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Stigmasterol: An adjuvant for beta lactam antibiotics against beta-lactamase positive clinical isolates

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ABSTRACT

The emergence of beta lactamase producing bacterial strains eliminated the use of beta lactam antibiotics as chemotherapeutic alternative. Beta lactam antibiotics can be coupled with non-antibiotic adjuvants to combat these multidrug resistant strains. We study the synergistic antibiotic effect of stigmasterol as adjuvant of ampicillin against clinical isolates. Ampicillin was used in this study as a beta lactam antibiotic model. All test bacteria were beta lactamase producing clinical isolates. The combination showed significantly better antibiotic activity on all bacteria tested. The two test substances have synergistic antibiotic activity, and the effect was observed in both Gram positive and Gram negative bacteria. The synergistic antibiotic effect of stigmasterol and ampicillin was evident by the low fractional inhibitory concentration (FIC) index on Checkerboard Assay. The results suggest that the combination of ampicillin and stigmasterol acts additively in the treatment of infections caused by beta-lactamase producing pathogens. In bacterial growth reduction assay, ampicillin and stigmasterol alone exhibited very weak inhibitory effect on the bacterial growth, relative to ethanol control. Comparatively, combination of stigmasterol-ampicillin greatly reduced the colony counts at least by 98.7%. In conclusion, we found synergistic effects of stigmasterol and ampicillin against beta lactamase producing clinical isolates. This finding is important as it shows potential application of stigmasterol as an antibiotic adjuvant.

1. Introduction

Antibiotic resistance can be literally defined as the acquired ability of a microorganism to resist the effect of chemotherapeutic agent to which it is normally susceptible to ensure a successful life history [1]. This phenomenon is frequently affecting critically-illed or immune-compromised patients which usually associate with hospital-acquired pathogens in intensive care unit specifically [2]. However, the incidence of drug-resistant pathogens in community-acquired infection has been rising in recent years and this led to increase in morbidity, mortality rate and also health care spending [3]. Within the past half century, the pharmaceutical industry, academic institutions and the government are investing necessary resources to develop new antibiotics. However, the vast investments did not give a fruitful outcome where the numbers of approved antibiotics are declining [4].

Beta lactam antibiotics are the most frequently prescribed class of drugs worldwide. The antibiotic inhibits bacterial cell wall synthesis and thus resulting in the lysis and deformation of the bacterium [5]. It is effective on both Gram positive and negative bacteria. The antibiotic binds to specific penicillin-binding proteins in the bacterial cell wall, and further inhibits the activity of trans-peptidase, an important enzyme in peptidoglycan cell wall synthesis [6]. The antibiotic is widely prescribed to treat skin and bladder infections, pneumonia, gonorrhoea, meningitis, and intestinal infections [7]. Beta lactam antibiotics are effective alternative to chloramphenicol, furthermore they are considered safe for neonates and children [5,6].

The efficacy of beta lactam antibiotics is reduced due to the beta lactamase enzyme produced by bacteria that hydrolyses the beta lactam ring of the antibiotics. It causes many failures of antimicrobial chemotherapy because it converts beta lactam to inert and ineffective structure [8]. Emergence of beta lactamase producing strains cause development of beta lactam resistant pathogens, which eliminated the use of beta lactam antibiotics as chemotherapeutic alternative. Many attempts have been made to combat these pathogens. The concept of

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synergism can be utilized to combat infections caused by complex multidrug resistant pathogens [9]. Combinational therapy of several clinical approved antibiotics from different subclasses has been successful in treating multidrug-resistant pathogens. Ampicillin in combination with ceftaroline, cefepime and ceftriaxone has become a prime chemotherapeutic choice for *Entericoccus faecalis* infection [10]. To further enhance the biocidal property while minimizing necessary antibiotic concentrations, the antibiotic can be coupled with non-antibiotic adjuvants to combat multidrug resistant strains [9]. In this study, we study the synergistic antibiotic effect of stigmasterol with ampicillin against clinical isolates. Ampicillin was used in this study as a beta lactam antibiotic model to study the drug synergism effect.

2. Methodology

2.1. Chemicals and drugs

Stigmasterol (95%) used in this study was purchased from Sigma-Aldrich. Ampicillin was purchased from Merck. The compound was dissolved in ethanol and filtered with membrane filter (PTFE, 0.2 μm) prior to use.

2.2. Test bacteria

The test bacteria used in this study were previously isolated from clinical samples in Hospital Seberang Jaya, Penang, Malaysia. The test bacteria include 2 Gram positive bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*) and 2 Gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). All test bacteria were identified as beta-lactamase producers. The microbial inoculums were prepared as per protocols described by Yenn et al. [11].

2.3. Disc diffusion assay

The assay was performed on Mueller Hinton agar (Merck) plates according to Panacek et al. [12]. Firstly, 100 μl of 10^6 cells/ml suspension was spread uniformly onto the agar plates. Then, 6 mm paper discs impregnated with ampicillin (Fisher) and stigmasterol alone at concentration 1 μg/disc, or in combination (1 μg/disc ampicillin + 1 μg/disc stigmasterol), were placed onto the inoculated medium. The plates were incubated at 37 °C for 24 h. The diameter of clear zone surrounding the paper disc were measured and recorded.

