THE SYNERGISTIC EFFECT OF *ALOCASIA LONGILOBA* MIQ. FRUIT'S EXTRACT WITH AMPICILLIN AND TETRACYCLINE AGAINST BACTERIA

by

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DECLARATION

I declare that this thesis entitled "The Synergistic Effect of *Alocasia longiloba* Miq. Fruit's Extract with Ampicillin and Tetracycline against Bacteria" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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APPROVAL

"I hereby declare that I have read this thesis and in our opinion this thesis is sufficient in terms of scope and quality for the award of the degree of Bachelor of Applied Science (Natural Resources Science) with Honors"

Signature : …….....
Name of Supervisor : ……..
Date : ……..

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The Synergistic Effect of *Alocasia longiloba* Miq. Fruit's Extract with Ampicillin and Tetracycline against Bacteria

ABSTRACT

The inappropriate usage of antibiotic is one of the factor of the emergence of the antibiotic resistance bacteria that will limit the effectiveness of the current antibiotic and lead to the treatment failure. In recent years, there has been growing interest to evaluate plant possessing antibacterial activity for various disease. The combination of plant extract with antibiotic approach may lead to the new ways in the treatment of the infectious diseases and this combination may reduce of bacterial resistance toward antibiotics. The objectives of this study was to determine the synergistic effect of *Alocasia longiloba* Miq. fruit extract with ampicillin and tetracycline against *Staphylococcus aureus* and *Escherichia coli.* The synergistic effect of *Alocasia longiloba* Miq. fruit extract and antibiotics was determined by using agar well diffusion and minimal inhibitory concentration (MIC) Resazurin 96-well micro-dilution methods. The fruit extract of *Alocasia longiloba* Miq. showed no inhibition against both gram positive and gram negative bacteria. The results of this study showed the increasing in the inhibition zone when the plant extract was combined with ampicillin against *E. coli*. The other combination showed no increasing in the inhibition zone and some of it will reduce the inhibition zone compared to the individual effect of the antibiotic. The value of MIC only showed by ampicillin on *E. coli* which was 12.5 µg/ml, and the combination of plant extract and ampicillin (2000+12.5 µg/ml). The fractional inhibitory concentration (FIC) index cannot be determined due to the lacked of MIC value. These results indicated that the fruit extract of *Alocasia longiloba* Miq. showed low antibacterial activity against *E. coli* and *S. aureus* and this plant extract may show the inhibition if the concentration is increase and test against the different microorganisms.

Kesan Sinergistic Ekstrak Buah *Alocasia longiloba* Miq. Dengan Ampicillin dan Tetracycline terhadap Bakteria

ABSTRAKS

Penyalahagunaan antibiotik merupakan salah satu faktor kemunculan bakteria yang kebal terhadap antibiotik, yang akan mengehadkan keberkesanan antibiotik dan membawa kepada kegagalan rawatan. Dalam kebelakangan ini, kajian aktiviti antibakteria menggunakan tumbuhan terhadap pelbagai penyakit semakin mendapat perhatian dari banyak pihak. Kombinasi antara ekstrak tumbuhan dan antibiotik merupakan salah satu pendekatan bagi mengatasi masalah immunisasi terhadap antibiotik. Objektif kajian ini adalah untuk menentukan kesan sinergi antara ekstrak buah *Alocasia longiloba* Miq. dengan ampicillin dan tetracycline terhadap *Staphylococcus aureus* dan *Escherichia coli*. Kesan sinergi ekstrak buah *Alocasia longiloba* Miq. dan antibiotik telah ditentukan dengan menggunakan kaedah 'agar well diffusion' dan 'minimal inhibitory concentration (MIC) Resazurin 96-well micro-dilution'. Ekstrak *Alocasia longiloba* Miq. tidak menunjukkan kesan perencatan terhadap bakteria gram positif dan gram negatif . Keputusan kajian ini menunjukkan peningkatan dalam zon perencatan apabila ekstrak tumbuhan digabungkan dengan ampicillin menentang *E.coli*. Gabungan lain tidak menunjukkan peningkatan dalam zon perencatan dan sesetengahnya menunjukkan pengurangan zon perencatan berbanding kesan individu antibiotik tersebut. Nilai MIC hanya ditunjukkan oleh ampicillin terhadap *E.coli* iaitu 12.5 µg/ml, dan kombinasi ekstrak tumbuhan dan ampicillin (2000:12.5 µg/ml). 'Fractional Inhibitory Concentration' (FIC) indeks tidak dapat ditentukan kerana tiada nilai MIC. Keputusan ini membuktikan bahawa ekstrak buah *Alocasia longiloba* Miq. mempunyai aktiviti antibakteria yang rendah terhadap *E. coli* dan *S. aureus* dan ekstrak tumbuhan ini mungkin dapat merencat pertumbuhan mikroorganisma yang berbeza.

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LIST OF ABBREVIATIONS

CFU/mL	Colony forming unit per milliliter
FIC	Fractional Inhibitory Concentration
MBC	Minimal Bactericidal Concentration
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimal Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
mL	Milliliter
mm	Millimeter
NA	Nutrient Agar
NB	Nutrient Broth
µL	Microliter
µL/well	Microliter per well

LIST OF SYMBOLS

%	Percentage
>	Greater than
<	More than
°C	Temperature (degree Celsius)
+	Addition

CHAPTER 1

INTRODUCTION

1.1 Background of study

The resistance of the pathogenic bacteria towards the antimicrobial agent is increasing due to the genetically ability of the bacteria to transmit and acquire resistance to the antibiotic. This will be a concern because the patient will suppress immunity due to this new bacteria strain which are multi-resistance and will cause new infection that will lead to mortality (Nascimento et al., 2000). Also, use of antibiotics can cause some unpleasant side effects, such as yeast overgrowth and gastrointestinal trouble. On the other hand, medicinal plant can promote beneficial effects on killing of bacteria with safer than that of antibiotics. To reduce the risk of side effect when use antibiotics, the combination of antibiotic at less than commonly use and the plant extract may increase the inhibition of the antimicrobial agents against the bacteria. A synergy effect can occur when the antibiotic are combined with an agent that antagonizes bacterial resistance mechanism (Wagner et al., 2009).

This study focused on the synergetic effect of *Alocasia longiloba* Miq. fruit extract with ampicillin and tetracycline against gram positive and gram negative bacteria. *A. longiloba* Miq. is the species from Araceae family are recommended as a plant of pharmaceutical importance based on its antioxidant potential and the properties bioactive compound present in it (Gengaderan, 2016). *A. longiloba* Miq. is believed to have antibacterial properties, because the stem part of this plant had been

used for treating pus in cattle and abdominal disease (Williams, 2012). The fruit of this plant has been used by the indigenous people in Malaysia as the natural remedies for treating the effect of gout. However, there was no study regarding the antibacterial activity and synergetic effect of *Alocasia longiloba* Miq. fruits.

Tetracycline is the family antibiotic that can inhibit the synthesis of bacteria protein by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. It has been widely used in the treatment of human and animal infection due to the antimicrobial properties and the absence of the major side effect. However, in the recent year, the emergence of the microbial resistance has limited their effectiveness (Chopra et al., 2001). Ampicillin is the antibiotic from penicillin group and it is responsible in the inhibition of the cell wall synthesis by interacting with the penicillin-binding proteins that are responsible in the synthesis of the peptidoglycan in the cell wall. The interruption of peptidoglycan will cause the cell lysis and cell death (Peechakara et al., 2018).

This study investigated the synergistic effect of ethanol extract from the fruits of *Alocasia longiloba* Miq. with tetracycline and ampicillin against the gram positive bacterium *Staphylococcus aureus* and gram negative bacterium which is *Escherichia coli.*

1.2 Problem Statement

The inappropriate usage of antibiotic is one of the factor of the antibiotic resistance and the emergence of the antibiotic resistance bacteria will limit the effectiveness of the current antibiotic and lead to the treatment failure (Wikaningtyas et al., 2016). In recent years there has been growing interest to evaluate plant possessing antibacterial activity for various disease (Perumal Samy et al., 2000). The

combination of plant extract with antibiotic approach may lead to the new ways in the treatment of the infectious diseases and this combination may reduce of bacterial resistance toward antibiotics (Moussaoui et al., 2016).

