of *Mycoplasma gallisepticum* colony growth conducted by using "dissecting" microscope. Small *Mycoplasma gallisepticum* colonies with a cross section of less than 1 mm, were oval or round with a middle section also oval and darker called

“bleb” that functioned to adhere to the media. The very specific form is known as ‘fried egg’. This colony consists of thousands of cells *Mycoplasma gallisepticum*, so if it required to be purified needed cloning technique by taking a colony using Pasteur pipette. The collected colony was then replanted in a liquid medium, shaken evenly by using a shaker and then grown on a solid medium. To get a pure colony required at least 3 times cloning. In addition, to identify whether the colonies produced was *Mycoplasma gallisepticum* or “*acholeplasma*”, the colonies of *Mycoplasma gallisepticum* growth were once recovered then cultured in liquid medium. After growing it was then diluted in liquid medium then poured on solid media. The culture on the solid medium was left drying in the sterile room cabinet for approximately 5 minutes. After 5 minutes, the discs containing “digitonin” were placed on a solid medium culture, then inserted in a bread tin and then incubated at 37°C. for 5 - 7 days. Whenever occurred barrier more than 5 mm then the cultured colonies could be confirmed *Mycoplasma gallisepticum* bacteria.

The growth resistance test was performed in the same way as in the microplasma bacteria detection test, except that the disc paper used contained *Mycoplasma gallisepticum*' antisera. Whenever occurred barrier more than 2 mm then the cultured colonies could be confirmed *Mycoplasma gallisepticum* germs [13].

2.4. Making Meniran Extract

Making *meniran* extract from dry *meniran* plants was conducted through pollinated and sifted to get its powder. One kilogram of powder extracted by maceration using 90% ethanol solvent as much 5 L. The stir was conducted twice, in the morning and afternoon, and after 3 × 24 hours continued with filtering. The waste was macerated again with 96% ethanol solvent as much 5 L. Maseration was conducted 3 times. The obtained filtrate was collected and then precipitated, filtered to subsequently evaporated by reducing pressure using rotary evaporator to obtain thick extract. It was calculated of 1kg powder resulted 74 grams *meniran* extract [24].

2.5. Activation Test or Potential Test of Meniran Against *Mycoplasma gallisepticum*

The method used in this study was dilution method which included ⁵ *Minimum Inhibitory Concentration (MIC)* and *Minimum Bactericidal Concentration (MBC)*. *Minimum Inhibitory Concentration (MIC)* was taken by making concentration of *meniran* extract as much

65%, 62,5%; 60%; 55%; 50%; 45%; 40%; then added bacteria of *Mycoplasma gallisepticum* as much as 5 colonies in 1ml *Mycoplasma Borth* media at each concentration then incubated at 37°C for 24 hours. The results were observed by looking cloudy or clear at each concentration that had been planted with *Mycoplasma gallisepticum* bacteria. Minimum bacterioside concentration (MBC) was taken by using *Mycoplasma Agar* media for 2 plates, each divided by 4 parts and given a writing concentration on the back of the media. The MIC test result was grown in *Mycoplasma Agar* medium by streaking on each concentration in the medium, then incubated at 37°C for 3x24 hours. The result of fertilization could be observed with no growth of *Mycoplasma gallisepticum* bacterial colonies on *Mycoplasma Agar* media [13].

