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Synergistic Antibacterial Activity of *Curcuma domestica* Val. Extract with Tetracycline Against Multidrug-resistant *Acinetobacter baumannii*

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ABSTRACT. Infections caused by multidrug-resistant (MDR) bacteria are on the rise globally. MDR is facilitated by overexpression of efflux pump and permeability changes of membrane. *Acinetobacter baumannii* is a pathogenic germ that causes a major problem in infection, also, there has been an increase in the incidence of resistance to various antibiotics. The present study highlights the synergistic of ethanolic extract of *Curcuma domestica* (EECD) rhizome with tetracycline against multidrug-resistant *A. baumannii* (MDR-Ab). Assessment of Minimum Inhibitory Concentration (MIC) was determined by microdilution using 96-well plates. The synergistic effect of EECD and tetracycline was determined by checkerboard method. The effect of EECD and tetracycline combination was investigated by bacteriolytic activity and inhibition of efflux pump by Ethidium Bromide (EtBr) accumulation assay. EECD presented the MIC value 250 μg/mL against MDR-Ab. Fractional Inhibitory Concentration Index (FICI) value of EECD and tetracycline combination was 0.4, which showed their synergistic effect. Additionally, the combination of EECD and tetracycline could inhibit the efflux pump in MDR-Ab. This combination can also compromise cell integrity by altering membrane permeability thus lysing the bacteria cells. According to these results, EECD and tetracycline combination has synergistic effects at some sites of action, and thus could be used as a breakthrough to overcome infection problems due to MDR-Ab.

Keywords: *Acinetobacter baumannii,* antibacterial, *Curcuma domestica*, multidrug-resistant, tetracycline

INTRODUCTION

Microbes, the most adaptable life forms on Earth, include organisms like bacteria, viruses, fungi, and parasites. These microscopic entities are remarkable but can cause various illnesses, often controlled by antimicrobials. However, when microbes become resistant to these medications, it leads to a global threat known as antimicrobial resistance, which is a crucial public health crisis that impacts everyone. This occurs when microorganisms, such as bacteria, adjust and become immune to the antibiotics used for treating infections. According to the World Health Organization, incomplete treatment allows bacteria to adapt and become resistant (WHO, 2014).

Acinetobacter baumannii is one of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) group pathogens, categorized by WHO as "priority status" (WHO, 2017). These bacteria have developed resistance mechanism against β-lactams, macrolides, tetracycline, fluoroquinolones, and other antibiotics groups (Naylor et al., 2018).

Currently, only a few are doing the development of antibiotics for these bacteria due to the high rate of antibiotic resistance. The discovery of natural compounds as antibacterial is one strategy that can be applied. Various new approaches, based on the mechanisms of bacterial resistance, are mainly being explored by scientists (Weledji et al., 2017).

The mechanisms of bacterial resistance include the production of enzymes that can modify antibiotics, the presence of efflux pumps, modification of cell membrane permeability, and modification of drug target sites of action (Lee et al., 2017). Secondary metabolites from plants mostly work in inhibiting these

mechanisms (Pancu et al., 2021). Based on the mechanism of action of secondary metabolites from plants, it is used as a potential approach to combine plant extracts with conventional antibiotics that would increase effectiveness in eradicating pathogenic bacteria, by providing access to these antibiotics to reach their site of action (Panichayupakaranant et al., 2019).

Curcuma domestica is one of the most used condiment in Indonesia, which has a distinctive yellow color due to the content of curcuminoid compounds consisting of curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Singh et al., 2017). The rhizome extracts of *Curcuma longa*, another species of *Curcuma* genus, have some pharmacological activities such as (Hatcher et al., 2005), antioxidant (Rudrappa and Bais, 2008), anti-inflammatory (Siddiqui et al, 2006), anticancer (LoTempio et al., 2005), and antibacterial (Mohammadi et al., 2005). *Curcuma mangga*, has an immunomodulatory effect and could be used as an immunotherapeutic agent (Yuandani et al., 2021). According to Singh et al. (2017), the rhizome extract of *Curcuma longa* has a more potent antimicrobial effect than the leaf extract against some bacteria species such as *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Bacillus subtilis*.

