Research Article

DETERMINATION OF FRACTIONAL INHIBITION CONCENTRATION INDEX OF ETHANOLIC EXTRACT OF KARAS LEAVES (*Aquilaria malaccensis* Lamk.) AND AMOXICILLIN AGAINST GRAM POSITIVE BACTERIA

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ABSTRACT

Combining antibiotics and natural ingredients is one of the way that can be used to decrease the case of antibiotics resistance. The aim of this research was to obtain the characteristics of combination of ethanolic extract of karas (*Aquilaria malaccensis* Lamk.) leaves with Amoxicillin against *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* bacteria. Kirby-bauer diffusion disk method was used in this research. The combination characteristics was analyzed from Fractional Inhibition Concentration Index (FICI) value which was calculated using Minimum Inhibitory Concentration (MIC) values. MIC of karas leaves ethanolic extract was 0.5 mg/ml and MIC of Amoxicillin for *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* respectively were 0.5; 0.0625; 0.0078125; and 0.001953125 mg/ml. The combination of ethanolic extract of karas leaves with Amoxicillin could inhibit the growth of *Bacillus cereus* bacteria (14.97 ± 2.09 mm); *Staphylococcus aureus* (7.7 ± 0.61 mm), and *Staphylococcus epidermidis* (6.183 ± 0.12 mm), but didn't show any inhibition in *Bacillus subtilis* bacteria. FICI value for each of the *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* respectively were 0.5 (synergistic), 4 (indifference), 2 (indifference), and more than 4 (antagonist). The conclusions of this research were combination of ethanolic extract of karas leaves and Amoxicillin can be used against *Bacillus cereus*, indifferent against *Staphylococcus aureus* and *Staphylococcus epidermidis*, and couldn't be used against *Bacillus subtilis*.

Keywords: Resistance, Amoxicillin, karas leaves (*Aquilaria malaccensis* Lamk.), MIC, FICI.

INTRODUCTION

Antibiotics are often used irrationally. The improper dosage, type and duration of use can cause the bacteria to become insensitive to antibiotics and is known as antibiotic resistance. The development of antibiotics is not proportional to the development of bacteria, so it will cause more bacteria to become resistant to antibiotics. Amoxicillin is one of the best known antibiotics and it's widely used by the wider community¹⁻³.

Gaharu or karas is a plant with high economic value and widely developed; one of this species is *Aquilaria malaccensis* Lamk. Local people usually just use the stem of this plant as hypertension drug, and the leaves are rarely used⁴. Yusuf *et al* found that gaharu leaves have antibacterial activity⁵.

Determination of FICI (Fractional Inhibitory Concentration Index) was to identify the characteristics of ethanolic extract of *Aquilaria malaccensis* Lamk. leaves and Amoxicillin combination. The results of calculations from FICI values showed that the combination of leaf-ethanol extract of karas and resistant antibiotics has synergistic, additive, indifferent or antagonist effects⁶. The combination of ethanolic extract of karas leaves and Amoxicillin is expected to be better activity than single activity. So it can be an alternative choice to prevent Amoxicillin resistance.

MATERIALS AND METHODS

Ethanolic extract of karas leaves, Amoxicillin from Shadong Bio-Technology, sterile distilled water, dimethyl sulfoxide from

Merck, McFarland 0.5 standard solution, Mueller Hinton Agar from Himedia, sterile sodium chloride 0.9% solution.

Karas Leaves Extraction

Karas leaves simplicia was extracted by maceration method, by soaking karas leaves powder for 1 x 24 hours in 96% ethanol. Solvent replacement was carried out every 24 hours with 1 litre 96% ethanol solvent for each solvent replacement and extraction was carried out with filter paper. All macerates were collected and inserted into the rotary evaporator. The remaining filtrate was evaporated using a water bath so that the thick extract obtained was weighed and the yield value was calculated⁷.

