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Antibacterial Inhibition Test Against the Combination Extract of Moringa Leaf (*Moringa oliefera*) and Basil Leaf (*Ocimum basilicum*) as a Substitute for Feed Additive

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Abstract

One of the efforts to increase poultry productivity is to provide feed additives that are mixed with feed. Feed additives to replace antibiotics such as probiotics, prebiotics, and acidifiers can be used as additives to feed because of their ability to regulate digestive tract conditions. Moringa leaves (*Moringa oliefera*) and basil leaves (*Ocimum basilicum*) contain active compounds that can used to inhibit the growth and extinguish *Escherichia coli* and *Staphylococcus aureus.* The purpose of this study was to determine the effectiveness of each of the bioactive substances produced from a combination of extracts of several plants which were combined to have a greater antibacterial inhibition compared to single plant extracts. The research design consisted of 5 treatments and 4 replications with treatment P0 (positive control, namely contrimexasol), P1 (Moringa leaves 55% + Basil leaves 25%), P2 (Moringa leaves 50% + basil leaves 30%), P3 (Kelor leaves 45 % + Basil leaves 35%), P4 (Moringa leaves 40% + Basil leaves 40%). The study used RAL (Completely Randomized Design) and further tests were carried out with the BNT test at 5% level to determine the difference between each replicate. The results was indicated that the highest inhibitory power was found in Gram positive bacteria and the lowest inhibitory power was found in Gram negative bacteria. The combination of Moringa leaves and Basil leaves reaches a concentration of 55% + 25% able to inhibit the growth of Gram positive bacteria and Gram negative bacteria, so that it is possible to improve the digestive system in poultry and can inhibit the bacteria that cause diarrhea.

Keywords : Probiotics, bioactive substance, inhibitory power, digestive system, *Staphylococcus aureus*

INTRODUCTION

One of the efforts to increase the productivity of poultry is by giving feed additives to poultry (Lavrinenko *et. al*., 2021). *Feed additives* to replace antibiotics such as probiotics (Krysiak *et. al*., 2021), prebiotics, and acidifiers (Rapiq *et. al*., 2021*)* can be used as additives to feed because of their ability to regulate the condition of the digestive tract to be healthier by inhibiting the

growth of harmful bacteria and increasing beneficial bacteria. However, the use of drugs, antibiotics, or animal growth-promoting hormones that are not as recommended and not in accordance with the prescribed dose can cause residues in the livestock products produced.

Moringa contains chemical compounds, namely flavonoids, saponins, tannins, and several other phenolic compounds (Riyadi *et. al.,* 2021) that have antimicrobial activity. In the study of Dima *et al.* (2016) which stated that with concentrations of 5, 10, 20, 40, and 80% had antibacterial power ranging from moderate to strong. The largest diameter of zona inhibition was at a concentration of 80% (21.50 mm in *Staphylococcus aureus* and 24.00 mm in *Eschericia coli*).

Basil leaves (*Ocimum basilicum*) contain essential oils that are antibacterial (Angraini *et al*., 2021). In addition to essential oils, basil leaves also contain flavonoids which are antibacterial. Flavonoids can inhibit nucleic acid synthesis, inhibit cytoplasmic membrane function, and inhibit cell energy metabolism.

Staphylococcus aureus are pathogenic bacteria or can harm humans and mammals because they can cause infection. How to control *Staphylococcus aureus* can use plants that contain natural antimicrobial chemicals that are expected to suppress bacterial growth. In addition, there are also *Escherichia coli* which are normal flora in the digestive tract but have the potential to cause disease. *Eschericia coli* become pathogenic if the number in the digestive tract increases, such as consuming contaminated water or food or entering the body. This study aims to determine the effectiveness of the combination of Moringa (*Moringa oleifera*) and basil (*Ocimum basilicum*) leaf extract against the cell life and growth *of Staphylococcus aureus* and *Eschericia coli* as bacteriostatic or bacteriocidal.

