Purified citrinin in combination with vancomycin inhibits VRE in vitro and in vivo

Kléber de Sousa Oliveira,1 Paulo Roberto Martins Queiroz,2 Isabel Cristina Marques Fensterseifer,1,3 Ludovico Migliolo,1,4 Aline Lima Oliveira5 and Octávio Luiz Franco1,4,*

Abstract
Gram-positive pathogens such as methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus faecium (VRE) have been frequently associated with bacterial resistance mechanisms. These mechanisms, in turn, restrict a range of therapeutic opportunities for the treatment of infections caused by these microorganisms. Faced with this problem, the present study aims to isolate and characterize molecules with antimicrobial activity derived from the fungus Penicillium citrinum isolated from Cerrado soil. Furthermore, we also tested possible antibacterial potential alone and in combination with commercial antimicrobial agents. In this context, citrinin was isolated and characterized by nuclear magnetic resonance and electrospray ionization. Functional analyses showed MIC of 128 µg ml−1 against S. aureus ATCC 25923, E. faecalis ATCC 29212 and a clinical isolate of vancomycin-resistant E. faecium (VRE01). However, for a clinical strain of methicillin-resistant S. aureus (MRSA01), the MIC was 256 µg ml−1. In order to avoid such high concentrations and reduce the collateral effects, additive effects were evidenced by combining citrinin with cefoxitin against MRSA01 (FIC index=0.5) and also citrinin with vancomycin toward VRE01 (FIC index=0.5). In vivo studies with BALB/c-tipe mice (MRSA assay) demonstrated a clinical ineffectiveness of cefoxitin associated with citrinin (9.8 mg kg−1 of cefoxitin +0.2 mg kg−1 of citrinin), with this combination being inefficient to increase animal survival. However, the combination used in the treatment of VRE (23.5 mg kg−1 of citrinin +1.5 mg kg−1 of vancomycin) sepsis model was extremely promising, leading to an animal survival rate of 80 percent. In summary, our data show, for the first time, the possible successful use of citrinin associated with vancomycin for pathogenic bacteria control.

INTRODUCTION
Infections caused by multidrug-resistant (MDR) bacteria are one of the worst medical problems faced today, increasing costs, morbidity and mortality rates and limiting the antibiotic therapy options [1–4]. In the past, infections caused by these bacteria were almost exclusively limited to hospital environments. Nevertheless, this situation is changing, and currently there are a number of reports of infections caused by these micro-organisms in community settings [5–7]. Amongst them are MDR Gram-positive bacteria that have included methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus faecium (VRE) [3, 8].

The treatments used against infections caused by these micro-organisms vary according to infection sites as well the antibacterial susceptibility profile, which is usually specific for each isolate [6, 9]. In addition to these peculiarities, only a few clinical studies have been conducted, with the aim of standardizing the most efficient therapeutic option for the control of these infections. In general, infections caused by MRSA are limited to treatment with glycopeptides, such as teicoplanin and vancomycin (gold standard) [9, 10]. Otherwise, VRE may not be treated with vancomycin, and only linezolid and daptomycin have been routinely used [11, 12]. New drugs for the treatment of these bacteria have entered the market, including tedizolid, dalbavancin...
and oritavancin, which are antibacterials recently approved by the US Food and Drug Administration (FDA) [13–16].

Citrinin, as well as several other secondary metabolites belonging to polyketides class, have several interesting biological activities such as antibacterial, antiviral, antitumor, antiparasitic, among others [17–19]. Among polyketides are included some important drugs such as amphotericin B, daunorubicin, erythromycin, rapamycin and lovastatin that are widely used in clinical practice [20].

With regard to these issues, pharmaceutical companies and research centers have been working on developing methodologies for prospecting novel antibiotic agents [21–23]. Among the strategies used, the synergistic and additive effects of existing drugs are of particular interest. The combined utilisation of antimicrobials may assist in the treatment of serious infections that could have no single therapeutic choice. Additionally, this behaviour could help to reduce the MDR-bacterial emergence [24]. Several studies have demonstrated the clinical efficacy of this type of methodology against producing Gram-negative bacteria carbapenemase [24], other β-lactamas, methicillin-resistant S. aureus [25], and vancomycin-resistant S. aureus [26]. Combined drugs such as amoxicillin/clavulanate, ampicillin/sulbactam and piperacillin/tazobactam have been successfully used in controlling bacteria with multiple resistance mechanisms [25].