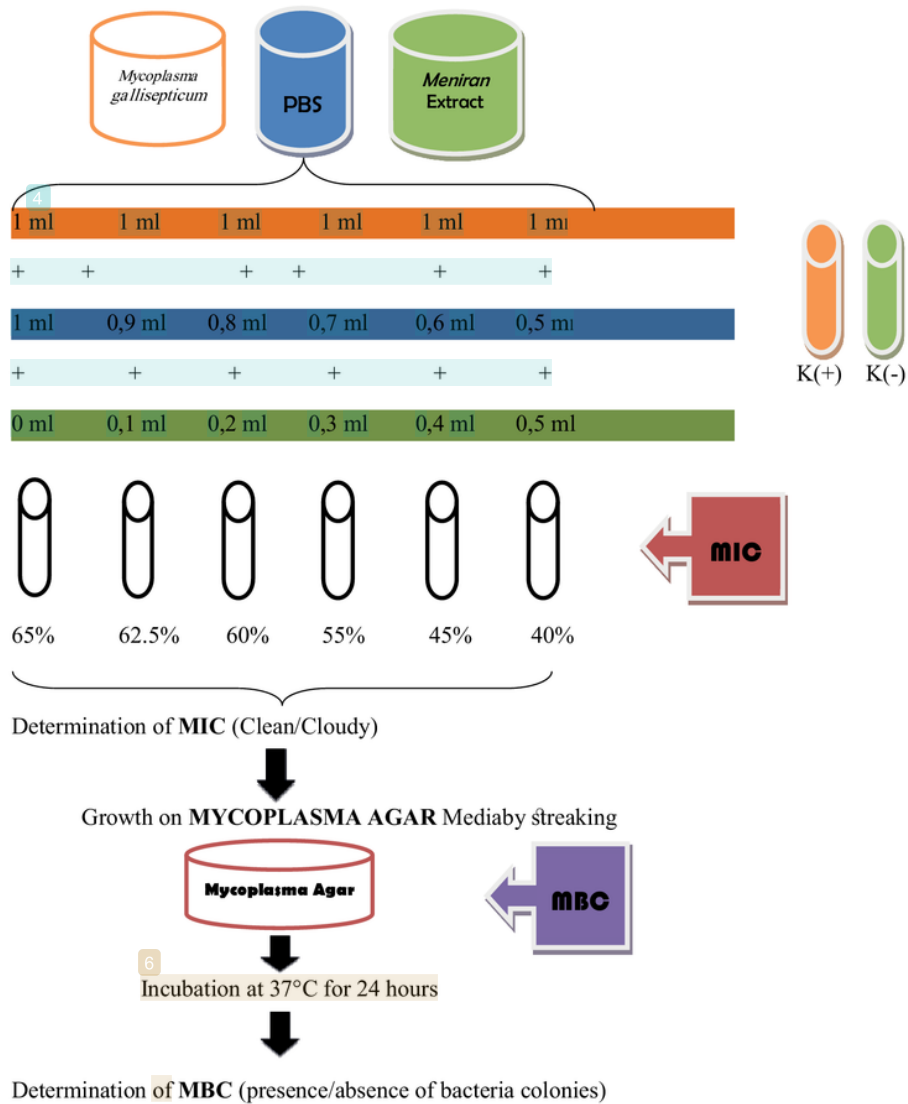
2.6. Data Analysis

Data analysis to identify the antibacterial effectiveness of *meniran* extract was tested by parametric using probit analysis.

3. Results

3.1. Isolation and Identification of *Mycoplasma gallisepticum*'s local isolates

The results of the isolation and identification of *Mycoplasma galisepticum* bacteria in the trachea and broiler lungs samples from broiler farms in Probolinggo and Blitar showing symptoms of *Mycoplasma galisepticum* infected with typical clinical symptoms preceded by the release of clear exudate fluid [*catarrhal*] from the nasal cavity, sneezing, coughing, snoring and inflammation of conjunctiva [*conjunctivitis*]. Bacteria of *Mycoplasma galisepticum* was isolated and identified from 23 samples of trachea and broiler lungs, 11 from Probolinggo farms and 12 from Blitar farms which resulted in positive rapid test using *Mycoplasma galisepticum* antigen showing 23 positive samples. Furthermore, conducted isolation and identification using *Mycoplasma broth* media was applied to the *Mycoplasma Agar* media which resulted of 23 samples showed positive results infected by *Mycoplasma galisepticum*.



3.2. Activation Test or Potential Test of *Meniran* Against *Mycoplasma galisepticum*

Dilution method to identify the sensitivity of *meniran* towards *Mycoplasma galisepticum* and to assess the dosage of *meniran* that can eradicate *Mycoplasma galisepticum* in *Chronic Respiratory Diseases* (CRD) case. The results obtained from the MIC and MBC tests was that a dose of 62,5% can eradicate the bacteria of *Mycoplasma galisepticum*.



Figure 1: Chicken Farms in Probolinggo Infected by CRD.



Figure 2: Chicken mortality caused by CRD (*Mycoplasma galisepticum*).

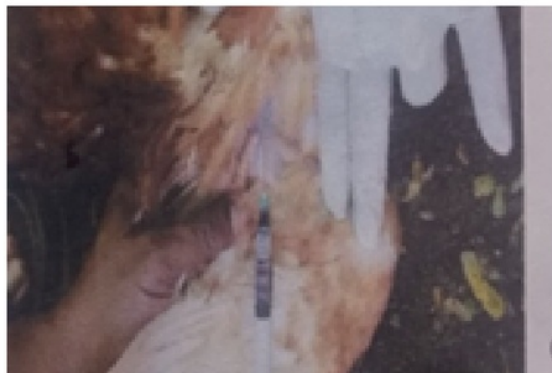


Figure 3: Blood-samples taken.