1.3 Objectives

1. To determine the synergistic effect of *Alocasia longiloba* Miq. fruits extract with ampicillin and tetracycline against the gram positive and gram negative bacteria.
2. To determine the Minimal inhibitory concentration (MIC) interaction of *Alocasia longiloba* Miq. fruits extract with ampicillin and tetracycline against bacteria.

1.4 Scope of study

This study focused on the synergistic effect of *Alocasia longiloba* Miq. fruit extract with tetracycline and ampicillin against *Staphylococcus aureus* and *Escherichia coli* by using agar well diffusion method, Minimal inhibitory concentration (MIC) Resazurin-based 96-well micro-dilution and Fractional inhibitory concentration (FIC) index.

1.5 Significance of Study

The final founding of this study was the discovery of the new plant-based antibiotic in the combination with the existing antibiotic that will reduce the effective doses of the antibiotic and decrease the side effect because the medicinal plant was widely used and found safe for the consumption for many years. Furthermore, the new combination

of antibiotic and plant extract can be more effective and cannot be resisted by the bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1 Antibiotic Resistance

Antibiotics have saved millions of lives and play a very vital role in preventing and treating infection that will decrease the morbidity and mortality caused by infection. Unfortunately, it is inevitable that, over time, bacteria have developed resistance to the existing antibiotic, making infection harder to be treated. Infection that were once easily curable with antibiotic are becoming difficult, even impossible to treat and an increasing number of people are suffering severe illness or dying as the result (Coates, 2012). *Figure 2.1* shown the key events of the development of antibiotic and antibiotic resistant identification.

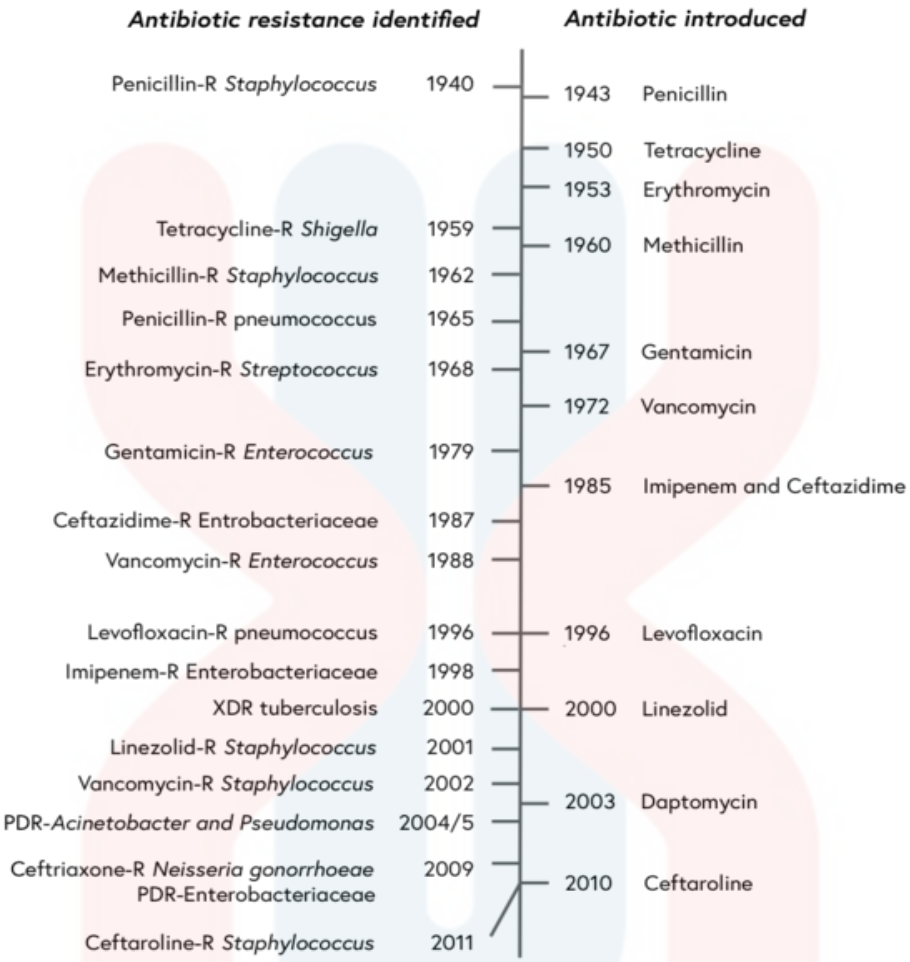


Figure 2.1: Developing Antibiotic Resistance: A timeline of Key Events (Source: Ventola, 2015)

The resistance of bacteria toward antibiotic has been seen to nearly all the antibiotic that have been developed. The first resistance is reported toward penicillin and after the discovering and developing of new β-lactam antibiotic, the first case of methicillin-resistant *Staphylococcus aureus* (MRSA) was identified, a few months after the development of the antibiotic. The introduction of vancomycin in 1972 for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) and the cases of vancomycin resistance were reported in coagulase-negative staphylococci in 1979 and 1983 (Ventola, 2015).

The discovery of new antibiotic is importance. However, due to the past record, the widespread emergence of resistance to newly introduced antimicrobial agents

indicates that even the new family of antimicrobial agents will have a short life expectancy. One of the approaches that can be done is the usage of combination therapy in order to achieve bactericidal synergism. The combination can be of different plant extracts or plant extract with standard antibiotic or antibiotic with some chemicals. This combination against the bacteria can create different mechanism of action and it may lead to new choice of infectious disease (Chanda et al., 2011).

2.2 Synergistic Effect

Synergy is the effect of the combined action of two or more substances, characterized by having a greater effect than that resulting from the sum of the effects of the individual substances. The synergistic effect happens when different constituent of an extract interacts to increase a specific antibacterial effect or when they act on the different targets, thus increasing the possibility of affecting several vital processes in bacterial reproduction or metabolism. This effect is most impactful when an antibiotic is combined with an agent that antagonizes bacterial resistance mechanism (Rueda, 2013).

The combination of plant extract with antibiotic approach may lead to the new ways in the treatment of the infectious diseases. Many studies have been conducted of the synergetic effect of the combination between different plant extract with antibiotics. The combination allowed the decreasing of bacterial resistance toward antibiotics (Moussaoui et al., 2016). Combination of plant extract with antibiotic will help to minimize the minimum inhibitory concentration (MIC), synergistic effect and reduce the side effects (Al-Alak et al., 2015). Some plant extract is found to be synergistic enhancer because they might not have any antimicrobial properties but

when they are taken concurrently with standard drugs, they will enhance the effect of the antibiotics (Chanda et. al, 2011).

The plant active substances reflects in modification or blocking of resistance mechanism so the bacteria will become more sensitive to antibiotics or the antibiotic can act in lower concentrations. This approach can reduce the doses of effectiveness of antibiotics and also reduce the side effects of the antibiotics (Stefanovic, 2018).

2.2.1 The Mechanism of Synergistic Effect

There are four mechanisms of synergistic effect which are (1) synergistic multi-target effects, (2) pharmacokinetic or physiochemical effects based on the improved solubility, resorption rate and enhance the bioavailability, (3) interaction of agents with resistance mechanism of bacteria and (4) the respective elimination or neutralization of adverse effects by agents contained in the extract, added to it, or achieved by heating, so that altogether a better effectiveness than without these addition or manipulation can be achieved (Wagner et al., 2009).

2.3 Medicinal Plant

Herbal and plant remedies have been used for centuries and have a huge potential for future antibacterial therapies. Many researchers have renewed their interest in these area and have focused on investigating the antimicrobial activities of medicinal plants and their extracts against multidrug-resistant bacterial strains in the hope of discovering new antibiotics.

Plant-derived compound can exhibit the direct antibacterial activity and indirect activity as antibiotic resistance modifying compound which in the combination with antibiotic will increase their effectiveness. Plant extract and their main components may exhibit the antibacterial activity by inhibiting bacterial growth and viability, targeting bacterial virulence factor or potentiating effectiveness of antibiotics as resistance modifying agents. The mechanism of the plant extract in the inhibition of bacterial growth can be through the disruption of membrane function and structure, interruption of DNA/RNA synthesis and function, intermediary metabolism interference and the induction of coagulation of cytoplasmic constituents (Stefanovic, 2018).

