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Synergistic activity of luteolin and amoxicillin combination against amoxicillin-resistant *Escherichia coli* and mode of action

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ABSTRACT

The purpose of this research was to investigate whether luteolin has antibacterial and synergistic activity against amoxicillin-resistant *Escherichia coli* (AREC) when use singly and in combination with amoxicillin. The primarily mode of action is also investigated. The susceptibility assay (minimum inhibitory concentration and checkerboard determination) was carried out by the broth macrodilution method's in Müeller–Hinton medium. MIC and checkerboard determination were carried out after 20 h of incubation at 35 °C by observing turbidity. The MICs of amoxicillin and luteolin against all AREC strains were >1000 and ⩾200 μg/ml respectively. Synergistic activity were observed on amoxicillin plus luteolin against these strains. Viable count of this combination showed synergistic effect by reducing AREC cell numbers. The results indicated that this combination altered both outer and inner membrane permeabilisation. Enzyme assay showed that luteolin had an inhibitory activity against penicillinase. Fourier Transform-Infrared (FT-IR) spectroscopy exhibited that luteolin alone and when combined with amoxicillin caused increase in fatty acid and nucleic acid, but decrease in amide I of proteins in bacterial envelops compared with control. These results indicated that luteolin has the potential to reverse bacterial resistance to amoxicillin in AREC and may operate via three mechanisms: inhibition of proteins and peptidoglycan synthesis, inhibition of the activity of certain extended-spectrum β-lactamases and alteration of outer and inner membrane permeability. These findings offer the potential to develop a new generation of phytopharmaceuticals to treat AREC.

1. Introduction

Bacterial resistance to β-lactam antibiotics has rapidly emerged to a global problem and posing a growing public health risk. The emergence of fecal *Escherichia coli* isolates exhibiting reduced susceptibility or resistance to extended-spectrum cephalosporins has been reported among pigs in Spain [1]. The prevalences of plasmid-mediated AmpC β-lactamase (pAmpC)-producers, exten ded-spectrum β-lactamase (ESBL)-producers and co-producers of pAmpC and ESBL were reported less than one-half of the pAmpC-producers were reported to be resistant to third-generation cephalosporins, cephamycins and β-lactam/β-lactam inhibitors [2]. High levels of antibiotic resistance were found in *E. coli* isolates in hospital sewage water and 19% were resistant to ampicillin, ceftazidime, ceftriaxone, cefotaxime, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid and trimethoprim-sulphamethoxazole among the resistant isolates from the wells [3]. In addition, the ESBL-producing clones of *E. coli* have a potential to translocate and cause septicemia in hospitalized patients with urinary tract infection [4]. The study analyzed antimicrobial resistance (AMR) and resistance genes in generic *E. coli* isolated from retail meat samples purchased suggested that the prevalence of AMR and select resistance genes were higher in poultry-derived *E. coli* and showed a complex nature of resistance in *E. coli* from retail meat. Hence, the use of a single antimicrobial could result in the selection of resistant *E. coli* not only to the drug being used but to other unrelated classes of antimicrobials [5]. The extended-spectrum β-lactamase (ESBL) and producing bacteria coexpressing AmpC type β-lactamase (ACBL)-producing isolates exhibited a lower susceptibility rate to fluoroquinolones, aztreonam, and β-lactam/lactamase inhibitors than those with either ESBL or ACBL alone [6]. Antibiotics available for the treatment of *E. coli* infection exhibit toxicity and their use is frequently associated with unwanted side-effects. The development of novel antibiotics and/or a new generation of phytopharmaceuticals that can reverse the resistance to well-established therapeutic agents that have lost their original effectiveness is of great importance [7]. Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a common flavonoid that exists in many types of plants including fruits, vegetables, and medicinal herbs. Dietary sources of luteolin include, for instance, carrots, peppers, celery, olive oil, peppermint, thyme, rosemary and oregano, hot peppers, parsley, broccoli, onion leaves, carrots,

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peppers, cabbages, apple skins, and chrysanthemum flowers. Preclinical studies have shown that this flavone possesses a variety of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial and anticancer activities [8,9]. Fourier Transform-Infrared (FT-IR) absorbance spectroscopy was used to study the effect of chlorine-induced bacterial injury on spectral features [10]. Furthermore, The deformation of macromolecules in the bacterial membrane, upon treatment with eugenol was verified by FT-IR spectroscopy [11]. Therefore, These investigations provide evidence that FT-IR spectroscopy can be used as an effective tool for an early evaluation of the efficiency of the anti-bacterial effect of other used drugs [12].

In this study, we investigated the in vitro activity and mode of action of the flavone, luteolin, against amoxicillin-resistant *E. coli* (AREC) strains when used alone and in combination with amoxicillin.

2. Materials and methods

2.1. β-Lactam antibiotics and bacterial strains

Luteolin (purity 97+%) was purchased from Indofine Chemical Company (New Jersy, USA). Amoxicillin, polymyxin B sulphate (PMX) and o-nitrophenol-β-D-galactoside (ONPG) were obtained from Sigma (Sigma–Aldrich, UK). Müeller–Hinton broth was obtained from Oxoid (Basingstoke, UK). AREC strains (DMST 20661, 20662, 20970, 20971) were obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. *E. coli* ATCC strain 25922, used as a positive control, was purchased from the American Type Culture Collection (ATCC).

