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Research Article

ANTIBACTERIAL INTERACTION OF COMBINATION OF ETHANOLIC EXTRACT OF *ZINGIBER OFFICINALE* VAR RUBRUM RHIZOME, *BOESENBERGIA PANDURATA* RHIZOME, AND *STEVIA REBAUDIANA* LEAVES WITH CERTAIN ANTIBIOTICS AGAINST INFECTIOUS MOUTH MICROBIAL

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ABSTRACT

Objectives: The present study was performed to show the *in vitro* activities of ethanolic extract of *Zingiber officinale var rubrum* rhizome, *Boesenbergia pandurata* rhizome, and *Stevia rebaudiana* leaves in combination with amoxicillin, vancomycin, or ketoconazole against infectious mouth microbial.

Methods: The antibacterial activities were calculated based on minimum inhibitory concentration (MIC) using microdilution method and minimum bactericidal concentration, and the antifungal activity were calculated using minimum fungicidal concentration using agar diffusion method. The extract-antibiotic interaction was performed using checkerboard method.

Results: Among the three extracts studied, *B. pandurata* rhizome extract had the best antimicrobial activity against *S. aureus* and *S. mutans* with MIC value at 128 µg/mL and 64 µg/mL, respectively while *Z. officinale* rhizome extracts had the best antifungal activity against *C. albicans* with MIC value at 2048 µg/mL. The antimicrobial interaction between vancomycin with *S. rebaudiana* against *S. aureus* and *S. mutans* showed a synergistic effect. Synergism also observed in the combination of amoxicillin with *B. pandurata* or *S. rebaudiana* against *S. aureus* and *S. mutans,* respectively. The combination of ketoconazole with each of all plant extracts studied against *C. albicans* showed the synergistic effect. The additive interaction was observed between amoxicillin with *B. pandurata* or *S. rebaudiana* against *S. aureus* and *S. mutans,* respectively. There was no antagonism being observed in this study.

Conclusion: There is a synergistic interaction between plant extract and the antibiotic against *S. aureus, S. mutans,* and *C. albicans*, therefore, the combinations may be useful for infectious mouth microbial treatment.

Keywords: Antimicrobial, Interaction, *Zingiber officinale var rubrum*, *Stevia rebaudiana*, *Boesenbergia pandurata*, Ketoconazole, Amoxicllin, Vancomycin, Minimum inhibition concentration, Microdilution, Synergistic, Additive.

INTRODUCTION

Mouth and dental infection caused by bacteria and fungus is very common but rarely recognized by patients. The patients will recognize the infection if the condition getting worse. Dental caries and candidiasis are the diseases due to microbial infection in the mouth. Dental caries is an infectious microbial disease that results in localized dissolution and destruction of the calcified tissues of the teeth. The early stage of dental caries is characterized by a destruction of organic compound resulted cavity caused by acids from substrates. This acid can disrupt the balance between enamel and its surrounding resulting enamel mineral dissolution [1]. Candidiasis is a fungal penetration of the epithelial cells facilitated by produced lipase, and the fungus should desquamate the surface of epitelial cells spontaneoulsy due to fungus survival stage in the epitelial [2]. The disrupted balance is caused by bacteria or fungus such as *Streptococcus mutans*, *Staphylococcus aureus*, and *Candida albicans*. However, various studies reported that *S. mutans* is the major pathogen causing dental caries, and *C. albicans* is the most common pathogen causing oral candidiasis [1-3]. Amoxicillin, vancomycin, and ketoconazole are the antimicrobial used to treat bacterial or fungal infection, respectively [4].

Traditionally, Indonesian people used plants since ancient times to treat diseases include mouth infection. This allows the possibility that the plants are used by patients while using antimicrobial agent simultaneously. The plants-antimicrobial agent's combination can modify the antimicrobial activity which potentially exhibiting synergism, antagonism, additive, or indifferent. The synergistic interaction provides increased antimicrobial activity using lower concentration when used together, as well as additive interaction does not provide increased nor decreased antimicrobial activity if used together, and antagonist interaction provides decreased antimicrobial activity. The synergism should be developed to enhance antimicrobial potentiation while antagonistic should be avoided due to emerging resistance bacteria.

