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ANTIMICROBIAL ACTIVITY TEST OF MEDICINAL PLANT EXTRACT USING ANTIMICROBIAL DISC AND FILTER PAPER

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ABSTRACT: Plant extracts contain secondary metabolites such as flavonoids and phenolic compounds with antibiotic activity. Antibiotic activity test can be done by using the Kirby-Bauer disc method which the antibiotic activity is indicated by the formation of a clear zone. This research was conducted using extracts of *Moringa oleifera* leaves and *Opuntia cochenillifera* cladode extracted using ethyl acetate as a solvent. Antibiotic activity test against *Staphylococcus aureus* bacteria was done in Mannitol Salt Agar and Luria Bertani Agar media. The Kirby-Bauer discs used are Antimicrobial Disc Oxoid® and Filter Paper. The results of this study show that the use of the Antimicrobial Disc Oxoid® and Filter Paper shows no statistical difference in clear zone results (t-value = 0,45; p-value = 0,655; with α = 95%). While the Clear Zone results on Mannitol Salt Agar gave better results than Luria Bertani Agar and were statistically significant (t-value = 2,46; p-value = 0,02; with α = 95%). These findings indicate that filter paper can be an inexpensive alternative for the antibiotic test with no significantly different result compared to commercial antibiotic disc. However, an antibiotic test against *Staphylococcus aureus* is better done on Mannitol Salt Agar (MSA) compared to Luria Bertani Agar (LBA).

Keywords: Antibiotic Test, Antimicrobial Disc, Kirby-Bauer Disc Method, Mannitol Salt Agar, *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus is a commensal gram-positive bacteria that colonize the human nasal mucosa and causes severe infection. The main problem in combating *Staphylococcus aureus* infection is the prevalence of multi-drug resistance, especially due to misuse (excess or abuse) of antibiotics (Eswari & Yadav, 2019). The increase in antibiotic resistance is estimated to cause the death of up to 10 million cases per year by 2050 (Jugreet & Mahomoodally, 2020). Exploration of alternative antibiotics needs to be done to solve the problem of the increase in antibiotic-resistant bacteria. Medicinal plant extract is one promising alternative antibiotics source (Langeveld *et al*., 2014). Medicinal plants such as *Moringa oleifera* are widely used for traditional medicine such as to treat dental caries, syphilis, typhoid, diarrhea, epilepsy, purgative, prostate cancer, fever, and

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HIV-AIDS (Dzotam *et al*., 2016). *Moringa oleifera* contains secondary metabolites including alkaloids, flavonoids (quercetin and kaempferol) (Lin *et al*., 2018; Wang *et al*., 2017), saponins, and tannins (Sulastri *et al*., 2018), which have antibiotic activity (Ilanko *et al*., 2019). Other used medicinal plant in this study, *Opuntia cochenillifera*, contain phenolic compounds, flavonoids, and anthocyanins (Alves *et al*., 2017), with antibacterial activity. Cladode is part of *Opuntia cochenillifera* that contain alkaloids, saponin, tannins, terpenes, flavonoid, and hydroxi flavones (Monrroy *et al*., 2017). One of the mechanisms of action of antibiotics from plant extract is through inhibition of peptidoglycan synthesis. Many of the proteins involved in this pathway are mur enzymes and the Penicillin Binding Proteins (PBPs), which are known as good targets for antibiotics (Liu & Breukink, 2016; Tomoda, 2016).

One of the important things to determine the antibiotic activity of a compound is the method of determining antibiotic activity. Kirby-bauer disc method is one of the major methods to test antibiotic susceptibility. The Kirby-bauer disc method relies on the diffusion of the test substance from the filter discs to the bacterial cultures (Kourmouli *et al*., 2018). The disk diffusion method is included in the agar diffusion method because the plant extract diffuses from its reservoir through the agar medium. The reservoir (Filter Paper Disc) is placed on top of an agar surface. If tested plant extracts compounds have antibiotic activity, the inhibition zone will be developed around the filter paper disk after incubation. The diameter of the inhibition zone describes the antimicrobial potency of plant extracts (Horváth *et al*., 2016). One of the most commonly used discs for antibiotic susceptibility test is the commercial Antimicrobial Disc. In this study, we tested the use of inexpensive filter paper as an alternative to commercial antimicrobial discs.