2.4. Antibiotic susceptibility test

The minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of the test substances against all test bacteria were determined according to CLSI-recommended procedure described by Hamoud et al. [13]. The bacterial inoculum was diluted to 5×10^5 cells/ml with double strength Mueller Hinton broth (Merck). Ampicillin was serially diluted with ethanol and mixed with the bacterial inoculum in a sterile 96-well plate (Fisher). The tested concentrations of ampicillin were ranged from 3.1 to 200 μg/ml. A negative control was included by replacing ampicillin with ethanol for all the test bacteria. The plate was incubated at 37 °C for 24 h. After that, 40 μl of 0.2 mg/ml Iodonitrotetrazolium Violet (Sigma) was added into each well and incubated for 30 min as a growth indicator. The color change of the broth from yellow to pink indicates bacterial growth. The MIC is defined as the lowest concentration of the test compound to prevent bacterial growth (no color change). To judge the viability of bacterial cells, one loopful of the sample in each well was streaked on Muller Hinton agar plates. The plates were then incubated at 37 °C for 24 h. MBC is defined as the lowest concentration of the test compound to kill the bacterial cells.

2.5. Checkboard assay

This assay was performed to study the synergistic antibiotic effect of stigmasterol and ampicillin. Stigmasterol was serially diluted to final concentrations ranged from 3.1 to 200 μg/ml and mixed with fixed amount of ampicillin (20 μg/ml). The MIC and MBC of the drug combinations were determined according to the protocol described in Section 2.4. The fractional inhibitory concentration (FIC) index was calculated based on the following equation: MIC of stigmasterol/ MIC of stigmasterol-ampicillin combination. Synergism is defined if FIC index ≤ 0.5; while antagonism is defined if FIC index > 0.5.

2.6. Bacterial growth reduction assay

Firstly, 0.1 ml of bacterial inoculum was added into 9.9 ml of Mueller Hinton broth. The test groups include: Stigmasterol (20 μg/ml), ampicillin at concentration of MIC, and stigmasterol-ampicillin combination at concentration of MIC. A negative control was included with 0.1 ml ethanol. The test substance was added into the flasks. After incubation with agitation speed 120 rpm at 37 °C for 24 h, aliquot was removed from each flask and serially diluted 10-fold with sterile saline. Then, 0.1 ml of the diluent was spread on Mueller Hinton agar plate. Colony counts were determined after incubation at 37 °C for 24 h. The percentage of growth reduction was calculated relative to ethanol control.

3. Results and discussion

Stigmasterol is a steroid derivative commonly found in plants with a molecular weight of 412.7 g/mol. The compound is commonly used as food additives due to its cholesterol-lowering property [14]. Stigmasterol also showed significant anticancer activity on several cell lines, including DMBA-induced skin carcinoma [15]. However, the potential of stigmasterol as antibiotic adjuvant is still unknown. Antibiotic is usually paired with non-antibiotic adjuvants to combat multidrug resistant strains [9]. Adjuvant is known as a substance that is not exhibiting antibiotic activity when administered alone, but increases the microbicidal effect when combines with antibiotic [16]. Beta lactam antibiotics are usually used with beta lactamase inhibitor, such as clavulanic acid. Clavulanic acid functions by inhibiting the beta lactamase enzyme produced by the bacteria, hence allows the antibiotic to inhibit bacterial cell wall synthesis [9,16]. This combination allows the continued used of beta lactam antibiotics in inhibiting the growth of beta lactamase producing pathogens.

The test bacteria used in this study were clinical isolates with extended spectrum beta lactamases. The bacterial strains were resistant to beta lactam antibiotics, including penicillin and ampicillin. The resistance is due to the ability of the enzyme to digest the beta lactam ring in the antibiotic [8]. Of all test bacteria, chloramphenicol inhibits only *S. pyogenes*. The antibiotic spectrum of all test bacteria was determined to compare the antibiotic efficacy of the test substance. The antibiotic efficacy of stigmasterol was tested in the same assay. Only *E. coli* is susceptible to stigmasterol. The antibiotic activity of stigmasterol is previously reported by Sharma [17]. However, the antibiotic activity reported here is weak, judging on the size of inhibition zone (≤ 9 mm).

The stigmasterol-ampicillin combination inhibits all test bacteria (Table 1). The combination showed significantly better ($p \leq .05$) antibiotic activity on all bacteria tested. The two test substances have synergistic antibiotic activity. Zygmunt and Tavormina [18] first reported the interference of stigmasterol with polyene antibiotics. They noticed a mild antagonistic anti-*Candida* effect after addition of stigmasterol. Our results do not match their study as we noticed a significant synergistic antibiotic effect when stigmasterol was used with beta lactam antibiotics. The synergistic effect was observed in both Gram positive and Gram negative bacteria. Beta lactamase produced by the bacteria can be deactivated by adenylation, phosphorylation or acetylation of