Since it is known that toxic drug effects could be directly related to dose, synergism with other molecules may decrease the dose needed for infection control as well their side effects. This study aims to examine the in vitro and in vivo antimicrobial activity of citrinin, derived from the fungus Penicillium citrinum isolated from Cerrado soil, alone and in combination with other antimicrobials, to provide new alternatives for the treatment of MRSA and VRE.

METHODS
Fungal strain and purification of citrinin
For this study P. citrinum (PC01) was used. This strain was obtained from Cerrado soil, located in the administrative region of Vicente Pires, Distrito Federal, Brazil (15° 50’ 08,11” S 48° 00’ 16, 89° W). To achieve the antimicrobial activity, a filtrate was obtained after fungal growth in potato dextrose liquid medium was subjected to liquid-liquid partition with 100 % ethyl acetate. The procedure was repeated twice more, and the fraction collected from the acetate was concentrated in a rotary evaporator Q344B (Quimis) at 85 °C. Then the filtrate from the partition was subjected to high-performance liquid chromatography (HPLC) using a reverse phase C8 column (4.6×250 mm 5 µm, Grace Vydac); as mobile phase water and acetonitrile were used. Further analysis of the purity of citrinin was performed in a fast liquid chromatograph (UFLC) (Shimadzu) using an XR-ODS C18 column 2.0×30 mm 2.2 µM, coupled to a mass spectrometer type micrOTOF ESI-Q III (Bruker Daltonics). The molecular formula of each compound in the spectrum obtained was deduced by SmartFormula program (Bruker Daltonics), and the data generated by the program matched the database of the Royal Chemical Society (www.chemspider.com) and literature data.

NMR characterization
All NMR measurements were carried out through on a Varian Mercury Plus spectrometer (7.05T) operating at 300 MHz for 1H and at 75.46 MHZ for 13C. All experiments were performed at 20 °C in a NMR tube of 5 mm diameter. The samples were prepared using deuterated chloroform (CDCl3) and tetramethylsilane (TMS) as internal reference. Spectrum analyses were carried out through the ACD/Spectrus Processor program (Advanced Chemistry Development).

Minimum inhibitory concentration (MIC)
In order to determine the citrinin MICs, a microdilution test was performed in accordance with the Clinical Laboratory Standards Institute [27]. Four micro-organisms were tested, including S. aureus ATCC 25923, E. faecalis ATCC 29212, a clinical isolate of methicillin-resistant S. aureus (MRSA01) and a clinical isolate of vancomycin-resistant E. faecium (VRE01). As positive control 1.5 % DMSO was used, and chloramphenicol (30 µg ml–1) was used as negative control. The absorbance values were automatically read every 60 min by the microplate reader (EON, BioTek) at a wavelength of 625 nm. Moreover our tests were always performed in technical and biological triplicates, in order to consolidate the results found, thus reducing the chances of obtaining a false positive result.

Checkerboard synergism test
Micro-organisms (MRSA01 and VRE01) were cultivated on BHI agar at 37 °C for 24 h. From these cultures, cell suspensions were prepared similarly to 0.5 MacFarland (1.0 to 2.0×108 c.f.u. ml–1), and this suspension was diluted in scale (1 : 10) in BHI broth in order to obtain a final bacterial concentration of 1.0 to 2.0×107 c.f.u. ml–1. The assay was performed in a 96-well plate with the final volume of 200 µl containing a suspension of 5.0×105 c.f.u. ml–1 per well and the following dilutions (µg ml–1) of 512, 256, 128, 64, 32, 16, 8, 4, 2 of citrinin, the same procedure performed for the two antibiotics used for the antibacterial synergism tests: cephotixin (Sigma Aldrich) and vancomycin (Sigma Aldrich); these latter two were used only for MRSA and VRE successively. As positive control 1.5 % DMSO was used, and as negative control chloramphenicol (30 µg ml–1) was used. The absorbance values were automatically read every 60 min by the microplate reader (EON, BioTek) at a wavelength of 625 nm. The MIC of the combinations was determined by no bacterial growth, with the results of the readings of the equipment, and by visual inspection of the lack of turbidity. The interaction results were performed by determining total fractional inhibitory concentration (FIC index). This was calculated by summing FICA + FICB, which in turn were calculated separately as follows: FICA=MIC of the combination/MIC of the antibiotic A alone, and FICB=B combined MIC/antibiotic MIC B alone (FIC index values ≤0,5 were
considered synergistic effects between 0.5–1.0 additives and indifferent 1–2) [28, 29].

