ANTIBACTERIAL EFFECT OF *Ocimum tenuiflorum* AND *Plectranthus amboinicus* EXTRACTS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AND *STAPHYLOCOCCUS AUREUS* (SA) ISOLATES

By

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, in partial requirement of the course DVT 55204 – Research Project. The growing frequency of antibiotic-resistant bacteria, including methicillin-resistant staphylococcus aureus (MRSA), warrants the investigation of alternative antimicrobial agents. This study evaluates the antibacterial inhibitory effects of methanolic extracts of *Ocimum tenuiflorum* and *Plectranthus amboinicus* against staphylococcus aureus (SA) and MRSA isolates using the disc diffusion method. Statistical analysis using one-way ANOVA revealed significant differences in the antibacterial inhibitory effects among treatment groups for both SA ($F(7,16) = 433.96$, $p < 0.001$) and MRSA ($F(7,16) = 304.70$, $p < 0.001$). Among individual extracts, *P. amboinicus* exhibited the highest antibacterial inhibitory effect, with inhibition zones averaging 1.1 cm for SA and 1.0 cm for MRSA, attributed to bioactive compounds such as thymol and carvacrol. Combined extracts displayed synergistic antibacterial inhibitory effects, particularly with higher concentrations of *P. amboinicus*, but their efficacy was lower compared to selected commercial antibiotics, including enrofloxacin, cefoxitin, and gentamicin. These findings highlight the potential of plant-based antimicrobials, particularly *P. amboinicus*, as alternative therapies for multidrug-resistant pathogens. Future studies should focus on optimizing extraction protocols and elucidating the molecular mechanisms underlying their antibacterial properties.

Keywords: *Ocimum tenuiflorum, Plectranthus amboinicus, staphylococcus aureus,* methicillin-resistant *staphylococcus aureus,* antibacterial effect, plant-based antimicrobials

ABSTRAK

Abstrak daripada kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai keperluan sebahagian daripada kursus DVT 55204 – Projek Penyelidikan. Peningkatan frekuensi bakteria tahan antibiotik, termasuk methicillin-resistant *staphylococcus aureus* (MRSA), memerlukan kajian terhadap agen antimikrob alternatif. Kajian ini menilai kesan perencatan antibakteria ekstrak metanol daripada *Ocimum tenuiflorum* dan *Plectranthus amboinicus* terhadap isolat *staphylococcus aureus* (SA) dan MRSA menggunakan kaedah resapan cakera. Analisis statistik menggunakan ANOVA sehala menunjukkan perbezaan yang signifikan dalam kesan perencatan antibakteria antara kumpulan rawatan untuk SA ($F(7,16) = 433.96$, $p < 0.001$) dan MRSA ($F(7,16) = 304.70$, $p < 0.001$). Antara ekstrak individu, *P. amboinicus* menunjukkan kesan perencatan antibakteria tertinggi dengan zon perencatan purata 1.1 cm untuk SA dan 1.0 cm untuk MRSA, disebabkan oleh sebatian bioaktif seperti timol dan karvakrol. Ekstrak gabungan menunjukkan kesan sinergistik antibakteria, terutamanya pada kepekatan tinggi *P. amboinicus*, tetapi keberkesanannya adalah lebih rendah berbanding antibiotik komersial yang terpilih seperti enrofloxacin, cefoxitin, dan gentamicin. Penemuan ini menyerlahkan potensi antimikrob berasaskan tumbuhan, khususnya *P. amboinicus*, sebagai terapi alternatif untuk patogen tahan pelbagai ubat. Kajian lanjut perlu memberi tumpuan kepada pengoptimuman protokol pengekstrakan dan penjelasan mekanisme molekul di sebalik sifat antibakterianya.

Kata kunci: *Ocimum tenuiflorum, Plectranthus amboinicus, staphylococcus aureus,* methicillin-resistant *staphylococcus aureus, kesan antibakteria, antimikrob berasaskan tumbuhan*

CERTIFICATION

This is to certify that we have read this research paper entitled **'Antibacterial effect of *Ocimum tenuiflorum* and *Plectranthus Amboinicus* against Methicillin Resistant Staphylococcus Aureus (MRSA) and Staphylococcus aureus (SA) isolates '** by **Santhini Venggadasalam**, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirements for the course DVT 55204 – Research Project.

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SA	-	Staphylococcus aureus
MRSA	-	Methicillin-resistant staphylococcus aureus
A	-	Plectranthus amboinicus
B	-	Ocimum tenuiflorum
ENR5	-	Enrofloxacin
FOX30	-	Cefoxitin
CN30	-	Gentamicin
DW	-	Distilled water
AST	-	Antimicrobial susceptibility test

CHAPTER 1

1.0 INTRODUCTION

The rapid rise of antibiotic-resistant bacteria is a global challenge, particularly the infections caused by *Staphylococcus aureus (SA)* and its methicillin-resistant variant, known as methicillin-resistant *Staphylococcus aureus (MRSA)* where in the United States alone, MRSA accounts for an estimated 94,360 invasive infections annually, resulting in approximately 18,650 deaths (Klevens *et al*., 2007) and MRSA has also been reported in various animal species, including horses, dogs, and cats, with prevalence rates ranging from 0.5% to 4.6% depending on the region and species (Hanselman *et al.*, 2006; Walther *et al*., 2012). These MRSA had developed resistance through horizontal acquisition of the mecA gene encoding PBP2a which has low affinity towards most beta-lactam antibiotics, hence conferring resistance not only to methicillin but to all members of beta-lactams (Vestergaard *et al*., 2019).