2.3.1 *Alocasia longiloba* Miq.

Alocasia longiloba Miq is the herb plant from the *Araceae* family. This species is also synonym as *Alocasia denudata* Engl., *Alocasia longifolia* Engl., *Alocasia lowii* Hook.f, *Alocasia watsoniana* Sander and *Alocasia korthalsii* Schott (Quattrocchi, 2016). The distribution of *Alocasia longiloba* Miq. is Borneo, Cambodia, China, Malaysia, Myanmar, Sulawesi, Sumatera, Thailand and Vietnam .

It can grow up to 150 cm tall with the diameter of the lower stem can be until 60 cm. The fruits are round, orange and contain one big seed, which can grow up to 4

until 7 mm long (National Park, 2013). According to Boyce (2008), the fruits of *Araceae* are typically juicy berries although rarely drier and leathery, the infructescence is usually cylindric and the berries are commonly red or orange.

Figure 2.2 : The Fruit of *Alocasia longiloba* Miq. (Source: Uforest.org, 2014)

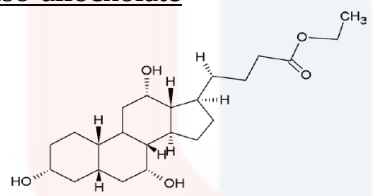
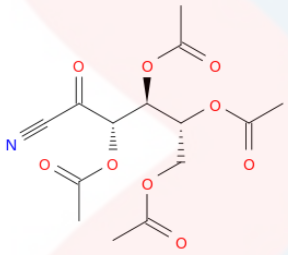
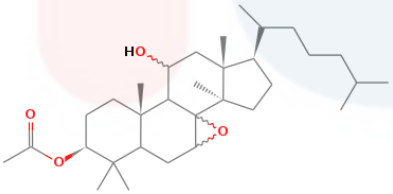
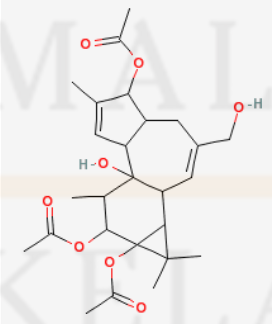
Figure 2.3: *Alocasia longiloba* Miq. (Source : Uforest.org, 2014)

2.3.2 Uses of *Alocasia longiloba* Miq.

Alocasia plants having many medicinal uses like external wound treating property, antitoxic reputation and antibacterial properties. *Alocasia longiloba* Miq. plants are recommended as a plant of pharmaceutical importance based on its antioxidant potential and the properties bioactive compound present in it (Gengaderan, 2016).The whole plant are applied as a poultice to relieve soreness and pain in the feet. The base of leaf stalk is used as the poultice for treatment of insect stings (Quattrocchi, 2016). *Alocasia longiloba* Miq. has the antibacterial properties, this is because it had been used for treating pus in cattle and abdominal disease (Williams, 2012). The fruits of *Alocasia longiloba* Miq. is used as the natural remedy for the treatment of gout disease by the Malaysian indigeneous people. This fruits will be eaten in the small quantity and there was no study regarding the antibacterial activity and the synergistic of the fruit extract of *Alocasia longiloba* Miq.

2.3.3 The Bioactive Compound of *Alocasia longiloba* Miq.

Table 2.1 : The Bioactive Compound of *Alocasia longiloba* Miq. by GC-MS (Gengaderan, 2016)

Name of the compound	Molecular formula	Plant part	Uses
Ethyl iso-allocholate 	$C_{26}H_{44}O_5$	Leaf Petiole	1.Antimicrobial 2. Diuretic 3.Dihydropteroate synthase Inhibitor (Malathi et al., 2016)
Tetraacetyl-d-xylonic nitrile 	$C_{14}H_{17}O_9$	Leaf Petiole	1. Antitumor, 2. Antioxidant 3. Antiinflammatory
Benzeneethanamine 	$C_8H_{11}N$	Petiole	Sympathomimetics
7,8-Epoxylanostan-11-ol, 3-acetoxy 	$C_{32}H_{54}O_4$	Leaf	Antimicobial
1H-Cyclopropa[3,4]benz[1,2e]azuulene-5,7b,9,9a-tetrol,3-[(acethyloxy)methyl]-1a,1b,4,4a,5,7a,8,9-octahydro-1,1,6,8-tetramethyl-9,9a-diacetate, [1aR(1aá,1bá,4aá,5á,7aá,7bá,8á,9á,9aá)] 	$C_{26}H_{36}O_8$	Leaf	1. Antiallergic 2. Antibacterial 3. Antihistaminic 4. Antiinflammatory 5. Hepatogenerative 6. Antiulcer

2.4 Antibiotics

According to Bhattacharjee (2016), antibiotic can be defined as a chemical that selectively inhibits a virulent (infectious) biological agents but causes minimal damage to the host cell. The antibiotic can be classify based on their targets in the microbial cell that they interact with to cause the growth inhibition. There are six major classes of antibiotics which are bacterial cell wall synthesis inhibitor, DNA synthesis (replication) inhibitor, RNA synthesis (transcription) inhibitor, protein synthesis (translation) inhibitor, the synthesis of important metabolites inhibitor and those that disrupt the cell membrane. Antibiotic can also be classified based on their effect on growth and survival of the bacteria which is bacteriostatic and bactericidal antibiotic. Bactericidal is the antibiotic that kill bacteria at a safe and practically achievable concentration and the bacteriostatic is antibiotic that inhibits growth of bacteria but does not kill bacterial cells at a safe and practically achievable concentration (Bhattacharjee, 2016).

2.4.1 Tetracycline

Tetracycline are the broad-spectrum agents, exhibiting the activity against a wide range of gram-positive and gram-negative bacteria, spirochetes, obligate intracellular bacteria and protozoan parasites. Tetracycline will act as protein synthesis inhibitor by preventing the association of the aminoacyl-tRNA with the bacterial ribosome. It will reversibly bind to the receptor of the bacterial ribosome and interact with a highly conserved 16S ribosomal RNA (rRNA) target in the 30S ribosomal subunit, preventing the translation by interfering with the attachment of aminoacyl-tRNA to the RNA-ribosome complex (Chopra et al., 2001; Grossman, 2016).

2.4.2 Ampicillin

Ampicillin is a β-lactam antibiotic, the first of the aminopenicillin to be developed is responsible in the inhibition of cell wall synthesis. The mode of action of β-lactam antibiotic can be considered as two steps process. The first step, ampicillin will bind to the primary receptors called membrane-bound penicillin-binding protein. Penicillin-binding protein is responsible in the cell cycle related, the morphogenetic formation of cell wall peptidoglycan. The inactivation of these protein by bound of antibiotic has immediate arresting action on their function. The second stage of this mechanism is the physiological effects caused by this receptor-ligand interaction. The penicillin-binding proteins are involved in the late stage of the peptidoglycan synthesis in the cell wall. Thus, the disruption of the peptidoglycan synthesis will cause lysis and cell death because peptidoglycan will maintain the integrity of the cell wall (Peechakara et al., 2018).

Aminopenicillin is responsible for the wide range of infection that made ampicillin is one of the most commonly prescribe agent, notably for the urinary and respiratory infection, as well as for gastrointestinal infection. However, the increasing frequency of isolation of β-lactamase-producing pathogens has resulted in the reduction of the effectiveness of this antibiotic as the monotherapy. Ampicillin also responsible in the treatment of severe infection including endocarditis, meningitis and septicemia, often in the combination with other antibacterial agent such as aminoglycosides (Finch et al., 2010).

2.5 Bacteria

2.5.1 *Escherichia coli*

Escherichia coli is very small, its cell is rod-shaped, about 2.5 µm long, 10,000 end to end span 1 inch by about 0.8 µm in diameter with hemispherical end caps. The cell has a thin three-layered wall enclosing the cytoplasm, the homogenous molecular and this bacteria cell does not have nucleus, other membrane-enclosed organelles, or any cytoskeletal elements (rope-like or rod-like components) typical of higher cells. However, some of this organelle are built into the cell wall and *E. coli* have the external organelles, thin straight filaments called pili that enable it to attach to specific substrata and thicker, longer helical filaments called flagella that enable this bacterial cell to swim (Berg et al., 2004).