2.2. Bacterial suspension standard curve

The bacterial suspensions of a known viable count was employed following the method of Richards and Xing [13]. Cation-adjusted Müeller–Hinton broth (CAMHB) were used as medium.

2.3. Minimum inhibitory concentration (MIC) and checkerboard determinations

MIC and checkerboard determinations of amoxicillin and luteolin against AREC were performed following the method of Liu et al. [14] and Clinical and Laboratory Standards Institute [15] by means of broth macrodilution method's in Cation-adjusted Müeller–Hinton medium. Bacterial suspension at 5×10^6 cfu/ml was prepared by bacterial suspension standard curve as above described. One ml of bacterial suspension was then added to tubes containing 9 ml of serially diluted antimicrobial agents to yield a final inoculum of approximately 5×10^5 cfu/ml in each tube. The working solutions of each amoxicillin and luteolin were prepared by means of seriated dilutions from 1024 μg/ml up to 0.5 μg/ml. MIC determination was carried out after 20 h of incubation at 35 °C by observing turbidity. The lowest concentrate on of each amoxicillin or luteolin that prevented bacterial growth was considered to be the MIC [16]. The interaction between the two agents was estimated by calculating the fractional inhibitory concentration of the combination (FIC) index. The FIC of each agent was calculated by dividing the concentration of the compound present in that tube in combination where complete inhibition of growth of the microorganism was observed by the MIC of that compound alone to inhibit the microorganism. The FIC index was calculated using the following formula: FIC of luteolin = MIC luteolin in combination/MIC of luteolin alone; FIC of amoxicillin = MIC of amoxicillin in combination/MIC of amoxicillin alone; hence FIC index = FIC of luteolin + FIC of amoxicillin. When the FIC index of the combination is equal to or less than 0.5, the combination is termed as synergistic; when FIC index falls between 0.5 and 4.0, it indicates 'no interaction' between the agents, and a value above four indicates antagonism between the two compounds [17–19]. *E. coli* ATCC 25922 was used as positive control. The MICs and FIC index are presented as the median values obtained in duplicates from three independent experiments.

2.4. Killing curve determinations

The methods of Richards and Xing [13] and Clinical and Laboratory Standards Institute [15] were followed for the determination of killing curves. Cation-adjusted Müeller–Hinton broth (CAMHB) were used as medium. After the FIC index was got, the killing curve determination was performed to confirm synergistic activity of the combination. The MIC of each compound that gave synergism FIC index of combination was chosen to investigate. The sub-inhibitory concentration (Sub-MIC) between FIC and MIC value of each compound was selected [20]. For this reason, the concentration of 100 μg/ml amoxicillin, 100 μg/ml luteolin, amoxicillin at 70 μg/ml plus luteolin at 80 μg/ml combination and control (without amoxicillin or luteolin) were tested.

2.5. Enzyme assays

Bacteria have evolved strategies to escape the activity of beta-lactam antibiotics by production of beta-lactamase enzymes that can inactivate beta-lactam antibiotics by hydrolyzing the peptide bond of the beta-lactam ring rendering the antibiotic ineffective [21]. The β-lactamase assay was investigated to clarify whether luteolin had inhibitory activity against this enzyme or not [22]. The β-lactamase type IV of *Enterobacter cloacae* (*E. cloacae*) were obtained from Sigma (Poole, England). Enzymes activities were performed as previously described by Richards et al. [23]. Enzymes activities were adjusted to concentrations sufficient to hydrolyse 50–60% substrate in 5 min. Luteolin was pre-incubated with enzyme in 50 mM sodium phosphate buffer (pH 7.0) at 37 °C for 5 min prior to substrate addition. Time-course assays were carried out using methanol/acetic acid (100:1) as stop reagent and analyses of the remaining substrate determined by reverse-phase HPLC with acetronitrile/acetate in the mobile phase [24].

2.6. Outer-membrane (OM) permeabilization assays

The method of alteration in outer membrane permeability was detected by crystal violet assay as previously described by Vaara and Vaara [25] and Devi et al. [11].

The amoxicillin-resistant *E. coli* DMST 20662 (AREC) strain was used in this investigation. The Sub-MICs and Sub-FICs of amoxicillin and luteolin were chosen because of these concentrations cannot kill bacteria but they have shown ability in vitro to affect bacteria in various ways, e.g. through suppression of bacterial growth, induction of morphological changes in bacteria, alteration of outer membrane protein (OMP) and the cell surface structure, inhibition of enzyme and toxin production and the loss of adherence capability [20,26,27]. Consequently, the concentration of 100 μg/ml amoxicillin, 100 μg/ml luteolin, amoxicillin at 50 μg/ml plus luteolin at 60 μg/ml combination and control (without amoxicillin or luteolin) were performed. The natural peptide, Polymyxin B (PMX), is a potent antibiotic that binds to and neutralizes LPS. It is a decapeptide cyclic cationic antibiotic containing lipophilic and hydrophilic groupment (lipophobic) that binds to lipid A [28]. Its mechanism of action has been related to its ability to disrupt phospholipid bilayers of the bacterial membrane [29]. Because of this, PMX at 7 μg/ml was used as positive control [30].