MATERIALS AND METHODS

Time and Place

Research on the antibacterial inhibition test of the combination of Moringa leaf extract (*Moringa oleifera)* and basil leaf extract (*Ocimum basilicum*) as an alternative *feed additive* was carried out at the Fishery Products Technology Laboratory, Faculty of Marine and Fishery Sciences, Hasanuddin University and Microbiology, Faculty of Medicine, Hasanuddin University, Makassar for bacterial activity test.

Research Material

The tools used in this research are analytical balance, blender, Erlenmeyer, autoclave, incubator, petri dish, test tube, test tube rack, ossicle needle, measuring cup, Bunsen, blender, container, caliper, glass funnel, stirring rod, *hot plate*, *rotary evaporator* and micropipette.

The materials used in this study were Moringa leaf flour, basil leaf powder, *Escherichia coli bacteria*, *Stapylococcus aureus*, antibiotic contrimexaxol, sterile distilled water, *Mueller-Hilton Agar* (MHA), 70% alcohol, filter paper, *sterile disc paper*, *labels*, markers, and aluminum foil.

Research Design

The design used in this study was a completely randomized design (CRD) with 5 treatments and each treatment consisted of 4 replications with the following treatments:

P0 : Antibiotic contrimexaxol
P1 : Moringa leaf extract 55%+Basil leaf extract 25%
P2 : Moringa leaf extract 50%+Basil leaf extract 30%
P3 : Moringa leaf extract 45%+Basil leaf extract 35%
P4 : Moringa leaf extract 40%+Basil leaf extract 40%

Research Implementation

The research method is the antibacterial inhibition test of Moringa leaves (*Moringa oleifera*) and basil (*Ocimum basilicum*) use *Kirby-Bauer disk* diffusion technique.

1. Making Moringa (*Moringa oleifera*) and basil (*Ocimumbasilicum*) leaf flour

Samples in the form of Moringa leaves were collected and then cleaned of residual dirt, then washed under running water until clean. After cleaning from impurities, the Moringa leaves are drained, and then dried by aerating. After that, the dried samples were mashed with a blender until they became powder. The resulting powder was sieved with a sieve, until a fine and homogeneous powder was obtained. The result is put in a closed glass container.

The process of making basil flour is that fresh basil is cleaned of soil and other impurities after the basil leaves are clean then withered at room temperature for 48 hours, then in the oven at 60^{o}C for 24 hours. Next, grind the ingredients in a blender until they become fine particles.

2. Tool Sterilization

Before use the tool must be sterilized first to avoid any contaminants. The sterilized tools include test tubes, petri dishes, and beakers. The sterilization process was using an autoclave at a temperature of 121^{0}C for 15 minutes. First, the tool to be sterilized was washed with running water, washed with 1% HCl solution, and then it was boiled over a bath using a mixture of 0.5% Na Carbonate and 1% tepol solution. The tool was rinsed using aquadest. Glass utensils are dried and wrapped in aluminum foil.

3. Preparation of Test Bacterial Suspension

The inoculated test bacteria were taken with sterile ose wire and then suspended into a tube containing 2 ml of 0.9% NaCl solution until the turbidity was the same as the standard turbidity of the solution. The same treatment was carried out for each type of bacteria to be used (Bayot and Bragg, 2021).

4. Making *Mueller Hinton Agar Media*

Mueller Hinton Agar powder as much as 60 grams was put into an Erlenmeyer tube and dissolved with 1000 mL of distilled water. The resulting suspension is heated to boiling. Next, the tube was put into an autoclave at 121°C for 15 minutes. Then, the Erlenmeyer tube was put in an incubator at 37°C for 24 hours.

