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Cucurbitacin B interacts synergistically with antibiotics against *Staphylococcus aureus* clinical isolates and exhibits antiviral activity against HSV-1

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ABSTRACT

The search for biologically promising compounds from natural sources against microbial diseases remains an important theme in drug discovery to overcome problems with drug-resistant strains. In this study, Cucurbitacin B (Cuc B), an active constituent of *Ecballium elaterium* L., has been investigated *in vitro* for its synergy effect with antibiotics against clinical isolates of *Staphylococcus aureus* (*S. aureus*) and anti-HSV-1 activity. Broth microdilution method was used to determine the antibacterial activity, while checkerboard assay was used to evaluate the synergy effect according to ΣFIC indices. The anti-HSV-1 activity was determined by the plaque number reduction assay, while cytotoxicity was evaluated by MTT assay. In this study, Cuc B exerted direct growth-inhibitory activity against all *S. aureus* strains tested with MICs values ranging from 0.12 to 0.44 μg/mL, as well as synergy effect with tetracycline or oxacillin against four of six *S. aureus* strains tested (ΣFIC ranging from 0.29 to 0.43). Cuc B showed remarkable anti-HSV-1 activity compared with that of acyclovir with IC_{50} values of 0.94 and 1.74 μM, respectively and selectivity indices SI = 127.7 and SI > 132.2, respectively. This study presents Cuc B as a promising therapeutic agent in the development of anti-staphylococcal and anti-HSV-1 drugs.

1. Introduction

Ecballium elaterium L. (*E. elaterium*, squirting cucumber, Cucurbitacae) is a wild-growing medicinal plant found abundantly in the Mediterranean region. The interior of the fruits contains black seeds and juice. In traditional folk medicine, *E. elaterium* fruits have been used as antimicrobial, analgesic, antipyretic and antiphlogistic agents. Moreover, *E. elaterium* is of interest in Mediterranean region due to the use of its fruits extracts in various medicinal uses (Khalil and Qaoud, 1993; Raikhlin-Eisenkraft and Bentur, 2000; Bohlooli et al., 2012). It has been reported that the main active compounds from the fruits that are responsible for the biological activities including antimicrobial properties were found to be fatty acids, proteins, cucurbitacins (B, D, E, I and L) and cucurbitacin derivatives such as glycosylcucurbitacins and triterpenoids glycosides (Rao et al., 1974; Attard et al., 2005; Chen et al., 2005). Cucurbitacin B (Cuc B, Fig. 1), an active constituent of *E. elaterium*, is a natural tetracyclic triterpene compound that belongs to Cucurbitacins (CUs) compounds, which are widely distributed in the family of Cucurbitaceae. Structurally, CUs are characterized by a tetracyclic cucurbitane nucleus skeleton, namely, 9β-methyl-19-nor lanosta-5-enea, which is traditionally divided arbitrarily into twelve categories, incorporating CUs A-T (Chen et al., 2005; Hassan and Žemlička, 2016). Numerous studies demonstrated that Cuc B possesses a variety of bioactivities, such as antibacterial, antifungal, anti-inflammatory, hepatoprotective and anticancer activities (Chen et al., 2005; Liu et al., 2008; Chen et al., 2012). For decades, plants remain the main source of biologically active compounds that have been used for the treatment of various diseases, including infectious diseases with reduced side effects, bioavailability, less resistance, low toxicity and various mechanisms of action (Cowan, 1999; Mabona et al., 2013; Hassan et al., 2015). In recent years, plant-derived chemicals are increasingly used in pharmaceutical industry (Gibbons, 2008; Fischer et al., 2013). *Staphylococcus aureus* (*S. aureus*), a Gram-positive bacterium has become one of the most serious human pathogens that cause serious infections, such as bacteraemia, severe pneumonia and skin infections, while methicillin-resistant *S. aureus* (MRSA) is considered one of the most main causes of antibiotic-resistant healthcare-associated infections worldwide (Cutler and Wilson, 2004; Appelbaum, 2007; Pandey, 2007; Sharifi-Rad et al., 2014). The intensive

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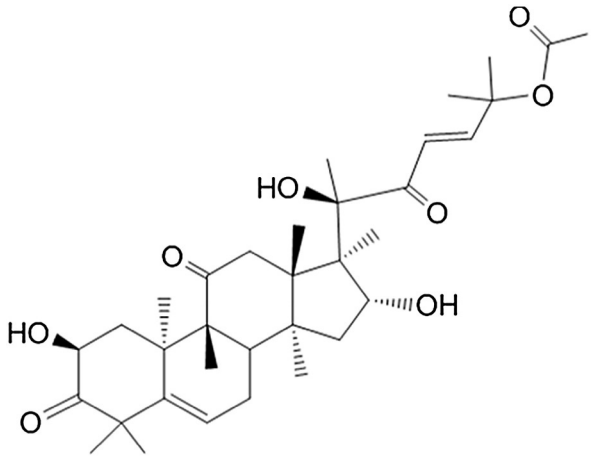

Fig. 1. Chemical structure of cucurbitacin B.

