INTRODUCTION

*Correspondence: L. V. Cordeiro. Department of Pharmaceutical Science. Federal University of Paraíba. 58033-455, João Pessoa, Brazil. Phone: +55 83 3216-7347. Fax: (83) 3216-7094. E-mail: laisavilar@gmail.com. ORCID: https://orcid.org/0000-0002-8884-7331

Pseudomonas aeruginosa is an important nosocomial pathogen and its clinical importance is mainly related to nosocomial infections. Increased rates of bacterial resistance in recent years has led WHO to publish a global priority list to guide research and discovery of new antibiotics, where *P. aeruginosa* is among the group of bacteria for which there is a critical level of priority for new drugs to be discovered. In this context, isoeugenol appears as an interesting alternative and the objective of this study was to investigate its action against *P. aeruginosa*. Isoeugenol presented significant antibacterial activity, with minimum inhibitory concentration (MIC) of 64µg/mL and minimum bactericidal concentration (MBC) of 128µg/mL, and was considered bactericidal against this species. Molecular docking revealed interactions that suggest that isoeugenol may bind to the enzyme Penicillin-Binding Protein 3 and interfere with the bacterial cell wall synthesis process. This study reinforces the antibacterial potential of this compound and emphasizes that more studies are needed in order to better investigate its mechanism of antibacterial action.

Key words: *Pseudomonas aeruginosa*. Isoeugenol. Antibacterial. Natural Product.
Listeria innocua through a non-disruptive detergent-like mechanism of action (Hyldgaard et al., 2015). In addition, another study proves that isoeugenol has a good antibacterial potential and is even more effective than eugenol against some microorganisms (Zhang et al., 2017).

In aim to investigate the antibacterial activity of isoeugenol, this study analyzed the minimal inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of this natural product against Pseudomonas aeruginosa clinical isolates. In addition, an in silico analysis was performed through molecular docking, to observe the interactions of isoeugenol with bacterial enzymes that can predict the molecular target where the substance may be acting to promote the antibacterial effect on P. aeruginosa.

MATERIAL AND METHODS

Cultures

This work investigated the antibacterial activity of isoeugenol against 10 clinical isolates of Pseudomonas aeruginosa obtained from different anatomical sites, as reported in Table I. All strains were isolated and gently provided by Pharmacist Darci de Magalhães Melo, in the Laboratory of Clinical Pathology “HEMATO”, located in João Pessoa-PB/Brazil. The cultures belong to the MICOTEC collection of the “Research Laboratory of Antibacterial and Antifungal Activity of Natural and Synthetic Bioactive Products” and the ethics committee of the Health Sciences Center of the “Universidade Federal da Paraíba” approved the accomplishment of this study with protocol approved the accomplishment of this study with the protocol 2.741.747. As control, two standard strains was used: ATCC-9027 and ATCC-27853.

The cultures were maintained at 4 °C in Nutrient Agar (NA) (DIFCO Laboratories/USA/France). For use in the tests, these cultures were reactivated in Brain Heart Infusion (BHI) agar (DIFCO Laboratories/USA/France) for 24 hours at 35 ± 2°C. The culture media were prepared according to the manufacturer’s instructions.

| Code | Anatomical site                  |
|------|----------------------------------|
| LM-136 | General culture                  |
| LM-163 | Gastrostomy secretion            |
| LM-230 | Left ear secretion              |
| LM-286 | Right nose secretion            |
| LM-297 | Urine                           |
| LM-356 | Urine                           |
| LM-359 | Gastrostomy secretion            |
| LM-362 | Tracheal secretion              |
| LM-375 | General culture                 |
| LM-410 | Right foot injury secretion     |

Bacterial inoculum

For preparation of the inoculum, colonies obtained from fresh cultures of P. aeruginosa in BHI agar were suspended in 0.85% sterile sodium chloride (NaCl) solution, and adjusted according to the McFarland standard 0.5, which corresponds 1.5 x 10^8 UFC/mL (CLSI, 2018).

Substances

In this work we used isoeugenol (Sigma-Aldrich/Meck®) and, for use in the tests, this compound was solubilized in dimethylsulfoxide (DMSO) in a proportion of up to 5%, 2% of tween 80 and distilled water in sufficient quantity to complete emulsion in a concentration of 1024μg/mL (Pinheiro et al., 2017a). As control, meropenem (Sigma-Aldrich/Meck®) 32μg/mL was used.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) of isoeugenol were determined by broth dilution as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines. MIC was defined as the lowest concentration of an antimicrobial that inhibited
the visible growth of a microorganism after 24h of incubation (CLSI, 2018). All experiments were performed in triplicate.

**Minimum bactericidal concentration (MBC)**

After MIC, 10μL aliquots of the supernatants were withdrawn from the wells of the microdilution plates at the concentrations corresponding to isoeugenol MIC, MICx2, MICx4 and MICx8 for each strain and inoculated into new microdilution plates containing only BHI medium. The assay was performed in triplicate. Plates were incubated at 35 ± 2°C for 24 hours and then bacterial growth was observed. CBM was defined as the lowest concentration capable of causing complete inhibition of bacterial growth (Pinheiro et al., 2017a).

**Molecular docking**

The structure of the enzyme Penicillin-Binding Protein 3 (PBP3) from Pseudomonas aeruginosa was acquired from Protein Data Bank (https://www.rcsb.org/), under code 3PBQ (Han et al., 2010) (R: 1.7Å), complexed with its inhibitor. For molecular docking, the Molegro Virtual Docker (MVD) software (v 6.0.1, Molegro ApS, Aarhus, Denmark) was used and water molecules were removed from the enzyme structure.