E. coli lives in the lower intestines of warm blooded organisms including human and animal. It can survive with and without oxygen outside the body, thus it can survive until it can find the host. *E. coli* can cause the urinary tract infection, diarrheal diseases and contribute to the infant mortality (Berg et al., 2004).

2.5.2 *Staphylococcus aureus*

Staphylococcus aureus belong to the Bacillales order in the *Staphylococcaceae* family in the Firmicutes (gram-positive) phylum and in the *staphylococcus aureus genus* (Werner , 2014). It is the part of the commensal flora of human skin and mucosal surface, in addition to being a pathogen capable of causing both superficial infection and invasive diseases with considerable associated morbidity and mortality. The main reservoir of *S. aureus* carriage in human is the anterior nares. The skin, pharynx,

perineum, vagina, axillae and gastrointestinal tract are the other carriage of *S. aureus* (Edgeworth et al., 2018).

S. aureus is a species that are naturally susceptible to antibiotic, however, over the years it has become resistant to almost every antibiotic that has entered clinical uses (Monaco et al., 2018). In 1960, the introduction of methicillin (a semisynthetic penicillin) has marked a major advanced in the treatment of staphylococcal infections resistant to penicillin, however after a few month of the clinical uses of methicillin, the first strain of methicillin-resistant *S. aureus* (MRSA) was identified. The main resistant mechanism of MRSA is the production of penicillin-binding protein (PBP) with the lower affinity for β-lactam (Rueda, 2013).

The infection of this bacteria will occur on the disturbance mucocutaneous barrier resulting in infection of the skin and subcutaneous tissue, wound infection and intravascular or urinary catheter-related infection. It also can cause the septic shock and severe metastatic infection such as acute endocarditis, arthritis, meningitis, myocarditis, pericarditis, pneumonia and osteomyelitis (Rueda, 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 The Plant Sample Collection

The plant materials in this study was the fruits of *Alocasia longiloba* Miq. had been collected at the area around Kelantan.

3.1.2 Microorganisms tested

The bacteria strains that were tested against the *Alocasia longiloba* Miq. fruits extract are *S. aureus* (ATCC25923) and *E. coli* (ATCC25922) are maintained at the Scientific Laboratory and Equipment Centre (SLEC), Prince of Songkla University, Surat Thani campus, Thailand.

3.1.3 Culture Media and Chemicals

Types of media that were used in this study are Nutrient agar, Nutrient broth, Mueller-Hinton agar and Mueller-Hinton broth. The chemical that are required in this study is 95% Ethanol, Resazurin, dimethyl sulfoxide (DMSO).

3.1.4 Antibiotic

The antibiotics that were used are Tetracycline and Ampicillin.

3.1.5 Apparatus and equipment

The apparatus and equipment that were used in the extraction of plant material are conical flask, inoculation loop, inoculation needle, cork-borer, filter paper Whatman no 1, spatula, shaker and rotary evaporator (BUCHI). For the microbial assay testing, the apparatus and equipment that have been used is conical flask (Pyrex), spatula, Scott Duran bottle, petri dish, centrifuge tube, 96-well plate, micropipettes, laminar air flow, hot air oven (WTB binder) , autoclave (TOMY SX-700), shaking incubator (N-BIOTEK) and incubator (WTB binder).

3.2 Methods

3.2.1 Preparation of the *Alocasia longiloba* Miq. fruit extract

The fruits of *Alocasia longiloba* Miq. were collected, cleaned, dried and powdered. The dried sample was suspended in 95% ethanol for 5 days and filtered by using two cotton layers. The ethanol filtrate was collected and concentrated by using rotary evaporator at 70ºC until a sticky mass was obtained. The extract was kept in the dark glass bottle and stored in the cool and dark place.

3.2.2 Determination of the combined activity using Agar well diffusion method

The antibacterial activity was measured by using the agar-well diffusion method on Mueller-Hinton Agar. The bacteria isolates was sub-cultured for 18 hours in the nutrient broth before transfer it into the MHA medium. The sterile cottons swab was dipped briefly in the bacteria strain suspension (1×10^7 CFU/mL) and the whole surface of the agar plate was inoculated (25mL medium). A 6 mm diameter hole was punched aseptically by using sterile cork-borer and 50 µL of the extract solution at concentration (3000, 4000, 5000 µg/well) and antibiotic which were tetracycline (3.75, 7.5 and 15 µg/well) and ampicillin (12.5, 25 and 50 µg/well). In case of the synergism effect 25 µL of each was introduced into the well. For 24 hours, the bacteria cultured plates was incubated at 37ºC and the antibacterial effect of the ethanol extract on bacteria was evaluated using Tetracycline (15 µg/well) and Ampicillin (50 µg/well) as the positive control. The antibacterial activity was accessed by measuring the inhibition zone around the well. Synergism effect was considered when the

combination exhibited with enlargement of the combined inhibition zone more than 5mm (Adwan et al., 2008).

3.2.3 Determination of Minimum inhibitory concentration (MIC) using Resazurin-based 96-well micro-dilution

The determination of minimum inhibitory concentration (MIC) was followed methods described by Elshikh et al. (2016) with slightly modification. Bacterial suspension at mid-log phase was used.

For the plant extract, 280 µL of mixed solution of Mueller Hinton Broth (MHB) and 2-fold extract with 8000 µg/mL concentration was filled in the first well and 140µL of MHB was filled in the wells 2 until 7. The preparation of the extract concentration from 8000-125 µg/mL was done by pipetting 140 µL aliquot from the first well into the next well to make the 2-fold serial micro-dilution in the 96-well plate. Each well was added with 50 µL of bacteria suspension except for well 8. Well 8 was the negative control which was without the bacteria suspension. The 96-wells was incubated for 24 hours at 37ºC, then 10 µL of 0.015% Resazurin was added and the plate was incubated for 1-2 hours before the evaluation of colour.

The same procedure was applied to antibiotic with the concentration of 15-0.234 µg/mL for tetracycline and 50-0.781 µg/mL for ampicillin. The concentration for the combination of plant extract with tetracycline was 8000:15 µg/mL until 125:0.234 µg/mL (plant extract concentration: antibiotic concentration) and 8000:50 µg/mL until 125:0.781 µg/mL for ampicillin. This procedure was done by using two antibiotics which were Tetracycline and Ampicillin against *E. coli* and *S. aureus*.

3.5 Fractional Inhibitory Concentration determination

Fractional inhibitory concentration (FIC) is the lowest concentration of the extract and the antibiotic in combination giving no detectable bacterial growth after incubation. FIC index value was calculated using formulae:

$$\text{FIC index} = \frac{\text{MIC extract in combination}}{\text{MIC of extract alone}} + \frac{\text{MIC antibiotic in combination}}{\text{MIC of antibiotic alone}} \qquad (3.1)$$

The combination defined synergy if $\Sigma FIC \leq 0.5$, addictive if $0.5 < \Sigma FIC \leq 1$, indifference if $1 < \Sigma FIC \leq 4$ and antagonism if $\Sigma FIC > 4$ (Noor, 2016).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Agar Well Diffusion Method

Table 4.1 showed the inhibition zone of the plant extract alone, tetracycline alone, ampicillin alone, the combination of plant extract and tetracycline and the combination of plant extract with ampicillin against *E. coli* and *S. aureus*.

