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Synergistic Interaction of Ethyl Acetate Fraction from *Melastoma malabathricum L.* Leaves in Combination with Ciprofloxacin and Gentamicin against *Eschericia coli* Isolated from Diabetic Foot Ulcer Patients

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ABSTRACT

Melastoma malabathricum L. leaves have active compounds, namely flavonoids, phenols, and terpenoids, that are thought to have potential as antibacterial. The objective of this study was to find effective drugs formulated from combination of the plant leaf fraction and antibiotics for diabetic foot ulcer therapy to prevent further complications and disability risks in diabetes mellitus patients. Ciprofloxacin and gentamicin were proven to be resistant antibiotics. In this study, the fraction from *Melastoma malabathricum L.* leaves was combined with those antibiotics. The minimum inhibitory concentration (MIC) was determined against bacterial strains. The determination of MIC value was done using Kirby-Bauer disk diffusion method. The interaction between the franction and the antibiotics was studied in vitro by calculating fractional inhibitory concentration index (FICI). The synergistic effects of the combination were then observed. The results showed that the combination of *Melastoma malabathricum L.* leaf fraction and the antibiotics, ciprofloxacin and and gentamicin, had a synergistic effect against *Escherichia coli* with a FICI value of 0.5. Thus, the combination had a synergistic effect on *Escherichia coli* inhibition.
Keywords: *Melastoma malabathricum*, synergistic, FICI, antibiotics, *Escherichia coli*

INTRODUCTION

Diabetic foot ulcer (DFU) is a complication of diabetes mellitus characterized by open sores on the surface of the skin and mucous membranes with extensive dead tissues and bacterial invasions. At Kitamura Care Specialist Clinic, among 800 diabetic patients, 470 of them suffered from DFU complications (Abidin *et al.,* 2013). Based on research in 2016, 14.3% of DFU patients ended with death a year after amputation, and 37% of them would die 3 years after amputation (Lina and Sholihatul, 2012). *Pseudomonas aeruginosa* found in DFU patients was one of bacteria found with the highest percentage of 30.57% (Manisha *et al.*, 2012). Found in DFU patients, *Escherichia coli* was as much as 8.33%. In the study related to antibiotics, ciprofloxacin was found to be resistant to *S. Epidermidis*, *M. Luteus*, and *Pseudomonas putida*. Gentamicin was resistant to *P. Mirabilis* bacteria (Sari and Apridamayanti, 2015). Antibiotic resistance is a major problem that causes long wound healing. The main causes of antibiotic resistance was caused by the improper use and long-term use of antibiotics. Likewise, organisms resistant to an antibiotic tended to become resistant to antibiotics (Cahyopoetro, 2014). In prior research, *Melastoma malabathricum L.* leaves extract was proven to be effective for treating wounds. Healing burns with formulations using ointment of ethanol extract from *Melastoma malabathricum* leaves was effective at a concentration of 5% (Izzati, 2015). Any infection was not found in injured mice given water extract from *Melastoma malabathricum* leaves (Nurdiana and Marziana, 2013). *Melastoma malabathricum L.* leaves have active compounds, namely flavonoids, phenols, and terpenoids, that are thought to have the potential as antibacterial. Quercetin had an antibacterial mechanism through inhibition of DNA gyrase in the process of bacterial protein synthesis. [8] The antibacterial mechanism of phenolic compounds was through the destruction of cell walls due to the formation of hydrogen bonds between phenols and proteins (Noventi and Carolina, 2016). The terpenoid action as an antibacterial through the interaction of terpenoid compounds with porins (transmembrane protein) in bacteria due to damaged porins made bacterial cells lack nutrients (Cowman, 1999). The purpose of this study was to find an effective drug formulated from *Melastoma malabathricum L.* leaf fraction combined with antibiotics for diabetic foot ulcer therapy to prevent further complications and disability risks in DM patients. There have been various studies

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that used a combination of natural ingredients and antibiotics, and they revealed a synergistic effect. The combination of lemongrass extract and antibiotics was synergistic and additive, and it did not have an antagonistic effect (Noor, 2016). The combination of gentamicin and natural compounds was a promising approach to combat microbial resistance (Bazzaz *et al.,* 2016). In this study, the fraction of *Melastoma malabathricum L.* leaves was combined with both types of antibiotics namely ciprofloxacin and gentamicin, and it was expected to kill bacteria causing diabetic foot ulcers, especially those that are resistant to the therapy of gentamicin and ciprofloxacin.