**RESULTS AND DISCUSSION**

Isoeugenol MIC was 64µg/mL against all *P. aeruginosa* strains used in this study (Table II), which demonstrates significant antibacterial activity, classified by Sartoratto et al. (2004) as strong activity (MIC < 600µg/mL).

| Pseudomonas aeruginosa | MIC Controls |  |
|------------------------|--------------|
|                         | Isoeugenol | Meropenem | Viability | Broth |
| ATCC-9027               | 64 µg/mL   | -         | +         | -     |
| ATCC-27853              | 64 µg/mL   | -         | +         | -     |

TABLE II - Isoeugenol minimum inhibitory concentration (MIC)

Another study that analyzed the activity of isoeugenol against bacteria obtained a MIC of 312.5 µg/mL against Gram-positive and negative strains: *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Escherichia coli, Salmonella typhimurium* and *Shigella dysenteriae* (Hyldgaard et al., 2015). Despite a strong antibacterial activity was also observed, our results show an even lower isoeugenol MIC against *P. aeruginosa*. It is suggested that isoeugenol acts by causing damage to the bacterial cell membrane in a non-disruptive manner (Zhang et al., 2017), but it remains unknown whether this compound also acts on intracellular targets or on the bacterial cell wall.

Isoeugenol MBC was 128 µg/mL against all strains in this study (Table III). As explained in Flamm et al. (2017) and Thwaites et al. (2018), a MIC/MBC ratio greater than 1:2 is indicative that the substance acts bacteriostatically. When this ratio is equal to or less than 1:4, the product is considered bactericidal. Then, since MBC was equivalent to isoeugenol MICx2, the results suggest this product is bactericidal against *P. aeruginosa*.
TABLE III - Isoeugenol minimum bactericidal concentration (MBC)

| Pseudomonas aeruginosa | MBC | MIC:MBC | Effect |
|------------------------|-----|---------|--------|
| ATCC-9027              | 128 µg/mL | 1:2 | Bactericidal |
| ATCC-27853             | 128 µg/mL | 1:2 | Bactericidal |
| LM-136                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-163                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-230                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-286                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-297                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-356                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-359                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-362                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-375                 | 128 µg/mL | 1:2 | Bactericidal |
| LM-410                 | 128 µg/mL | 1:2 | Bactericidal |

In addition to being antibacterial, isoeugenol also has activity against filamentous fungi such as *Penicillium* spp., *Fusarium* spp., *Aspergillus* spp. and yeasts such as *Cryptococcus neoformans* (Zabka, Pavela, 2013; Pinheiro *et al*., 2017b; Ferreira *et al*., 2018).

Molecular docking is an *in silico* method that assists in the study of new drug development, as it can predict the anchoring of molecules in the active site of the target protein and estimate the interactions involved in this process (Surabhi, Sing, 2018). In the present study, the interaction of Isoeugenol with PBP3 was verified, which is considered an important therapeutic target, since β-lactam antibiotics inhibit this enzyme, preventing the formation of peptidoglycan and, consequently, interfering in the synthesis of the bacterial cell wall. The analysis of this molecular docking was validated through re-docking, and the RMSD (Root Mean Standard Deviation) value must be less than 2 Å (Thomsen, Christensen, 2006; Kaushik *et al*., 2014). Thus, the enzyme used for the tests presented the RMSD value within the acceptable range, confirmed by the overlap of the ligand and the best conformation obtained by re-docking (Table IV).

TABLE IV - Information about the target protein of *P. aeruginosa* and their respective ligand

| Enzima Ligante | Classificação | RMSD | Moldock Score |
|----------------|---------------|------|---------------|
| 3PBQ Imipenem  | Hidrolase      | 0.36 | -98.4         |

The PBP3 enzyme complexed with the inhibitor imipenem, presents a direct hydrogen bonding interaction with the amino acid residues Ser 294, Thr 487 and hydrophobic interactions with the amino acid residues Tyr 409, Val 333, Tyr 532 and Asn 242 (Han *et al*., 2010). Although isoeugenol presents binding energy lower than imipenem (-62.0 kJ/mol), the molecule showed hydrogen binding interactions with the amino acid residues Thr 487 and Ser 294, as well as hydrophobic interactions of the Van der Walls type with the amino acids Val 333 and Tyr 532 (Figure 1A and 1B), indicating that isoeugenol can anchor in PBP3. Thus, this fact suggests that the mechanism of action of isoeugenol may be related to interference with cell wall synthesis, and *in vitro* and *in vivo* studies are necessary to confirm whether the substance actually acts as an inhibitor of this enzyme.
Due to their antimicrobial potential, studies suggest a wide range of applications for isoeugenol. As, for example, the use in a functional polymer coating with antimicrobial properties against various most prominent oral pathogens (including Streptococcus mutans, Staphylococcus aureus, Actinomyces viscosus, Enterococcus faecalis, and others) using nanogels with surfacegrafted antibacterial molecules of isoeugenol instead of eugenol due to its higher antibacterial activity and the fact that it is not genotoxic, in contrast to eugenol (Kather et al., 2017). Another research highlights the possibility of using isoeugenol and other compounds derived from molecular modifications as food preservatives since these have activity against Escherichia coli, Listeria monocytogenes, Salmonella enteritidis and Staphylococcus aureus (Resende et al., 2017). In addition, the encapsulation of isoeugenol has been shown to increase its efficacy and this can be used for future studies of viability of a new antibacterial drug (Nielsen et al., 2016).

Further studies are needed to fully clarify the mechanism of antibacterial action of isoeugenol and verify the viability of its application in clinical practice. Faced with the need to develop new antibacterial drugs, isoeugenol is an interesting alternative to be better understood and to explore, especially, its activity against P. aeruginosa.

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