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Research Article

EVALUATION OF THE COMBINED ANTIBACTERIAL ACTIVITY OF *KAEMPFERIA PANDURATA* RHIZOME AND *SENNA ALATA* LEAVES AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Objectives: The present study was performed to show the combination *in vitro* activities of ethanolic extract and ethyl acetate fraction of *Kaempferia pandurata* and *Senna alata* against methicillin-resistant *Staphylococcus aureus* (MRSA).

Methods: The antibacterial activities were calculated based on minimum inhibitory concentration (MIC) using microdilution method and minimum bactericidal concentration. The antimicrobial interaction between plant extract with antibiotic was performed using the paper disc and checkerboard method.

Results: The similar MIC value was displayed by ethyl acetate fraction of *K. pandurata* and *S. alata* (128 µg/mL) while the ethanolic extract of *K. pandurata* showed lower than *S. alata*, 512 µg/mL and 256 µg/mL, respectively. The combination between the ethanolic extract of *K. pandurata* with *S. alata* showed synergism in all MIC tested and its fraction combination showed synergism in selected MIC value.

Conclusions: The observed antimicrobial efficacy and synergistic interactions indicate the beneficial aspects for the MRSA treatment.

Keywords: Antimicrobial, Interaction, *Kaempferia pandurata, Senna alata*, Minimum inhibition concentration, Checkerboard, Synergism.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterial pathogen responsible for a variety of infections especially skin and nose which commonly seen in patients of all ages. MRSA infection is an important cause of nosocomial infections worldwide which showed raising morbidity and mortality [1]. MRSA infections are more difficult to treat than ordinary staphilococci type infections. This is because of MRSA strain do not respond well to many common antibiotics used to kill bacteria [2].

The antibiotic sometimes have considerable limitations regarding antimicrobial spectrum, side effects, and their inappropriate use has led to increasing clinical resistance of previously sensitive microorganisms and to the occurrence of uncommon infections [3]. This perspective has put pressure on pharmaceutical research to obtain its goals concerning antimicrobial activity, especially in MRSA treatment. One of the alternatives is exploring and discovering pharmacological effect from traditional plants. The plants have been used since ancient times to treat disease including infection because they contain many bioactive compounds that can be of interest in therapeutic. *Kaempferia pandurata* extract and *Senna alata* have been reported to have antibacterial activity. The previous study showed that *K. pandurata* and *S. alata* were susceptible to selected bacteria in a varied value of minimum inhibitory concentration (MIC) [4,5]. However, none of the investigations on these plants extract and fraction combination have been conducted. The extract combination is one of the strategies to overcome the increasing emerging infectious and antibiotic resistance. Combinations of extracts can modify the antimicrobial activity which potentially exhibiting synergism, antagonism, additive, or indifferent effects. Synergy, the interaction of compounds to create more profound microbial action, may be an important factor in using spices for antimicrobial actions. The additive effect is equal to the individual effects, whereas the antagonistic effect is less potent than individual effects [6]. The synergism should be developed to enhance antimicrobial potentiation while antagonistic should be avoided due to emerging resistance bacteria. Hence, the objective of this present study is to determine the *in vitro* activities of ethanolic extract of *K. pandurata* rhizome in combination with ethanolic extract of *S. alata* leaves against MRSA.

METHODS

Materials

Plants grinder, rotavapor, autoclave, microplate 96-wells, shaker, laminar air flow, Eppendorf, micropipette, separation funnel, glass set, chromatography set, cuved, ethanol, dimethyl sulfoxide (DMSO), Mueller-Hinton Broth, Mueller-Hinton Agar (MHA), vancomycin, tetracycline.

Plants

The dried *K. pandurata* (Roxb.) rhizome and *S. alata* leaves collected from Manoko field in Bandung, respectively. The collected plants were identified and classified according to the herbarium Bandungense at the School of Technology and Life Science Research Centre.

Preparation of plant extract and fraction

K. pandurata (Roxb) and *S. alata* were extracted by the reflux method using ethanol 96%. The solvent contained in the extracts was completely removed by a rotary evaporator to obtain a semi-solid mass, and the yield was calculated based on the weight of the dried plants. Then, extracts were filtered using separation funnel. Three repetitions were performed. After filtration, each mixture was evaporated under reduced pressure (at 60°C and 50 rpm) using a rotary evaporator to obtain crude extracts. A portion of resulting crude extract was fractioned by separation funnel using solvent ethyl acetate. Eluates were collected in 1-L Erlenmeyer flasks, and each fraction was subjected to evaporation under reduced pressure in a rotary evaporator. Fractions were stored at 4°C until assayed.