Consequently, numerous studies have been conducted to find new alternative sources of antimicrobial agents, especially from plants (Yamani *et al*., 2016) where medicinal plant usage as a part of traditional medicine has been described in literature dating back to several 1000 years ago (Chang *et al*., 2016). Moreover, medicinal plants have numerous benefits, the main ones being their cost-effectiveness, global availability, lack of major side-effects and more sustainable compared to conventional antibiotics (Niu *et al.*, 2011), thereby reducing the risk of antibiotic resistance development. In modern medical practice, plants are the primary source of therapeutics and all parts of plants, including the seeds, root, stem, leaves, and fruit contain bioactive components (Jiang *et al*., 2014, 2015; Mandave *et al*., 2014; Sun *et al.*, 2014). Two such plants with potential antibacterial activity against MRSA are *O.tenuiflorum* commonly

known as Tulsi and *P.amboinicus* known as Indian borage. Both plants contain bioactive compounds such as phenol, flavonoids (Satheesh *et al*.,2022), camphor, eugenol (Raina *et.al,* 2013) that have proven to exhibit antibacterial properties. This proposed study seeks to evaluate and compare the antibacterial efficacy of individual and combined plant extracts against SA and MRSA isolates using disc diffusion method and additionally, it explores the possibility of potential alternative treatment against multidrug resistant infection.

1.1 Research Problem Statement

Staphylococcus aureus is a ubiquitous bacterium capable of causing infections ranging from superficial skin infections to life-threatening conditions like sepsis. The emergence of antibiotic-resistant strains, such as MRSA, complicates treatment options and highlights the need for alternative antimicrobial strategies. This growing threat is not confined to human medicine but extends to veterinary contexts, where MRSA have been reported with increasing frequency worldwide (Deshpande *et al*., 2004). In Malaysia, the national prevalence rate of MRSA ranges from 17.2% to 28.1%, with the rates recorded at 18% and 19.8% for the years 2016 and 2017, respectively (Chai *et al*., 2022).

Moreover, in a study conducted at veterinary hospitals in Malaysia, the prevalence of MRSA was 19.8% (26/131) in veterinary personnel and students revealed a higher carriage percentage, particularly in the latter which suggests that animal health professionals, animal attendants, and veterinary medicine students are at risk of MRSA colonization which can be attributed to occupational exposures (Aklilu *et al*., 2013). If the rising AMR threat is not addressed effectively, the annual death toll attributed to infections caused by AMR pathogens is expected to reach 10 million by the year 2050 (O'Neill, 2014). Along with conventional antibiotics losing their efficacy , there's a dire need for alternative treatment options such as plant based antibiotics. Therefore, the findings are expected to provide a groundwork for developing new plant-based antibiotics that are effective, accessible, affordable with fewer side effects compared to conventional antibiotics.

1.2 Research Question

1. What are the antibacterial efficacies of *O.tenuiflorum* and *P.amboinicus,* individual extracts against SA and MRSA isolates ?
2. What are the potential synergistic effects of combining *O.tenuiflorum* and *P.amboinicus* extracts against SA and MRSA isolates ?
3. What is the difference between the antibacterial efficacy of individual and combined plant extracts against SA and MRSA isolates ?
4. What is the difference between the antibacterial effect exhibited by an individual and combined plant extracts with the selected commercial antibiotics ?

1.3 Research Hypothesis

- There is a significant difference in the antibacterial inhibitory effects of individual and combined extracts of *O.tenuiflorum* and *P.amboinicus* against SA and MRSA isolates.

- When compared with individual *O.tenuiflorum* and *P.amboinicus extracts*, the synergistic antibacterial inhibitory effect of combined extracts against SA and MRSA isolates will be higher.

1.4 Research Objective

1.4.1 General Objective

To compare the antibacterial efficacy of *O.tenuiflorum* and *P.amboinicus* extracts, individually and in combination, against the *Staphylococcus Aureus* (SA) and Methicillin resistant *Staphylococcus aureus* (MRSA) isolates.

1.4.2 Specific Objectives

1. To determine the antibacterial effect of *O.tenuiflorum* and *P.amboinicus* individual extracts against the SA and MRSA isolates.
2. To assess the potential synergistic antibacterial effect of *O.tenuiflorum* and *P.amboinicus* combined extracts against the SA and MRSA isolates.
3. To compare the antibacterial efficacy *O.tenuiflorum* and *P.amboinicus,* individually and in combined extracts against SA and MRSA isolates.
4. To compare the antibacterial efficacy of *O.tenuiflorum* and *P.amboinicus* individually and in combined extracts with the selected commercial antibiotics against SA and MRSA isolates.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Antimicrobial Resistance

Antimicrobial resistance (AMR) is not a new issue, this global health problem has in fact soared since the 1970s and it is now named by the World Health Organization as one of the primary public threats of the 21st century due to its rapid and ongoing spread. If AMR spreads unchecked, many infectious diseases will become untreatable which in turn leads to increase in mortality. A report from the Review on Antimicrobial Resistance by Jim O' Neill projected that if rising AMR is not addressed, the annual death toll could reach 10 million by the year 2050. While AMR is known as a public health threat, it is also a major economic concern due to the high cost of treating AMR (Smith & Coast, 2013). With no major new class of antimicrobials discovered since the late 1980s and limited new drugs in the pipeline, this severely restricts future therapeutic options (World Health Organization, 2019).

2.2 Background on MRSA

Staphylococcus aureus is a facultative anaerobic, gram-positive bacterium which colonizes the skin and mucosal surfaces of humans and animals but can cause infections ranging from superficial skin conditions to life-threatening diseases such as bacteremia, endocarditis, and osteomyelitis (Liu, 2015). As for methicillin resistant *staphylococcus aureus* (MRSA) is a nonmotile, Gram-positive, non capsulated, coccus (spherical to ovoid) bacterium belonging to Micrococcaceae (Liu, 2015).

In the 1960s, MRSA emerged as a major clinical problem following the introduction of methicillin where the transition from methicillin-sensitive Staphylococcus aureus (MSSA) to MRSA occurs through the acquisition of the mecA gene, located on the staphylococcal cassette chromosome mec (SCCmec), which encodes an altered penicillin-binding protein (PBP2a). This PBP2a has a low affinity towards beta-lactam antibiotics thus disrupting the antibiotic binding. The SCCmec is a mobile genetic element that can be transferred horizontally between bacterial populations, facilitating the spread of resistance (Vestergaard et al., 2019). In Malaysia, the national prevalence rate of MRSA among *S. aureus* clinical isolates ranged from 17.2% to 28.1%, with the rates recorded at 18% and 19.8% for the years 2016 and 2017, respectively (Chai *et al*., 2022).

