Antimicrobial effect of Morita-Baylis-Hillman adducts against oral pathogens and cellular viability in human leukocytes

Efeito antimicrobiano de adutos de Morita-Baylis-Hillman contra patógenos orais e viabilidade celular em leucócitos de humanos

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ABSTRACT
The aim of this work was to evaluate Morita-Baylis-Hillman adduct (MBHA) antimicrobial effect against oral pathogens and related cell viability in human leukocytes. Minimum inhibitory concentration (MIC) was determined using microdilution method. Cell viability was assessed in human peripheral blood mononuclear cells (PBMCs) using the resazurin assay. For S. aureus (ATCC 15656) and S. mutans (UA 159) MIC values of 2,000 μg/mL were reported for the A1 Morita-Baylis-Hillman adducts. The MICs of the A2 and A3 adducts were not found for the bacterial strains. MIC values for the A1 adduct was 125 μg/mL, A2 1,000 μg/mL and A3 to 15.6μg/mL against the C. albicans strain (ATCC 11006). PBMCs showed cell viability greater than 50 % when in contact with concentrations 10x higher than MIC of MBHA. It is concluded that MBHA A1, A2 and A3 present potential antimicrobial effects against C. albicans without presenting substantial cytotoxic effects in human cells, highlighting adduct A3 for future therapeutic applications.

Keywords: Microbiology, Organic Chemicals, Cytotoxicity Tests, Immunologic, Leukocytes.
Many of the diseases that affect the oral cavity are associated with infectious agents. The microorganisms that colonize the oral cavity contribute to the development of numerous clinical conditions: such as dental caries, periodontal, endodontic, and periapical diseases (Foschi et al. 2006), oral candidiasis (Basavaiah, Reddy, and Badsara 2010), and others. The pathogenicity of microorganisms depends on their virulence and the state of the host (Foschi et al. 2006; Fauci et al. 2018).

The microorganisms found in hospitals appear to be more resistant because they promote high morbidity and difficult eradication of these infections (Singh and Bhat 2011; Fauci et al. 2018). Due to excessive and indiscriminate use of first-generation drugs and the consequent growing increase of bacterial (Toma and Deyno 2015) and fungal (Cuenca-Estrella 2014; Scorzoni et al. 2017) resistance, the antimicrobial effects of many substances for clinical use are being intensely studied. Some new options for treatment of these diseases have been proposed including modified molecules. Morita-Baylis-Hillman adducts (MBHA) are molecules derived from Morita-Baylis-Hillman reactions (MBHR), which consist in alkene group coupling between molecules containing electron attracting groups (EAG), and aldehydes, ketones and imines in the presence of a nucleophilic catalyst, typically a tertiary amine (Barbosa et al. 2009), such as the 1,4-diazabicyclo-[2.2.2]-octane (DABCO) is an example of MBHR (Basavaiah, Reddy, and Badsara 2010). This reaction results in different molecules that can have biological effect.

These MBHA have been employed in several applications as starting material for organic synthesis products, such as antiparasitics (Barbosa et al. 2009; Lima Junior et al. 2010), neoplastics (Kohn et al. 2006; Lima-Júnior et al. 2016; Faheina-Martins et al. 2017), antioxidant (Elleuch et al. 2018) and anti-inflammatory (Faheina-Martins et al. 2017; França et al. 2021). Although some molecules of MBHA have been previously evaluated in parasites and bacteria, different molecules need to be studied. In addition, there are no reports of the antimicrobial evaluation against oral pathogens including fungal species.

Thus, the objective of this study was to evaluate the antimicrobial effect of Morita-Baylis-Hillman adducts against common oral pathogens and their cell viability in human leukocytes, aiming at the future use of these compounds in antimicrobial therapy.

2 MATERIALS AND METHODS

2.1 MORITA BAYLIS HILLMAN ADDUCTS (MBHA)
In this study we used three Morita-Baylis-Hillman reaction synthesized MBHA (A₁, A₂ and A₃), using the corresponding aldehyde and acrylonitrile or methyl-acrylate in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) as nucleophilic catalyst at 0°C (Table 1).