Medicinal plants have potential source for treatment of disease include infection because they contain many bioactive compounds that can be of interest in therapeutic. The antimicrobial activity of *Boesenbergia pandurata* rhizome, *Zingiber officinale* rhizome, and *Stevia rebaudiana* has been studied in several microorganism, but none of this study includes an interaction effect with antibiotic [5-7]. Hence, the objective of the present study is to determine the *in vitro* activities of ethanolic extract of *Z. officinale* rhizome, *B. pandurata* rhizome, and *S. rebaudiana* leaves in combination with amoxicillin, vancomycin, or ketoconazole against infectious mouth microbial such as *S. aureus, S. mutans*, and *C. albicans* using the checkerboard method.

MATERIALS AND METHODS

Materials

Plants grinder, rotavapor, autoclave, microplate 96-wells, shaker, laminar air flow, Eppendorf, micropipette, separation funnel, glass set, chromatography set, curved semimicro, ethanol, dimethyl sulfoxide (DMSO), Mueller-Hinton Broth (MHB), Mueller-Hinton Agar

(MHA), Potato Dextrose Agar, (PDA) ammonia, trihydrate amoxicillin, clindamycin, vancomycin, chloroform, chloride acid, and sulfate acid.

Plants

The dried *Z. officinale* rhizome, *S. rebaudiana* leaves, and *B. pandurata* were collected from Bumi Herbal Dago field, Research and Development Agency for Medicinal Plants and Traditional Medicine in Tawangmangu, and 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

96% ethanol was used for the maceration extraction procedure. Two hundred gram of plants powder was macerated using 800 mL of ethanol and kept for 24 hrs at room temperature. 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.

Preliminary phytochemical screening

The extracts were subjected to preliminary phytochemical testing to detect the presence of different chemical groups of compounds including alkaloids, flavonoids, saponins, tannins, quinones, and steroid/triterpenoids as described in the literature [8].

Test microorganism preparation

The microorganism preparation was carried out according to the method of Bhalodia and Shukla [9]. In this, the human dental caries pathogens, *S. mutans, S. aureus,* and *C. albicans* were used in this study. The bacterial strains were grown in MHA at 35±2°C, whereas the yeasts were grown inPDA media, respectively, at 28°C. The bacterial cell were overnight cultured (18-24 hrs) at 35±2°C on MHB for the preparation of cell suspensions. The bacterial cell suspension was homogenized and adjusted to 0.5 McFarland standards (5×10^5 CFU/mL) using spectrophotometry. The fungal were cultured in PDB adjusted to 0.5 McFarland standards (1-5 × 10^3 CFU/mL) using spectrophotometry. The bacterial cell and fungal suspension were further used to test the antimicrobial activity of the plant extracts and their combination with antibiotic.

Antimicrobial activity

Determination of minimum inhibitory concentration (MIC)

The MIC was initially determined using MHB Microdilution. MIC determination was performed by a serial dilution technique using 96-well microtiter plates. The 200 µL MHB and PDB were put into the first column used as negative control and 100 µL microbial suspension were put into the second column used as positive control. The 100 µL extract were put into the column and row number 12 of the well. Then, 100 µL microbial suspension were put into each well/plates. Microplates were incubated for 24 hrs at 37°C [10]. The lowest concentrations without visible growth were defined as concentrations which completely inhibited bacterial (MICs) [11]. DMSO was used as a control while trihydrate amoxicillin and vancomycin were used as a positive control. The assay was repeated twice for the control and three replicate for the extract 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±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 minimum fungicidal concentration (MFC)

The MFC was determined by sub-culturing the test dilution onto a fresh drug-free solid medium (PDA) and incubated further for 24 hrs at 35°C. The highest dilution that yielded no signal fungal colony on the solid medium was taken as MFC. Two repetitions were performed [10].

Determination of combination interaction using microdilution checkerboard method

The extract-antibiotic combination effect was determined using microdilution checkerboard method. Each antibiotic and extract concentration to at least double the MIC and double dilutions of both the antibiotic as well as the plant extracts in each well. The antibiotic was serially diluted along the abscissa while the plant extracts of was diluted along the ordinate. Each suspension well was inoculated with 100 µl of the culture. All the tubes were incubated at 35±2°C for 18-24 hrs for bacteria and 35°C for fungal. After incubation, the growth was observed by visual observation with eye naked detection for the test organism growth. Fractional inhibitory concentration index (FICI) 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 while 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 [11,12].

RESULTS

Phytochemical screening

Dried plants and extracts were subjected to a preliminary phytochemical screening for various constituents. The results (Table 1) revealed the presence of flavonoid, alkaloid, quinone, steroid/triterpenoid in dried plants, and extracts.

The antimicrobial activity

The results of the antibacterial activity of ethanol extracts are presented in Table 2. Among all plant extracts studied, *B. pandurata* had the lowest MIC value against *S. aureus* and *S. mutans* which are128 and 64 µg/µL, respectively. *Z. officinalis* had the lowest MIC against *C. albicans* which performed at 2048 µg/µL.