Another factors that influence the clear zone results are the media used when testing antibiotic activity. The agar media must allow free diffusion of the antimicrobial from the disc. Mannitol Salt Agar (MSA) is used as a selective and differential medium for the isolation and identification of *Staphylococcus aureus*. Mannitol Salt Agar (MSA) contains peptones and beef extract, which essential nutrients for growth. The 7,5% concentration of sodium chloride results in the partial or complete inhibition of bacterial organisms other than *Staphylococci*. Fermentation of mannitol (in MSA) leads to acid production, detected by phenol red indicator, aids in the differentiation of *Staphylococcal* species. Coagulase positive *Staphylococci* (e.g., *Staphylococcus aureus*) produce yellow colonies and a surrounding yellow medium (Aryal, 2019), Luria Bertani Agar (LBA) consists of tryptone, yeast extract, NaCl, and agar (Macwilliams & Liao, 2006). This study aimed to compare the use of commercial antimicrobial disc and filter paper, as well as the use of Mannitol Salt Agar (MSA) and Luria Bertani Agar (LBA) media in susceptibility testing of medicinal plant extracts against *Staphylococcus aureus*.

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METHODS

This study uses an experimental approach in the laboratory. This research method consists of several stages listed in Figure 1.

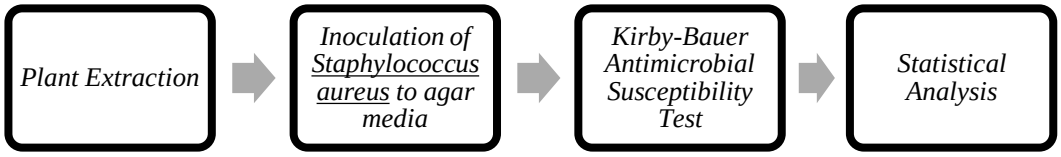

Figure 1. Research Work Scheme.

Plant Extraction

The plant extraction process is done based on (Kher *et al*., 2019), with modifications. *Moringa oleifera* leaves and *Opuntia cochenillifera* cladode was extracted using ethyl acetate as a solvent. The plant samples were oven-dried at 50°C until they reached a constant weight. After drying, the sample is crushed into a powder. Then, the plant samples were macerated using ethyl acetate solvent with a mass ratio of plant samples to the solvent volume of 1:10 for 24 hours. The filtrate is concentrated in a rotary evaporator until crude extract is obtained.

Inoculation of *Staphylococcus aureus* to Agar Media

Inoculation of *Staphylococcus aureus* was done using the swab technique based on Weme (2018), with modifications. One loop from a single colony of *Staphylococcus aureus* culture was inoculated aseptically into luria bertani broth media then incubated for 24 hours at 37º C. The sterile cotton is dipped in overnight-culture broth, then swab evenly over the surface of the agar medium.

Kirby-Bauer Antimicrobial Susceptibility Test

The antimicrobial activity assay is done based on (Yu *et al*., 2019), with modifications. Each of the Antimicrobial Disc Oxoid® and filter paper with a diameter of 5 mm was immersed in a plant extract with a concentration of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6,25 mg/mL, and amoxicillin 25 mg/mL as a positive control and DMSO 1% as a negative control. After immersing for 1 minute, each disc was placed on the surface of the media that had been inoculated with *Staphylococcus aureus*. Culture on solid media with a disc was incubated for 24 hours at 37º C.