The result of a study conducted against ¹⁰ *Minimum inhibitory concentration* (MIC) of *Meniran extract* (*Phyllanthus niruri L*) was determined by observing the fluid changed in the tube becoming cloudy or clear. The cloudy fluid showed that *Mycoplasma galisepticum* bacteria in the tube was alive, while the clear fluid showed that *meniran* extract



Figure 4: Positive Rapid Test.

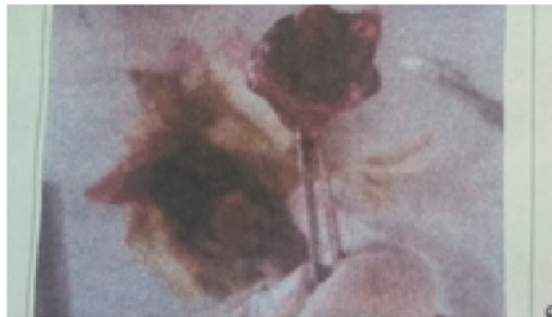


Figure 5: Lungs samples taken.



Figure 6: Test Results of *Mycoplasma gallisepticum* Bacteria.

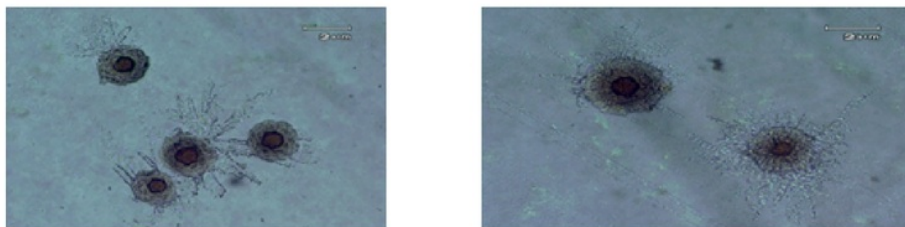


Figure 7: The shape of *Mycoplasma gallisepticum* colonies were small and round with also round and darker middle section called "bleb", functioned to adhere to the media. The very specific form is known as 'fried egg'.



Figure 8: Submersion of *Meniran*.



Figure 9: Extraction process Extractusing evaporator.



Figure 10: Extract *Meniran*.

(Phyllanthus niruri L) was able to inhibit the growth of local isolate's *Mycoplasma galisepticum* bacteria.

TABLE 1: Minimum Bactericidal Concentration (MBC) in extract *meniran* (*Phyllanthus niruri* Linn.)

| | Concentration of <i>Meniran infusum</i> | | | | | |
|---|---|--------|--------|--------|--------|--------|
| | 45% | 50% | 55% | 60% | 62,5% | 65% |
| Bacteria Growth of <i>Mycoplasma galisepticum</i> | Cloudy | cloudy | cloudy | cloudy | cloudy | cloudy |

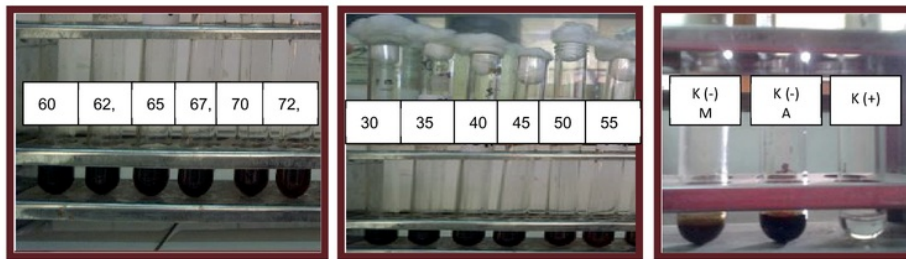


Figure 11: Minimum Inhibitory Concentration (MIC).