Antimicrobial interaction of combination of plant extract studied and antibiotic

The FICC index for the combination of all plant extracts and antibiotic combinations against microbial resulted in synergistic, additive, and indifferent activity. No antagonism being observed in those combinations. The details FIC index can be seen in Table 3. The antimicrobial interaction of vancomycin with *S. rebaudiana* or *B. pandratae* against *S. aureus* and *S. mutans* showed the synergistic effect where FICC index of those combinations was ranged between 0.062 and 0.75. Synergism also observed in the combination of amoxicillin with *S. rebaudiana* or *B. pandurata* against *S. mutans* and *S. aureus* by FICC index were 0.75 and 0.375, respectively. The combination of ketoconazole with each of all plant extracts against *C. albicans* showed synergistic effect with FICC index ranged from 0.197 to 0.312. The additive interaction as indicated by the FICC index value of 1 was observed from a combination of amoxicillin with *B. pandurata* or *S. rebaudiana* against *S. aureus* and *S. mutans,* respectively.

DISCUSSION

The combination of drug used is occasionally recommended to prevent resistance emerging during therapy and to achieve higher efficacy in the treatment of infections. The traditional plants had shown activity against the bacteria isolates. In this study, the antimicrobial activity and interaction between plant extract and antimicrobial had been investigated.

Preliminary phytochemical analyzes revealed that the ethanolic extracts of *Z. officinalis*, *S. rebaudiana*, and *B. pandurata* consisted of flavonoid, alkaloids, and steroid/triterpenoid as shown in Table 1. These bioactive compounds have been reported to be used by plants for protection against bacterial and responsible for antimicrobial activity [13-15].

In our study, antimicrobial activity using microdilution method showed that each of the extracts tested in the present study displayed antibacterial activity against *S. aureus*, *S. mutans,* and *C. Albicans* in

Table 1: Results of phytochemical analysis of dried plants and extracts

Secondary metabolites	*Z. officinale*		*S. rebaudiana*		*B. pandurata*	
	Dried	Extracts	Dried	Extracts	Dried	Extracts
Alkaloids	+	+	+	+	+	-
Flavonoids	+	+	+	+	-	+
Tannins	-	-	-	-	-	-
Saponins	-	-	-	-	-	-
Quinones	+	+	-	-	-	-
Steroid/triterpenoids	+	+	+	+	+	+

+: Present, -: Absent, *Z. officinale: Zingiber officinale, S. rebaudiana: Stevia rebaudiana, B. pandurata: Boesenbergia pandurata*

Table 2: The antimicrobial activity of plant extracts against microorganisms

The extracts/ antibiotics	Microorganisms (µg/mL)					
	S. aureus		*S. mutans*		*C. albicans*	
	MIC	MBC	MIC	MBC	MIC	MBC
Z. officinalis	>2048	>2048	>2048	>2048	2048	>2048
B. pandurata	128±0	>2048	64±0	>2048	>2048	>2048
S. rebaudiana	256±0	>2048	512±0	2048	>2048	>2048
Trihydrate amoxicillin	1024	>1024	512±0	>1024	ND	ND
Clindamycin	>4096	>4096	>4096	>4096	ND	ND
Vancomycin	256±0	512±0	128±0	512±0	ND	ND
Ketoconazol	ND	ND	ND	ND	8±0	8±0

Experiments were conducted in triplicate. ND: Not done, *Z. officinale: Zingiber officinale, S. rebaudiana: Stevia rebaudiana, B. pandurata: Boesenbergia pandurata, S. aureus: Staphylococcus aureus, S. mutans: Streptococcus mutans, C. albicans: Candida albicans*, MBC: Minimum bactericidal concentration, MIC: Minimum inhibitory concentration

Table 3: The *in vitro* antimicrobial interaction of plant extracts-antibiotic combination

