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The Highest Dosage Combination Activity Screening from the Leaf Fraction of *Melastoma malabathricum* with Antibiotic Gentamicin and Ciprofloxacin

Rafika Sari*, Liza Pratiwi, Pratiwi Apridamayanti

Faculty of Medical Science, Tanjungpura University, Pontianak, Indonesia

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***Corresponding Author**
Rafika Sari
Faculty of Medical Science, Tanjungpura University, Pontianak 78124, Indonesia
Tel: +62-085849682284
E-mail: rafikasari@pharm.untan.ac.id

Objectives: This study aims to determine the Fractional Inhibitory Concentration Index (FICI) of combinations of *Melastoma malabathricum* leaf fraction with ciprofloxacin or gentamicin against pathogenic bacteria, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, isolated from Diabetic Foot Ulcer (DFU) patients.
Methods: We tested concentrations of 45%, 55%, 65%, and 75% of gentamicin and ciprofloxacin using dilution and agar diffusion methods. The combination of *M. malabathricum* leaf extract with these antibiotics was tested *in vitro* against all three bacteria.
Results: The combination of *M. malabathricum* leaf extract and ciprofloxacin gave a FICI value of 0.5, indicating synergistic antibacterial activity against the test bacteria.
Conclusion: The results show that the antibacterial effect of a combination of high doses of the leaf extract with either antibiotic is greater than that of the leaf extract and the antibiotics in single use.

Keywords: melastoma malabathricum, antibacterial effect, fici, combination, mbc

INTRODUCTION

Diabetic Foot Ulcer (DFU) is a complication of diabetes mellitus (DM) characterized by open sores on the surface of the skin or mucous membranes and extensive tissue death accompanied by bacterial invasion [1-4]. The impairment of wound healing is caused by misuse or long-term use of antibiotics and the development of cross-resistance to several antibiotics by organisms [5-7]. The specific aim of this study is to develop effective formulations from the leaf fraction of *Melastoma malabathricum* in combination with antibiotics as diabetic ulcer therapy to prevent further complications and the risk of disability in DM patients [8]. Topical antibiotics known to cause resistance are gentamicin and ciprofloxacin. Previous research has shown the efficacy of the leaf fraction of *M. malabathricum* in treating wounds. An ointment formulation of an ethanolic extract of *M. malabathricum* leaf, effective at 5% concentration [9], was applied to mice with injuries, leading to healing without any infection [10].

One approach to preventing and controlling resistance is through the use of a combination of natural antibacterial compounds with antibiotics in topical dosage forms that have proven effective against DFU wounds with bacterial infections. Drug formulations in the form of nanosprays based on self nanoemulsifiying drug delivery systems (SNEDDS) have been prepared based on the optimal dose of the leaf fraction of *M. malabathricum* with antibiotics, gentamicin and ciprofloxacin. Such formulations aim to reduce the severity of the continued infection of DFU wounds and can be effective in the treatment of resistant bacteria [11]. These nanospray preparations are based on the dosages of antibiotics and their combinations with the extract determined based on the value of the Fractional Inhibitory Concentration Index (FICI) used to define additivity or synergism [12]. The synergistic combination of the leaf fraction of *M. malabathricum* with gentamicin and ciprofloxacin was then tested *in vitro* against pathogenic bacteria isolated from DFU patients, namely, *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*.

MATERIALS AND METHODS

1. Research ethics code

This study was approved by the Ethics Review Division of the Faculty of Medicine, Tanjungpura University No.3002/UN22.9/DL/2019.

2. Tools and materials

The equipment used in this study included autoclaves (All American Model no. 75X), incubators (Memmert®), Laminar Air Flow (MINIHELIC®II), Ovens (Memmert), analytical scales (BEL model M254Ai), calipers (Memihert®) Vernier Caliper), micropipette (Rainin®), water bath (Memmert®), and glassware (Pyrex®Iwaki).