Bacteria Media and Inoculum Preparation

38 g of Mueller Hinton Agar was dissolved in 1 L of distilled water and heated. pH was checked and sterilization done using autoclave at 121°C for 15 minutes⁸. Inoculum was made from bacteria stock culture, then suspended into 10 ml of sodium chloride 0.9% solution then incubated at 37°C until the turbidity was obtained. Compared the turbidity with McFarland 0.5 solution. It was equivalent to 1.5×10^8 bacterial cell/ml.⁹

Minimum Inhibitory Concentration (MIC) Determination

Minimum Inhibitory Concentration (MIC) values were determined using the Kirby-Bauer diffusion disc method. Determination of MIC consisted to find out MIC of ethanolic extract of karas leaves, Amoxicillin and the combination of them. The bacterial suspension was inoculated using a sterile ose needle on the surface of the MHA medium and placed a 6 mm paper disk.

The paper disk was immersed in the sample solution (extract solution, Amoxicillin solution, and combination solution) and then placed on the surface of the MHA medium and pressed gently using sterile tweezers. After that, incubated the medium in incubator at $35 \pm 2^\circ\text{C}$ for 18-24 hours and the inhibitory zone diameter was measured.¹⁰

Fractional Inhibitory Concentration Index (FICI) Determination

Fractional Inhibitory Concentration Index (FICI) values were calculated based on the following formula¹¹:

$$FICI = \frac{MIC_{ec}}{MIC_e} + \frac{MIC_{ac}}{MIC_a}$$

MIC_{ec} was MIC of the extract in combination, MIC_{ac} was MIC of the antibiotic in combination, MIC_e was MIC of the extract, and MIC_a was MIC of the antibiotic. $FICI \leq 0.5$ indicates the combination effect was synergist, additive if > 0.5 and < 1 , indifferent if > 1 and ≤ 4 , and antagonist if > 4 ¹².

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration (MIC)

The MIC of ethanolic extract of *Aquilaria malaccensis* leaves for *Bacillus subtilis*, *Bacillus cereus*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* bacteria was determined from previous research that is 0.5 mg/ml. The MIC of Amoxicillin was different for each bacteria, it was depending of the characteristics of the bacteria. Diameter of inhibition could be seen in the Table 1 and Table 2.¹³

From the tables, we could conclude that the MIC of *Bacillus subtilis*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, and *Bacillus cereus* was 0.001953125, 0.0078125, 0.0625 and 0.5 mg/ml respectively. The MIC that was obtained above more than the resistant MIC according to CLSI, which is 0.00025 mg/ml. It meant that Amoxicillin had been resistant to tested bacteria¹⁴.

The Amoxicillin molecule had an β -lactam ring. Bacteria could produce β -lactamase enzymes that can attack and destroy the β -lactam ring in Amoxicillin molecule so that Amoxicillin can not work as an antibacterial. This was a process of resistance to Amoxicillin. Gram-positive bacteria released β -lactamase enzymes into the medium and destroyed antibiotics before reaching the cell. The mechanism of Amoxicillin as an antibiotic was to prevent crosslinking of peptidoglycan by inhibiting proteins in the plasma membrane of bacterial cells and block the activity of transpeptidase enzymes so that bacterial cells become fragile and lysis¹⁵.

Aquilaria malaccensis leaves extract has compounds which act as antibacterial that are flavonoids, phenols, steroids and saponins. Flavonoids as antibacterials that work by forming complexes with extracellular proteins that will damage the cell membrane so that bacterial intracellular fluid will come out. Flavonoids can also inhibit the use of oxygen in bacteria that will inhibit energy metabolism and impact on the process of bacterial development. Phenol is an acidic alcohol and work as an antibacterial by denaturing proteins and destroying cell membranes from bacteria. Steroids can decrease the integrity of bacterial cell membranes so that their morphology changes and the cells become brittle and easy to lysis. Saponins can cause leakage of proteins and enzymes from within the bacterial cell and can increase the permeability of the cell membrane so that it will cause the bacteria to have hemolysis in the cells and bacteria will lysis¹⁶.