Cytotoxic evaluation of purified and combined citrinin

The concentrations of the isolated citrinin (256 and 128 µg ml⁻¹) and combined citrinin (64 µg ml⁻¹ cefoxitin +1 µg ml⁻¹ citrinin) (4 µg ml⁻¹ vancomycin +64 µg ml⁻¹ citrinin) were evaluated for their cytotoxic potential against the cell line RAW 264.7 monocyte. These cells are well characterized and are standard for cytotoxic tests. They were plated in a 96-well microplate at a concentration of 1.0×10⁵ cells ml⁻¹, using Dulbecco’s modified medium (DMEM-s) supplemented by agar medium, making the final volume of the well 200 µl. They were incubated at 72 h at 37 °C in an oven with 5 % CO₂. At the end of 72 h, 155 µl supernatant was removed from each well and 10 µl of 3-(4,5-di-methylazol-2-il)-2,5-difenil tetrazolium bromide (MTT) added (5 mg ml⁻¹). The plate was incubated in a CO₂ incubator at 37 °C for 3 h, and after that period 60 µl of DMSO 100 % was added. Finally, the plate was read by a microplate reader (EON, BioTek) at a wavelength of 575 nm to check cytotoxic activity. As a positive control we used DMEM-s and as a negative control the lysis solution (50 mM Tris, pH 7.4, 1 mM EDTA and 0.1 % Triton X-100) [30, 31].

Animals

Pathogen-free female BALB/c mice (aged 4 to 6 weeks) were obtained from Bioassays Labs (Distrito Federal, Brazil). The animals were kept in polypropylene cages (five animals per cage) at a temperature of 23±2 °C with 12:12 h dark-light cycle and food and water ad libitum. All procedures with the animals were approved by the Local Committee for Institutional Animal Care and Uses (CEUA UCB 026/2014) and according to international ethical standards.

In vivo evaluation of combined citrinin

A sepsis model was used for in vivo experiments [32, 33]. We performed the assay with a biological quintuplet for each treatment. Forty animals (body weight approximately 18–21 g) were randomly divided into two groups. Twenty animals (body weight approximately 18–21 g) were intraperitoneally infected with MRSA (5×10⁶ c.f.u. ml⁻¹) and another 20 were infected with VRE (2×10⁸ c.f.u. ml⁻¹). We first performed the in vitro checkerboard assay to determine the percentage of each drug required to have a potentiating effect against the bacteria tested. After the bulk dilution procedure we identified that it would be necessary to use 94 % citrinin +4 % vancomycin (23.5 mg kg⁻¹ of citrinin +1.5 mg kg⁻¹ of vancomycin) to have the desired effect against VRE and 98.5 % of cefoxitin +1.5 % citrinin (9.8 mg kg⁻¹ of cefoxitin +0.2 mg kg⁻¹ of citrinin) for MRSA. The experimental design is illustrated in Fig. S1 (available in the online Supplementary Material). These groups of 20, in turn, were divided into smaller groups (five animals) and treated separately. Mice were treated with a single dose 30 min after infection. The monitoring of deaths was carried out for 7 days.

Statistical analysis

The results observed in the in vitro cytotoxicity test were represented with their respective means and standard deviations. Statistically significant results were determined by student’s t-test or an analysis of variance (ANOVA). The percentage of survival of mice was analyzed by the Kaplan-Meier test. The statistical significance of the results was calculated using the log-rank test. P values ≤0.05 were considered to indicate statistical significance. All statistical analysis was performed using GraphPad 6.0 PRISM.