As urgency of multi-drug resistant (MDR) bacteria rises, and the potential of secondary metabolite in *Curcuma* species as an antibacterial agent, this present study is conducted to explore the synergistic activity between ethanolic extract of *Curcuma domestica* (EECD) and tetracycline in order to inhibit the resistant mechanism of bacteria include the bacteriolytic activity and inhibition of efflux pump existed on multidrug-resistant *A. baumannii* (MDR-Ab).

EXPERIMENTAL SECTION

Chemical and Media

Tetracycline powder as antibiotic standard, sodium chloride, and crystal violet were purchased from Sigma-Aldrich, United Kingdom. Phosphate buffer saline (PBS) tablets were obtained from Oxoid Limited, UK. Dimethyl sulfoxide (DMSO) was from Merck, while brain heart infusion (BHI) broth and agar base were purchased from HiMedia, India. Ethanol 96% and distilled water were obtained from National Research and Innovation Agency.

Plant Materials

Rhizomes of *C. domestica* (voucher number 192/MEDA/2022) were collected from a central market in Medan, North Sumatera, Indonesia. This plant was identified by Medanense Herbarium (MEDA), Universitas Sumatera Utara.

Bacterial Strains and Growth Conditions

The MDR-Ab strains were collected from several clinical isolates conducted by Marine Education and Research Organization Foundation in Bali, Indonesia. The strain was stored in a specialized bacterial storage refrigerator added with glycerol stock at −80 °C and re-grown in BHI agar at 37 °C for 18-20 hours before being used for analysis in this study.

Extraction Process

Fresh rhizome of *C. domestica* were cleaned, cut into small pieces, and put in the oven at temperature 45-50 °C. Dried samples were grounded and passed through 20 mesh sieve to obtain a fine samples powder. Extraction was carried out by maceration using ethanol solvent (1:10 w/v) with a sample weight of 200 g. Then the extract was concentrated using a rotary evaporator maintained at 50-60 °C.

Antibacterial Assay with Microdilution Method

Antibacterial activity assay was carried out using the microdilution method to obtain Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values. The determination of MIC was carried out according to the Guidelines of Clinical Laboratory Standard Institute M7-A6 (2008). Bacterial suspension was prepared on BHI broth (BHIB) and adjusted to 0.5 McFarland standard (- 1.5 x 10^8 CFU/mL), and then diluted with 0.9% NaCl sterile solution to obtain 1.5 x 10^6 CFU/mL bacterial suspension. Briefly, 100 µL BHI broth) was added to each well, then the test sample was added and diluted with the mix solution using two-fold dilution method to obtain serial dilutions in the range of 500 µg/mL to 3.9 µg/mL. Then, 100 µL of the previously prepared bacterial suspension was added to each well. Tetracycline solution was used as a comparison and DMSO 0.5% v/v as a negative control. Microplates were incubated at 37 °C for 24 hours. MIC value shown by the smallest concentration which gave a clear solution without precipitation in each well.

MBC is the minimum concentration of an antimicrobial agent that does not show bacterial growth on BHI agar media. This assay was done by transferring colonies at MIC concentrations into a petri dish containing solid media, then incubated for 24 hours at 37 °C. After incubation, the bactericidal concentration was determined from the smallest concentration that showed no microbial growth in petri dish.