Novelty in this research was the combination of *Melastoma malabathricum L.* leaf fraction and antibiotics namely ciprofloxacin and gentamicin to overcome the resistance of DFU patients from MIC values, and bacteria used in this research were isolated from DFU patients.

METHODOLOGY

Materials

Leaves of *Melastoma malabathricum L.* selected for the study were freshly obtained from Sekajang village, Sanggau Regency. West Kalimantan, Indonesia. The other materials were ciprofloxacin (Sigma-Aldrich 98%), gentamicin sulfate (Kimia Farma), n-hexane (Merck), ethyl acetate (Merck), methanol (Merck), ethanol 96% (Bratachem), and aquades (Dwicentra).

Methods

Preparation of Plant Extract

Melastoma malabathricum L. leaves were extracted by maceration using ethanol 96%. Simplicia of the leaves that have been sieved with no. 40 mesh was put into a glass vessel, then was poured and soaked with ethanol 96% until it was completely submerged; it was covered and left to stand for 24 hours while being repeatedly shaken and macerated. Furthermore, the extracts of maceration mixed with the solvent were evaporated with a rotary evaporator to obtain a thick extract of the leaves. The filtrate was then evaporated further on a hot plate. The remaining solvent was removed by placing the remaining residue in the desiccator containing silica or dryer for ± 24 hours.

Bacteria Samples from Patients

Bacteria were from the third and fourth Wagner degree of DFU patients in Pontianak's Kitamura clinic. The results of the identification were then tested for antibiotic sensitivity.

MIC of *Melastoma malabathricum L.* Fraction against *Escherichia coli*

The concentrations of the plant leaf fraction were: 100; 50; 25; 12.5; 6.25; 3.12; 1.56; 0.78; and 0.39 mg/mL. Preparing solution series of the fraction concentration was very difficult; to help to dissolve it in water, 20% DMSO was needed.

MIC of Ciprofloxacin against *Escherichia coli*

The concentration of ciprofloxacin solution used in bacteria was determined after a preliminary test. It was carried out at the concentration of the mother liquor which was 10 ppm, and the concentration was lowered based on the results of the inhibition zone diameter produced from the bacteria. The ciprofloxacin solution was made with a concentration of 0.039; 0.019 mg/mL, 0.0097 mg/mL, and 0.0048 mg/mL.

Determination of the MIC Value of Gentamicin against *Escherichia coli*

The gentamicin stock solution was made 10 at ppm, and concentration variations of 2.496 made were: 1.248; 0.624; 0.312; 0.156; 0.078; 0.039; 0.0195 mg/mL. Fresh Bacteria (24 hours) were for bacterial culture with inoculum density equivalent to 0.9×10^9 cell/ml using Mc Farland III.

Value of Fractional Inhibitory Concentration Index (FICI) of Ciprofloxacin - *Melastoma malabathricum L.* Fraction against *Escherichia coli*

Determination of FICI values was with the Kirby-Bauer diffusion disk method, and there were 8 concentrations tested: 32 x MIC, 16 x MIC, 8 x MIC, 4 x MIC, 2 x MIC, 1 x MIC, ½ x MIC, and ¼ x MIC with a comparison volume of 1:1.

