

SYNERGISM OF ISOPTEROPODINE AND PTEROPODINE WITH ANTIBIOTICS AGAINST SELECTED STREPTOCOCCI

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ABSTRACT

We have previously reported the isolation of four compounds from Malaysian *Uncaria tomentosa*. In view of the lack of specific antibacterial studies reported on the isolated compounds, this study was undertaken to evaluate its antibacterial potential against selected *Streptococcus* species. The antibacterial activity of phytochemicals (isopteropodine and pteropodine) as well as antibiotics (amoxicillin, cephadrine, cloxacillin, oxacillin and penicillin) was evaluated with three oral bacteria (*Streptococcus mitis*, *Streptococcus mutans* and *Streptococcus oralis*). In addition, the possible synergistic effects when associated with antibiotics were studied. For the antibacterial assay, the agar-well diffusion method has been employed. The antibacterial activity was expressed as the average diameter of the clear zone inhibition of bacterial growth around the well. The minimum inhibitory concentration (MIC) of the compounds and antibiotics was determined by broth microdilution assay utilizing 96-well microtitre plate. For the synergistic activity, checkerboard assay has been employed. In this study, only isopteropodine was found to be active against *S. mutans* with inhibition zone of 13 mm. No growth inhibition was found on pteropodine. Upon MIC determination, the isopteropodine was found to show strong inhibitory properties against *S. mutans* with MIC value of 31.25 mg/mL. Association of antibiotics and isopteropodine showed synergistic antibacterial activity against *S. mutans*. The FICs values obtained by Checkerboard Assay were 0.09 for all tested combinations.

Keywords: Isopteropodine; Pteropodine; MIC; FIC

INTRODUCTION

Infectious diseases have been recognised as the leading cause of death worldwide [1]. Infective endocarditis (IE) for example, can be described as a microbial infection of the endothelial lining of the heart, in further lead to cardiovascular diseases (CVD). In Malaysia itself, CVD has been accounted for 25.4% of all deaths in 2010. Viridans staphylococci, enterococci and, streptococci are among the causative microorganisms

for this illness. Streptococci, spherical Gram-positive bacteria have emerged as significant pathogens of immunocompromised patients and those with haematological malignancies including infective endocarditis, adult respiratory distress syndrome and streptococcal shock resulting in mortality rates of up to 30% [2,3]. Despite an overwhelming number of studies on the antimicrobial activities of plant and natural product derivatives, infections have increased to a great extent and antibiotic resistance has become a serious therapeutic problem. The success of chemotherapy lies in the continuous search for new drugs to counter the challenge posed by resistant strains of pathogens. Minimum inhibitory concentration (MIC) has been used as a guide in seeking the most efficient solution in treating endocarditis [4] and plays a crucial role for the determination of antibiotic resistance of bacteria.

Previous studies on isopteropodine (7S) and its epimer pteropodine (7R) isolated from the Peruvian medicinal plant *Uncaria tomentosa* have shown that these bioactive oxindole alkaloids possessed immunomodulatory properties and showed good inhibitory activity against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis* [5,6]. Our preliminary work on isopteropodine and pteropodine isolated from the Malaysian plant *Uncaria longiflora* variety pteropoda (Miq.) Ridsd. has shown the compounds' inhibitory potential against some Streptococci species (unpublished work). However, no report has been published on antimicrobial properties of these compounds against microorganisms causing IE.

This study aims to evaluate the potential of isopteropodine and pteropodine against a panel of oral streptococci via antimicrobial activity, antimicrobial susceptibility test and synergy test. Therefore objectives of the study are i) to determine the resistance of oral streptococci against commonly used antibiotics such as penicillin, oxacillin, amoxicillin, cloxacillin and cephradine, ii) to evaluate antimicrobial activities of isopteropodine and pteropodine against a panel of oral streptococci (*S. mutans*, *S. oralis* and *S. mitis*) and iii) to test the ability of isopteropodine and pteropodine to modify streptococci resistance to selected antibiotics in vitro.

It is expected that the output of the study will bring us a step closer towards the discovery of novel anti-oral streptococci agents in overcoming IE and explain stereochemistry-activity relationship exemplified by activities of stereoisomeric isopteropodine and pteropodine.

EXPERIMENTAL

Microbial Samples

Three microbial species were analyzed which are *Streptococcus mitis* (ATCC 6249), *Streptococcus mutans* (ATCC 25175) and *Streptococcus oralis* (ATCC 35037). All bacterial strains were purchased from Oxoid (UK). The control used for this test was chlorhexidine. The microorganisms studied are clinically important as they associated to infective endocarditis (IE).