Test microorganism preparation

The MRSA was taken from isolated specimens, which exhibited resistance to some antibiotics in hospitalized patients. They were taken based on ethical clearance approval from the ethical committee in the hospital. The bacteria were cultured overnight (18-24 hrs) at 37°C on nutrient broth for the preparation of cell suspensions. The bacteria cell suspensions were homogenized and adjusted to 0.5 McFarland standards (5×10^5 CFU/mL) using spectrophotometry.

Antimicrobial activity

Determination of MIC

The MIC of *K. pandurata* and *S. alata* was initially determined using Mueller-Hinton Broth Microdilution [7]. MIC determination was performed by a serial dilution technique using 96-well microtiter plates. The extract was dissolved in broth medium with inoculum to achieve the desired concentrations (0.05-20 mg/ml). Microplates were incubated for 24 hrs at 37°C. The lowest concentrations without visible growth were defined as concentrations which completely inhibited bacterial (MICs). DMSO was used as a control while tetracycline and vancomycin were used as a positive control. The assay was repeated twice with three replicate per assay.

Determination of minimum bactericidal concentration (MBC)

The MBC was determined by sub-culturing the test dilution onto a fresh drug-free solid medium (MHA) and incubated further for 18-24 hrs at 35°C±2°C. The highest dilution that yielded no signal bacterial colony on the solid medium was taken as MBC. Two repetitions were performed.

Determination of combination interaction using double disc synergy method

The inoculum was grown in MHB for 4-6 hrs. at 370C and then lawn culture was made on Mueller Hinton Agar plate. After drying of inoculum the antibiotic discs are placed at a distance of sum of zone radii for each antimicrobial's zone of inhibition, which was obtained when antimicrobials were tested alone and incubated for 24 hr at 37 0C. The data were analysed according to CLSI standards for the antagonism, indifference and synergism after visualization of the pattern of inhibited zone [8].

Determination of combination interaction using microdilution checkerboard method

The extract combination effect was determined using microdilution checkerboard method. Each extract concentration to at least quadruple the MIC and quadruple dilutions of each plant extracts in each well. The *K. pandurata* extract was serially diluted along the abscissa while the *S. alata* extract was diluted along the ordinate. Each suspension well was inoculated with 100 µl of the culture. All the tubes were incubated at 35°C±2°C for 18-24 hrs for bacteria. After incubation, the growth was observed by visual observation with naked eye detection for the test organism growth. Fractional inhibitory concentration index was used to interpret the results. The combination is considered synergistic when the fractional inhibitory concentration of combination (FICC) is <1. Additive was indicated by an FICC=1, whereas antagonism when the FICC is >1. The FICs were calculated as follows: FICC=FIC A+FIC B, where FIC A is the MIC of drug A in the combination/MIC of drug A alone, and FIC B is the MIC of drug B in the combination/MIC of drug B alone [9].

RESULTS

The antimicrobial activity

The results of the antibacterial activity of each plant extracts are presented in Table 1. Among both plant extracts studied, *K. pandurata* extract had the same MIC value with its ethyl acetate fraction while *S. alata* fraction had lower MIC value than its extract.

Antimicrobial interaction of combination of plant extract and fraction

The FICC index for the combination of both plant extracts or fraction combinations against MRSA resulted synergistic activity. No additive, indifference or antagonism being observed in those combinations. The details FIC index can be seen in Table 2. The antimicrobial interaction of *K. pandurata* and *S. alata* extracts showed a synergistic effect from paper disc method and confirmed using checkerboard method while *K. pandurata* and *S. alata* extracts showed no interaction in paper disc method but showed various interactions in different MIC value.

DISCUSSION

In light of the emergence of infections and increase of bacteria drug-resistance especially caused by MRSA infection in clinical setting new approaches to overcome this problem. One of the strategies employed in traditional herbal medicine to overcome these mechanisms is the combination of herbal remedies. Plant-derived antimicrobials have a long history of providing the much-needed novel therapeutics [10]. The pharmacological effects of such mixtures could be as a result of the total sum of different classes of compounds with diverse mechanisms of action. The antimicrobial activities of *K. pandurata* rhizome and *S. alata* leaves against several bacteria have been reported by several research groups [11-13]. In this study, we investigated the antimicrobial activity and interaction effect of plant extract and also its fraction.

In our study, antimicrobial activity using microdilution method showed that each of the extracts and fraction tested in the present study displayed antibacterial activity against MRSA in various MIC values. *K. pandurata* ethanolic extract showed the lower MIC value than *S. alata* ethanolic extract. This was due to panduratin A as an active compound which responsible in antibacterial activity [14]. In contrast, the ethyl acetate fraction from each plant showed the similar result (128 µg/mL).