Determination FICI

FICI value could be calculated using formulas (Morgan, 2014):

$$\sum FICI = FICI\ A + FICI\ B$$

$$\sum FICI = \frac{MIC\ Combination}{MIC\ A} + \frac{MIC\ B\ Combination}{MIC\ B}$$

Note : A : *Melastoma malabathricum L.* fraction; B : Ciprofloxacin/ gentamicin sulfate

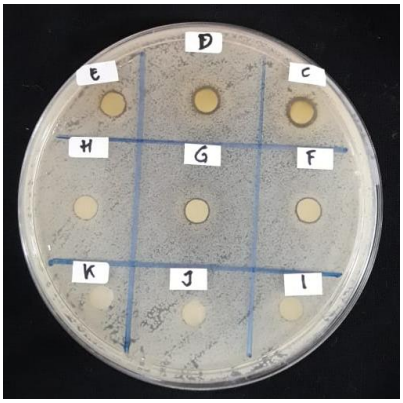

Figure 1. MIC of *Melastoma malabathricum L*. Fraction against *Escherichia coli*

Table I. Average Zones of Inhibition from *Melastoma malabathricum L*. Fraction against *Escherichia coli*

Concentration (mg/mL)	*Escherichia coli* I	*Escherichia coli* II	*Escherichia coli* III	Average ± SD (mm)
100	7.35	6.85	6.9	7.03 ± 0.27
50	6.7	7.55	8.6	7.62 ± 0.95
25	6.6	6.6	6.1	6.43 ± 0.29
12.5	0	7.1	6.6	4.57 ± 3.96
6.25	0	7	7.6	4.87 ± 4.22
3.125	0	6.9	6.1	4.33 ± 3.77
1.562	0	0	0	0
0.781	0	0	0	0
0.390	0	0	0	0

Determination of Fractional Inhibitory Concentration Index (FICI) Value of Gentamicin - *Melastoma malabathricum L.* Fraction against *Escherichia coli*

The FICI value was determined based on the MIC values of a single *Melastoma malabathricum L*. fraction, ciprofloxacin, and single gentamicin which were then combined. The method applied in this study was Kirby-Bauer diffusion disk method. The revealed parameters were the formation of a clear zone around the disk and measured inhibition zones formed around the disk. The *Melastoma malabathricum L*. fraction was stocked with 1000 mg/mL. The solvent used was 20% DMSO. Based on previous research, DMSO at a concentration of 50% showed no antibacterial activity (Rifani *et al.,* 2017). DMSO was used because the fraction of *Melastoma malabathricum L*. was difficult to dissolve in aquadest. Variations of concentration made were: 800; 400; 200; 100; 50; 25; 12,5; 6,25 mg/mL.

RESULT AND DISCUSSION

MIC of *Melastoma malabathricum L.* Fraction against *Escherichia coli*

MIC of *Melastoma malabathricum L*. leaves fraction was at a concentration of 25 mg/mL with inhibition zone of 6.43 mm ± 0.29. For interpretation of MIC results in the category of bacterial inhibition, if the diameter of the bright inhibition zone was ≥ 20 mm, the response to growth resistance in the category was very strong; diameter 10-20 mm was in strong category, diameter 5-10 mm was in medium category; if the diameter was ≤ 5 mm, the growth inhibition response was in the weak category (Davis and Stout, 1971). Based on Piatek jacek *et al.,* experiment that was in vitro pathogen inhibition with 6 mm-8 mm inhibition zone showed the weak inhibition (Piatek jacek *et al.,* 2020). The MIC results of *Melastoma malabathricum L*. fraction were 25 mg/mL with *Escherichia coli*. The activity of the plant fraction on *Escherichia coli* was considered in the medium category. The inhibition was produced from the fraction because there were secondary metabolites which played an important role as antibacterial. The results of phytochemical screening of *Melastoma malabathricum L*. fraction revealed that the plant contains tannins, flavonoids, steroids, saponins, and quinones. The obtained results were also supported by previous studies in which the ethyl acetate fraction of *Melastoma malabathricum L.* leaves could inhibit the growth of *Escherichia Coli.*