Table 4.1: The Inhibition Zone of *A. longiloba*, antibiotics and the combination against *E. coli* and *S. aureus*

Treatment	**Inhibition Zone (mm)**	
	Staphylococcus aureus	*Escherichia coli*
***Alocasia longiloba* extract**		
3000	0.00±0.00^{b}	0.00±0.00^{b}
4000	0.00±0.00^{b}	0.00±0.00^{b}
5000	0.00±0.00^{b}	0.00±0.00^{b}
10% DMSO	0.00±0.00^{b}	0.00±0.00^{b}
Tetracycline	25.33±0.58^{a}	26.00±1.00^{a}
***Alocasia longiloba* + Tetracycline**		
3000 + 15	25.67±0.58^{ab}	19.00±0.00^{a}
4000 + 15	26.33±0.58^{a}	19.33±0.58^{a}
5000 + 15	26.33±0.58^{a}	19.67±0.58^{a}
10% DMSO	0.00±0.00^{c}	0.00±0.00^{c}
Tetracycline (15)	25.00±0.00^{b}	17.67±0.58^{b}

***Alocasia longiloba* + Ampicillin**		
3000 + 50	34.33±3.79^{ab}	27.33±0.58^{ab}
4000 + 50	35.67±4.16^{a}	26.67±0.58^{a}
5000 + 50	35.67±3.21^{a}	28.00±0.00^{a}
10% DMSO	0.00±0.00^{c}	0.00±0.00^{d}
Ampicillin (50)	32.00±3.61^{b}	24.33±0.58^{c}
Tetracycline		
3.75	22.00±1.00^{d}	17.33±0.58^{c}
7.50	24.67±0.58^{c}	18.67±0.58^{b}
15.00	26.33±1.15^{b}	19.67±0.58^{b}
10% DMSO	0.00±0.00^{e}	0.00±0.00^{d}
Ampicillin (50)	36.33±1.53^{a}	24.67±0.58^{a}
Ampicillin		
12.5	34.67±0.58^{b}	19.00±0.00^{c}
25	35.67±1.15^{ab}	21.33±0.58^{b}
50	36.33±1.15^{a}	24.33±0.58^{a}
10% DMSO	0.00±0.00^{d}	0.00±0.00^{e}
Tetracycline (15)	25.00±0.00^{c}	17.67±0.58^{d}

Value are expressed as mean of three replicates. Values with different letters in the same column indicated statistically significant differences.

The plant extract showed no inhibition against both *S. aureus* and *E. coli* at the concentration of 3000, 4000 and 5000 µg/well as shown in Figure 4.1 and Figure 4.2, there were no inhibition zone around the well.

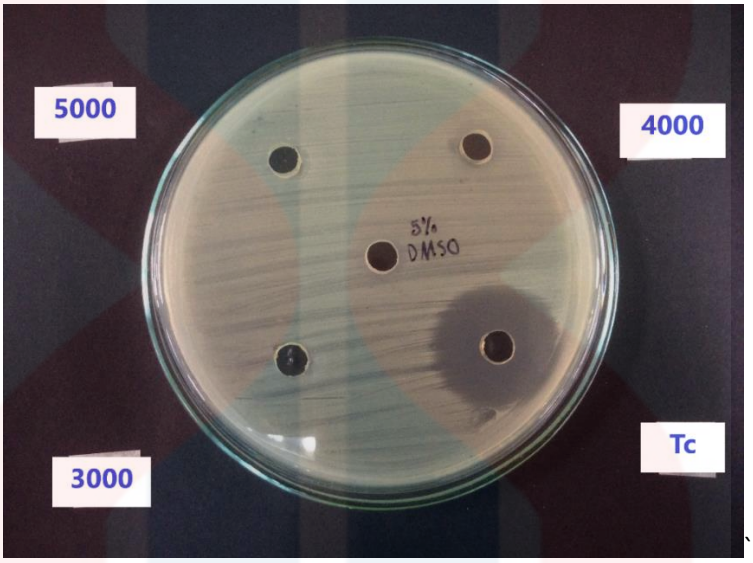


Figure 4.1: The Inhibition Zone of *Alocasia longiloba* extract against *S. aureus*

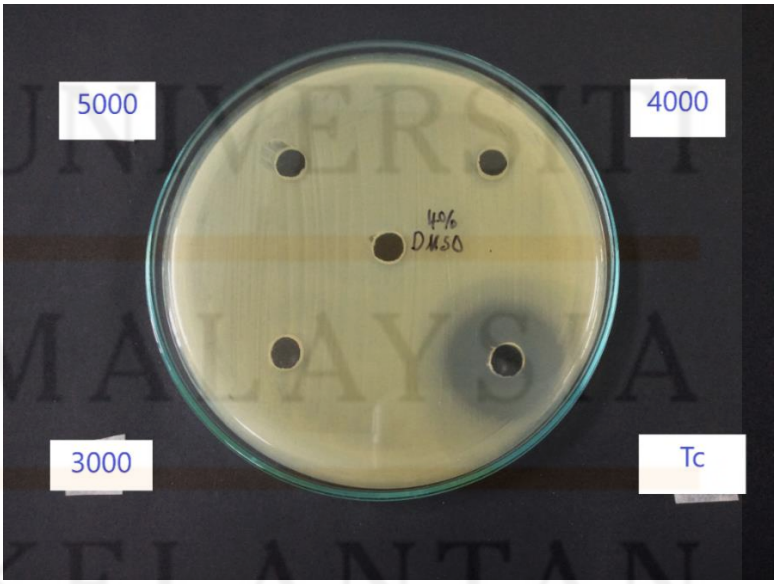


Figure 4.2*:* The Inhibition Zone of *Alocasia longiloba* extract against *E. coli*

For the combination of plant extract and tetracycline against *S. aureus*, the highest inhibition shown by the concentration of 4000+15 µg/well and 5000+15 µg/well which were 26.33 mm. The inhibition zone of tetracycline without extract at the same concentration as the combination which was 15 µg/well, showed the same diameter of inhibition zone indicated that there was no synergistic effect between the combination of tetracycline and plant extract against *S. aureus*. The combination of antibiotic and the plant extract with the concentration of 4000+15 µg/well showed the reduction in the inhibition zone. The inhibition zone of the combination of tetracycline and plant extract as well as tetracycline alone on *S. aureus* shown in Figure 4.3 and Figure 4.4.

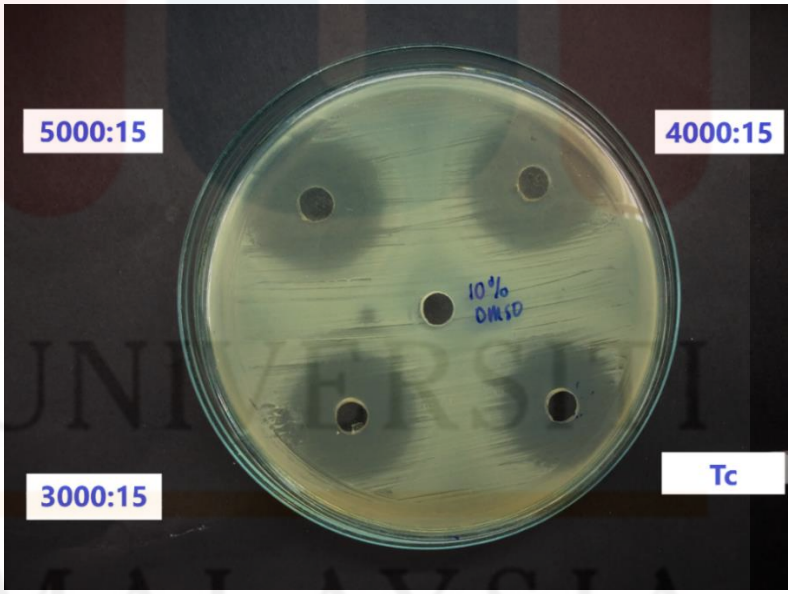


Figure 4.3: The Inhibition Zone of Combination of *Alocasia longiloba* extract and Tetracycline against *S. aureus*