5. Extract Making

Moringa leaf powder (*Moringa oleifera*) and basil leaves (*Ocimum basilicum*) weighed as much as 250 g. Then soaked in ±2.5 L of methanol until all samples were submerged and left for 24 hours. The maserate was filtered and the methanol extract of Moringa leaves was obtained. Moringa leaf methanol extract was concentrated by evaporation to obtain a solid methanol extract. Then precede with the antibacterial test against *Staphylococcus aureus* and *Eschericia coli.*

6. Antibacterial Test Procedure

A total of 1 mL of the test bacterial suspension was put into a petri dish, then +15 mL of MHA and MRS media were added and allowed to solidify. *Blank disks* were immersed in each test sample for +15 minutes. Blank disk that has been soaked is placed on the surface of the agar media. As a positive control, the antibiotic cotrimoxazole was used and the negative control was sterile distilled water. The media was incubated at 37˚C for 1x24 hours, observing and measuring the diameter of the barrier (clear zone) formed around *the blank disk.*

7. Measured Parameter

Parameter which be measured on study this are a resistance which seen of the clear zone formed around the previously placed disc paper on surface media MHA. Zone clear (zone resistor) in the diameter around the disk paper formed after incubation is measured using a caliper, it will get the results of measurement.

RESULTS AND DISCUSSION

Based on the results of the research that has been done, the average inhibitory power of Moringa leaf extract and basil leaf extract as an alternative *feed additive* in feed against contaminant *Escherichia coli* and *Staphylococcus aureus* is presented in Table 1.

Table 1. Average diameter of antibacterial inhibition of Moringa leaf extract and basil leaf extract against *Staphylococcus aureus* and *Escherichia coli bacteria*.

Treatment	Diameter of Bacterial Growth Inhibitory Zone (mm)	
	Escherichia coli (mm)	*Staphylococcus aureus* (mm)
P0	16.22 ± 2.31^{b}	28.12 ± 0.29^{e}
P1	$17.45 \pm .052^{b}$	20.92 ± 0.17^{d}
P2	13.05 ± 0.82^{a}	13.62 ± 0.28^{a}
P3	14.05 ± 0.33^{a}	14.55 ± 0.33^{b}
P4	13.72 ± 0.38^{a}	17.45 ± 0.38^{c}

Description: Different superscript ^{abc}in the same column showed significant differences ($P<0.05$).

The results of the BNT test in Table 1 show that the antibacterial inhibition of Moringa leaf extract and basil leaf extract significantly ($P<0.05$) against *Staphylococcus aureus* and *Escherichia coli*. The results of this study showed that the combination of Moringa leaf extract and basil leaf extract with a concentration of 55%+25% had the highest inhibition zone diameter (20.92 mm) in *Staphylococcus aureus* while *Escherichia coli bacteria* had a diameter of (17.45 mm) compared with the treatment of Moringa leaves and basil leaves with a concentration of 50%+30% as well as the treatment of 45%+35% and 40%+40%. The average diameter of the inhibition zone formed in the P1, P2, P3, and P4 treatments was included in the strong category. According to Puspita *et al.* (2021) the diameter of the inhibition zone between 11-20 mm is categorized as strong.

Inhibition Test

The analysis results of the various antibacterial inhibition tests of the combination of Moringa leaves and basil leaves with different concentrations showed a significant effect

(P<0.05) on the growth of *Escherichia coli* and *Staphylococcus aureus*. The choice of cotrimoxazole as a positive control was because *cotrimoxazole* is a fixed combination preparation of trimethoprim and sulfamethoxazole with a ratio of 1: 5 which has broad spectrum bactericidal activity so that it can kill Gram-positive and Gram-negative bacteria. In the use of *cotrimoxazole*, the diameter of the inhibition zone is in the strong category for *Escherichia coli* while *Staphylococcus aureus* has an inhibition zone diameter in the very strong category. According to Morales *et al.* (2003), the activity of the antimicrobial inhibition zone was grouped into four categories, namely weak activity (<5 mm), moderate (5-10 mm), strong (>10-20 mm), very strong (>20-30 mm). The antimicrobial inhibitory activity was expressed based on the clear zone produced around the paper disc. The diameter of the zone of inhibition of bacterial growth was measured in mm. Co-trimoxazole is a combination of sulfamethoxazole and trimethoprim which trimethoprim has an anti-bacterial spectrum of action similar to that of sulfonamides, which is effective against most Gram-positive and Gram-negative bacteria. The mechanism of action is that sulfamethoxazole interferes with bacterial folic acid synthesis and growth by inhibiting the formation of dihydrofolic acid and trimethoprim inhibits the reduction of dihydrofolic acid to tetrahydrofolate, so the combination of the two is very suitable (Fernandez-Villa *et al.,* 2019).