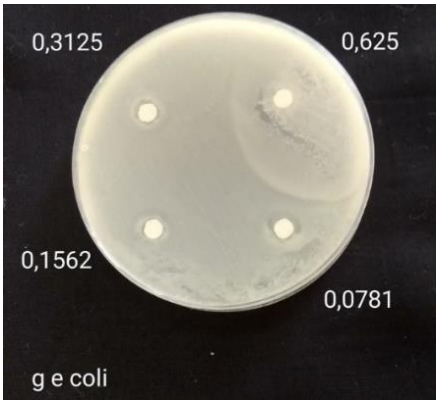

Figure 2. Result of the Gentamicin MIC against *Escherichia coli*

Table II. Result of the Ciprofloxacin MIC against *Escherichia coli*

Concentration (mg/mL)	Inhibition Zone (mm)			Average ± SD (mm)
	I	II	III	
0,039	8.55	7.9	6.3	7.6 ± 1.15
0,019	0	0	0	-
0,0097	0	0	0	-
0,0048	0	0	0	-

The MIC of Ciprofloxacin was at a concentration of 0.039 mg/mL with a inhibition zone of 7.6 mm ± 1.15.

MIC of Ciprofloxacin against *Escherichia coli*
Table II shows the results of observations.

MIC of Gentamicin against *Escherichia coli*

Based on the study, the value of gentamicin was 0.0781 mg/mL with a 7.37 mm ± 0.15 inhibition zone in *Escherichia coli*. The value of gentamicin was 0.0781 mg/mL with a 7.37 mm inhibition zone in *Escherichia coli*. The inhibition was produced from gentamicin because gentamicin has 3 rings: ring 1 (purpurosamine), ring II (deoxystreptamine), and ring III (gentosamine). Ring 2-deoxystreptamine (2-DOS) had glycosidic bonds in C numbers 4 and 6, and it played an important role in antibacterial activity. Gentamicin contained hydroxyl (OH) and amine groups (NH2) in which these two groups interacted with receptors on bacterial cells (Wang *et al.*, 2014).

Determination of Fractional Inhibitory Concentration Index (FICI) Value

The characteristics of combinations can be seen by determining the FICI value. This is useful for interpreting the results of the combination of two antibacterial compounds to inhibit bacterial growth.

Based on Table IV above, the combination of ¼ times MIC of *Melastoma malabathricum L.* fraction and ciprofloxacin can still inhibit the growth of *Escherichia coli*. Thus, the MIC combination is ¼ times the MIC.

Determination of Fractional Inhibitory Concentration Index (FICI) Value of *Melastoma malabathricum L.* Fraction - Gentamicin against *Escherichia coli*

Based on the study, the MIC results of gentamicin inhibiting *Escherichia coli* were at a concentration of 0.078 ppm. The MIC results of the tested *Melastoma malabathricum L.* fraction in inhibiting *Escherichia coli* were at a concentration of 25 mg /mL. Antibiotics and natural ingredients were combined with a ratio of 1: 1 where each concentration was raised and lowered from a single MIC value. The concentrations combined were 32, 16, 8, 4, 2, 1, ½, ¼, of the MIC values respectively.

This study then combined ciprofloxacin and gentamicin with *Melastoma malabathricum* fraction which was also tested for antibacterial activity and potential *Escherichia coli* inhibition. The results of combinations were expected to have a better effect than single-use and to be able to overcome the incidence of bacterial resistance to gentamicin. The presence of bacteria that have been resistant to antibiotics is one of the causes of treatment failure. This encourages the discovery of solutions in therapy by combining natural compounds and antibiotics. The combination

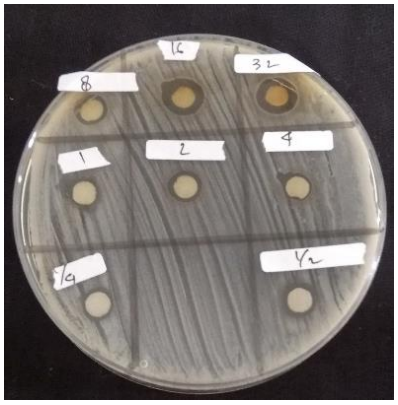

Figure 3. Combination of *Melastoma malabathricum L.* Fraction and Ciprofloksasin against *Escherichia coli*