Table 1. Synthesis of the Morita-Baylis-Hillman (MBHA) adducts used in this study, where (AIR) is the aromatic ring and (EAG) represents the electron-attractor group.

<table>
<thead>
<tr>
<th>MBHA</th>
<th>AIR</th>
<th>EAG</th>
<th>Molecules obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>P-(Br)C₆H₄</td>
<td>Lu</td>
<td>C₁₉H₂BrNO</td>
</tr>
<tr>
<td>A₂</td>
<td>2-C₅H₄N</td>
<td>Lu</td>
<td>C₈H₆N₂O</td>
</tr>
<tr>
<td>A₃</td>
<td>2-C₁₀H₇</td>
<td>CO₂CH₃</td>
<td>C₁₂H₁₄O₃</td>
</tr>
</tbody>
</table>

The compounds were obtained ready-for-use and were pre-purified through simple filtration in silica gel, using ethyl acetate/hexane (2:8) as the solvent, and then subjected to chromatography to verify their purity. The spectroscopic study data for the adducts used are described in the literature (Barbosa et al. 2009; Lima Junior et al. 2010).

The MBHA(s), obtained in powder form were dissolved in a sterile distilled water solution, with 10% dimethylsulfoxide (DMSO - Casa da Química Ltda, Diadema, Brazil), and 5% Tween 80 ® (Dynamic Contemporary Chemistry Ltda, São Paulo, Brazil), resulting in the stock solutions with concentrations of 8 mg/mL.

2.2 MICROBIOLOGICAL TEST

2.2.1 Microbial Strains and preparation of the inoculum

The reference strains (AU - University of Alabama - and ATCC - American Type Culture Collection) used for evaluation of the MBHA antimicrobial effect were Streptococcus mutans (AU 159), Staphylococcus aureus (ATCC 15656), seeded in Brain Heart Infusion (BHI-Broth (Brain Heart Infusion Broth - HiMedia Laboratories Pvt Ltd), and Candida albicans (ATCC 11006), seeded in Sabouraud Dextrose Broth (CSD).
(HiMedia Laboratories Pvt Ltd). The bacteria were anaerobically cultured at 37°C (5% CO$_2$). *Candida albicans* strain was grown at 37°C in aerobic conditions. The strains were standardized by means of a spectrophotometer (GloMax® Multi Microplate Reader, Promega, Madison, USA), at a wavelength of 625 nm for bacteria and 560 nm for the fungus, with absorbance values ranging from 0.080 to 0.100 corresponding to concentrations of $10^8$ CFU/mL and $10^6$ CFU/mL respectively. These concentrations were equivalent to 0.5 McFarland Scale, and diluted to 1:1000 for microbiological testing, in accordance with the Clinical and Laboratory Standards Institute methodology (CLSI 2008, 2015).

### 3 MICRODILUTION

Determination of Minimum Inhibitory Concentrations (MIC) for the adducts evaluated was performed using broth microdilution technique (CLSI 2008, 2015). Initially, were distributed 100 µL of medium per hole in the broth microdilution U bottom plate. Then, 100µL of the evaluated MBHA went to the first hole and diluted serially through withdrawal of a 100µL aliquot transferred from more concentrated cavities to successor cavities continuing thus to the last hole. Afterwards, 100µl of each inoculum was added to each well: at the concentrations of $10^5$ CFU/mL for bacteria; and $10^3$ CFU/mL for fungi (CLSI 2008, 2015).

In parallel with the microdilution, growth controls (GC – 100 µL of broth and 100 µL of the inoculum), sterility of the medium (MS – 100 µL of broth), and negatives: (CN – 100 µL of DMSO solution at 10 %, and Tween 80 to 5 % at 100µL of broth medium) were carried out. The assay was performed in triplicate with microdilution plates incubated at 37 °C for 48 hours.
The readings for determinations of the adduct MICs on the strains of *S. mutans*, *S. aureus*, and *C. albicans* were made using visual methods, taking into account cell cluster formation in the bottom of the microdilution plate cavity (CLSI 2008, 2015). To confirm the absence of viable microorganisms for inhibitory concentrations, resazurin sodium dye was used (Sigma-Aldrich® St. Louis, USA) at 0.01 % for bacteria; and 2,3,5-triphenyl tetrazolium chloride (TCT) (Sigma-Aldrich®, St. Louis, USA) at 1% for *C. albicans* (Gomes et al. 2015). The MIC was considered as the lowest concentration of the product in the test, capable of inhibiting the growth of the strain used in each microbiological assay (Andrews 2001).