Statistical Analysis

Each plate consists of 6 test points that have been given a number code (1-6) consisting of plant extracts of 50 mg/mL (1), 25 mg/mL (2), 12.5 mg/mL (3), 6.25 mg/mL (4), amoxicillin 25 mg/mL (5), and DMSO 1% (6). The data entered are test points number 1 to 5, while negative control is not included in the statistical test because as a negative control it should be 0 in clear zone diameter. DMSO is widely used as a solvent for antibiotic compounds (Camp *et al*., 2020), and is known to have low toxicity (Phaechamud & Setthajindalert, 2018). Each test consists of 2 plates for *Moringa oleifera* leaves and 1 plate for *Opuntia cochenillifera* cladode so that each treatment consisted of 15 data replications. The t-test statistical test was used to compare whether the clear zone diameter

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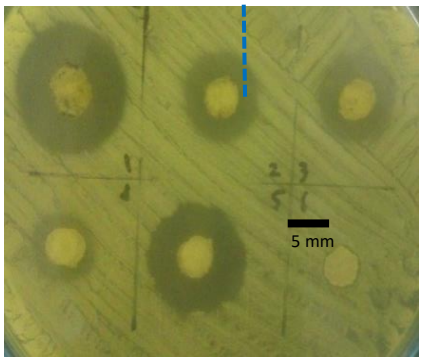
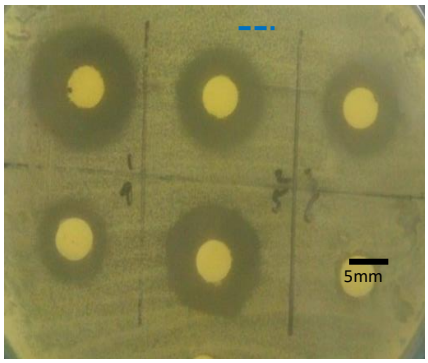
value between the two treatments was significantly different or not. Statistical analysis was done using minitab® with a confidence level of 95%.

RESULTS AND DISCUSSION

Comparison of Using Commercial Antimicrobial Disc and Filter Paper

Disc diffusion is an excellent, inexpensive, and flexible method for Antibiotic Susceptibility Test (AST) and its quality is determined by the quality of discs and media (Ahman *et al*., 2019). The clear zone results after 24 hours incubation (Table 1) showed that the use of filter paper and commercial antimicrobial disc was not statistically significant (t-value = 0,45; p-value = 0,655; with α = 95%).

Table 1. Clear Zone Comparison of Disc.

	Filter Paper	**Antimicrobial Disc Oxoid®**
Clear Zone Average (mm)	11.27 ± 4.91	12.07 ± 4.79
Image		
Statistical Analysis	T-Value = 0.45 P-Value = 0.655 with α = 95%	

*__Additional Information:__ Clear Zone is Indicated by a Blue Dotted Line.

The commercial oxoid antibiotic discs are made from paper which conforms to the WHO and FDA standards (Joshi *et al*., 2008). During the incubation period, the plant extract diffuses from the disc into the agar medium seeded with the test microorganism. The active antimicrobial extracts or compounds result in zones of inhibition around the disc, which give information about the value of the Minimum Inhibitory Concentration (MIC). *Moringa oleifera* contains bioactive compounds such as 9-Octadecenoic acid (z)-, heptadecanoic acid and phytol acetate (Syeda & Riazunnisa, 2020). *Opuntia cochenillifera* cladode contains flavonoids, phenols, alkaloids, tannins, and saponins (Pooja & Vidyasagar, 2016).

Factors influencing the size of inhibition zones are the size of the filter paper disk, the amount of compound placed onto the disk, the type and concentration of the agar, the thickness and pH of the medium, the microbial strain tested, and the incubation temperature (Horvath *et al*., 2016). The average clear zone value of the filter paper (11,27 ± 4,91 mm) was smaller than the Antimicrobial Disc Oxoid® (12,07 ± 4,79 mm) but did not differ significantly.

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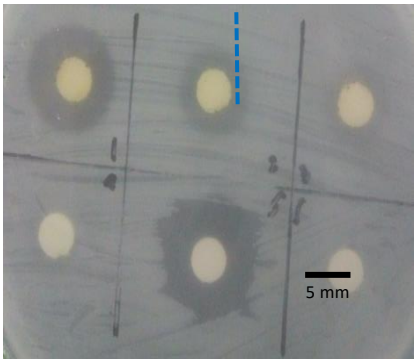
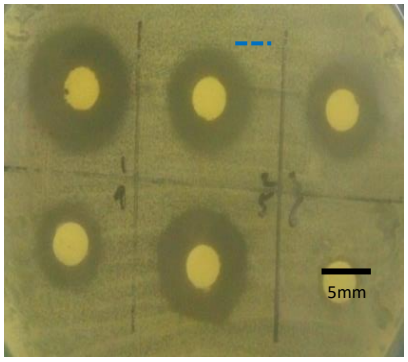
Antimicrobial Disc Oxoid® is made of unique inert material which enhances their absorption hence allowing faster adheration of discs to the media (Justesen *et al*., 2013).