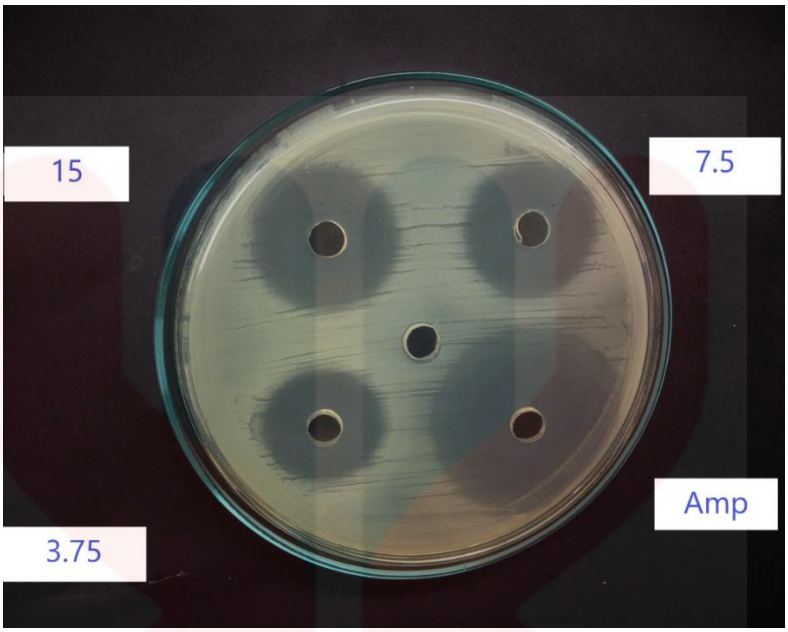


Figure 4.4: The Inhibition Zone of Tetracycline against *S. aureus*

The highest inhibition of the combination between plant extract and tetracycline against *E. coli* showed by the concentration of 5000+15 µg/well which was 19.67 mm. The same diameter of inhibition zone shown by the treatment of tetracycline alone against *E. coli* indicated the combination of tetracycline and plant extract against *E. coli* showed no synergistic effect as the inhibition zone of the combination same with the individual effects. The inhibition zone were slightly decrease when the combination of tetracycline and plant extract with the concentration of 3000+15 µg/well and 4000+15 µg/well were tested against *E. coli* as shown in Figure 4.5 and Figure 4.6.

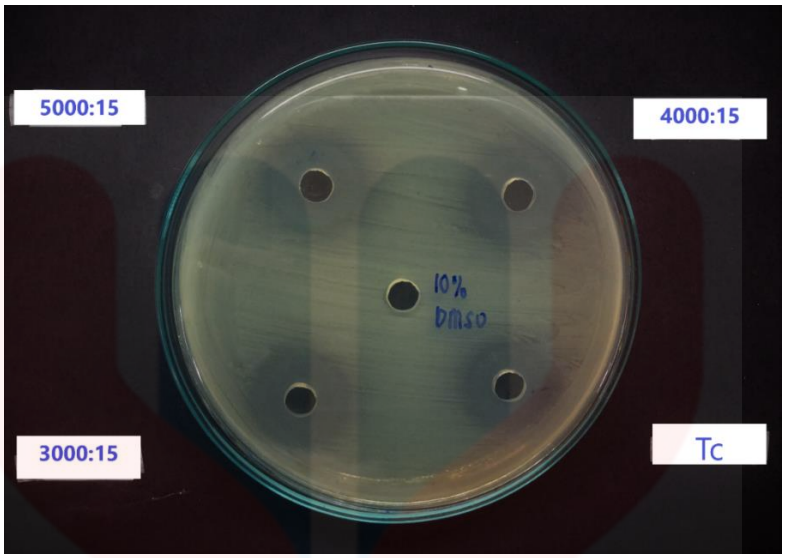


Figure 4.5: Combination of *Alocasia longiloba* extract and Tetracycline against *E. coli*

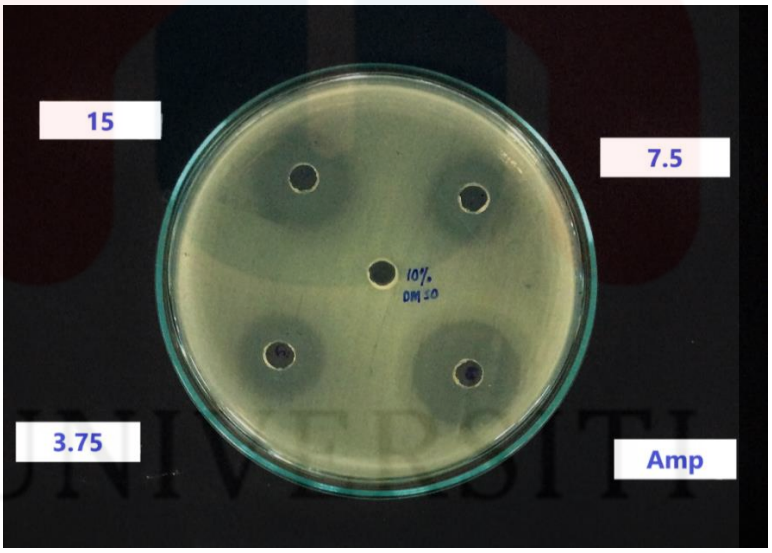


Figure 4.6: The Inhibition Zone of Tetracycline against *E. coli*

For the combination of the plant extract and ampicillin against *S. aureus*, the highest inhibition shown by the concentration same as combination with tetracycline, 4000+50 µg/well and 5000+50 µg/well with the inhibition zone of 35.67 mm. The inhibition zone of ampicillin against *S. aureus* was 36.33 mm showed that the inhibition of the combination of ampicillin and plant extract was lower than the

inhibition of ampicillin alone. The inhibition zone of the combination of ampicillin and plant extract with the concentration of 3000+50 µg/well and 4000+50 µg/well also slightly decreased which were 34.33 mm and 35.67 mm respectively. The inhibition zone of the combination of ampicillin with plant extract and ampicillin alone shown by Figure 4.7 and Figure 4.8.

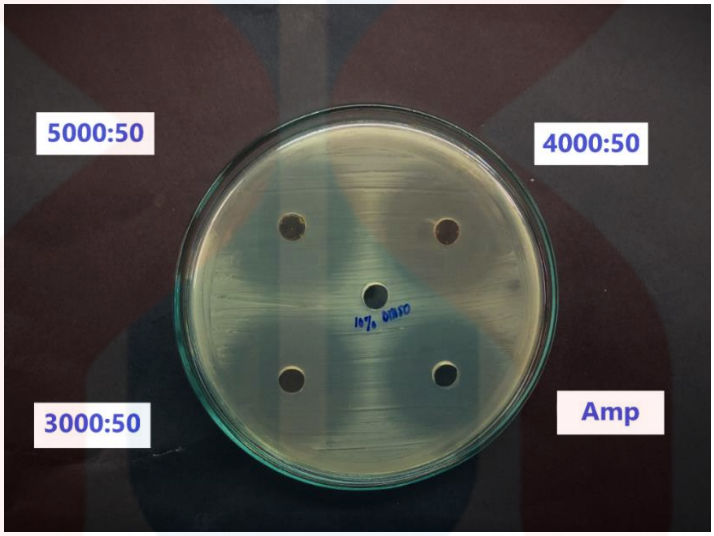


Figure 4.7: The Inhibition Zone of Combination of *Alocasia longiloba* extract and Ampicillin against *S. aureus*

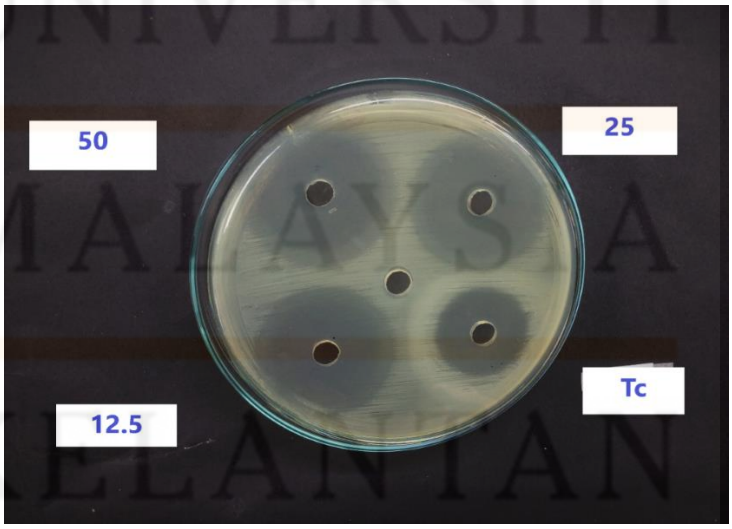


Figure 4.8: The Inhibition Zone of Ampicillin against *S. aureus*

The highest inhibition of the combination of ampicillin against *E. coli* was 28.00 mm, shown by the highest concentration of the combination which was 5000+50 µg/well. The inhibition zone 3000+50 µg/well and 4000+50 µg/well were 27.33 mm and 26.67 mm respectively. The inhibition zone of the combination of plant extract and ampicillin against *E. coli* showed the increasing in the inhibition compared to the inhibition by ampicillin alone which was 24.33 mm as shown in Figure 4.9 and Figure 4.10. The increasing of the inhibition zone of the combination of plant extract and ampicillin compared to ampicillin alone indicated there was synergistic effect of the combination of *A. longiloba* and ampicillin against *E. coli*.