The materials used in this study included the leaf fraction of *M. malabathricum*; stock cultures of *E. coli*, *S. aureus*, and *B. cereus*; gentamicin sulfate (Yantai Justaware Pharmaceutical); ciprofloxacin; distilled water; sterile distilled water (IKA); sulfuric acid (Merck); barium chloride (Merck); 96% ethanol (Brataco); glycerol (Merck); Whatmann filter paper; DMSO solution (Merck); 0.9% NaCl solution (Otsu-Ns); Mueller Hinton Agar (Oxoid); Nutrient Broth (Oxoid); n-Hexane; and ethyl acetate.

3. Test microorganisms

Bacillus cereus, *Escherichia coli*, and *Staphylococcus aureus* were isolated from the wound isolates of patients with III and IV Wagner diabetic ulcers, as they comprise the highest percentage of infectious bacteria in DFU patients.

4. Culturing *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*

One sengkelit each from *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus* colonies from 24-h-old cultures was suspended in 10 mL of sterile, 0.9% NaCl solution and incubated at 37℃ until turbid. The obtained turbidity was then compared to that of Mc Farland III's standard solution, which is equivalent to growth of 0.9×10^9 bacterial cells per mL. If the turbidity of the culture was equivalent to that of Lar Mc Farland III, the bacterial suspension was used as a test culture. Observation of turbidity was done visually with the help of black and white background paper.

5. Extraction and fractionation of *Melastoma malabathricum* leaves

Melastoma malabathricum leaves were extracted with 96% ethanol for 24 h. The maceration product was filtered, and the filtrate was concentrated using a rotary evaporator until a thick extract was obtained. This extract was fractionated in stages until an ethyl acetate fraction was obtained. The ethyl acetate fraction was separated from the aqueous fraction repeatedly until the ethyl acetate phase was clear. All these ethyl acetate fractions were collected and concentrated using the rotary evaporator.

6. Determination of antibiotic MBC and MBC of the leaf fraction of *Melastoma malabathricum*

The Minimum Inhibitory Concentration (MBC) value was determined using the tube dilution method based on turbidity observations and confirmed using the disk diffusion method with agar media. As the lowest concentration not associated with bacterial growth is the MBC value for that bacterium, all test tubes containing concentrations above the MIC showed no signs of growth or turbidity. To confirm these MIC values, the cultures were inoculated by the streak method and aseptically grown with each concentration in MHA media in Petri dishes. These Petri dishes were incubated for 24 h for 37℃. Nutrient Broth (NB) liquid media (0.5 mL/tube) were added to 36 diluted tubes, and 0.1 mL each of the *E. coli*, *S. aureus*, and *B. cereus* cultures was added to the 36 tubes.

Gentamicin sulfate and ciprofloxacin were dissolved in sterile water, while the leaf fraction of *M. malabathricum* was dissolved in 20% DMSO as no interference with the antibacterial activity of the leaf fraction of *M. malabathricum* was noted for up to 50% DMSO [13]. Next, 0.4 mL of gentamicin sulfate at concentrations of 75%, 65%, 55%, and 45%; ciprofloxacin at concentrations of 95%, 85%, 75%, and 65%; or the leaf fraction of *M. malabathricum* at concentrations of 65%, 55%, 45%, and 35% were added to the tubes containing the bacteria. The tubes were vortexed until homogeneous.

The antibiotics and leaf extract were then tested using the diffusion method. Mueller Hinton's medium was poured into a Petri dish, and 20 μL of the above-mentioned solutions of the antibiotics and leaf extract were spread on the surface of the respective MHA media plates and incubated at 37℃ for 24 h. The lowest concentration that did not show bacterial growth was defined as the MBC.

Table 1. FICI values and combination characteristics

No	FICI values	Characteristics
1	≤ 0.5	Synergistic
2	> 0.5-≤ 1	Additive
3	> 1-≤ 4	No different
4	> 4	Antagonist

7. Determination of FICI value

The FICI (Fractional Inhibitory Concentration Index) value was determined based on the combinations of the leaf fraction of *M. malabathricum* with gentamicin sulfate and the leaf fraction of *M. malabathricum* with ciprofloxacin. The results are shown in Table 1 [2].