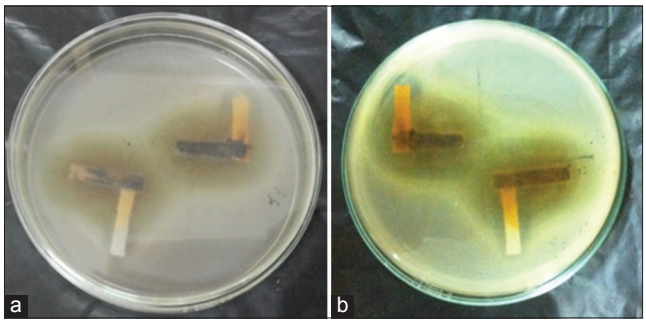

Fig. 1: The pattern of the plants combination, (a) The combination of ethanolic extract of *Kaempferia pandurata* and *Senna alata*, (b) The combination ethyl acetate fraction *K. pandurata* and *S. alata*

Table 1: The antimicrobial activity of plant extract and fraction against MRSA

Plants	Antimicrobial activity			
	MIC (µg/mL)		MBC (µg/mL)	
	Ethanolic extract	Ethyl acetate fraction	Ethanolic extract	Ethyl acetate fraction
K. pandurata	256	256	128	128
S. alata	512	256	>2048	1024
Tetracycline HCl	32		ND	ND
Vancomycin HCl	1		ND	ND

ND: Note done, MRSA: Methicillin-resistant *Staphylococcus aureus*, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *K. pandurata: Kaempferia pandurata, S. alata: Senna alata*

Table 2: The *in vitro* antimicrobial interaction of plant extracts or fraction combination

Combination		FICK	FICS	FICC	Interpretation
K. pandurat ethanolic extract	*S. alata* ethanolic extract	0.5	0.5	1	Additive
K. pandurata ethyl acetate fraction	*S. alata* ethyl acetate fraction	2	1	3	Antagonism

K. pandurata: Kaempferia pandurata, S. alata: Senna alata, FICK: Fractional inhibitory concentration of *K. pandurata*, FICS: Fractional inhibitory concentration of *S. alata*, FICC: Fractional inhibitory concentration of combination

These results demonstrate that ethyl acetate fraction of *K. pandurata* and *S. alata* present a high antibacterial activity. By this result, we combined the two plant fraction compared to the extract form to determine the interaction effect. Surprisingly, the extraction combination showed additive using paper disc method (Fig. 1). These results were confirmed using checkerboard method and found that the extraction combination showed synergism in all experiments. This result revealed that even though a single *K. pandurata* had good antibacterial activity, but the activity can be enhanced if it was combined to *S. alata*. In contrast with the fraction combination, antagonism is showed by FICC value is 3. The results probably indicate that the efficacious interaction effect may be dependent on the certain compounds in an extract combination. From this study showed the single use of the extract showed better efficacy than its combination form.

The aspect of synergistic mechanisms becomes the apparent strategy employed by plants; hence, the improved efficacy demonstrated by combining the within plants extracts in this study [15]. Synergism is the most desirable effects of combination and beneficial to treat bacteria infection [16]. The synergistic effect of plant-antimicrobial combination probably due to the active phytochemicals in the plant that acted synergistically with each of the antibiotics to produce significant antibacterial effects at their supposed target sites.

This study provides novel information about the antimicrobial activity of *K. pandurata* and *S. alata* against MRSA infection. The single use of each extract especially *K. pandurata* alone showed best result towards MRSA. The panduratin A has been reported to have the ability to reduce the biofilm of multispecies oral bacteria in vitro [14]. Biofilm is a complex agglomeration of microbes adhering to a solid surface and to one another, all encased in a scaffold of self-produced extracellular polymeric substances. Several pathogenic bacteria are capable of forming biofilms including S. aureus [17,18]. Thus, further pharmacological tests using in vivo models are therefore necessary to help confirm and further ascertain the efficacious properties of such combinations in living systems.

CONCLUSION

The results of this study provide clear evidence that the full potential therapeutic value from synergistic interaction which observed in the combination of *K. pandurata* and *S. alata* against MRSA. This study reveals that the combined use of plant extracts and antimicrobial agent can be useful in eradicating MRSA strain. Further, pharmacological tests using *in vivo* models are, therefore, necessary to help confirm and further ascertain the efficacious properties of such combinations in living systems.

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