Based on the results of the study, the antibiotic cotrimoxazole was able to inhibit the growth of *Escherichia coli* at all concentrations with16.22 mm of zona inhibitions while in *Staphylococcus aureus* it has higher than *E. coli* was 20.1 mm. The purpose of the observation test on the positive control was to prove that the *Mueller Hinton Agar* (MHA) media used in the test of Moringa leaf extract and basil leaf extract contained the growth of *Escherichia coli* and *Staphylococcus aureus.*

The use of Moringa leaf and basil leaf was too compared with antibiotics in was not only for comparison in samples but also because these antibiotics-like were often used to treat diarrheal diseases which were often caused by bacterial contamination. Because for the treatment of diarrhea caused by bacteria, cotrimoxazole antibiotics can be used which are broad spectrums for gram-positive and gram-negative bacteria. The mechanism of action of the antibiotic cotrimoxazole is the inhibition of DNA/RNA protein synthesis, may be the mechanism same with the Moringa and Basil leaf extract. The result is the cessation of the synthesis of float acid which is the base material for the synthesis of purines and DNA/RNA, so that bacterial cell division is stopped.

Combination Inhibitory Power

The results of the analysis of variance (Table 1) was describes that the antibacterial inhibition of the combination of Moringa leaf extract and basil leaf extract had a significant effect (P<0.05) on *Escherichia coli* and *Staphylococcus aureus*. The diameter of the antibacterial inhibitory power of the combination of Moringa leaf extract and basil leaf extract with the highest strength of the inhibitory zone activity was found in the treatment with a concentration of 55% Moringa leaves+25% basil leaves 17.45 mm in *Escherichia coli* and 20.92 mm in *Staphylococcus aureus.* The high inhibition zone produced was influenced by the higher concentration given to the treatment of Moringa leaves than basil leaves so that bioactive compounds such as phenols and chemical compounds pterygospermin contained in Moringa leaves were more dominant where these compounds both played a role in forming the inhibition zone. Makarewicz *et al.,* (2021) reported that one of the phenolic compounds that damage and penetrate the walls and precipitate bacterial cell proteins and large molecule phenolic compounds is able to inactivate essential enzymes in bacterial cells even in very low concentrations.