Microorganisms	Combination		FICA	FICE	FICC	Interpretation
	Plant extracts	Antibiotics				
S. aureus	*Z. officinalis*	Trihydrate amoxicillin	1	-	1	Indifference
	S. rebaudiana	Trihydrate amoxicillin	0.25	0.5	0.75	Synergism
	B. pandurata	Trihydrate amoxicillin	0.5	0.5	1	Additive
	Z. officinalis	Vancomycin	1	-	1	Indifference
	S. rebaudiana	Vancomycin	0.5	0.125	0.625	Synergism
	B. pandurata	Vancomycin	0.25	0.5	0.75	Synergism
S. mutans	*Z. officinalis*	Trihydrate amoxicillin	1	-	-	Indifference
	S. rebaudiana	Trihydrate amoxicillin	0.5	0.5	1	Additive
	B. pandurata	Trihydrate amoxicillin	0.125	0.25	0.375	Synergism
	Z. officinalis	Vancomycin	1	-	-	Indifference
	S. rebaudiana	Vancomycin	0.031	0.031	0.062	Synergism
	B. pandurata	Vancomycin	0.25	0.25	0.5	Synergism
C. albicans	*Z. officinalis*	Ketoconazol	0.031	0.25	0.281	Synergism
	S. rebaudiana	Ketoconazol	0.125	0.062	0,197	Synergism
	B. pandurata	Ketoconazol	0.25	0.062	0.312	Synergism

*Experiments were conducted in quadruplicate. FICA: Fractional inhibitory concentration of antibiotics, FICE: Fractional inhibitory concentration of extracts, FICC: Fractional inhibitory concentration of combination, *Z. officinale: Zingiber officinale, S. rebaudiana: Stevia rebaudiana, B. pandurata: Boesenbergia pandurata, S. aureus: Staphylococcus aureus, S. mutans: Streptococcus mutans, C. albicans: Candida albicans*

various MIC value. Table 2 showed that among the extracts, *B. pandurata* extracts had the lowest value of MIC against both *S. aureus* and *S. mutans*. These results are similar with has been reported that *B. pandurata* had strong activity against various strains of bacteria include *S. aureus* and *S. mutans* even in low concentration [16,17]. Among the three extracts studied, *Z. officinalis* showed the lowest MIC value against *C. albicans* (2048 µg/mL) but showed no antibacterial activity against *S. aureus* and *S. mutans*. However, the previous study reported by Giriraju and Yunus showed that methanol extract of the same plant had antibacterial activity against the same bacteria used in this study [18]. Although the antimicrobial activity of these plants has been previously reported, their interaction with antimicrobial agents which are commonly prescribed is not widely explored yet. The FICC showed that interactions between the extracts and antibiotics were synergistic, additive, and indifferent. Synergism is the most desirable effects of combination and beneficial to treat bacterial infection [19]. The synergistic interaction was found in the combination of vancomycin with the *S. rebaudiana* against *S. aureus* and *S. mutans* as well as amoxicillin with *B. pandurata* or *S. rebaudiana*. The combination of ketoconazole with each extract against *C. albicans* showed synergism. 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. Other study reported that synergistic effect also observed in the combination of *Carica papaya* leaves extract with cephalothin or ofloxacin against *Bacillus subtilis* [20]. The synergistic indicated increasing of activity and a decreased risk of emergence of resistant strains and also could shorten the total duration of therapy and decrease toxicities by allowing the use of lower doses [21].

This study provides novel information about the antimicrobial potential of *B. pandurata* and *S. rebaudian a*against infectious mouth microbial.

The mechanism in synergism combination is not established yet but probably the plants enhance the mechanism of action of the antibiotics. As has been reported that the observed synergistic effects of essential oils from *Salvia officinalis* and oxacillin could be theoretically the results of the perturbation of the cell membrane coupled with the action of oxacillin. β-lactam antibiotic oxacillin inhibits the final stage involved in the synthesis of peptidoglycan of the cell wall (transpeptidation reaction), which occurs outside the cell membrane and is mediated by alternative protein binding protein PBP2a encoded by *mec*A gene [22]. Thus, further studies investigating the mechanism of synergistic action are important to prove candidacy of these substances for antimicrobial therapy.

CONCLUSION

Synergistic interaction is observed in the combination of vancomycin or amoxicillin with *S. rebaudiana* or *B. pandurata* against *S. aureus* and *S. mutans*. Moreover, all plants extracts show synergism against *C. albicans*. This study reveals that the combined use of plant extracts and antimicrobial agent can be useful in treating mouth infection as well as fighting emerging drug-resistance problem and *in vivo* experiments are needed to confirm microbial eradication using these combinations.