Culture Media, Antibiotics and Phytochemical Solution

Brain heart infusion (BHI) agar and broth were used. The following antibiotic solutions were prepared: Amoxicillin (100 mg/mL), Cephadrine (100 mg/mL), Cloxacillin (100 mg/mL), Oxacillin (100 mg/mL) and Penicillin (100 mg/mL). The phytochemical solutions used were isopteropodine and pteropodine dissolved in dimethyl sulfoxide (DMSO) and prepared as 1 mg/mL.

Antibacterial Assay

Bacteria colonies from brain heart infusion (BHI) agar plates were transferred into brain heart infusion (BHI) broth and grown 24 hours at 37°C. The turbidity of the cultures was adjusted with BHI broth to absorbance (A) reading within the range of 0.08-0.10 at OD_{625 nm} equivalent to 1-2x10⁸ CFU/ml (Shafie et al., 2012) with matched to the turbidity 0.5 McFarland standards [7]. The antibacterial tests were performed using agar-well diffusion assay. 25 ml of liquefied Brain Heart Infusion agar (45°C) was aseptically mixed with 1 ml bacterial strains of standardized cultures and poured immediately into sterile petri dishes. Then, the plates were left solidify in a laminar flow cabinet for 20 minutes. Once the agar was hardened, four wells (8 mm) were bored using a sterile cork borer. A 100-μL volume of pteropodine and isopteropodine (1 mg/ml, 0.5 mg/ml and 0.1 mg/ml) were introduced into the wells. Chlorhexidine was used as positive control and solvent dimethyl sulfoxide (DMSO) as negative control. The agar plates were then covered with lids and incubated at 37°C for 24 hours.

The plates were observed for the appearance of inhibition zone of bacterial growth indicated by a clear zone around the wells. The sizes of inhibition zone were measured and the antibacterial activity was expressed in terms of the average diameter of inhibition zone to the nearest millimeters. The tests were carried out in triplicates.

Minimum Inhibitory Concentration (MIC)

MIC was determined by the microbroth dilution method using 96-well microtitre plate. 100 μL of samples were two-fold serially diluted with Brain Heart Infusion broth in the test wells. 50 μL of standardized bacterial culture suspension was added to 100 μL of samples containing culture medium. The following controls were prepared: wells containing broth only (sterility control), bacterial strain with no test sample (inoculum viability), serial dilution of chlorhexidine (positive control) and DMSO (negative control). The plates were incubated at 37°C overnight. A solution of resazurin (30 μL) was then added to each well and plates were incubated for an additional one to two hours. The MIC was determined as the lowest sample concentration at which no pink color (signifying live growth) appeared.

Checkerboard Assay

The combinations tested (in triplicate) against each microorganism were isopteropodine plus amoxicillin, isopteropodine plus cephradine, and isopteropodine plus cloxacillin were used in further calculations. The concentration ranged from 1/32 times the MIC (1/32 x MIC) to 4 x MIC. Two-fold dilutions of drugs A and B were made. The combination of the drugs in which the growth is completely inhibited was considered as

effective MIC for the combination. To evaluate the effect of the combinations, the fractional inhibitory concentration (FIC) was calculated for each antibiotic in each combination.

The Σ FICs were calculated as follows: Σ FIC = FIC A + FIC B, where FIC A is the MIC of drug A (Antibiotic) in the combination/ MIC of drug A (Antibiotic) alone, and FIC B is the MIC of drug B (phytochemical) in the combination/ MIC of drug B (phytochemical) alone. The combination is considered synergistic when the Σ FIC is ≤ 0.5 , indifferent when the Σ FIC is >0.5 to <2 , and antagonistic when the Σ FIC is ≥ 2 [8].

RESULTS AND DISCUSSION

In this study, two phytochemicals from Malaysia *Uncaria tomentosa* were tested against three different bacteria which are *Streptococcus mitis*, *Streptococcus mutans* and *Streptococcus oralis*. *S. mitis* and *S. oralis* have been reported as the leading causes of endocarditis and bacteraemia in neutropenic patients while *S. mutans* is associated with pyogenic and other infections in various sites including mouth, heart, joints, skin, muscle, and central nervous system [9,10]. Results of the evaluation of antimicrobial activities of isopteropodine and pteropodine obtained by agar well diffusion method, showed significant antimicrobial activity of isopteropodine against *S. mutans* by giving a zone of inhibition of 13 mm and was inactive against *S. mitis* and *S. oralis*. On the other hand, for pteropodine, no antibacterial activity was found against all bacteria tested (Table 1). An inhibition zone of less than 9 mm diameter is considered as not active, 9-12 mm as moderately active, 13-18 mm as active and >18 mm as very active [11].