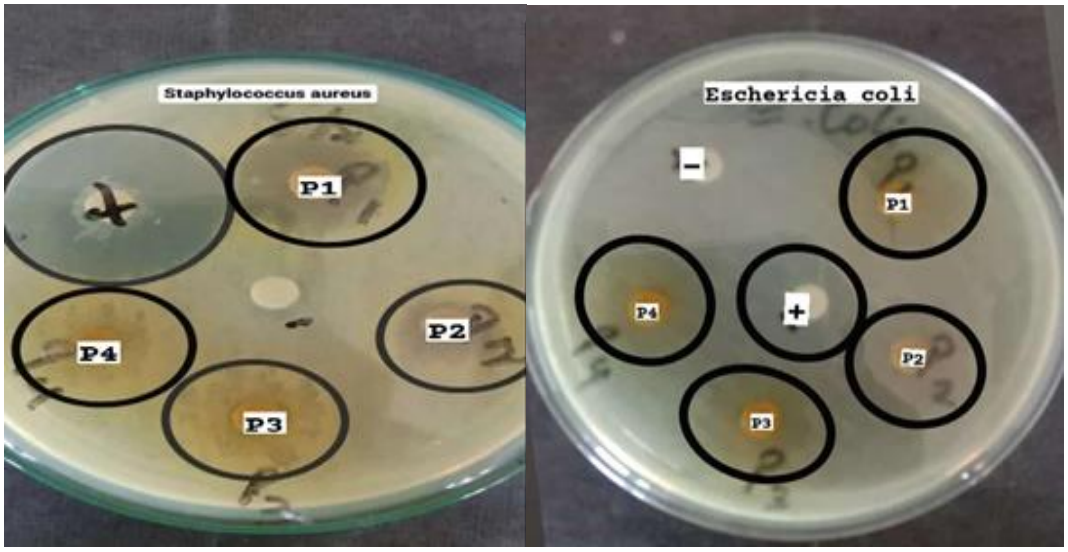

Figure 1.Diameter of inhibition zone combination of Moringa and basil leaves against *Escherichiacoli* and *Staphylococcus aureus.*

Based on this study, it was shown that the combination of Moringa leaf extract and basil leaf extract resulted in an inhibition zone. (Figure 1). The resulting inhibition zone against *Escherichia coli* bacteria showed that there was a significant difference (P<0.05) between P1. While the P2 treatment was not significantly different from the P3 and P4 treatments, but numerically there was an increase in. Combination of Moringa leaf extract and basil leaf extract was showed a good resistance response at P1. The clear zone formed due to the bioactive substances contained in Moringa leaves and basil leaves which act as antibacterial as in the study of Fitri *et al.*, (2020) stated that the inhibition zone formed by each extract occurred due to the content of secondary metabolites that it has which can work. Bacterial growth can be inhibited by saponin compounds where the main mechanism of action of saponins on the surface of bacterial cells is to reduce the voltage so that cell permeability increases which will cause cell leakage so that intracellular compounds will come out which can cause death.

The combination of Moringa leaves and basil leaves with different concentrations showed a significant difference (P<0.05), namely *Escherichia coli* had a lower inhibition zone diameter when compared to *Staphylococcus aureus*, but the difference was not too much. This is because the cell wall structure between the two bacteria is different, where *Escherichia coli* have a more complex cell wall structure while *Staphylococus aureus* have a simpler bacterial cell wall besides that it can also be influenced by treatment concentration, the type of solvent used in the treatment, the type of bacteria used, the metabolic activity of bacteria, and the sensitivity of different bacteria. This is in accordance with the review article by Manandhar *et al.* (2019) which states that the difference in the results of the inhibition zones produced by each treatment can be caused by the concentration of the treatment, the type of solvent used in the treatment, the type of bacteria used, the metabolic activity of the bacteria, and the sensitivity of the bacteria different.

Inhibitory activity of *Escherichia coli* and *Staphylococcus aureus* by basil leaf extract and Moringa leaf extract has a better inhibition zone on the effect of giving the highest concentration of extract to Moringa leaves which causes Moringa leaf extract to be more dominant in inhibiting bacterial growth than basil leaf extract as evidenced by the presence of substances. The most

dominant bioactive in Moringa leaves which can support the process of inhibiting bacterial growth as in Rahmawati's research (2014) which states that the greater the interaction concentration of a given extract, the greater the diameter of the inhibition formed, because the more bioactive components contained in the extract.