8. Data analysis

MBC results were analyzed using the Tukey's Multiple Comparison Statistical Analysis a = 0.05, the results were considered significant if p value < 0.05.

RESULTS

This study examined the antibacterial activity of gentamicin sulfate, ciprofloxacin, and the leaf fraction of *Melastoma malabathricum*. MBC or Minimum Bactericidal Concentration is the lowest concentration that can kill bacteria. The results show that 45% gentamicin sulfate inhibited the growth of *E. coli*, *S. aureus*, and *B. cereus*. Badenoch et al. showed that gentamicin sulfate is effective in killing *P. aeruginosa* with an MBC value of 4 µg/mL [12], while Bazzaz et al. reported MBC values of 80 µg/mL and 0.625 µg/mL for gentamicin sulfate against *S. aureus* and *P. aeruginosa*, respectively [11]. Riwom et al. reported an MBC value of 30 µg/mL gentamicin sulfate against *B. cereus*, *S. typhi*, *S. aureus*, *Shigella* spp, and *E. coli* [8].

Ciprofloxacin showed an MBC value of 65% against *E. coli*, *S. aureus*, and *B. cereus*. Previous studies had shown MBC values of 25 µg/mL, 10-25 µg/mL [10], and 1.83 µg/mL [14] against *S. aureus*, *E. coli*, and *B. cereus*. The leaf fraction of *M. malabathricum* inhibited the growth of *S. aureus* and *B. cereus* at a concentration of 35% and of *E. coli* at a concentration of 55%.

DISCUSSION

The results of this study show that gentamicin sulfate, ciprofloxacin, and the leaf extract of *Melastoma malabathricum* have the ability to kill *E. coli*, *S. aureus*, and *B. cereus*. Combinations of the leaf extract with each antibiotic were tested against all three bacteria to identify synergistic effects.

1. Determination of FICI value

Ciprofloxacin showed an MBC value of 55% against *E. coli*, *S. aureus*, and *B. cereus*, while the leaf extract of *M. malabathricum* inhibited the growth of *S. aureus* and *B. cereus* at a concentration of 35% and of *E. coli* at a concentration of 55%.

Antibiotics and natural ingredients are typically combined in a ratio of 1:1 and the concentration of each component is raised and lowered from its MBC value. The concentrations for the combinations were 8, 4, 2, 1, ½, ¼ from the MBC value of gentamicin sulfate and the leaf extract of *M. malabathricum*, and 2.1, ½, and ¼ from the MBC value of ciprofloxacin and the leaf extract of *M. malabathricum*. Gentamicin sulfate was diluted as follows: 180%, 90%, 45%, 22.5%, and 11.25%; ciprofloxacin was diluted as follows: 110%, 55%, 27.5%, 13.75%; the dilutions of the leaf extract of *M. malabathricum* were 280%, 140%, 70%, 35%, 17.5%, and 8.75% for *S. aureus* and *B. cereus* and 440%, 220%, 110%, 55%, 27.5%, and 13.75% for *E. coli*. Each antibiotic solution was combined with each solution of the extract, and discs were dipped in the resulting mixtures and placed on MHA media plates inoculated with the test bacteria.

The combination of the leaf extract of *Melastoma malabathricum* and gentamicin sulfate gave a FICI value of 0.5, suggesting synergy against both bacteria (Table 2).

Both gentamicin sulfate and the leaf extract by themselves showed bactericidal activity; therefore, their combination showed synergistic antibacterial activity. A similar trend was observed for the combination of ciprofloxacin with the leaf extract of *M. malabathricum*. The FICI value of 0.5 indicated synergistic antibacterial activity against both bacteria (Table 3).