At present, vancomycin remains the drug of last resort for the treatment of MRSA infections (Rodvold & McConeghy, 2013). However, reports regarding MRSA strains that had developed resistance to vancomycin have emerged in many parts of the world, with the first of such strains reported in the United States almost two decades ago (Chai *et al*., 2022). Furthermore, MRSA has also been considered as an emerging problem in veterinary settings and various studies have reported MRSA in different species of animals and veterinary professionals (Duquette and Nuttall, 2004, Hanselman *et al*., 2006, Weese *et al.*, 2006). Some reports have also indicated that veterinarians have higher MRSA carriage rates than the general population (Hanselman *et al.*, 2006; Wulf *et al*., 2006; Williams *et al.*, 2007; Anderson *et al.*, 2008)

2.3 Alternative treatment for MRSA

There are several alternative treatments that have been studied previously such as bacteriophage therapy, silver nanoparticle and medicinal plant studies. Bacteriophages are viruses that specifically attack the bacteria and it has shown promising results against MRSA (O'Flaherty *et al*. 2009). However, there are risks for bacterial resistance and regulatory hurdles since the specificity of phages requires specific matching of the phage to the bacterial strain which is strenuous. (O'Flaherty *et al.* 2009).

As for silver nanoparticles, it will continually release silver ions that adhere to the cell wall and cytoplasmic membrane resulting in disruption of bacterial cell membrane and cellular process, leading to bacterial inhibition (Yin *et al*., 2020). However, it imposes a significant concern of cellular toxicity and lack of studies on protocol to detect and investigate the risk of nanoparticles in the environment where it imposes a threat towards the aquatic and terrestrial populations of microbes which could disrupt the ecosystems at massive scale (*Nanosilver: Environmental Effects*, 2021).

Meanwhile, the medicinal plant studies have shown several benefits over non-plant-based alternatives, in terms of safety, accessibility, cost effectiveness and sustainability (Niu *et al* 2011). For instance, Tulsi is commonly used in traditional medicine with minimal reported adverse effects (Jamshidi & Cohen, 2017). In contrast, the long-term safety of silver nanoparticles and the potential for phage-induced immune responses require further study. Moreover, medicinal plants are widely available and cost-effective since mass production of these plants is relatively easy compared to the production of bacteriophages or the

synthesis of silver nanoparticles. Furthermore, it is generally more sustainable and environmentally friendly compared to non-plant-based alternatives where the silver nanoparticle production and disposal pose environmental challenges due to potential contamination and bioaccumulation (*Nanosilver: Environmental Effects*, 2021.)

2.4 *Ocimum tenuiflorum*

Ocimum tenuiflorum, commonly known as Tulsi. It is classified under the lamiaceae family in the plantae kingdom which is widely distributed over Asia, Africa, Central and Southern America (Joseph and Nair, 2013). This *O. tenuiflorum* thrives in warm and temperate climates since it requires warmth for growth and is vulnerable in the cold climates (Joseph and Nair, 2013). *O. tenuiflorum* has been cultivated extensively for its medicinal value (Ravi *et.al*, 2012) where it contains numerous bioactive compounds such as eugenol, carvacrol, rosmarinic acid, camphor, eucalyptol, β-bisabolene and β caryophyllene which are proposed to exhibit strong antibacterial activities. Based on previous studies, it has shown that methanol extracts of *O.tenuiflorum* could inhibit MRSA growth with inhibition zones averaging 15 mm, making it a promising candidate for alternative antimicrobial treatments (Prakash & Gupta, 2005; Agarwal *et al*., 2010)

2.5 *Plectranthus amboinicus*

Plectranthus amboinicus, commonly known as Indian borage belongs to the family Lamiaceae. It is a herb which is aromatic and has fleshy leaves. This is a native to southern and eastern Africa but it has been widely distributed to tropical and subtropical regions around the world including India, Southeast Asia and the Caribbean (Padalia *et al.*, 2013; Al-Sereiti *et al*.,

2013). *P. amboinicus* has been traditionally used in various cultures for its medicinal properties (Kumar *et al*., 2017) since the numerous bioactive compounds such as flavonoids, phenolic acids and essential oils found in the plant have therapeutic properties. Based on previous studies, it shows that *P. amboinicus* exhibits significant antibacterial activity especially against *Escherichia coli, Staphylococcus aureus*, and *Pseudomonas aeruginosa* due to the presence of thymol and carvacrol which aids in the disruption of the bacterial cell membranes resulting in the bacterial inhibition (Vasina *et al.* 2018). In another study, methanol extracts of *P.amboinicus* have demonstrated strong antibacterial activity against MRSA, with inhibition zones averaging 18 mm (Lee *et al*., 2014), highlighting its potential as an alternative antimicrobial agent (Kuo *et al*., 2011).

2.6 Potential Combined Therapy

Synergistic effects of two substances are normally greater than the sum of their individual effects where studies have shown that combining plant extracts can enhance antibacterial efficacy and reduce resistance, suggesting that similar synergistic interactions might be observed with *O. tenuiflorum* and *P. amboinicus* (Ahmad *et al.*, 2010). While individual studies have explored the antimicrobial effects of *O.tenuiflorum* and *P.amboinicus*, the research on comparing their individual and combined efficacy against SA and MRSA isolates in Malaysia remains limited. This forges knowledge gaps in the data concerning potential alternative treatment for multidrug resistant pathogens. Realizing this knowledge gap, the proposed study will be carried out with the aim to compare the antibacterial effect of *O.tenuiflorum* and *P.amboinicus extracts*, individually and in combination against SA and MRSA isolates. Additionally, it also explores the possibility of potential alternative treatment for multidrug infection with combined therapy.

Chapter 3

3.0 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

This study does not require any ethical approval since no animal or humans will be involved in this study.

3.2 SAMPLE COLLECTION AND PREPARATION

The sampling method used is convenience sampling to collect adequate *O. tenuiflorum* and *P. amboinicus* plants from my garden. Then, the leaves were separated from its stem respectively before it was washed thoroughly with clean water to remove any dust or contaminants. Then, the leaves are air dried in a shaded area at room temperature for a week until it's suitable to be grounded. Dried leaves are then pulverized separately using a domestic blender until a homogenous fine powder is obtained.