Checkerboard Assay

The assay was planned to examine the interactions between the extract and antibiotic against the tested bacteria (Septama et al., 2022). The EECD was diluted with BHI broth media by two-fold dilution that was performed along the x-axis of 96-well plate. Then, tetracycline was prepared by two-fold dilution along the y-axis. The MDR-Ab bacterial suspension with a concentration of approximately 1×10^6 was added to each well and incubated at 37 °C for 24 h. Following incubation, the fractional inhibitory concentration index (FICI) was calculated using the formula below to determine the interaction between EECD and tetracycline.

$$\text{FICI} = \frac{\text{MIC of extract in combination}}{\text{MIC of extract alone}} + \frac{\text{MIC of tetracycline in combination}}{\text{MIC of tetracycline alone}}$$

The index was categorized into synergistic (FICI ≤ 0.5), additive (0.5 ≤ FICI ≤ 1), indifferent (1 ≤ FICI ≤ 4), and antagonistic (FICI > 4) (Septama et al., 2017).

Bacteriolytic Activity Assay

This assay was performed with several modification from previous reported studies to analyze the bacteriolytic properties of EECD on MDR-Ab proliferation (Li et al., 2022). The bacteriolytic activity is related to the ability of the extract to alternate the permeability of bacterial cell membrane, measured by determining the maximum absorption of intracellular material out of the cell due to the damaged cell membrane integrity, and also by measuring the reuptake of crystal violet used as indicators that bind to the cell membrane. The dose of tested samples was referred to the results of MIC and checkerboard assay. The bacterial suspension was prepared in normal saline to an optical density (OD) of approximately 0.4 at 600 nm. As much as 500 µL of the sample solution in various concentrations was put into the Eppendorf Tube with 250 µg/mL of EECD, 31.25 µg/mL of tetracycline, and the combination of EECD and tetracycline with concentration 31.25 µg/mL and 7.8 µg/mL respectively. The 500 µL of the prepared bacterial suspension was then added to the tubes before being centrifuged at 13000 rpm for 1 h. Furthermore, the supernatant was transferred to a microplate to measure OD_{260}. This analysis also aims to measure the absorbance of nucleic acids that come out because of the leakage of the bacterial cell membrane. In addition, the crystal violet uptake assay was performed to confirm the effect of EECD on the membrane destabilization. The precipitate obtained from the previous treatment, was added with 500 µL of 0.001% crystal violet. Afterwards, the tube was centrifuged at 13000 rpm for 15 min. The OD_{590} of the supernatant was measured to determine the absorption of crystal violet that is not bound to the bacterial cell membrane or the free crystal violet.

Efflux Pump Inhibitor Assay

This assay was performed in modest modification referring to the method by Tran et al., (2020) to analyze the potential of EECD to accumulate EtBr in MDR-Ab. The MDR-Ab was cultivated in 10 mL of BHIB at a temperature of 37 °C. After centrifugation of the bacterial suspensions (3000 rpm for 15 min), the supernatant was discharged, and the pellet was rinsed with PBS pH 7.3 and diluted in normal saline until OD_{600} of 0.4 was achieved. A 50 µL of test solution in various concentrations (same as the previous assay) and 100 µL the cultures were combined in each well in the microplate. The negative control used was DMSO 0.5% v/v. The microplate was incubated for 30 min at 37 °C. Afterward, 50 µL EtBr 0.5 mg/L was added to each well in a suitable condition to keep the stability of EtBr solution.

Efflux pump inhibitory activity was determined by measuring the fluorescence accumulation of EtBr. The fluorescence was measured at 37 °C by a fluorimeter with 530 nm excitation and 600 nm emission wavelengths as a parameter set periodically at 0, 5, 15, and 45 min. The assay was repeated three times and the results were reported in Ratio Fluorescence Unit (RFU) of EtBr.

Data Analysis

The experiments were performed in triplicate. Data were expressed as mean ± standard deviation (SD) and analyzed using SPSS v.26 program. The comparison between groups were assessed by ANOVA with post-hoc Tukey HSD test where p-value <0.05 indicating a significance.