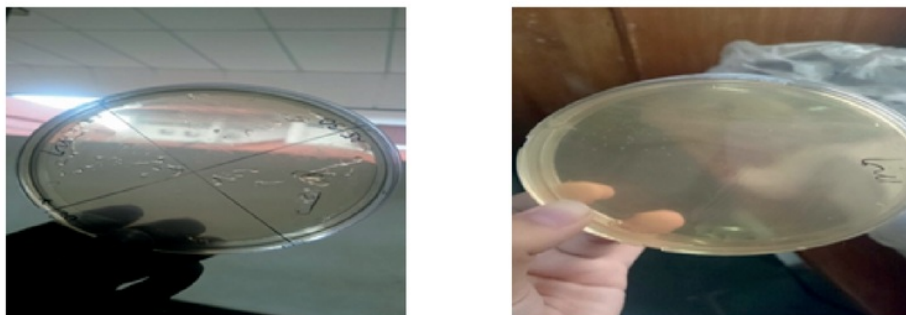


Figure 12: Result of Minimum Bactericide Concentration (MBC) from *meniran* extract against *Mycoplasma gallisepticum* bacteria.

The results of the study could not be able to determine the proper concentration to inhibit the growth of *Mycoplasma galisepticum* bacteria because all the tubes appeared cloudy caused by the *meniran* extract (*Phyllanthus niruri* L) used colored dark brown to make determination of Minimum inhibitory concentration (MIC) against *Mycoplasma galisepticum* bacteria became difficult to do. The observation result of minimum inhibition level on *meniran* extract described in Figure 1 and table 2.

4. DISCUSSION

Based on the data in this study can be concluded that the higher concentration of extract *meniran* can affect the inhibition and eradicate *Mycoplasma gallisepticum* bacteria. It is because the higher concentration the higher active substances content of

TABLE 2: Minimum Bactericidal Concentration (MBC) in extract *meniran* (*Phyllanthus niruri* Linn.)

| | Concentration of <i>Meniran infusum</i> | | | | | |
|---|---|---------|---------|---------|-----------|-----------|
| | 45% | 50% | 55% | 60% | 62,5% | 65% |
| Bacteria Growth of <i>Micoplasma galisepticum</i> | Growing | Growing | Growing | Growing | No growth | No growth |

extract *meniran*, otherwise the lower concentration of *meniran* extract the lower ability of antibacterial solution. In general, high concentrations of antibacterial substances will become bactericidal, while at low concentrations will become bacteriostatic [22]. Bacteriostatic worked to inhibit protein synthesis and bond with ribosomes. In contrast to the way bactericidal substances to eradicate bacterial cells [17].

Growth of bacteria can be inhibited and eradicated by *meniran* extract because *meniran* plants are wealth of chemical compounds like *tannins compounds*, *saponins*, *flavonoids*, and *alkaloids*. Based on the screening results, the highest content of *meniran* extract was *tannin*. *Tannin* is useful to prevent the growth of microorganisms by precipitating proteins from enzymes produced by microorganisms that become inactive and cause the growth of bacteria inhibited. *Tannin* is easily soluble in water, glycerol, alcohols, liquid alkali and acetone, and also insoluble in ether and benzene. *Tannin* is phenolic, astringent and has a tanning effect. *Tannin* is hydrolyzed in the form of amorphous, hygroscopic, yellow-brown and water-soluble (mainly hot water) to form colloids. Phenol compounds and its derivatives when interacting with bacterial cells at low levels will form a protein complex that can cause protein denaturation and damage cell membranes [22]. *Saponin* is a compound suspected as an antibacterial compound because it has the ability to inhibit cell membrane function, thus destroying membrane permeability resulting in damaging or destroying cell wall [23].

Another useful chemical content of *meniran* is *flavonoids*. In other plants there is also flavonoid content, except in *meniran*, the increasing activity of immune system is better. Flavonoid is antibacterial and antioxidant and able to enhance immune system performance because leukocytes as antigen-eaters are produced faster and lymphoid systems are activated faster [19]. *Meniran* plants also contain *alkaloids* that are toxic to microbes, therefore effectively eradicating and inhibiting Gram-negative and Gram-positive bacteria. *Alkaloids* worked as antibacterial by destroying the peptidoglycan component of the bacterial cell, so that the cell wall layer will not completely be formed and caused the mortality of the cell [2].

5. CONCLUSION

Based on the study conducted, It has been isolated and identified *Mycoplasma galisepticum* bacteria from broiler chickens in Probolinggo and Blitar broiler farms respectively 23 samples and from result of activation test or potential test obtained *meniran* extract with a dose of 62,5% could eradicate *Mycoplasma galisepticum* bacteria.

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