RESULTS

Isolation and purification of citrinin

Twelve chromatographic fractions (Fig. 1a) were obtained from the filtrate M01. Each fraction was tested by the disc diffusion method against Gram-positive bacteria, including S. aureus ATCC 25923, MRSA01, E. faecalis ATCC 29212, and VRE01. Only fraction 8 was capable of inhibiting all of the bacteria tested (data not shown). Fraction 8 was chromatographed onto an UFLC, showing a single chromatographic peak (Fig. 1a). The ESI-MS analysis (Fig. 1b) revealed the presence of the protonated molecule [M+H]⁺ (273.07 Da) equal to 251.09 Da, sodium ion [M+Na]⁺ (273.07 Da) and dehydrated ion [M-H₂O]⁻ (233.08 Da). Through the SmartFormula program (Bruker Daltonics), it was possible to propose that the metabolite molecular formula was C₁₃H₁₂O₅ for 251.09 Da, C₁₃H₁₄NaO₅ for 273.07 Da and C₁₃H₁₃O₄ for 233.08 Da, suggesting that the molecular ion m/z is equal to 250 Da and molecular formula C₁₃H₁₂O₅. The data obtained by ESI-MS when compared with data in the literature and the ChemSpider masses bank revealed that this compound is citrinin. This identification was confirmed by NMR data evidencing the chemical shifts: ¹H NMR (300 MHz, CDCl₃): δ 8.23 (3H, s, H-8), δ 7.77 (3H, s, H-13), δ 1.34 (3H, d, H-11, J=6.7), δ 1.22 (3H, d, H-12, J=7.2) and two hydrogen signals with chemical shifts in very de-blinded δ 15.11 and δ 15.86 ppm, relative to the hydroxyl chelated with carbonyls and ¹³C NMR (75 MHz, CDCl₃): δ 183.76 (C-8), δ 177.17 (C-6), δ 174.49 (C-14), δ 162.76 (C-10), δ 139.04 (C-1), δ 123.05 (C-5), δ 107.38 (C-8a), δ 100.29 (C-7), δ 81.61 (C-3), δ 34.57 (C-4), δ 18.48 (C-12), δ 18.22 (C-11), δ 89.43 (C-13) (Fig. 1c).

Antibacterial activities of citrinin and further combinations

The MICs 100, complete bacterial inhibition, of citrinin were determined for S. aureus ATCC 25923 (128 µg ml⁻¹), E. faecalis ATCC 29212 (128 µg ml⁻¹), MRSA01 (256 µg ml⁻¹) and VRE01 (128 µg ml⁻¹) by the broth microdilution method. MICs 100 of cefoxitin (128 µg ml⁻¹) against MRSA, and of vancomycin (512 µg ml⁻¹) toward VRE. Furthermore, synergism was determined for MRSA01 (64 µg ml⁻¹ of cefoxitin +1 µg ml⁻¹ of citrinin) and for VRE01 (4 µg ml⁻¹ of vancomycin +64 µg ml⁻¹ of citrinin). The results of all FIC indices were both considered, and the additive effects were 0.5 for MRSA01 and VRE01 (Fig. 2).
Viability of MTT assay

Cell viability tests showed that citrinin, when used alone at high concentrations (256 µg ml\(^{-1}\)), has deleterious effects on RAW 264.7 cells growth (\(P=0.004\)) when compared with negative control (DmeM-s). However, when citrinin was combined with vancomycin and cefoxitin, no harmful effects were observed, suggesting that this combination is a very promising alternative to use (Fig. 3).

In vivo assays

The results of in vivo tests for association showed that the in vivo combinatory effect observed with cefoxitin and citrinin was insufficient to control the MRSA infection in a sepsis model (9.8 mg kg\(^{-1}\) of cefoxitin +0.2 mg kg\(^{-1}\) of citrinin), since all the animals treated with this combination died on the second day (Fig. 4a). However, the combination of vancomycin with citrinin for the sepsis model treatment with VRE showed higher effectiveness (23.5 mg kg\(^{-1}\) of citrinin +1.5 mg kg\(^{-1}\) of 232 vancomycin). When animals were treated only with the vehicle or vancomycin alone, 80% of the animals died on the first day, whereas when the animals were treated with the combination of citrinin plus vancomycin, no animal died on the first day. After 7 days, 80% of survival (\(P=0.0005\)) of BALB/c mice was observed. These concentrations were defined with the percentage of each drug obtained in the synergism test in vitro; for example, we used 4 µg ml\(^{-1}\) vancomycin +64 µg ml\(^{-1}\) citrinin (94% citrinin +4% vancomycin). These data were similar to results obtained with the treatment with positive control (5.0 mg kg\(^{-1}\) of vancomycin +5.0 mg kg\(^{-1}\) of gentamicin) (\(P=0.0005\)) (Fig. 4b).

DISCUSSION

Although several previous studies have described the nephrotoxic and teratogenic citrinin effects [34–36], beneficial effects such as antimicrobial, antitumoral, antiviral among others have been described since their discovery. In our study, we observed the cytotoxic citrinin effects only when used at a high dose of 256 µg ml\(^{-1}\). A similar study with Vero cells, from green monkey kidney challenging high citrinin doses (55 053 µg ml\(^{-1}\)) was performed showing a cell viability decrease [36]. These data consolidate our theory that the citrinin cytotoxicity may be directly related to its concentration.
against the cell tested. Thus, studies aimed reducing the citrinin dose as an alternative to decrease the citrinin side effects.