RESULTS AND DISCUSSION

An antibacterial assay was conducted to establish the MIC value of EECD against MDR-Ab, which was 250 µg/mL, as shown in **Table 1**. A previous study has also documented that the ethanolic extract derived from the rhizome of *C. longa* has a MIC value of 25 mg/mL against *Pseudomonas aeruginosa* (Singh et al., 2017). The antibacterial activity of *C. longa* has been tested not only in bacteria, but also in the case of fungal infections produced by dermatophytes in guinea pigs. Topical use of turmeric oil on experimental animals demonstrated the inhibition of dermatophytes and growth of harmful fungus. There was an improvement in dermatophyte lesions in guinea pigs infected by fungi after 7 days of turmeric application, and the lesions disappeared (Dujic et al., 2009). Negi et al. (1999) have previously reported data on secondary metabolites in *C. domestica* that exhibit antibacterial activity. The chemicals curlone and turmerone found in *C. domestica* exhibit antibacterial properties against a range of pathogens including *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Furthermore, it has been documented that the antibacterial efficacy of *C. domestica* is associated with the existence of valeric acid, turmerol, curcuminoids, essential oils, and turmeric oil (Momoh et al., 2022).

Interaction between EECD and tetracycline was determined by checkerboard assay and the result was presented in **Table 1**. This combination against MDR-Ab produced a synergistic effect with FICI value of ≤ 0.5. This finding demonstrated that the required dose in their combination was less than the dose of single compound to inhibit the growth of bacterial cells. Based on FICI value, the combination of EECD and tetracycline against MDR-Ab had a very potent activity. Previous study also reported that combination of curcumin with ampicillin and gentamycin significantly improved the efficacy of antibiotic against *Enterococcus faecalis*. Similar result was also observed in the combination of curcumin with gentamycin and ciprofloxacin against MDR *S. aureus* (Górski et al.,

2022). This combined activity can enhance the effectiveness of antibiotics by leveraging their distinct targets of action. Flavonoids interacting with lipophilic membranes cause a reduction in membrane fluidity, leading to the occupancy of the target site of action (Górski et al., 2022). Additional research on the combination of two substances has found that Cellulose nanofiber/AgNp-chitosan at a ratio of 80:20 exhibits the most effective antibacterial capabilities against *Pseudomonas aeruginosa* and *Bacillus subtilis* (Hasibuan et al., 2021).

Antibiotic resistance arises from the transmission of resistance genes through plasmids, as well as from changes in target genes (Andersson and Hughes, 2010). The efficacy of the EECD against a range of antibiotic-resistant bacteria was assessed using optical density measurements at 260 nm, which can quantify the release of nucleic acid components from cells, and at 590 nm, which can quantify the presence of free crystal violet in a solution test. Compared to the negative control (DMSO 0.5% v/v), the combination of EECD (31.25 μg/mL) and tetracycline (7.8 μg/mL) showed significantly improved results against MDR-Ab, as depicted in **Figure 1A**. The proportion of crystal violet absorption exhibits a nearly identical correlation with the results of the cell membrane permeability assay (**Figure 1B**). Nevertheless, the group that received EECD treatment had a greater proportion of crystal violet absorption in comparison to both the untreated group and the group treated solely with tetracycline. The concurrent administration of EECD and tetracycline to MDR-Ab demonstrated synergistic effects in the assessment of cell membrane integrity and permeability.

Table 1. MIC and FICI value of EECD and tetracycline against MDR-Ab

Samples	MIC a (μg/mL)	MBC (μg/mL)	MIC c (μg/mL)	FICI	Interaction
EECD	250	500	31.25	0.4	Synergistic
Tetracycline	31.25	31.25	7.8		

Note : MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration, a = substance alone; c = substance in combination, EECD = ethanolic extract of *Curcuma domestica*

