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Article

Antibacterial effect of isoeugenol against *Pseudomonas aeruginosa*

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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.

INTRODUCTION

Pseudomonas aeruginosa is found as a part of normal intestinal microbiota and a significant pathogen responsible for wide range of infections acquired in intensive care unit (ICU) in critically ill patients, including gastrointestinal infection, urinary tract infections and bloodstream infection (Pachori, Gothalwal, Gandhi, 2019).

The spread of antibiotic resistance in this species is a serious concern. Antibiotic resistant *P. aeruginosa* are strongly associated with nosocomial infections, and are a worldwide health problem due to the increasing development of multidrug resistant (MDR) strains (Streeter, Mohammad, 2016).

In 2017 the World Health Organization (WHO) released for the first time a list of resistant microorganisms that threaten human health and for which it is a priority need for the development of new antibiotics. The specialists used as basis for the construction of this document criteria such as mortality, prevalence of resistance and transmissibility. The list was divided into three levels of need for antibiotic development: critical, high and medium. Comprising the critical group are Gram-negative bacteria, more specifically *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and Enterobacteriaceae resistant to carbapenems and third generation cephalosporins. In this context, has been shown that isoeugenol, which is an essential oil constituent, has a strong antibacterial activity and apparently acts against *Escherichia coli and*

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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 MICOTECA 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.

TABLE I - Anatomical sites of *Pseudomonas aeruginosa* clinical isolates

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 ware 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).

TABLE II - Isoeugenol minimum inhibitory concentration (MIC)

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)

Pseudomonas aeruginosa	MIC	Controls		
	Isoeugenol	Meropenem	Viability	Broth
LM-136	64 µg/mL	-	+	-
LM-163	64 µg/mL	-	+	-
LM-230	64 µg/mL	-	+	-
LM-286	64 µg/mL	-	+	-
LM-297	64 µg/mL	-	+	-
LM-356	64 µg/mL	-	+	-
LM-359	64 µg/mL	-	+	-
LM-362	64 µg/mL	-	+	-
LM-375	64 µg/mL	-	+	-
LM-410	64 µg/mL	-	+	-

(-) Inhibition of bacterial growth. (+) Bacterial growth.

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 peptideoglycan 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.

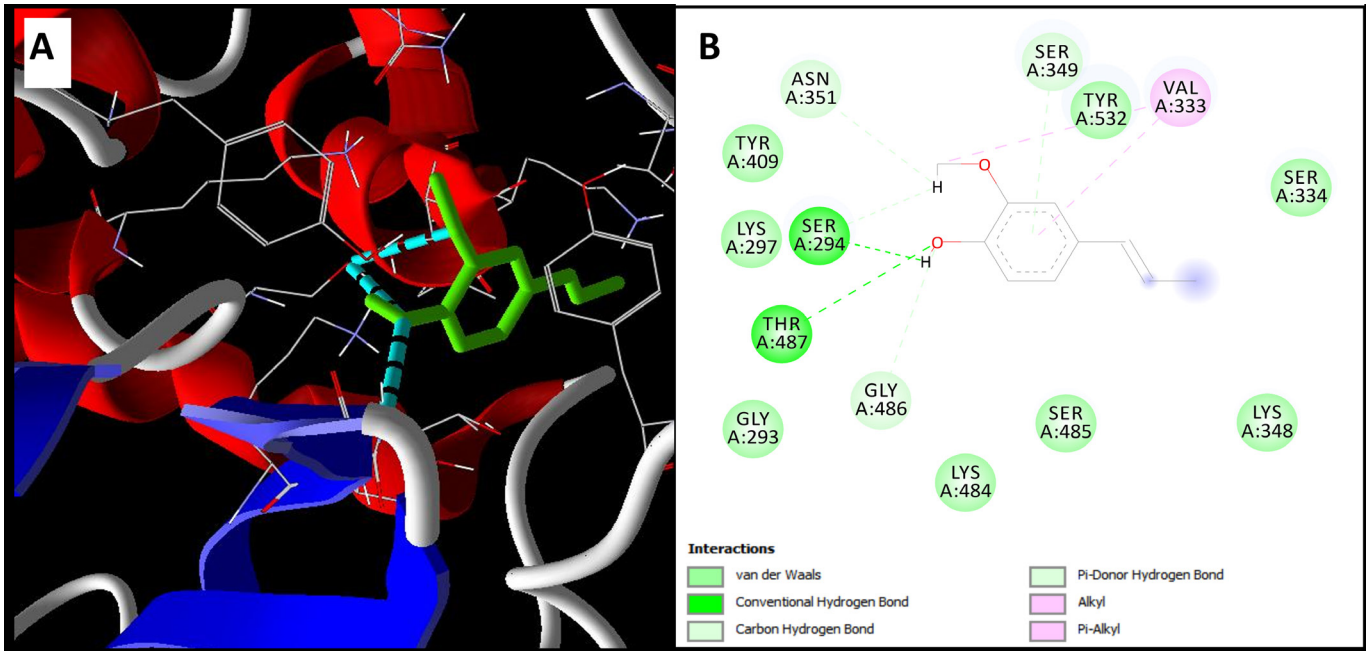

FIGURE 1 - Interactions of isoeugenol with the Penicillin-Binding Protein 3 (PBP3). A) Three-dimensional view of the anchorage of isoeugenol with PBP3. B) Types of interactions that occur between isoeugenol and the amino acid residues of PBP3.

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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