use of antibiotics has led to the problem of drug-resistant strains, and thus has led to the failure of current treatment regimens of staphylococcal infections in humans (Drago et al., 2014; Farrell et al., 2014). Therefore, a new treatment strategy has been devoted to overcome the problem by using plant-derived products in combination with antibiotics to enhance the treatment efficacy (Aiyegoro and Okoh, 2009; Wagner, 2011). Herpes simplex virus (HSV) infections are quite common in humans. HSV is a member of Herpesviridae, a wide family of enveloped-DNA viruses that cause several clinically significant syndromes in both adults and neonates. HSV-1 is mainly connected with oral or facial infection and encephalitis (Field, 1989; Paludan et al., 2011; Hassan et al., 2015). Treatment of HSV infection remains a main target for many researchers worldwide, where it cannot be managed by vaccination. Acyclovir and related nucleoside analogs have been widely used in the treatment of HSV, but the intensive use of such drugs has led to several undesirable effects including drug-resistant strains (Piret and Boivin, 2011; Evans et al., 2013). Despite few studies have reported that crude extracts of *E. elatrium* and various fractions of cucurbitacins have been shown to possess direct anti-staphylococcal growth inhibitory activity (Dogruoz et al., 2008; Oskay et al., 2009; Adwan et al., 2011), these studies did not evaluate the possibility of antimicrobial combinatory effect of Cuc B with standard antibiotics against *S. aureus*. Therefore, in this study, we report the synergistic effect of Cuc B with tetracycline (TET) or oxacillin (OX) as well as the direct inhibitory effect against *S. aureus*. In addition, we report for the first time the antiviral activity of Cuc B against HSV-1.

2. Materials and methods

2.1. Anti-Staphylococcus aureus activity

2.1.1. Bacterial strains, cultures, chemicals and antibiotics

Tetracycline, oxacillin and Cuc B were purchased from Sigma-Aldrich (Prague, Czech Republic). *S. aureus* (ATCC 29213) and methicillin-resistant *S. aureus* (MRSA-ATCC 43300) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Four clinical isolates of *S. aureus* (SA1, SA2, SA3, and SA4; isolated from patients with skin infection) were obtained from U Sv. Anny Univesity Hospital, Brno, Czech Republic. Dimethyl sulfoxide (0.05% DMSO; Sigma-Adrich, Czech Republic) was used to dissolve Cuc B. For antimicrobial assay, the strains were grown in cation-adjusted Mueller–Hinton broth (MHB; Oxoid, Basingstoke, UK) equilibrated with Tris–buffered saline (Sigma-Aldrich, Prague, Czech Republic).

2.1.2. Antimicrobial assay

For antibiotic susceptibility testing, *S. aureus* (ATCC 29213) was used as a reference strain. Cuc B at concentrations ranging from 0.25 to 3 μg/ml was used. Oxacillin-supplemented with 2% NaCl and tetracycline were used as standard antibiotics at concentrations ranging from 0.25 to 3 μg/mL. 0.05% DMSO and deionized water were used as negative controls that did not inhibit any strain tested. The broth microdilution method using 96-well microtiter plates was performed to determine the minimum inhibitory concentrations (MICs) following the recommendation of Clinical and Laboratory Standards Institute (CLSI, 2009). Briefly, the samples were two-fold diluted in MHB (100 μL), and inoculated with bacterial suspension to reach the density of 5×10^5 CFU/mL. Microtiter plates were incubated at 37 °C for 24 h, and bacterial growth was determined as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were subjected as the lowest concentrations that inhibited the growth of the test bacteria by ≥80% compared with that of negative controls. MICs obtained from three parallel experiments, each performed in triplicate.

2.1.3. Combinatory effect of Cuc B with antibiotics

The checkerboard assay was used to evaluate the combination effect of antibiotics with Cuc B, and the sum of the fractional inhibitory concentration (ΣFIC) indices have been evaluated as previously described (Vuuren and Viljoen, 2011; Hassan et al., 2016). Briefly, two-fold serial dilutions of oxacillin-supplemented with 2% NaCl or tetracycline prepared in horizontal rows of microtiter plate (at concentrations ranging from 0.25 to 3 μg/mL) were subsequently cross-diluted vertically by two-fold serial dilutions of Cuc B (at concentrations ranging from 0.25 to 3 μg/mL). The one-half MIC of Cuc B, oxacillin and tetracycline was used as a starting concentration in combinations. For evaluation of antibacterial combination effect of Cuc B (A) with antibiotic tested (B), the following equation $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_A$ (in the presence of B) / MIC_A (alone), and $FIC_B = MIC_B$ (in the presence of A) / MIC_B (alone), was used to calculate ΣFIC. The MICs used in this equation are the averages of MICs obtained from three parallel experiments, each performed in triplicate. The interpretation of the *in vitro* antibacterial interactions was determined as follows: synergistic effect if ΣFIC ≤0.5; additive if ΣFIC > 0.5 and ≤1; no interaction if ΣFIC > 1 and ≤4; and antagonistic if ΣFIC > 4.

2.2. Anti-HSV-1 activity

2.2.1. Viral strains, cultures, cell lines and reagents

For antiviral activity, Vero cells (ATCC: CCL 81, UK; were obtained from the Motol University Hospital, Prague, Czech Republic) were grown in Eagle's minimum essential medium (MEM; Cultilab, Campinas, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, UK), 100 U/ml penicillin G, 100 μg/ml streptomycin and 25 μg/ml amphotericin B (Sigma-Aldrich, Germany) and maintained at 37 °C in a humidified incubator with 5% CO_2. HSV-1 [KOS strain] was obtained from The Motol Univesity Hospital, Prague, Czech Republic, and propagated in Vero cells. Viral stocks were stored at −80 °C and titrated based on plaque forming units (PFU) count by plaque assay as previously described (Burleson et al., 1992).

2.2.2. Determination of cytotoxicity

MTT [3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay was used to determine the cytotoxic effect as previously described (Mosmann, 1983). In brief, confluent Vero cells were exposed to different concentrations of Cuc B for 72 h, and after incubation, the 50% cytotoxic concentration (CC_{50}) of Cuc B was calculated as the concentration that reduces cell viability by 50%, when compared to the untreated controls.

2.2.3. Antiviral assay

For antiherpetic activity, acyclovir was used as a positive control and the plaque number reduction assay was performed to evaluate the anti-HSV-1 activity as previously described (Silva et al., 2010). Briefly, cell