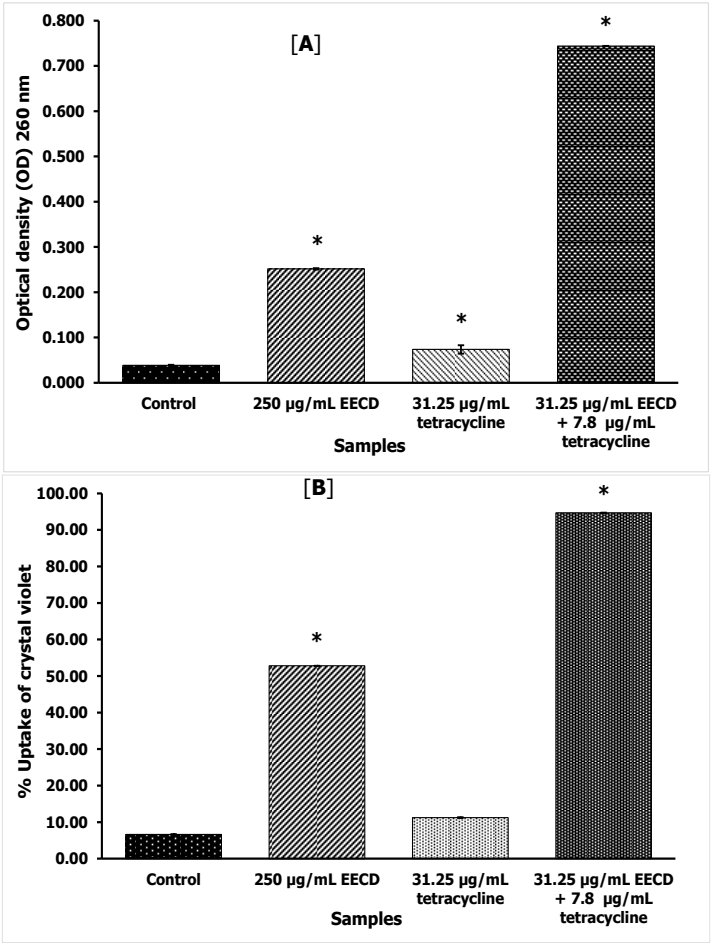

Figure 1. Effect of combined EECD and tetracycline on alteration of permeability of MDR-Ab membrane cell [**A**] OD_{260}, [**B**] % uptake of crystal violet. *samples present significant differences compared to control ($p < 0.05$)

Curcumin, a significant component found in turmeric, is deemed safe for oral administration in the treatment of bacterial infections. Multiple investigations have demonstrated that curcumin possesses antibacterial properties against both Gram-negative and Gram-positive bacteria. Curcumin exerts its antibacterial effects by disrupting the bacterial membrane, inhibiting the generation of bacterial virulence factors and biofilm formation, and inducing oxidative stress. These properties also help to explain how curcumin functions as a broad-spectrum antibacterial adjuvant. This is supported by the significantly enhanced or synergistic effects shown when combined with several types of conventional antibiotics (Dai et al., 2022). Flavonoids are also one of the secondary metabolites found in EECD, which have the mechanism to disrupt the integrity of the bacterial cell membrane thus increasing the permeability of the cell membrane. Hence, the synergistic impact of EECD and tetracycline can facilitate the cellular penetration of tetracycline and its occupancy of the target location (Moghadam et al., 2010). *C. domestica* possesses a high concentration of essential oil. According to a study conducted by Kebede et al. (2021), the essential oil derived from the rhizomes of *C. domestica* contains various significant components. These include oxygenated sesquiterpenes such as α-Turmerone, ar-Turmerone, and curlone, as well as sesquiterpenes like α-Curcumene, α-Zingiberene, and β-Sesquiphellandrene. This study supports the findings of Oyemitan et al. (2017) which revealed a significant resemblance in the predominant constituents of the volatile oil, specifically α-Turmerone, curlone, and ar-turmerone. The essential oil found in *C. domestica* can disturb the structure of cells, leading to the disruption of cell membranes integrity, including the proton pump in bacterial cells (Saad et al., 2013).