3.1 CELL VIABILITY

Cell viability was performed in peripheral blood mononuclear cells (PBMCs) from three healthy volunteers. The PBMCs were collected through peripheral venous puncture in heparinized tubes, and isolated using the Ficoll-Paque™ 1077 protocol (GE Healthcare, Little Chalfont, United Kingdom). The initial viability of the cells was determined using the exclusion method with Trypan blue 0.4% (Sigma-Aldrich®) in a Neubauer chamber, and optical microscope; presented ≥ 95%. The cells were re-suspended at a concentration of 2 x 10^6 cells/mL in RPMI 640 (GIBCO) supplemented with 10 % fetal bovine heat inactivated serum, 1 % penicillin (100 UI/mL) and streptomycin (100 µg/mL), 20 mM HEPES, and 5 µg/mL phyto-hemagglutinin (PHA-P, Sigma-Aldrich, St. Louis, USA).

PBMC (100 µl) aliquots (1:1) with the MBHA samples in concentrations 10 times higher than the MIC were incubated for 24 hours at 37 °C, at 5 % CO_2_ atmosphere. Cell viability was assessed according to the protocol taken from the Resazurin (AlamarBlue® reagent, Bio-Rad, Hercules, USA) kit. The fluorescence was measured on a GloMax® Multi Microplate Reader (Promega, Madison, USA), (with a 570 nm excitation / emission 590 nm) filter, and the viability percentage was determined from the fluorescence readings and calculated using the kit provided formula being; % cell viability = [(FI of samples treated / FI of cells not treated) (100)], where FI is equivalent to the fluorescent emission intensity at 590 nm. This study was approved by the Ethics Committee for Research Involving Human Beings at the Federal University of Paraiba (CEP/CCS/UFPB) under protocol CAAE 66128417 8 0000 5188.
4 RESULTS AND DISCUSSION

4.1 ANTIMICROBIAL EFFECT

The Morita Baylis Hillman reaction (MBHR) has been previously used to obtain different molecules with potential biological activity (Basavaiah, Reddy, and Badsara 2010; Faheina-Martins et al. 2017; Elleuch et al. 2018; França et al. 2021). The antiparasitic action of certain adducts is described in the literature. We observed anti-Plasmodium effect for adduct A2 (Kundu et al. 1999), and anti-Leishmania for adduct A1 (Barbosa et al. 2009; Lima Junior et al. 2010; de Souza et al. 2007), adduct A2 (Barbosa et al. 2009; Lima Junior et al. 2010), and adduct A3 (Lima Junior et al. 2010); demonstrating IC50 values (Inhibitory Concentration) in the range of 9.58 µg/mL to 62.75 µg/mL (Lima Junior et al. 2010). The antibacterial and antifungal effects of compounds originating in the MBHR has been reported (Sá et al. 2014; Singh and Bhat 2011), but MBHA A1, A2 and A3 as presented here have not been thus assessed. In the present study, MICs values of 2,000 µg/mL for MBHA A1 were demonstrated for S. aureus (ATCC 15656) and S. mutans (AU 159) strains. The MICs of the A2 and A3 adducts were not determined for bacterial strains (Table 2). For the C. albicans strain (ATCC 11006) MIC values of 125 µg/mL for A1, 1,000 µg/mL for A2 and 15.6 µg/mL for A3 were observed (Table 2).

Table 2. Minimum inhibitory concentrations (MIC) of the MBHA compounds A1 to A3 in µg/mL compared for S. aureus (ATCC 15656), S. mutans (AU 159) and C. albicans (ATCC 11006). N.E. means that the MIC was not found.