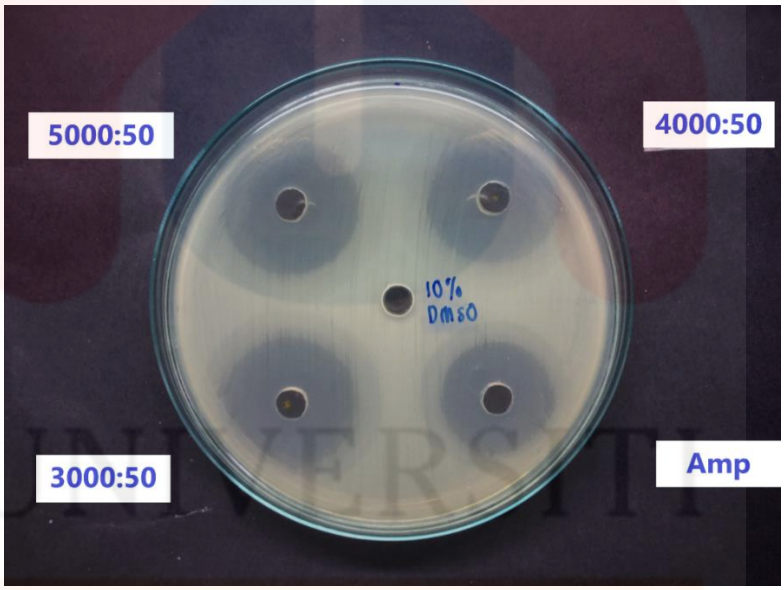


Figure 4.9: The Inhibition Zone of Combination of *Alocasia longiloba* extract and Ampicillin against *E. coli*

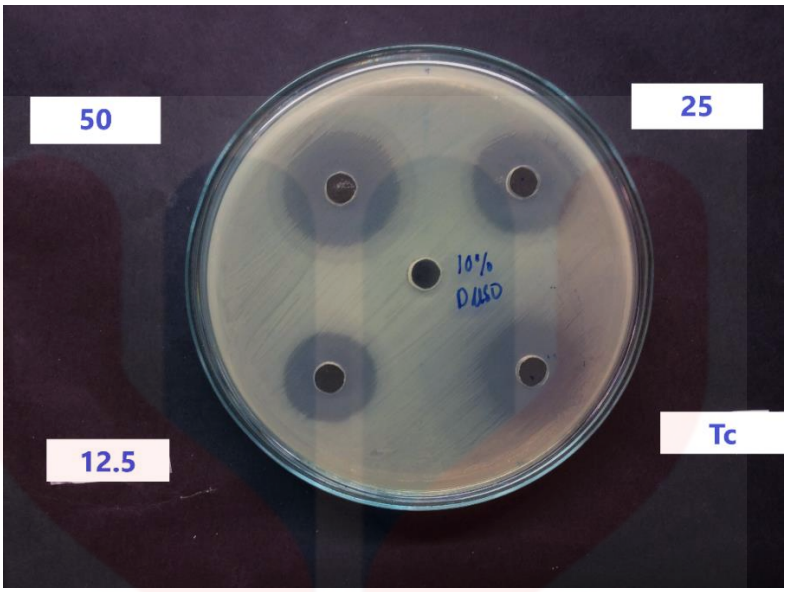


Figure 4.10: The Inhibition Zone of Ampicillin against *E. coli*

From the results, it can be concluded that the synergistic effect only happened with the combination of the ampicillin and plant extract against *E. coli.* The other combination showed the same inhibition as the antibiotic alone and some of the combination showed the decreasing of the inhibition zone when it was combined compared to the individual effects of the antibiotics.

The results also indicated that *S. aureus* are more sensitive to antibiotics compared to *E. coli*. This is because of gram positive bacteria does not have the outer membrane unlike the gram negative bacteria that will prevent certain antibiotic and drug from penetrating bacterial cell and make gram negative bacteria more resistant to antibiotic compared to gram positive bacteria (Delcour, 2009).

4.2 The Minimal Inhibitory Concentration (MIC)

From Table 4.2, the MIC values are not detected for any treatments on *S. aureus* and the MIC value on *E. coli* only for ampicillin and the combination of *A. longiloba* and ampicillin.

Table 4.2: MIC of *Alocasia longiloba*, antibiotics and their combination on Bacteria

Treatment	**The Minimal Inhibitory Concentration (MIC) (µg/ml)**	
	Staphylococcus aureus	*Escherichia coli*
Alocasia longiloba extract	nd	nd
Tetracycline	nd	nd
Ampicillin	nd	12.5
Alocasia longiloba + Tetracycline	nd	nd
Alocasia longiloba + Ampicillin	nd	2000 + 12.5

nd: not detected

The MIC values on *S. aureus* cannot be detected for all treatment which were plant extract alone, tetracycline alone, ampicillin alone, the combination of plant extract and tetracycline and lastly the combination of plant extract with ampicillin. All the Resazurin blue dye decolourized into pink and purple colour indicated the present of active cell in the 96-well plate as shown in Figure 4.11. The well 8 was the negative control without the bacteria suspension.

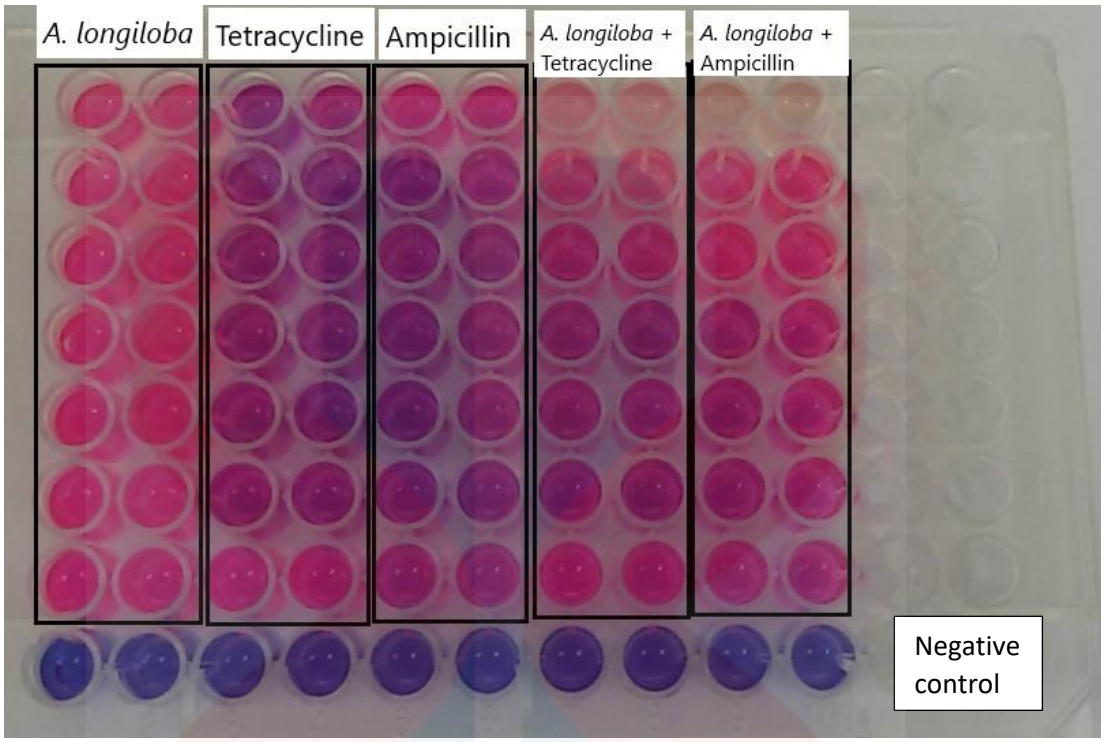


Figure 4.11: MIC of *Alocasia longiloba*, antibiotics and their combination on *S. aureus*

The MIC values of the treatments on *E. coli*, only for the treatment ampicillin alone and the combination of ampicillin with plant extract showed the MIC value which were 12.5 µg/ml and 2000+12.5 µg/ml respectively. The other treatments which were plant extract, tetracycline and combination of tetracycline and plant extract showed no inhibition against the bacteria as shown in Figure 4.12.