We also demonstrated the capability of citrinin to inhibit growth of MDR bacteria in in vitro assays, including MRSA01 and VRE01. Since our MIC 100 values were considered high for MRSA01 (256 µg ml⁻¹) and VRE01 (128 µg ml⁻¹), we decided to study a way to reduce the citrinin concentrations required to prevent the growth of those bacteria. A similar study was published by [21], which tested citrinin isolated from Penicillium sp. against MRSA and VRE, obtaining the MICs 3.9 and 7.81 µg ml⁻¹, respectively. This discrepancy may be due to the difference in the bacterial inocula used for microdilution tests. Given the high values of MICs found, we then decided to seek viable alternatives for reducing the amount of citrinin to be used in tests against MRSA and VRE. Thus, we tested the combinatorial effects of citrinin with cefoxitin for MRSA01 and citrinin with vancomycin for VRE01 (in both cases, the bacteria show intrinsic resistance to the respective antibiotics). The association results indicated an additive effect. We hypothesised that the additive effect may occur since citrinin, vancomycin and cefoxitin act on very similar cell wall components present including the mesosomes and also in peptidoglycan formation. The effects evidenced in our work are possibly related to mesosome activity inhibition including a reduction in Fe³⁺ and lipid peroxidation amount available, thus interfering with NADPH cytochrome P₄₅₀ reductase activity [37, 38]. This, in turn, may be associated with cell wall synthesis inhibition, commonly caused by cefoxitin and vancomycin. Similar combinatorial effects, as obtained in this study, have been described for a combination of azonapyrone with vancomycin against E. faecalis and E. faecium [39]. Furthermore, a fungal extract of Colletotrichum gloeosporioides associated with vancomycin was also effective against S. aureus [40].

In possession of in vitro association results, the in vivo combinatorial effects of citrinin combined with cefoxitin and vancomycin were challenged. In vivo studies showed that cefoxitin and citrinin (9.8 mg kg⁻¹ of cefoxitin + 0.2 mg kg⁻¹ of citrinin) in the treatment of a sepsis model were not enough to increase the survival of animals infected with MRSA01 (Fig. 4a). In contrast, the combination used in the treatment of a VRE (23.5 mg kg⁻¹ of citrinin + 1.5 mg kg⁻¹ of vancomycin) sepsis model was extremely desirable, with an animal survival rate of 80% (Fig. 4b), similar data were found in the group of animals treated with vancomycin associated with gentamicin (positive control). Although this combination is not routinely used, it has been adopted in some cases in which there is no response to treatment. Some studies report that the use of vancomycin in combination increases the entry of gentamicin into the cell, thus favoring the increase of their activity [41–43]. In view of the excellent outcome obtained in the treatment of the sepsis model, similar effects are likely to be obtained from studies of limited infections caused by such bacteria, such as skin and soft tissue infections, which are widely caused by these microorganisms. These effects are expected because citrinin is extremely apolar, so it can easily bind to plasma proteins and
be carried by the various anatomical sites. Similar *in vivo* combinatorial effects have been described for penicillin associated with streptomycin and penicillin associated with gentamicin against *Streptococcus viridans* [44], which led to an improvement of 100% in animal survival.

As observed in our study, some care should be taken in the citrinin utilisation for infection diseases treatment such as which dose should we use for treatment, should we differ in dosages for individuals with hepatic and renal dysfunction, and what is the periodicity of administrations? Further studies of pharmacokinetics and pharmacodynamics of vancomycin-associated citrinin should be performed to determine the optimal plasma dosage for MDR-related diseases treatment. Since citrinin is a highly hydrophobic molecule, its plasma concentration should remain stable for a long time, even with low administered concentrations.

In summary, our data show for the first time the possible successful use of citrinin associated with vancomycin for VRE control, demonstrating an attractive novel treatment for persistent infections caused by this organism. In addition, we hope that further studies with citrinin will be developed to verify its possible synergistic or additive effects with other commercial antimicrobials, thus increasing the choices for the treatment of infections caused by MDR in sites other than those studied by our group.

**Funding information**

This work was supported by Universidade Católica de Brasília (UCB), Distrito Federal State Agency for Research and Development (FAPDF), Foundation to Support the Development of Education, Science and Technology (FUNDECT) and the National Council for Scientific and Technological Development (CNPq).

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**


