Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.journals.elsevier.com/revista-brasileira-de-farmacognosia

Original article

Study of the antimicrobial activity of metal complexes and their ligands through bioassays applied to plant extracts

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ARTICLE INFO

Article history:
Received 16 Ocotber 2013
Accepted 4 April 2014

Keywords:
Bacterial resistance
Antibiotics
Metal complex
Antibacterial activity
Disk diffusion

A B S T R A C T

The appearance of resistant bacteria was found to reduce the efficiency of antimicrobial therapies with the current antibiotics, thereby increasing the need for more efficient drugs for the treatment of infections. Several studies have demonstrated an increase in antimicrobial activity following the interaction of several compounds with metal ions. The present study used a methodology adapted for antimicrobial bioassays using plant extracts, in compliance with the standards of the Clinical and Laboratory Standards Institute against Gram-positive and Gram-negative bacteria. The results obtained were considered appropriate for determining MIC, MBC as for performing antimicrobial sensitivity testing with good efficiency and reproducibility. The bacteria *Pseudomonas fluorescens* exhibited high sensitivity to the tested compounds, being efficient to evaluate the antibacterial activity. The bioassays with the metal complexes of flavonoid quercetin and Ga(III) ions, and synthetic ligand H2bbppd and Cu(II) ions showed a greater inhibitory effect than their individual ligands, thus, the addition indicated an increase in the antimicrobial activity after the coordination. Both metal complexes exhibit good antimicrobial performances, such as low minimum inhibitory concentration (MIC ≤ 250 μg/ml), bactericidal effect and a broad activity spectrum, which qualify these compounds as suitable candidates to the next step of drugs fabrication. Nevertheless, further studies on the mechanism of growth inhibition and toxicity are needed, in order to evaluate the potential of therapeutic application.

Introduction

Annually, millions of people die due to infections caused by microorganisms resistant to current antibiotics (WHO, 2012). When an antibiotic is discovered and commercially available, the appearance of resistant strains begins to reduce its clinical utility after a period of indiscriminate use, leading to future use restriction (Rocha et al., 2011). The use of antibiotics with broad spectrum of action and low toxicity can reduce the efficacy of future antimicrobial therapies, leading to the use of drugs

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http://dx.doi.org/ 10.1016/j.bjp.2014.07.008

with larger selective toxicity (Tortora et al., 2003; Willey et al., 2008). In addition, the use of restricted antibiotics can cause the failure of antimicrobial therapy, thereby increasing the rate of morbidity and mortality, along with the treatment costs (Garcia, 2011).

Microbiologists acknowledge that Gram-negative bacteria own mechanisms specialized in the extrusion of strange substances out of the cell (efflux bomb), limiting the access of antimicrobial agents to its active site. Consequently, it prevents the accumulation of antibiotics in the interior of the cell, and inhibits the action of antimicrobial agents (Reichling et al., 2005). Furthermore, the enzymes are known to be capable of rendering drugs inactive (β-lactamases), making the cell resistant to them in their periplasmic space (Silva, 2011).

In an analogous way, the Gram-positive bacteria protect their cytoplasmic membrane with a thick cell wall. The myriad layers of peptidoglycans hinder the passage of hydrophobic compounds owing to the presence of sugars and amino acids (Sartori, 2005). By virtue of that , there has been a rise in research studies aiming at producing compounds with a broad spectrum of activity, and with action mechanisms unknown to the pathogenic bacteria. In spite of its low virulence in the human, *Pseudomonas fluorescens* (a Gram-negative bacterium) is considered as a health problem, due to its resistance against antiseptics and antibiotics used in medical centers (Gershman et al., 2008). Given the scarcity of reports regarding its use in the study of antimicrobial activity, the strain was selected purposefully for its evaluation among other bacteria of clinical interest.

In the search for new antimicrobials, effective in the treatment of infections caused by multiresistant bacteria, due consideration ought to be given for the synthesis of drugs with new activation targets, as well as the potentialization of the activity of compounds with known antimicrobial activity (Schaechter et al., 2002; Masunari and Tavares, 2006). A new strategy in the production of drugs proposes the interaction of metal ions for antibiotics within three study fields: the first one aiming at creating a reversed mechanism of microbial resistance; the second one seeking to promote the development of new drugs with an action mechanism unknown to the pathogenic bacteria; and a third one aiming at reducing the toxicity of the metal ion in the form of a complex (Rocha et al., 2011).