While the administration of basil and moringa leaf extracts with the same concentration, the diameter of the inhibition zone of *Escherichia coli* was lower than that of *Staphylococcus aureus*. This is due to the content of volatile oil compounds that can damage the cell walls of Gram-positive bacteria (*Staphylococcus aureus*) which have peptide chains that are arranged tightly and regularly between one glycan chain and another, causing the cell wall structure to be more difficult to damage. Although compounds are more difficult to penetrate from the outside, it is predicted that the essential oil solution of basil leaves can damage the peptide chains that make up the peptidoglycan, so that the bacterial cell wall becomes weak and under goes lysis. Types of bacteria in Gram-positive bacteria, most of the cell walls consist of a layer of peptidolian and teichoic acid (components of bacterial cell walls) so that hydrophilic extract components are easy to pass where hydrophilic compounds usually cannot bind to fat, oil or nonpolar molecules. According to Lingga *et al*. (2016) stated that the difference in the size of the inhibition of each solvent was due to differences in the active compounds dissolved in each solvent. The level of polarity affects the inhibition of the cell. The lower the polarity (closer to polar) the more effective it will be to inhibit Gram-positive bacteria than Gram-negative bacteria. Angane *et al.* (2022) also proved that the components of essential oils, which are semipolar to nonpolar, have stronger antibacterial properties against Gram-positive bacteria than Gram-negative bacteria. This is due to differences in the structure of the bacterial cell wall (Dorr *et al*., 2019).

The clear zone formed as evidence that there is active substance activity in Moringa and basil leaves which acts as an antibacterial. The ability of Moringa and basil leaves to inhibit bacteria due to the presence of saponins, flavonoids, and tannins. This is also due to the presence of essential oils in these two types of plants. The essential oil content of basil leaves is up to 60% higher than the essential oil content of Moringa leaves. This essential oil is able to inhibit the bacteria that cause diarrhea and improve the digestive tract. This is supported by the opinion of Setyanto (2012) which states that the effect of essential oils can stimulate digestive tract enzymes, so that essential oils can also inhibit bacteria that cause diarrhea so that the digestive process and absorption of nutrients become more perfect and can improve the digestive system. Therefore food is digested more quickly; a fast feed rate can cause the stomach to empty quickly and the birds to be hungry more quickly.

Extraction of antibacterials contained in Moringa leaves and basil leaves with ethanol solvent containing quercetin-type flavonol compounds which are proven to be antibacterial at the right concentration is predicted to produce effective extracts to kill *Escherichia coli* and *Staphylococcus aureus serovars*, so that they can be used as feed additives to replace AGP in feed chicken. According to Mahfuz and Piaoi (2019), one type of herbal feed known as a substitute for growth *promoter antibiotics* is Moringa leaves. Pankey and Sabath (2004) describes that antibiotics have the property of stopping growth or killing other microorganisms that are pathogenic by bacteriostatic and bactericidal mechanisms. This results in an increase in the population of beneficial bacteria in the digestive tract. Purwanto *et al.* (2021) was explained that Moringa leaves and N-hexane fraction have function as antioxidant with very strong activity.

While the content of basil leaf extract such as alkaloids, steroids, flavonoids, and polyphenols are active compounds that have been proven to be antioxidants, anti-inflammatory, and immunomodulators. Additives such as aromatic herbs or essential oils can help animals to grow better, free from stress during critical situations, and increase the availability of essential nutrients in the gut. Plant extracts can stimulate the immune system; suppress harmful microorganisms such as *Escherichia coli* (Mandey and Pontoh, 2020).

CONCLUSION

The results showed that the combination of Moringa leaf extract and basil leaf extract had inhibitory power as an antimicrobial against *Escherichia coli* and *Staphylococcus aureus*. Giving a combination of Moringa leaf extract and basil leaf extract up to a concentration of 55% + 25% can inhibit the growth of Gram positive bacteria (*Staphylococcus aureus)* and Gram negative bacteria (*Escherichia coli*. The diameter of the highest inhibition was found in Gram-positive bacteria (*Staphylococcus aureus)* with an inhibition zone of 20.92 mm and the lowest inhibition was found in Gram-negative bacteria (*Escherichia coli)* with an inhibition zone of 17.45 mm.

SUGGESTION

In vivo research is needed on livestock to see the effect of the combination of Moringa (*Moringa oliefera*) and basil (*Ocimum basilicum*) leaves as an alternative *feed additive* for poultry.

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