3.3 PLANT EXTRACTION METHOD (MACERATION TECHNIQUE)

A 200g of powdered *O. tenuiflorum* and *P. amboinicus* was soaked in 2 liters of 70% methanol (ratio 1:10 w/v) in a large glass container respectively. The mixture was stirred occasionally and sealed before it was left under the fume hood at room temperature for 72 hours. After 72 hours, the mixture was filtered through tea strainer twice followed by Whatman No.1 filter paper to remove any plant debris. Then, the filtrate was poured into the rotary evaporation flask and the rotary evaporator water bath was set at 45°C to obtain the extract. Later, the concentrated extracts were collected in the universal bottle which was then covered

with parafilm (with holes) and left under the fume hood for drying. The semi-dried extract was stored at 4°C until further usage.

3.4 BACTERIAL CULTURE AND CONFIRMATION

A confirmed ATCC 33591 methicillin-resistant *staphylococcus aureus* (MRSA) and ATCC 33862 *staphylococcus aureus* (SA) bacterial sample was obtained from the centralized lab management centre (CLMC), campus Besut of Universiti Sultan Zainal Abidin (UniZa). However, these bacterial samples were then cultured and confirmed to reduce the probability of culturing the contaminants which could affect the results. Therefore, a primary and secondary culture using nutrient agar (NA) was performed which has resulted in yellowish, mucoid, ball-point colonies, followed by that a selective media using mannitol salt agar (MSA) was used which has resulted in the formation of ball-point, yellowish colony surrounded by yellow region on the medium, thus confirming it as SA and MRSA. Then, gram staining and biochemical test was performed for further confirmation. For gram-staining, gram positive coccoid shaped bacterial colonies were seen for both SA and MRSA culture. As for the biochemical test, only urease, simon's citrate and methyl red test were positive, whereas the oxidase and catalase test were negative and positive respectively.

3.5 ANTIBACTERIAL SUSCEPTIBILITY TEST (DISC DIFFUSION METHOD)

A well-isolated colony was taken from the cultured SA and MRSA media for bacterial suspension which was standardized using 1.0 McFarland solution. The standardized bacterial suspension was then streaked onto the Mueller Hinton agar (MHA) using sterile cotton swabs. The plate was dried for 15 minutes, meanwhile a sterile disc was placed into a decided volume

of plant extracts for 5 minutes until it was soaked. Then the disc was tapped on a tissue paper gently until no excessive extracts were dripping from the disc. It was then placed on the agar as stated below :

A	10 ml *P. amboinicus*
B	10 ml *O. tenuiflorum*
AB_1 (1:1)	5 ml *O. tenuiflorum* + 5 ml *P. amboinicus*
AB_2 (1:3)	2.5 ml *P. amboinicus + 7.5 ml O. tenuiflorum*
ENR5	Enrofloxacin (Positive control)
FOX30	Cefoxitin
CN30	Gentamicin
DW	10 ml Distilled water (Negative control)

Then, all 6 MHA agars were incubated in an upright position at 37°C for 24 hours. After 24 hours, the diameter of zones of inhibition (mm) was measured using a ruler. Then the antibiotic susceptibility of commercial antibiotics was determined according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Three replicates were carried out to obtain accurate results with minimal test error. All the results obtained were tabulated and expressed in mean±standard deviation values.

3.6 CALCULATION AND INTERPRETATION

Once the mean and standard deviation values were calculated based on the obtained results, it was then assessed for normality using the Shapiro-Wilk test where the p value obtained was more than *0.05* indicating that the data is distributed normally and for homogeneity of variances, Levene's test was used. This test yielded a p-value more than *0.05* indicating that the equal variance was met as well. Since both assumptions were satisfied, therefore a parametric test like one-way ANOVA was used to analyze the data.

CHAPTER 4

4.0 RESULTS

4.1 *STAPHYLOCOCCUS AUREUS (SA)*

Treatment	Zone of inhibition (cm)			Average (cm)	Standard deviation
	Trial 1	Trial 2	Trial 3		
A	1.0	1.1	1.1	1.1	0.06
B	0.9	0.9	0.8	0.9	0.06
AB_1 (1:1)	1.1	0.9	1.0	1.0	0.10
AB_2 (1:3)	0.8	0.7	0.7	0.7	0.06
ENR5	2.1	2.0	2.1	2.1	0.06
FOX30	2.5	2.4	2.5	2.5	0.06
CN30	2.0	1.9	1.8	1.9	0.10
DW	0.0	0.0	0.0	0.0	0.00

Table 1 : *Staphylococcus aureus* result. A, *P.amboinicus.* B, *O.tenuiflorum*. AB_1, 1:1 ratio of *P.amboinicus* and *O.tenuiflorum*. AB_2, 1 : 3 ratio of *P. amboinicus* and *O.tenuiflorum*. ENR5, enrofloxacin of 5µg. FOX30, cefoxitin of 30µg. CN30, gentamicin of 30µg. DW, distilled water.