The selection of antibacterial agents is important in the development of cosmetics and health products where the smaller the MBC means higher efficiency of bacterial killing. Tristram et al. [15] showed that the successful eradication of *Hemophilus influenza* by sulfamethoxazole-trimethoprim could be predicted through the MBC test. Future studies will investigate the antibacterial activity of antibiotics that have experienced thera-

Table 2. The diameter of MBC inhibition zones of the leaf fraction of *Melastoma malabathricum* and gentamicin sulfate against bacteria

Combination	The diameter of inhibition zones (mm)											
	Escherichia coli				*Staphylococcus aureus*				*Bacillus cereus*			
	I	II	III	Averages	I	II	III	Averages	I	II	III	Averages
8 + 8	35.8	34.6	42.5	37.63	42	42.8	41.9	42.23	36.5	37.6	38.3	37.47
4 + 4	36	36.8	43.7	38.83	42.1	42.5	44.3	42.97	32.7	35.6	37.6	35.3
2 + 2	38.3	36.9	39.2	38.13	39.8	43.6	36.7	40.03	33.5	39.2	36.5	36.4
1 + 1	36.8	37.6	38.4	37.6	38.7	41.6	35.4	38.57	35.6	36.6	34.8	35.67
½ + ½	34.7	38.3	34.6	35.86	36.8	39.6	32.9	36.43	22.7	31.5	31.8	28.67
¼ + ¼	35.8	35.4	35.4	35.53	35.4	38.6	31.3	35.1	21.9	29.4	28.7	26.67

Table 3. The diameter of MBC inhibition zones of the leaf fraction of *Melastoma malabathricum* and Ciprofloxacin against bacteria

Combination	The diameter of inhibition zones (mm)											
	Escherichia coli				*Staphylococcus aureus*				*Bacillus cereus*			
	I	II	III	Averages	I	II	III	Averages	I	II	III	Averages
2 + 2	35.7	37.7	38	37.13	36.9	34.8	39.3	37	42.8	46.8	33.8	41.13
1 + 1	33.8	36.9	33.8	34.83	37.4	35.7	36.7	36.6	41.6	46.4	33.6	40.53
½ + ½	34.6	35.6	35.4	35.2	33.8	36.7	36.8	35.77	36.9	38.9	32.9	36.23
¼ + ¼	29.5	28.9	34.9	31.1	29.4	33.6	37.6	33.53	35.4	36.4	33.4	35.07

peutic failure due to resistance to bacteria, especially in diabetic ulcer patients.

2. Data analysis

The MBC results were analyzed using the Tukey's Multiple Comparison Statistical Analysis method with $\alpha = 0.05$. The results were considered significant if $p \leq 0.05$. Almost all tests of the combination of the leaf extract of *M. malabathricum* and gentamicin on the three bacteria showed a value of $p > 0.05$; only a few concentrations showed $p < 0.05$, such as for *S. aureus* at concentrations of "4 + 4" and "¼ + ¼". The results were significant for *B. cereus* at concentrations of "¼ + ¼", "8 + 8", "2 + 2", and "1 + 1". The combination of the leaf extract of *M. malabathricum* and ciprofloxacin showed significant antibacterial activity at concentrations of "2 + 2" and "¼ + ¼" against only *E. coli*.

CONCLUSION

Our results show that the nanospray formulation based on the self-nanoemulsifying drug delivery system (SNEDDS) containing the combination of the leaf extract of *Melastoma malabathricum* with gentamicin and ciprofloxacin has the potential to be a superior antibacterial agent against three pathogenic bacteria isolated from DFU wounds, indicating that it can reduce the severity of the infection of DFU wounds.

CONFLICT OF INTEREST

During the research, there is no conflict of interest in this research.

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ORCID

Rafika Sari, https://orcid.org/0000-0002-8477-119X
Liza pratiwi, https://orcid.org/0000-0003-3359-2025
Pratiwi Apridamayanti, https://orcid.org/0000-0001-5620-9813

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