Table 1: Antibacterial activity of phytochemicals through agar well diffusion method

Bacterial strains	Zone of inhibition (mm)		
	Isopteropodine	Pteropodine	Chlorhexidine
<i>S. mitis</i> ATCC 6249	-	-	28
<i>S. mutans</i> ATCC 25175	13	-	29
<i>S. oralis</i> ATCC 35037	-	-	28

(-) absence of susceptibility

Phytochemicals, isopteropodine and pteropodine as well as five antibiotics were screened for their antibacterial potential. Table 2 summarized minimal inhibitory concentration (MIC) of those compounds and selected antibiotics against the tested streptococci. The MIC is the lowest concentration of drug that inhibits the growth of the organism. In this study, the MIC was determined only for phytochemicals that presented positive in antibacterial assay as well as the antibiotics. MIC values between 0.05-0.50 mg/mL is considered as strong activity, moderate activity MIC values between 0.60-1.50 mg/mL and weak activity above 1.50 mg/mL [12].

Isopteropodine showed promising activity against *S. mutans* (MIC 31.25 mg/mL) while no antibacterial activity shown by pteropodine against all three pathogens (Table 2). The results of this MIC appear to agree fairly well with results from Garcia et al. [6] in which they found that the isopteropodine was active only against the Gram positive bacteria *Staphylococcus aureus* (ATCC 6538P) and *Bacillus subtilis* (ATCC 6633), with a MIC value of 150 mg/mL (408 mM) and 250 mg/mL (679 mM), respectively. All the five antibiotics (amoxicillin, cephradine, cloxacillin, oxacillin and penicillin) showed MICs in the range of 0.013-1.563 mg/mL against the bacterial strains used in the study. Chlorhexidine which is used as positive control showed MIC of 0.586 mg/mL. After the overall observation of the MIC results, it is concluded that isopteropodine showed strong activity and could be potential source of antimicrobial compound.

Table 2: Minimal inhibitory concentration (MIC) of phytochemicals and antibiotics against bacteria

Antibacterial agent	MIC (mg/mL)		
	<i>S. mitis</i> ATCC 6249	<i>S. mutans</i> ATCC 25175	<i>S. oralis</i> ATCC 35037
Isopteropodine	Na	31.25	Na
Pteropodine	Na	Na	Na
Amoxicillin	0.098	0.391	0.049
Cephradine	0.195	1.563	1.563
Cloxacillin	0.195	0.391	0.098
Oxacillin	0.098	0.195	0.049
Penicillin	0.063	0.013	0.031
Chlorohexidine	0.586	0.586	0.586

(Na) Not active

Checkerboard assay was performed to study the synergistic activity between isopteropodine and selected antibiotics. The selection of these antibiotics was made upon the MICs values. Synergistic effects of isopteropodine with the three antibiotics, including amoxicillin, cephradine and cloxacillin against *S. mutans* by the checkerboard method and the fractional inhibitory concentration indices (FICIs) are demonstrated in Table 3. The MICs of isopteropodine/amoxicillin combination, isopteropodine/cephradine combination and isopteropodine/cloxacillin combination were reduced by 94-97% compared to the agents used alone, which demonstrated significant synergistic interaction against tested pathogenic strain. The FICIs for all combinations were less than 0.5.

Different rates of synergy have been reported with various antibiotic combinations employing checkerboard technique. These differences might be attributed to the different features of the strains. Although there is a standard procedure for checkerboard synergy testing, it fails to exhibit standard results owing to different methods of

interpretation method for checkboard synergy testing [13].

Table 3: Synergy method results

Organism	Drugs combination	Checkerboard FIC index (interpretation)
<i>S. mutans</i> ATCC 25175	Isopteropodine + Amoxicillin	0.09 (S)
	Isopteropodine + Cephadrine	0.09 (S)
	Isopteropodine + Cloxacillin	0.09 (S)

Abbreviation for interpretation: S, Synergy

CONCLUSION

The rate of emergence of resistant and multiresistant bacteria has increased during the past two decades, especially among Gram-positive microorganisms, and evaluation of new treatment regimens is needed. In many published *in vitro* studies, combinations of antibiotics with natural products have demonstrated synergistic activities against the pathogens that commonly cause infective endocarditis (IE). The synergistic effect from the association of antibiotics and isopteropodine against *S. mutans* leads to new alternative for the treatment of IE. From this study, our results revealed the importance of isopteropodine when associated with antibiotics, to control resistant bacteria, which are becoming a threat to human health. Furthermore, the isopteropodine was active against the tested bacteria under very low concentration, thus minimizing the possible toxic effects. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment. It is hoped that the inhibitory and/or synergistic effects of isopteropodine against IE might be a way of overcoming this deficiency.

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