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REFERENCES

1. Jebashree HS, Kingsley SJ, Sathish ES, Devapriya D. antimicrobial activity of few medicinal plants against clinically isolated human cariogenic pathogens - An *in vitro* study. ISRN Dent 2011;2011:541421.
2. Grubb SE, Murdoch C, Sudbery PE, Saville SP, Lopez-Ribot JL, Thornhill MH. *Candida albicans*-endothelial cell interactions: A key step in the pathogenesis of systemic candidiasis. Infect Immun 2008;76(10):4370-7.
3. Marrelli M, Tatullo M, Dipalma G, Inchingolo F. Oral infection by *Staphylococcus aureus* in patients affected by white sponge nevus: A description of two cases occurred in the same family. Int J Med Sci 2012;9(1):47-50.
4. Greenberg MS, Glick M. Burket's Oral Medicine. 11th ed. Ontario: BC Decker Inc.; 2008. p. 79-82.
5. Sukandar EY, Sunderam N, Fidrianny I. Activity of *Kaempferia pandurata* (Roxb.) rhizome ethanol extract against MRSA, MRCNS, MSSA, *Bacillus subtilis* and *Salmonella typhi*. Pak J Biol Sci 2014;17(1):49-55.
6. Karuppiah P, Rajaram S. Antibacterial effect of *Allium sativum* cloves and *Zingiber officinale* rhizomes against multiple-drug resistant clinical pathogens. Asian Pac J Trop Biomed 2012;2(8):597-601.
7. Sichani M, Karbasizadeh M. Effect of different extracts of *Stevia rebaudiana* leaves on *Streptococcus mutans* growth. J Med Plants Res 2012;6(32):4731-4.
8. Wadood A, Ghufran M, Jamal SB, Naeem M, Khan A, Ghaffar R, *et al*. Phytochemical analysis of medicinal plants occurring in local area of Mardan. Biochem Anal Biochem 2013;2:144.
9. Bhalodia NR, Shukla VJ. Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* l.: An ethnomedicinal plant. J Adv Pharm Technol Res 2011;2(2):104-9.
10. Sen A, Batra A. Evaluation of antimicrobial activity of different solvent extracts of medicinal plant: *Melia azedarach* L. Int J Curr Pharm Res 2012;4(2):67-73.
11. Clinical and Laboratory Standards Intitute. M07-A08 – Methods Dilution Antimicrobial Succeptibility Tests for Bacteria That Grow Aerobically; Approve Standard. 8th ed. Pennsylvania: CLSI; 2009.
12. Meletiadis J, Pournaras S, Roilides E, Walsh TJ. Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and *in vitro-in vivo* correlation data for antifungal drug combinations against *Aspergillus fumigatus*. Antimicrob Agents Chemother 2010;54(2):602-9.
13. Doughari J, Okafor B. Antimicrobial activity of *Senna alata* Linn. East Central Afr J Pharm Sci 2007;10:17-21.
14. Najafi S. Phytochemical screening and antibacterial activity of leaf Extract of *Ziziphus mauritiana* Lam. Int Res J Appl Basic Sci 2013;4(11):3274-6.
15. Kumar S, Pandey AK. Chemistry and biological activities of flavonoids: An overview. ScientificWorldJournal 2013;2013:162750.
16. Norajit K, Laohakunjit N, Kerdchoechuen O. Antibacterial effect of five *Zingiberaceae essential* oils. Molecules 2007;12(8):2047-60.
17. Phongpaichit S, Subhadhirasakul S, Wattanapiromsakul C. Antifungal activities of extracts from Thai medicinal plants against opportunistic fungal pathogens associated with AIDS patients. Mycoses 2005;48(5):333-8.
18. Giriraju A, Yunus GY. Assessment of antimicrobial potential of 10% ginger extract against *Streptococcus mutans*, *Candida albicans*, and *Enterococcus faecalis*: An *in vitro* study. Indian J Dent Res 2013;24(4):397-400.
19. Adwan G, Mhanna M. Synergistic effect of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens. Asian Pac J Trop Med 2009;2(3):46-51.
20. Rakholiya K, Chanda S. *In vitro* interaction of certain antimicrobial agents in combination withplant extracts against some pathogenic bacterial strains. Asian Pac J Trop Biomed 2012:S876-80.
21. Olajuyigbe OO, Afolayan AJ. Evaluation of combination effects of ethanolic extract of *Ziziphus mucronata* Willd. subsp. Mucronata Willd. and antibiotics against clinically important bacteria. ScientificWorldJournal 2013;2013:769594.
22. Pinho MG, Lencastre H, Tomasz A. An acquired and a native penicillin- binding protein cooperate in building the cell wall of drug-resistant Staphylococci. In: Chovanova R, Mikulasova, Veverkova S, editors. In Vitro Antibacterial and Antibiotic Resistance Modifying Effect of Bioactive Plant Extracts on Methicillin-Resistant Staphylococcus Epidermidis. 2013. p. 1-7.