Fractional Inhibitory Concentration Index (FICI)

Combining characteristics can be seen based on FICI values. FICI values are derived from single extract MIC values and combined MIC antibiotics. Combined solutions were tested by Kirby-bauer disc diffusion method with 1: 1 volume ratio. The concentrations are 1, $\frac{1}{2}$, and $\frac{1}{4}$ of the MIC value of extracts as well as antibiotics. The observations found that there was a presence of the inhibit zone formed at the combined concentration used.

The combination of leaf-ethanol extract of carcass with Amoxicillin against *Bacillus cereus* bacteria has inhibition zone up to $\frac{1}{4}$ times concentration of MIC extract and Amoxicillin with a volume ratio of 1:1 (Figure 1 and Table 3). It is based on the FICI calculation that the value is 0.5 so it can be concluded that it has synergistic characteristics. Synergistic means the combination can produce a zone of resistor greater than a single concentration.

Staphylococcus aureus and *Staphylococcus epidermidis* bacteria in combination of MIC concentrations did not show any inhibitory zone. If at a combination MIC concentration has no inhibit zone it is certain that its characteristics are not synergistic. This study was also tested at concentrations 2 and 4 times of MIC values to ascertain the characteristics of the combination. Characteristics based on FICI calculations obtained are 4 for *Staphylococcus aureus* (Figure 2 and Table 4) and 2 for *Staphylococcus epidermidis* (Figure 3 and Table 5), which means no different from single use.

The combination of ethanolic extract of karas leaves and Amoxicillin in *Bacillus subtilis* bacteria did not have inhibition zone at MIC concentration as well as at concentrations 2 and 4 times of MIC. So, it can be concluded that the characteristics of this combination was antagonist which is better to single use than a combination (Figure 4 and Table 6).

The result of combination characteristics varies because there are differences in the way extract and antibiotic work as antibacterials. The synergistic character of *Bacillus cereus* bacteria with $FICI=0.5$ indicates that the action of the combination is greater than single use. This may occur because of the role of the compounds contained in the extract of phenol that can denature the proteins and saponins that can cause enzyme leakage so that the enzyme β -lactamase in the bacteria becomes damaged and Amoxicillin can enter into the bacterial cell, and the content in the extract is also suspected to help the work of Amoxicillin that will make the bacterial cell becomes brittle and easy to lysis.

Indifferent means the action of the combination is not different from the action of extract or Amoxicillin single use. This indifferent character occurs in *Staphylococcus aureus* bacteria with $FICI=4$ and *Staphylococcus epidermidis* values of $FICI=2$. This occurs because suspected flavonoid compounds in extracts that have the same target as Amoxicillin is the extracellular protein in bacteria. The same target causes the antibacterial action of each extract and Amoxicillin does not support each other. An antagonist FICI characteristic occurs in bacteria of *Bacillus subtilis*, $FICI>8$, this may occur probably because the presence of other non-antibacterial compounds inhibits the action of antibiotics in inhibiting bacterial growth.

Antagonist characteristic occurred on *Bacillus subtilis*, but had synergic characteristic on *Bacillus cereus*. This result was different on bacteria with the same genus. It is because there were structural differences of the bacteria so that would affect the penetration ability of the antibacterial compounds of the karas leaves ethanolic extract and Amoxicillin. According to previous studies, *Bacillus subtilis* could produce lipoprotein aterimine and

bacitracine that could work as antibiotic, thus *Bacillus subtilis* was allegedly to be more resistant against the antibacterial compound¹⁷. *Bacillus cereus* is a gram-positive bacteria that is more sensitive against non-polar antibacterial compound because it has alanine which is hydrophobic that constructs the peptidoglycan of the bacteria.¹⁸ Amoxicillin could easily

penetrate through the bacteria cells because there were porin. Porin is a channel that helps the hydrophobic antibiotics penetrate through the bacteria cells¹⁹. Amoxicillin is hard to dissolve in water or aquabidest which has polar characteristic, so it could be concluded that Amoxicillin is non-polar, thus *Bacillus cereus* was allegedly more sensitive against Amoxicillin.