Table III. Result of the Gentamicin MIC against *Escherichia coli*

Concentration (mg/mL)	Inhibition Zone (mm)			Average ± SD (mm)
	I	II	III	
0.625	10.1	10	13.3	11.13 ± 1.,87
0.312	8.2	9.5	10.6	9.,43 ± 1.20
0.156	8.5	7.1	9.1	8.23 ± 1.02
0.078	7.5	7.4	7.2	7.37 ± 0.15

Table IV. Result of the Combination of *Melastoma malabathricum L.* Fraction and Ciprofloksasin against *Escherichia coli*

Combination	Inhibition Zone (mm)			Average (mm)
	I	II	III	
32+32	9.2	12.2	12.2	11.2
16+16	8.1	11.3	13.2	10.67
8+8	9.5	9.3	11.3	10.33
4+4	9.2	8.2	8.2	8.53
2+2	7.1	9.4	10.5	9
1+1	8.1	7.1	9.4	8.2
½ + ½	7.1	7.5	0	7.3
¼ + ¼	8.1	7.2	7	7.43

characteristics can be seen by determining the FICI value. The determination of the value is useful to interpret the results of the combination of two antibacterial compounds in inhibiting bacterial growth. The FICI value was obtained from a combination of *Melastoma malabathricum L.* fraction and ciprofloxacin. The concentration used was based on the MIC value or the smallest concentration that can inhibit bacterial growth. The determination of FICI values was with Kirby-Bauer disk diffusion method, and there were 8 concentrations tested:32 x MIC, 16 x MIC, 8 x MIC, 4 x MIC, 2 x MIC, 1 x MIC, ½ x MIC, ¼ x MIC with a comparison volume of 1: 1.

The combination results with ¼ times MIC of *Melastoma malabathricum L.* fraction and ciprofloxacin can still inhibit the growth of *Escherichia coli*, so that the MIC combination is ¼ times the MIC. The results of the combination FICI produced synergistic characteristics that was a FICI value of 0.5. The synergistic results showed that the combination of *Melastoma malabathricum L.* fraction and ciprofloxacin had a greater effect than the second amount of antimicrobials. Ciprofloxacin worked to inhibit DNA replication by binding itself to an enzyme called DNA gyrase which causes double cracks in bacterial chromosomes (Sumampouw and Oksfriani, 2018). *Melastoma malabathricum L.* fraction can inhibit bacterial growth caused by the presence of the fraction compounds, namely flavonoids, phenols, and terpenoids which have antibacterial abilities. Flavonoids could denature bacterial cell proteins that change the structure of these bacteria (Tjay, 2002). The mechanism of terpenoids as an antibacterial was that they can lyse bacterial cells

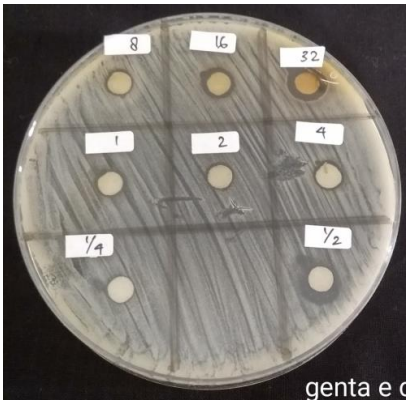

Figure 4. Combination of *Melastoma malabathricum L.* Fraction and Gentamicin against *Escherichia coli*

Table V. Result of the Combination of *Melastoma malabathricum L.* Fraction and Gentamicin against *Escherichia coli*

Combination	Inhibition Zone (mm)			Average (mm)
	I	II	III	
32+32	9.2	9.3	10.5	9.67
16+16	9.1	8.2	10.1	9.13
8+8	8.5	7.5	9.2	8.4
4+4	8.3	7.2	7.1	7.53
2+2	8.2	7.1	6.5	7.27
1+1	7.93	6.95	6.2	7.07
½ + ½	7.65	7.2	0	7.42
¼ + ¼	6.95	6.4	0	6.67

by damaging the porins (Cowan, 1999). Mechanism of phenol as an antibacterial by changing the permeability of the cytoplasmic membrane caused intracellular leakage (Setyaningsih *et al.,* 2009). Both mechanisms can increase their antibacterial activity against *Escherichia coli*. Thus, combining *Melastoma malabathricum L.* fraction and ciprofloxacin can have a synergistic effect in inhibiting the growth of *Escherichia coli*.