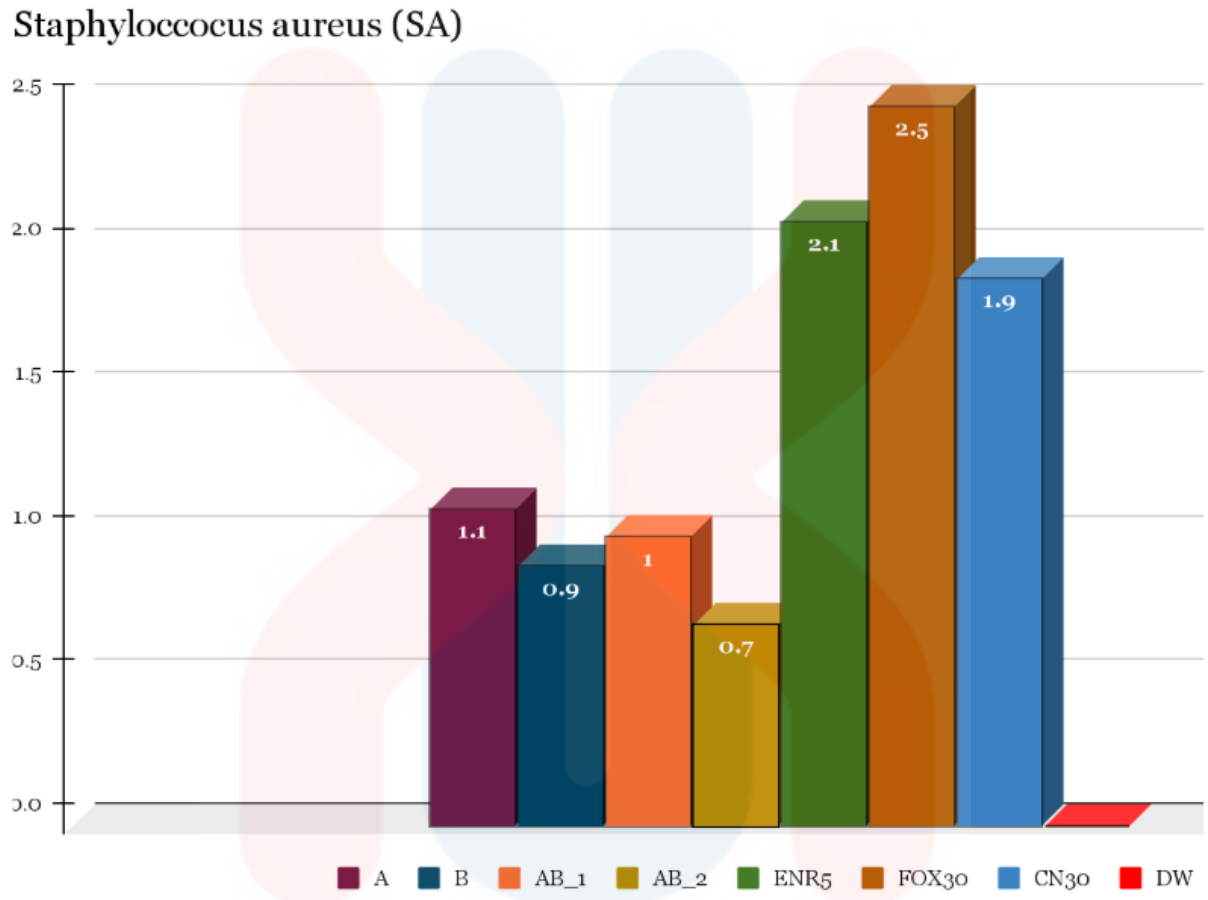

Figure 1 : Bar chart representing SA zone of inhibition zone (mm). A, *P.amboinicus*. B, *O.tenuiflorum*. AB_1, 1:1 ratio of *P.amboinicus* and *O.tenuiflorum*. AB_2, 1 : 3 ratio of *P. amboinicus* and *O.tenuiflorum*. ENR5, enrofloxacin of 5µg. FOX30, cefoxitin of 30µg. CN30, gentamicin of 30µg. DW, distilled water.

4.2 METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA)

Treatment	Zone of inhibition (cm)			Average (cm)	Standard deviation
	Trial 1	Trial 2	Trial 3		
A	1.0	1.0	1.0	1.0	0.0
B	0.8	0.8	0.9	0.8	0.07
AB_1 (1:1)	0.9	1.0	1.0	1.0	0.07
AB_2 (1:3)	0.7	0.6	0.7	0.7	0.07
ENR5	1.8	2.0	1.9	1.9	0.1
FOX30	0.0	0.0	0.0	0.0	0.0
CN5	1.0	1.1	1.2	1.1	0.1
DW	0.0	0.0	0.0	0.0	0.0

Table 2 : Methicillin-resistant staphylococcus aureus (MRSA) result. A, *P.amboinicus*. B, *O.tenuiflorum*. AB_1, 1:1 ratio of *P.amboinicus* and *O.tenuiflorum*. AB_2, 1 : 3 ratio of *P. amboinicus* and *O.tenuiflorum*. ENR5, enrofloxacin of 5µg. FOX30, cefoxitin of 30µg. CN30, gentamicin of 30µg. DW, distilled water.