Table 1: MIC Inhibition Zone of *Aquilaria malaccensis* Lamk.¹³

Bacteria	MIC (mg/ml)	Diameter (mm)
<i>Bacillus subtilis</i>	0,5	6,68 ± 0,07
<i>Bacillus cereus</i>		6,81 ± 0,03
<i>Staphylococcus aureus</i>		6,73 ± 0,13
<i>Staphylococcus epidermidis</i>		6,86 ± 0,05

Table 2: MIC Inhibition Zone of Amoxicillin

Bacteria	MIC (mg/ml)	Diameter (mm)
<i>Bacillus subtilis</i>	0,001953125	6,753 ± 0,386
<i>Bacillus cereus</i>	0,5	9,183 ± 1,944
<i>Staphylococcus aureus</i>	0,0625	8,267 ± 0,473
<i>Staphylococcus epidermidis</i>	0,0078125	9,753 ± 0,735

Table 3: Inhibition Zone of the Combination against *Bacillus cereus*

No	Antibiotic Concentration (mg/ml)	Extract Concentration (mg/ml)	Diameter (mm)			
			I	II	III	Mean ± SD
1	0,5	0,5	14,5	13,15	17,25	14,97 ± 2,09
2	0,25	0,25	12	14,075	14,175	13,42 ± 1,23
3	0,125	0,125	11,5	10	12,75	11,42 ± 1,38

Table 4: Inhibition Zone of the Combination against *Staphylococcus aureus*

No	Antibiotic Concentration (mg/ml)	Extract Concentration (mg/ml)	Diameter (mm)			
			I	II	III	Mean ± SD
1	0,25	2	9,55	8,93	8,53	9,003 ± 0,51
2	0,125	1	7	8,05	8,05	7,7 ± 0,61
3	0,0625	0,5	-	-	-	-
4	0,03125	0,25	-	-	-	-
5	0,015625	0,125	-	-	-	-

Table 5: Inhibition Zone of the Combination against *Staphylococcus epidermidis*

No	Antibiotic Concentration (mg/ml)	Extract Concentration (mg/ml)	Diameter (mm)			
			I	II	III	Mean ± SD
1	0,03125	2	8,45	9,375	8,25	8,692 ± 0,6
2	0,015625	1	6,35	6,68	6,68	6,57 ± 0,19
3	0,0078125	0,5	6,05	6,25	6,25	6,183 ± 0,12
4	0,00390625	0,25	-	-	-	-
5	0,001953125	0,125	-	-	-	-

Table 6: Inhibition Zone of the Combination against *Bacillus subtilis*

No	Antibiotic Concentration (mg/ml)	Extract Concentration (mg/ml)	Diameter (mm)			
			I	II	III	Mean ± SD
1	0,03125	2	-	-	-	-
2	0,015625	1	-	-	-	-
3	0,0078125	0,5	-	-	-	-
4	0,00390625	0,25	-	-	-	-
5	0,001953125	0,125	-	-	-	-

Figure 1: Combination of Ethanolic Extract of Karas Leaves and Amoxicillin against *Bacillus cereus*

Figure 2: Combination of Ethanolic Extract of Karas Leaves and Amoxicillin against *Staphylococcus aureus*

Figure 3 Combination of Ethanolic Extract of Karas Leaves and Amoxicillin against *Staphylococcus epidermidis*

Figure 4 Combination of Ethanolic Extract of Karas Leaves and Amoxicillin against *Bacillus subtilis*

CONCLUSION

The combination of ethanolic extract of *Aquilaria malaccensis* Lamk. leaves and Amoxicillin had a synergist characteristic against *Bacillus cereus* bacteria (FICI=0.5); indifferent against *Staphylococcus aureus* (FICI=4) and *Staphylococcus epidermidis* (FICI=2); and antagonist characteristic against *Bacillus subtilis* (FICI > 4) bacteria.

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