The combination of *Melastoma malabathricum L.* fraction and gentamicin had synergistic characteristics in inhibiting *Escherichia coli*. The synergistic characteristics was due to the secondary metabolites such as tannins and flavonoids in *Melastoma malabathricum L.* Fraction; they were important compound in antibacterial activities as they had mechanisms to destroy bacterial cell membranes so that intracellular compounds are released, and this will cause the cell wall layer intact (Suryaningsih *et al.,* 2010; Robinso, 1995; Kiyama *et al.,* 2001). Gentamicin as an antibacterial mechanism got into the cell and bound strongly to the tRNA side component in the 16S RNA section on the 30S ribosome subunit which caused errors in translation results and interfered the cell's working system in the bacteria (Wattimena *et al.,* 1991). Merging these two mechanisms woud increase synergistic antibacterial activity. Several other studies have shown that a synergistic combination of non-antibiotic drugs and ciprofloxacin and gentamicin can overcome resistance (Bazzaz *et al.,* 2019). Likewise, in the combination of verbascoside, lemon verbena extract, and caffeine and gentamicin, higher activity and synergistic combinations were obtained (Bazzaz *et al.,* 2018).

Based on the results of the study, it was expected that in the future a preparation formulation be developed into one which consists of *Melastoma malabathricum L.* fraction in combination with ciprofloxacin and gentamicin that can overcome bacterial resistance to antibiotics. Ethyl acetate fraction of *Melastoma malabathricum L.* could be improved for its bioavailability, the ability to penetrate the skin layer and the ability to protect active compounds contained in it so that it could use the drug delivery system such as SNEDDS (Pratiwi *et al.*, 2017). Formulations with the drug delivery system would support increasing bacterial sensitivity to antibiotics and could reduce the death rate from bacterial infections (Bazzaz *et al.*, 2018).

MIC of the plant leaf fraction was at a concentration of 25 mg/mL with an inhibition zone of 6.43 mm ± 0.29. For interpretation of MIC results in the category of bacterial inhibition, if the diameter of the bright inhibition zone was ≥ 20 mm, the response to growing resistance in the category was very strong; diameter 10-20 mm was in the strong category, diameter 5-10 mm was in the medium category; if the diameter was ≤ 5 mm, the growth inhibition response was in the weak category (Davis and Stout, 1971). The MIC results of *Melastoma malabathricum L.* fraction were 25 mg/mL. The activity of the plant fraction against *Escherichia coli* was considered in the medium category in inhibiting the pathogenic bacteria. The inhibition was produced from the plant fraction because there were secondary metabolites which played an important role as antibacterial. The phytochemical screening results of *Melastoma malabathricum L.* fraction revealed that *Melastoma malabathricum* contains tannins, flavonoids, steroids, saponins, and quinones. The results of the research obtained were also supported by previous studies in which the ethyl acetate fraction of *Melastoma malabathricum L.* leaves could inhibit the growth of *Escherichia coli*.

CONCLUSION

The synergistic combination of antibacterial activity can be done through combining antibiotics and compounds derived from natural ingredients. Ethyl acetate fraction shows a synergistic combination with increasing inhibition zones in the combination of *Melastoma malabathricum* with ciprofloxacin as well as with gentamicin compared with non-combined use. This synergistic combination has been proven to overcome antibiotic resistance and increase antibacterial activity against E. coli bacteria isolated from diabetic foot ulcer patients.

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