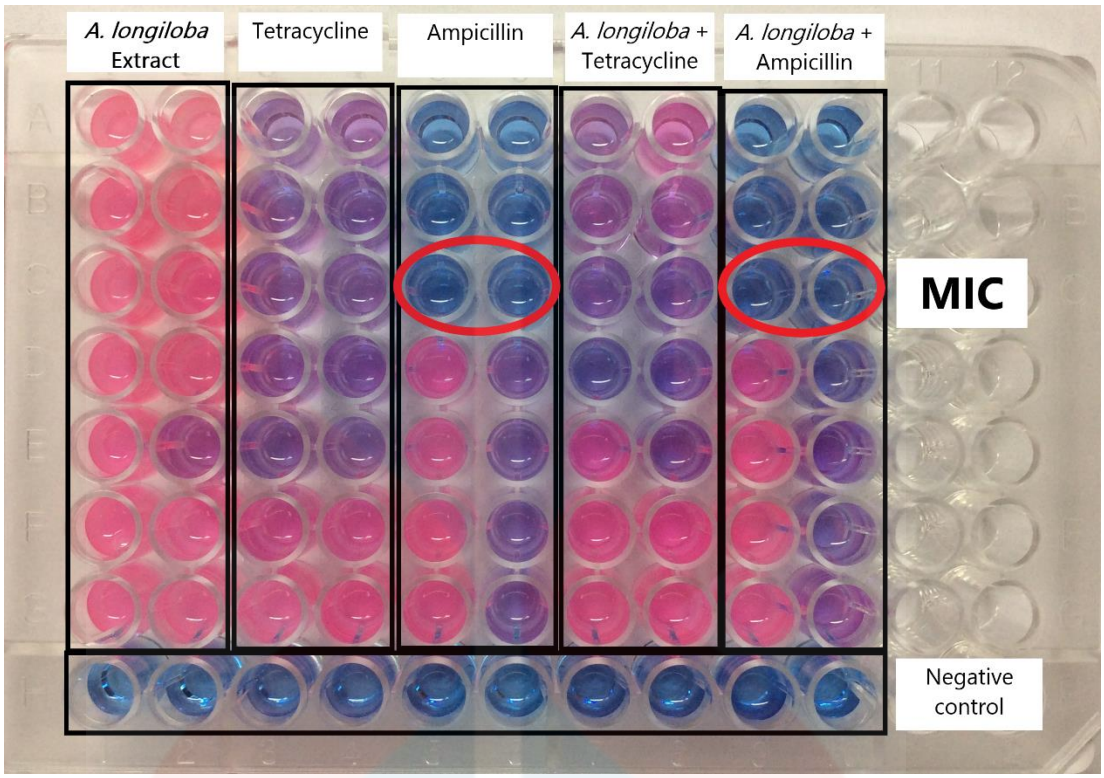


Figure 4.11: MIC of *Alocasia longiloba*, antibiotics and their combination on *E. coli*

The MIC value of treatment on *S. aureus* should be lower than *E. coli*, however in this study, the MIC value on *S. aureus* cannot be detected and there was MIC value on *E. coli*. *S. aureus* should be more susceptible to antibiotics compared to *E. coli* and showed lower resistance toward bacteria. The synergistic effect cannot be shown by both of the combination with ampicillin and tetracycline. The combination of the plant extract with antibiotic showed no reduction in the MIC value compared to the individual effects. Thus, no synergistic effect shown by the combination with both antibiotic against *S. aureus* and *E. coli*.

4.3 Fractional Inhibitory Concentration (FIC)

The FIC index for this study cannot be determined due to the lack of MIC values. The only available MIC values was the MIC for ampicillin and the combination of ampicillin and *Alocasia longiloba.* Thus, there is no synergistic effect of *Alocasia longiloba* with ampicillin and tetracycline against *S. aureus* and *E. coli.*

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

Medicinal plant has been used for century as the natural to the antibiotic remedy for the treatment for many diseases and found safe for human consumption and give less side effects compared to the antibiotic. The combination of plant extract and antibiotic is one of the approaches in combating the antibiotic resistance that will cause the difficult to treat or untreatable disease and cause mortality. The combination of this two substances will decrease the effectiveness dosses of antibiotic and reduce the side effect to the consumers.

In this study, the synergistic effect of *Alocasia longiloba* fruit extract were studied with the combination of tetracycline and ampicillin. *Alocasia longiloba* is the medicinal important plant. However, in this study, it showed low inhibition against gram positive and gram negative bacteria. This plant extract showed no inhibition against *E. coli* and *S. aureus* when it tested alone and tested together with the antibiotics except for the combination of ampicillin and plant extract against *E. coli*. The MIC value of this plants also cannot be detected against both of the bacteria and only the combination with ampicillin against *E. coli* showed the MIC value.

5.2 Recommendation

The fruits part of *Alocasia longiloba* should be studied more in the future. For the antibacterial activity of this plant, this plants might unable or showed low inhibition against *E. coli* and *S. aureus* but it might inhibit the activity against different microorganisms. The study on this plant against different microorganism should be done to see the potential of this species.

Other than that, the combination of this extract with tetracycline and ampicillin might not compatible, thus the study on the combination of this plant with other antibiotic should be done because it might show the antibacterial effect. The concentration of this plant extract should be increase, however the increasing of the concentration of this plant extract might give the side effects to the consumer if the concentration is too high.

The next recommendation is the identification of the bioactive compound in this fruit part of the plant should be done in the future. Furthermore, the fruits part of *Alocasia longiloba* is used as the natural remedy in the gout treatment by the Malaysian indigenous people. So, the study regarding the in vitro xanthine oxidase inhibitory activity should be done to see the potential of this plant as the gout treatment remedy.

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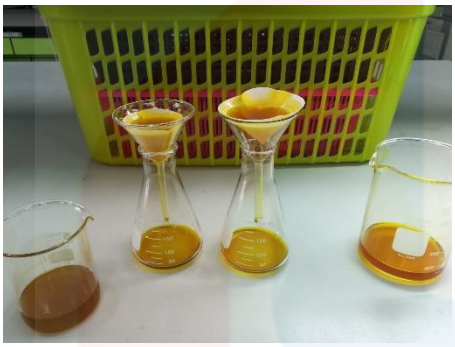
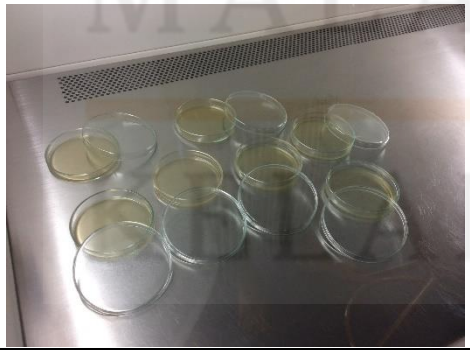
APPENDIX A

Raw Material	Description
	The fruits of *Alocasia longiloba* Miq.

APPENDIX B

Chemical	Description
 	**Antibiotic:** Ampicillin sodium salt and Tetracycline hydrochloride
	**Media:** Nutrient agar (Oxoid), Nutrient broth (Oxoid), Mueller-Hinton agar (Oxoid), Mueller-Hinton broth (Oxoid).
 	**Solvents:** Ethanol (Fisher Chemical) and Dimethyl sulfoxide (Fisher Chemical)

APPENDIX C

Laboratory work	Description
	The extraction of *Alocasia longiloba* sample by using maceration method. The sample was immersed in the ethanol for 5 days.
	The filtration of ethanol extract
	The ethanol filtrate was concentrated by using rotary evaporator.
	The preparation of the media.