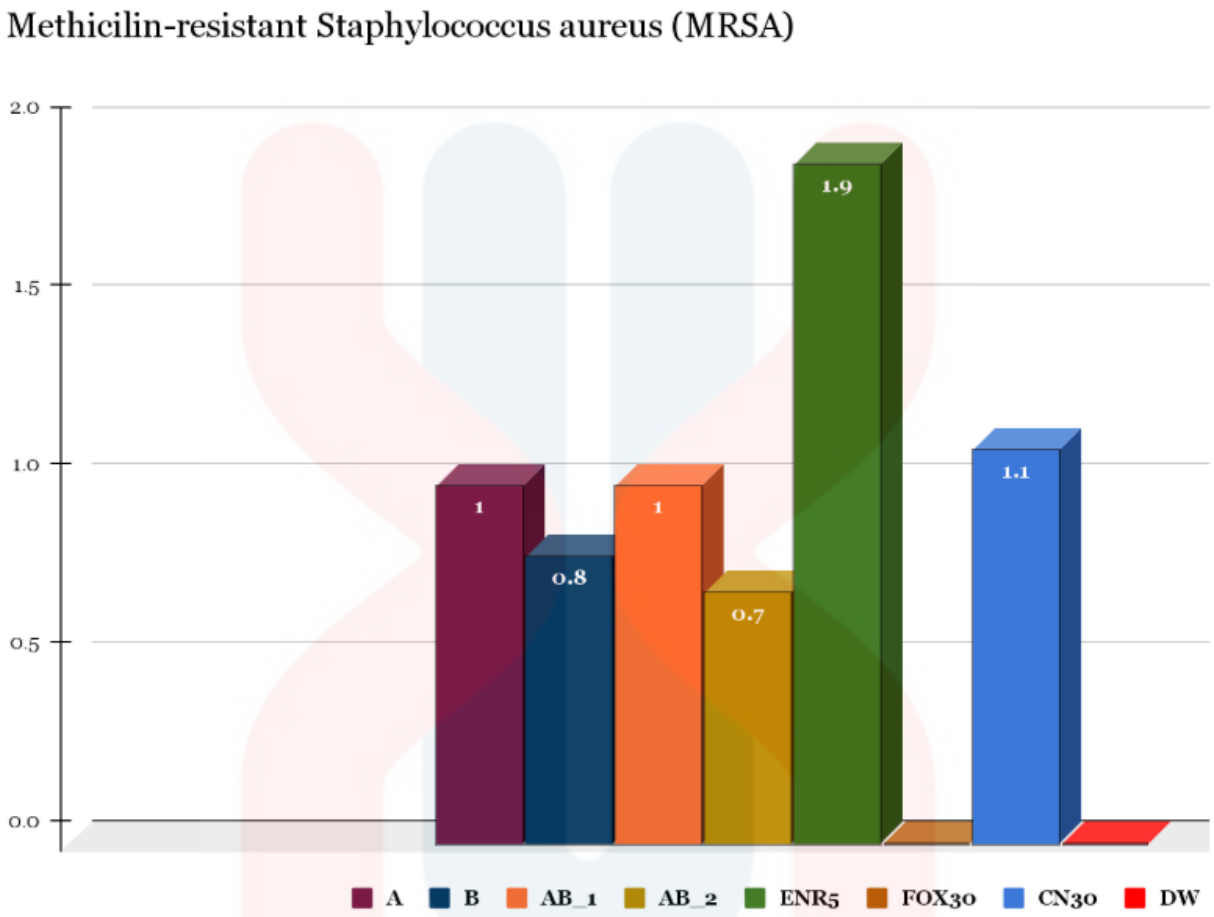

Figure 2 : Bar chart representing MRSA zone of inhibition (mm). A, *P.amboinicus*. B, *O.tenuiflorum*. AB_1, 1:1 ratio of *P.amboinicus* and *O.tenuiflorum*. AB_2, 1 : 3 ratio of *P. amboinicus* and *O.tenuiflorum*. ENR5, enrofloxacin of 5µg. FOX30, cefoxitin of 30µg. CN30, gentamicin of 30µg. DW, distilled water.

4.3 STATISTICAL ANALYSIS

The mean inhibition zones for each treatment were analyzed using a one-way ANOVA test to compare the antibacterial effect of *O. tenuiflorum*, *P. amboinicus*, and their combined extracts against SA and MRSA.

Based on the figure 1 and 2, the results showed a statistically significant difference in zone of inhibition among the different treatment groups against SA, $F(7,16) + 433.961$, $P < 0.001$ and MRSA, $F(7,16) = 304.698$, $P < 0.001$ respectively.

ANOVA

Zone_of_Inhibition

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.923	7	1.989	433.961	<.001
Within Groups	.073	16	.005		
Total	13.996	23			

Figure 3 : Staphylococcus aureus (SA) ANOVA result

ANOVA

Zone_of_Inhibition

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	7.998	7	1.143	304.698	<.001
Within Groups	.060	16	.004		
Total	8.058	23			

Figure 4 : Methicillin-resistant staphylococcus aureus (MRSA) ANOVA result

CHAPTER 5

5.0 DISCUSSION

The main objective of this study is to compare the antibacterial efficacy of *O.tenuiflorum* and *P.amboinicus* extracts, individually and in combination, against the *staphylococcus aureus* (SA) and methicillin resistant *staphylococcus aureus* (MRSA) isolates. Additionally, also to compare with the selected commercial antibiotics such as cefoxitin, gentamicin and enrofloxacin.

Therefore, based on the results shown in table 1, A (*P.amboinicus*) has the highest antibacterial inhibitory effect against staphylococcus aureus (SA) showing an average inhibition zone of 1.1 cm compared to AB_1 , AB_2 and B (*O. tenuiflorum*) with the average inhibition zone of 1.0 cm, 0.7 cm and 0.9 cm respectively.

Comparison of combined plant extract, AB_1 and AB_2 of different concentrations shows different antibacterial inhibitory effects against SA with an average inhibition zone of 1.0 cm and 0.7 cm respectively. This indicates a potential role played by *P.amboinicus influencing the* synergistic effects of combined plant extracts. As the concentration of *P.amboinicus* decreases, the synergistic effect of combined plant extract also decreases.

As for the commercial antibiotics, cefoxitin (FOX30), enrofloxacin (ENR5) and gentamicin (CN30) show an average inhibition zone of 2.5 cm, 2.1 cm and 1.9 cm respectively. According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), SA was

susceptible towards all three commercial antibiotics mentioned earlier. When compared, the antibacterial effect exhibited by selected commercial antibiotics was greater compared to the individual and combined plant extracts. In addition, the distilled water (negative control) showed no inhibition, thus confirming the reliability of the test as well.

The extracts and controls were also tested against MRSA as well. Based on the results shown in table 2, A (*P.amboinicus*) has the highest antibacterial inhibitory effect against methicillin-resistant staphylococcus aureus (MRSA) too showing an average inhibition zone of 1.0 cm compared to AB_1 , AB_2 and B (*O. tenuiflorum*) with the average inhibition zone of 1.0 cm, 0.7 cm and 0.8 cm respectively.

Comparison of combined plant extract, AB_1 and AB_2 of different concentrations shows different antibacterial inhibitory effects against MRSA with an average inhibition zone of 1.0 cm and 0.7 cm respectively. This indicates a potential role played by *P.amboinicus influencing the* synergistic effects of combined plant extracts. As the concentration of *P.amboinicus* decreases, the synergistic effect of combined plant extract also decreases.

As for the commercial antibiotics, cefoxitin (FOX30), enrofloxacin (ENR5) and gentamicin (CN30) show an average inhibition zone of 0 cm, 1.9 cm and 1.1 cm respectively. According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), MRSA was resistant towards gentamicin and cefoxitin whereas it was intermittent with enrofloxacin. Resistance towards cefoxitin is an indicator for methicillin-resistant staphylococcus aureus too.