The active compounds of the extract are less retained in the filter paper than a commercial antimicrobial disc (Royo *et al*., 2010), so that in this study, 2 sheets of filter paper were placed on the media. Research from Ogba *et al*. (2017), shows that locally prepared discs using whatman filter paper 3 are more effective than commercial discs. These results indicate that the use of filter paper (2 layers) can provide clear zone results that are not significantly different from commercial antimicrobial discs. Filter paper can be an inexpensive alternative to commercial antimicrobial discs with results that are not significantly different.

Comparison of Using Mannitol Salt Agar and Luria Bertani Agar

Media is one of the factors that influence clear zone results (Ahman *et al*., 2019). The results of this study indicate that the use of Mannitol Salt Agar (MSA) is better than Luria Bertani Agar (LBA) for antibiotic susceptibility for *Staphylococcus aureus*.

Table 2. Clear Zone Comparison of Media.

	Mannitol Salt Agar	**Luria Bertani Agar**
Clear Zone Average (mm)	13.47 ± 3.81	9.73 ± 4.46
Image		
Statistical Analysis	T-Value = 2.46 P-Value = 0.020 with α = 95%	

***Additional Information:** Clear Zone is Indicated by a Blue Dotted Line.

Clear zone results on Mannitol Salt Agar (MSA) gave better results than Luria Bertani Agar (LBA) and were statistically significant (t-value = 2,46; p-value = 0,02; with α = 95%). MSA has been used for many years as a selective, differential medium for the isolation of *Staphylococcus aureus* (Sharp & Searcy, 2006). Several factors that affect the accuracy and repeatability of disc diffusion methods include bacterial inoculum preparation, manual streaking of media plates, disk content, agar medium, nutritional requirements, incubation temperature and atmosphere, incubation time, and subjectivity of inhibition zone reading (Cherkaoui *et al*., 2020). Fermentation of mannitol (in MSA) leads to acid production, detected by phenol red indicator, aids in the differentiation of

Staphylococcal species. Coagulase positive *Staphylococci* (e.g., *Staphylococcus aureus*) produce yellow colonies and a surrounding yellow medium (Aryal, 2019).

The 7,5% concentration of sodium chloride results in the partial or complete inhibition of bacterial organisms other than *Staphylococci*. Luria Bertani Agar (LBA) consists of tryptone, yeast extract, NaCl, and agar (Macwilliams & Liao, 2006). The use of MSA will limit the contamination of other bacteria than *Staphylococcus aureus*. The results of this study indicate that the use of selective media provides the best clear zone results for the antibiotic susceptibility test. Mannitol Salt Agar (MSA) is the right medium for the antibiotic susceptibility test for *Staphylococcus aureus*.

CONCLUSIONS

In conclusions, the use of the Antimicrobial Disc Oxoid® and filter paper shows no statistical difference in clear zone results (t-value = 0,45; p-value = 0,655; with α = 95%). While the clear zone results on Mannitol Salt Agar (MSA) gave better results than Luria Bertani Agar (LBA) and were statistically significant (t-value = 2,46; p-value = 0,02; with α = 95%). These findings indicate that filter paper can be an inexpensive alternative for the antibiotic test with no significantly different result compared to commercial antibiotic disc. However, an antibiotic test against *Staphylococcus aureus* is better done on Mannitol Salt Agar (MSA) compared to Luria Bertani Agar (LBA).

RECOMMENDATIONS

Further research could focus on identifying the active compounds in medicinal plant extracts and testing their efficacy against a broader range of antibiotic-resistant bacteria. Moreover, additional studies may explore the use of other cost-effective and accessible alternatives to commercial antimicrobial discs. Finally, future research could investigate the optimal agar media and testing conditions for accurate and reliable testing of antibiotic susceptibility.

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