The interaction of metal ions with organic ligands shows better antimicrobial activity compared to free ligands (not coordinated), and as such, it justifies the investigation of new drugs with unknown mechanism of action against pathogenic bacteria. The use of these new compounds is likely to have great potential against pathogenic bacteria, nonetheless, the need for new methodologies of evaluation of antimicrobial activity cannot be relegated to the background. Admittedly, owing to the innovative character of this approach, we have not found enough information in the literature regarding the use of specific bioassays involving metal complexes. Although, there is a great variety of laboratory methods that can be used to measure the susceptibility of bacteria to antimicrobial agents *in vitro*, these are applied, for example, to plant extracts in different fractions of solvent (Mamidala and Gujjeti, 2013; Soniya et al., 2013).

The above mentioned methods are effective for the determination of parameters such as the minimal inhibitory concentration (MIC), the minimal bactericidal concentration (MBC), as well as performing the susceptibility test to antimicrobials. These methodologies are known to have, undoubtedly, good efficiency and reproductivity, but in a mixture of compounds it turns too hard to establish which is the active substance acting as the antimicrobial agent (Gonçalves et al., 2011; Sá et al., 2011; Prasannabalaji et al., 2012). Therefore, the purpose of this research was to evaluate the antimicrobial activity of some metal complexes, and to compare their performance against free ligands. The response to standardized Gram-positive and Gram-negative bacteria was taken as a reference; including the applicability of the use of *Pseudomonas fluorescens* and the methodologies for bioassays, as a proper methodology for the evaluation of antimicrobial activity of plant extracts.

Materials and methods

Materials

The bacterial strains *Staphylococcus aureus* (ATCC SP 25923); *Enterococcus faecalis* (ATCC SP 19433); *Escherichia coli* (ATCC SP 11229), and *Pseudomonas fluorescens* (ATCC SP 13525), purchased for this work, were reconstituted in sterile distilled water, cultured in Muller-Hinton medium, and incubated at 37°C for 24 h. The standardized antimicrobial CEFAR discs, containing Aztreonam (AZT) µg; Ceftazidime (CAZ) 30 µg; Chloramphenicol (CLO) 10 µg; Imipenem (IPM) 10 µg; Tetracycline (TET) 30 µg; Vancomycin (VAN) 10 µg.

Synthesis of the chemical compounds

Metal Complex 1 - Ga(III)-Quercetin

Ga(III) ions were complexed with quercetin ligand using the methodology proposed by Simões et al. (2013), which involves the reaction between the flavonoid quercetin with gallium(III) nitrate salt, in a 3:1 stoichiometric proportion. The results obtained through CNH and infrared spectroscopy indicate the formation of a mononuclear metal complex with adequate purity degree for the bioassays experiments. The elemental analysis of CNH for $GaC_{45}H_{39}O_{27}$: MM 1,081.51 g mol^{-1}; calculated: C 49.98 % e H 3.63 %; found: C 49.41 % e H 3.51 %.

Synthetic ligand - H2bbppd

The synthetic ligand N,N´,N,N´-bis[2-hydroxi-3,5-di-tert-buthylbenzyl)(2-pyridylmethyl)]-1-3-diaminopropane, identified in this work as H2bbppd was synthesized in line with the methodology proposed by Cabeza et al. (2010). The CNH and infrared spectroscopy results indicate the formation of a mononuclear metal complex with a purity degree adequate for its use in bioassays and for the synthesis of the Cu(II) complex. The elemental analysis of CNH for $C_{45}H_{64}N_4O_2$: MM 693 g mol^{-1}; C 77.99; H 9.31; N 8.08 %. Found: C 77.51; H 9.42; N 8.20 %. IV (KBr), in cm^{-1}: 3400-3300 (ν_{O-H}); 2956 (ν_{C-H}, *tert*-buthyl); 1596, 1477 ($\nu_{C=N,C=C,}$ aromatics); 1394 (δ_{O-H}, phenol); 1362 (δ_{C-H}, *tert*-buthyl); 1237 (ν_{C-O}, phenol); 879 (δ_{C-H}, aromatics); 756 (δ_{C-H} pyridine).