Even though MRSA was not susceptible towards any of the selected commercial antibiotics, the antibacterial effect exhibited was still far more superior compared to the individual and combined plant extracts. In addition, the distilled water (negative control) showed no inhibition too, thus confirming the reliability of the test once again.

As an overall view, both results showed a significant difference in the antibacterial inhibitory effects of individual and combined extracts of O.tenuiflorum and P.amboinicus against SA and MRSA isolates. When compared with individual and combined O.tenuiflorum and P.amboinicus extracts, the *P.amboinicus* showed a higher antibacterial inhibitory effect individually and also signifies an important role in influencing the synergistic antibacterial inhibitory effect of combined extracts against SA and MRSA isolates as well.

The high antibacterial effect of *P.amboinicus* can be attributed to their bioactive compounds such as thymol and carvacrol, which aids in the disruption of the bacterial cell membranes resulting in the bacterial cellular component leakage followed by death. (Vasina *et al.* 2018). However, the exact mechanism of action of these bioactive components requires further elucidation.

This is crucial in the context of addressing antimicrobial resistance (AMR) issues. While the inhibitory effects of the plant extracts were less potent compared to the selected commercial antibiotics, however their inhibitory effect against both SA and MRSA highlights their relevance especially *P. amboinicus* which had demonstrated a strong inhibitory effect, suggesting its potential as a key component in alternative therapies. These findings can

contribute to the body of knowledge on plant-derived antimicrobials and their potential role in reducing reliance on synthetic antibiotics in this era.

However, there are several limitations to such studies that should be acknowledged as well such as limited bacterial strains related studies. In this study, it was solely focused on SA and MRSA strains which might not be sufficient to represent the broad spectrum of bacterial pathogens that are developing resistance in this era. Moreover, there's also a concern for standardization issues where the variability in plant extract composition due to external factors such as age of the plant, environmental growing condition, soil used, extraction method used, types of solvent used in a controlled laboratory setting could also affect the reproducibility, thus the urge for further studies in this field for better understanding of developing plant-based formulations to address AMR issues.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

This study highlights the antibacterial effect of *P.amboinicus* and *O.tenuiflorum* extracts against *staphylococcus aureus* (SA) and methicillin-resistant *staphylococcus aureus* (MRSA). Among the individual plant extracts, *P. amboinicus* demonstrated the highest antibacterial inhibitory effect, with significant inhibition zones for both bacterial strains, suggesting the presence of potent bioactive compounds such as thymol and carvacrol.

However, when compared with combined extracts, the antibacterial inhibitory effect was much higher thus rejecting my second hypothesis. As for the comparison to commercial antibiotics such as cefoxitin, enrofloxacin, and gentamicin, the plant extracts exhibited lower antibacterial inhibitory effects. Notably, MRSA showed resistance to cefoxitin and gentamicin, thus highlighting the limitations of current antibiotic options and the urgent need for alternative antimicrobial strategies.

While the results are promising, further studies are essential to optimize extraction methods and explore the molecular mechanisms underlying the observed antibacterial inhibitory effect. In conclusion, *P. amboinicus* and *O. tenuiflorum* hold significant potential as an alternative treatments for bacterial infections, thus offering a sustainable and natural approach to combat the rising AMR issue. This study lays the foundation for future research on integrating plant-derived antimicrobials into mainstream therapeutic applications.

7.0 APPENDIX

Figure 5 : Maceration Technique (Soaking process of the plant powder)