Another method of bacterial resistance is the upregulation of efflux pumps. The EtBr accumulation experiment was employed to verify that EECD directly inhibited the efflux pump in MDR-Ab. **Figure 2** demonstrated that EECD at a concentration of 250 μg/mL enhances the accumulation of EtBr in MDR-Ab when combined with tetracycline. The *Curcuma* genus is characterized by a high concentration of essential oils, which are volatile and complex compounds with a characteristic odor produced by aromatic plants as secondary metabolites. They have been commonly employed for antibacterial, antiviral, antifungal, antiparasitic, and therapeutic applications (Alam et al., 2022). Monoterpenes, such as menthol, geraniol, and thymol, exert an influence on both Gram-positive and Gram-negative bacteria. Their efficacy stems from their ability to block the efflux pump (Mahizan et al., 2019).

Furthermore, this collaborative impact can be ascribed to the suppressive influence of curcumin on the functions of efflux pumps. The effect of curcumin and other antibiotics against bacterial resistance has been reported in many other studies. The combination of curcumin and polymyxin for treating bacterial infections offers an additional benefit: it significantly improves the therapeutic index of polymyxins by inhibiting polymyxin-induced cytotoxicity, neurotoxicity, and nephrotoxicity (Dai et al., 2020). Studies have indicated that the combination of curcumin and vancomycin has a synergistic impact on multidrug-resistant clinical *Klebsiella pneumoniae* isolates. This putative method may rely on the combined impact on cell membrane permeability (Ahmida, 2012). The mechanism observed in this research is highly likely to have a comparable interaction. EECD enhances cell membrane permeability and inhibits efflux pumps in bacteria, facilitating the entry of tetracycline into bacterial cells and maintaining a concentration that effectively hinders the growth of pathogenic bacteria. Combining plant extracts

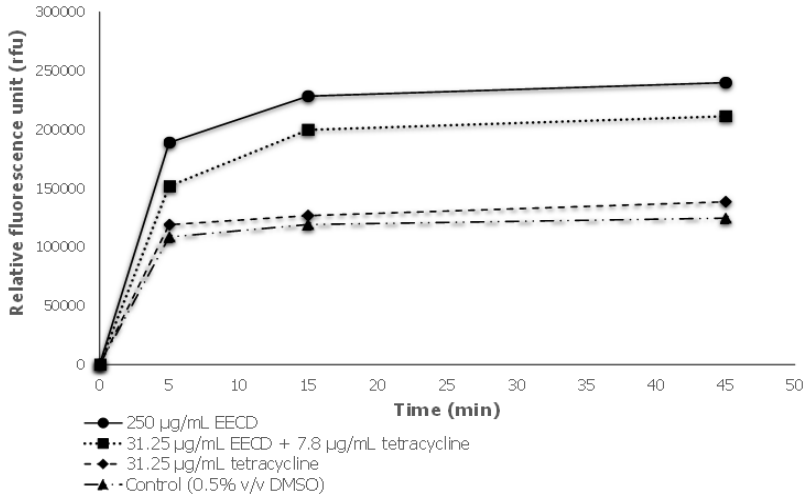

Figure 2. Effect of EECD in accumulation of EtBr in MDR-Ab bacterial cells

with antibiotics has been effective in treating bacterial infections and addressing drug resistance. Combining natural antibacterial compounds with antibiotics can help lower antibiotics consumption and address global resistance.

CONCLUSIONS

The study demonstrated the possibility of combining EECD and tetracycline to block resistance mechanisms in bacterial strains, particularly in MDR-Ab. In addition, the combination of EECD and tetracycline can hinder the production of the efflux pump in MDR-Ab. This combination also induces cell death and compromises cell integrity by modifying membrane permeability. The findings provide a potential strategy to address the issue of drug resistance, by utilizing the combination of natural substances with antibiotics. Additional research is required to provide comprehensive data on the toxicity and safety of the substances, as well as to investigate the molecular-level effects of secondary metabolites in the extracts.

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