<table>
<thead>
<tr>
<th>Compounds (µg/mL)</th>
<th>S. aureus</th>
<th>S. mutans</th>
<th>C. albicans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MIC</td>
<td>MIC</td>
<td>MIC</td>
</tr>
<tr>
<td>A1</td>
<td>2,000</td>
<td>2,000</td>
<td>125</td>
</tr>
<tr>
<td>A2</td>
<td>N.E.*</td>
<td>N.E.</td>
<td>1,000</td>
</tr>
<tr>
<td>A3</td>
<td>N.E.</td>
<td>N.E.</td>
<td>15.6</td>
</tr>
</tbody>
</table>

The antimicrobial potential of MBHA was determined in analogy to the study of Gomes et al. (2015), who considered: strong activity as MIC values lower than 500 µg/mL, moderate activity as values between 500 to 1,500 µg/mL, and weak or inactive...
as values above 1,500 µg/mL. The MBHAs A₁, A₂ and A₃ were considered inactive against the *S. aureus* and *S. mutans* strains. Against *C. albicans* the MBHAs demonstrated strong activity for A₁ and A₃ and moderate activity for the MBHA A₂.

Singh and Bhat (2011) evaluated the activity of different MBHAs showing strong activity against *S. aureus* (ATCC 9144). However, adducts A₁, A₂ and A₃ were considered inactive for the *S. aureus* (ATCC 15656) strain. This can be explained by differences in the reference strains, and in the molecules used in these studies. In the present study, a potential MBHA effect was verified against *C. albicans*; highlighting adduct A₃, yet with little or no effect against the bacterial strains, which may be related to MBHA’s mechanism of action, the fungal structure, and to the significant differences in both composition and cellular structure of fungi and bacteria (Ghannoum and Rice 1999). The cellular or molecular mechanism involved in this process should be addressed in further studies.

In a study conducted by Sá et al. (2014) evaluating the antimicrobial activity of different compounds originated from MBHR showed that the most active compounds showed bromine and chlorine groupings in their structures. This data corroborates the present study since the adduct A₁ (C₁₀H₈BrNO) was alone among the tested adducts; whose MIC was determined for the bacterial strains, even though being considered weak.

### 4.2 CELL VIABILITY

The mononuclear cells stimulated with phytohemagglutinin (PHA-P) showed cellular viability of 53.2 %, 63.6 %, and 51.0 % when in contact with MBHA A₁, A₂ and A₃, respectively, at concentrations being 10 times higher than the corresponding MIC of each adduct (Figure 1).

Figure 1. Viability of human PBMCs incubated for 24 hours with MBHA in concentrations 10 times higher than the corresponding MIC.
The bars represent mean values and standard deviation. Testing done in triplicate (n=9).

With the aim of ensuring dosage safety margins, and to enable future clinical applications of these products, concentrations of ten times higher than MIC were chosen for cytotoxicity tests. Another important point considered is that the biofilm inhibitory action is revealed by concentrations of compounds greater than those required for inhibition of planktonic microorganisms (Moreira et al. 2006). In addition, the incorporation of substances into controlled and slow-release systems, in many cases might require higher concentrations of the drug.

Cytotoxicity testing using peripheral mononuclear blood cells for A_1, A_2 and A_3 adducts has not been reported previously. The present study demonstrated cellular viability of greater than 50% for all three tested compounds, and non-cytotoxic in accordance with cytotoxicity index of (IC 50%), represents the substance concentration that reduces cellular viability by 50%. Values of cell viability above 50% are considered non-cytotoxic to lymphocytes and values below this are considered cytotoxic (Rogero and Ikeda 2003). Considering the compatibility of adducts at concentrations 10 times higher than corresponding to their MICs, the potential for therapeutic application of formulations containing these adducts to treat infections becomes apparent.

5 CONCLUSION

The MBHA A_1, A_2, and A_3 presented antimicrobial effect against *C. albicans* and were considered non-cytotoxic in accordance with the IC_{50} results. Adduct A_3 is noteworthy with high antifungal potential and adequate cellular viability
at concentrations ten times greater than its MIC. We suggest further research to prove antifungal activity against other *Candida* species, as well as the mechanism of action for these substances against pathogens. More surveys are needed to secure future clinical use of MBHAs against fungal infections.

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**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interest.
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