Figure 6 : Filtration process of the soaked plant powder

Figure 7 : Rotary Evaporator for plant extraction process

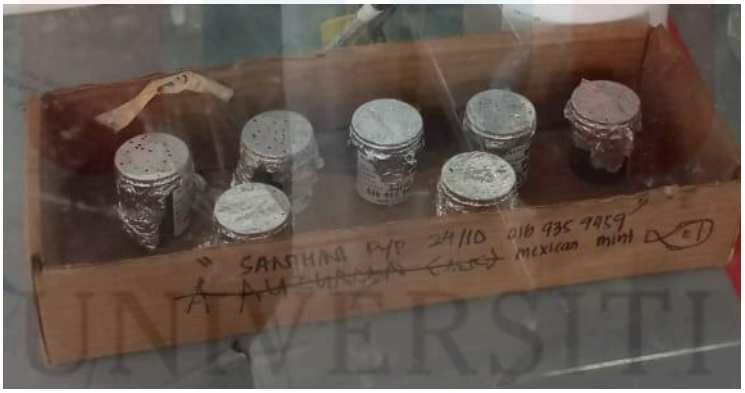

Figure 8 : Plant Extract Drying Process

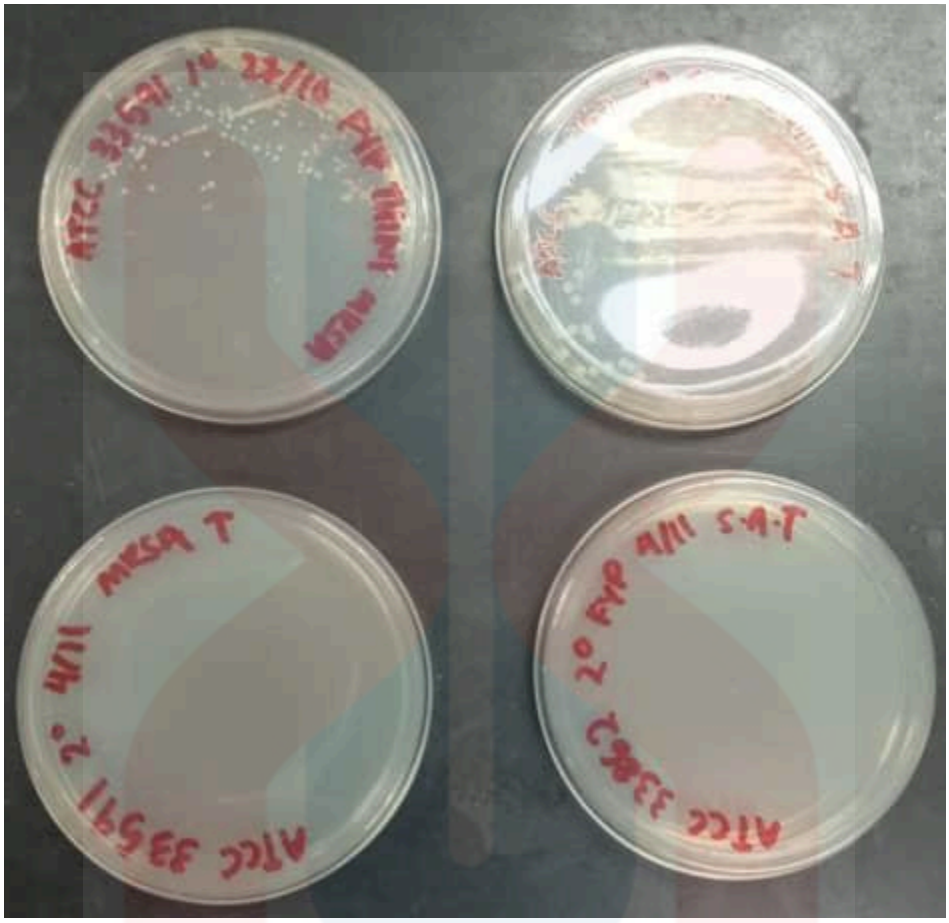

Figure 9 : Primary and secondary culture of SA & MRSA in NA media

Figure 10 : Gram staining of SA & MRSA (Gram positive, basophilic & coccoid shape)

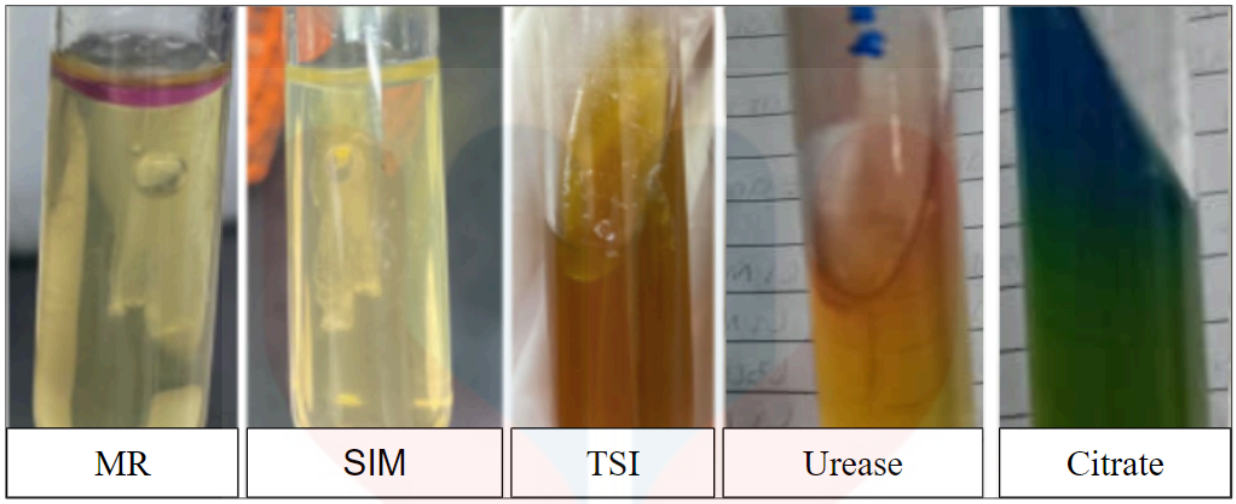

Figure 11 : Biochemical test result for SA & MRSA (All negative except for urease, citrate and methyl red)

Figure 12 : Catalase and Oxidase Test Result for SA & MRSA (Only catalase positive)

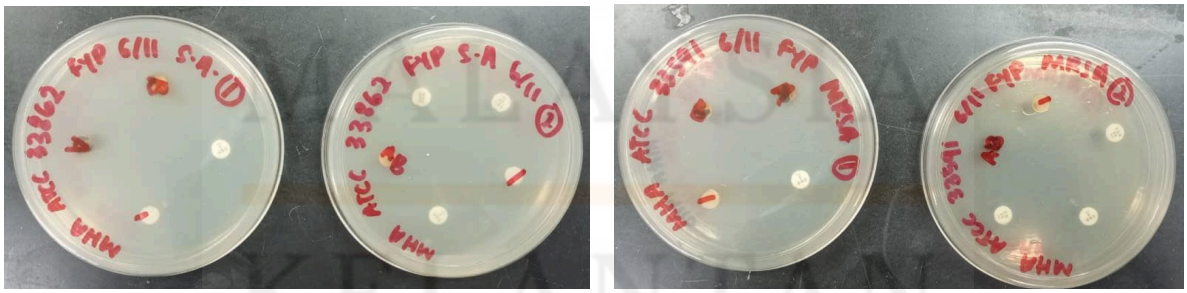

Figure 13 : AST test for SA & MRSA

Figure 14 : AST test result for SA

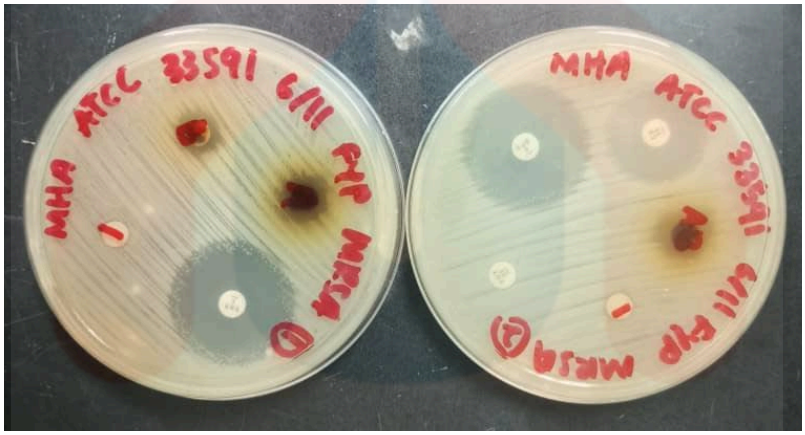

Figure 15 : AST test result for MRSA

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