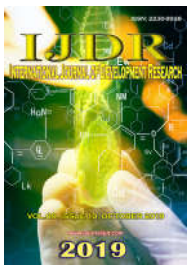
ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 09, Issue, 10, pp. 30877-30879, October, 2019



RESEARCH ARTICLE OPEN ACCESS

ISOEUGENOL EFFICACY AGAINST *Staphylococcus aureus*

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ARTICLE INFO

Article History:
Received 17th July, 2019
Received in revised form
21st August, 2019
Accepted 03rd September, 2019
Published online 30th October, 2019

Key Words:

Staphylococcus aureus,
Medicinal plants, Gentamicin.

ABSTRACT

Background: *Staphylococcus aureus* is a Gram-positive bacterium responsible for the development of infections of varying degrees of complexity. In addition, due to resistance phenomena, treatment against this pathogen has become increasingly ineffective, making it necessary to search for new molecules with antibacterial activity**. Objective**: The present study aims to evaluate isoeugenol antibacterial activity against clinical strains of *Staphylococcus aureus* through in vitro assays. **Methodology**: It was held Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Time-kill curve and checkerboard association of isoeugenol with standard antibiotic (gentamicin). **Results**: Isoeugenol presented 512 µg/mL MIC, > 4096 µg / mL in the MBC assay and demonstrated non-concentration-dependent bacteriostatic activity. The substance had synergistic and additive results when associated with gentamicin. **Conclusion**: Isoeugenol is an interesting alternative to be better understood, and further studies are needed to better investigate its mechanism of antibacterial action.

Citation: Lyvia Layanne Silva Rosa, José Lucas Ferreira Marques Galvão, Hermes Diniz Neto, *et al.* 2019. "Isoeugenol efficacy against Staphylococcus aureus", *International Journal of Development Research*, 09, (10), 30877-30879.

INTRODUCTION

Staphylococcus aureus is an important human pathogen and causes community and hospital infections. In addition, several resistance phenomena have been associated with this pathogen, making it necessary to search for new drugs with antibacterial activity (MCGUINNESS; MALACHOWA; DELEO, 2017). Among the drug candidates, it is possible to highlight the phenylpropanoid isoeugenol, which has shown antimicrobial activity against several pathogens (HYLDGAARD *et al*., 2015; ZHANG *et al*., 2017). However, there are only few studies on the microbicide role of this phytoconstituent against *S. aureus*. Thus, the present study aimed to evaluate the *in vitro* antibacterial activity of isoeugenol against clinical strains of *S. aureus*.

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MATERIAL AND METHODS

Cultures: This work investigated the antibacterial activity of isoeugenol against 15 clinical isolates of *Staphylococcus aureus* obtained from different anatomical sites, as reported in Table 1. All strains were isolated and kindly provided by Darci de Magalhães Melo, Pharmacist at 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/ Universidade Federal da Paraíba". As control, one standard strains was used: ATCC-13150. The cultures were maintained at 4°C in Nutrient Agar (NA) (DIFCO Laboratories/ USA /France). For use in the tests, these cultures were reactivated on 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 1. Anatomical sites of *Staphylococcus aureus* clinical isolates

Code	Anatomical site
SA-02	Nasal discharge
SA-40	Nasal discharge
SA-45	Oropharyngeal secretion
SA-116	Oropharyngeal secretion
SA-182	Nasal discharge
SA-220	Nasal discharge
SA-232	Nasal discharge
SA-262	Tracheal secretion
SA-297	Nasal discharge
SA-314	Tibial injury secretion
SA-349	Skin injury secretion
SA-356	Tracheal secretion
SA-418	Ophthalmic secretion
SA-419	Leg ulcer secretion
SA-443	Leg injury secretion

Bacterial inoculum: For inoculum preparation, colonies obtained from fresh cultures of *S. aureus* 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 CFU/mL (CLSI, 2018).

Substances: In this work, we used isoeugenol and gentamicin (Sigma-Aldrich/Meck®). For use in the tests, this compound was solubilized in dimethylsulfoxide (DMSO) in a ratio of up to 5%, 2% of tween 80 and and enough distilled water to complete the emulsion at a concentration of 1024µg/mL (Pinheiro *et al*., 2017).

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

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

Time-kill analysis: The determination of time kill curve of isoeugenol and gentamicin action against *S. aureus* SA-116 and ATCC-13150 was performed using microdilution plates with BHI broth and 10µl of the bacterial inoculum (1 x 10^7 CFU/mL), in the presence and absence of the products (isoeugenol and gentamicin) (Wang *et al*., 2018). At intervals corresponding to 0h, 2h, 4h, 8h and 24h, 1µL aliquots were taken from the wells using sterile calibrated bacterial loop and seeded in Mueller-Hinton agar Petri dishes, which were incubated in a bacteriological oven at 35 ± 2ºC. To interpret the results, growth curves were constructed by plotting the mean $\log_{10}$CFU/mL colonies as a function of time (hours).

Checkerboard Assay: The checkerboard assay was performed as detailed by Wu *et al*. (2017). The most effective combination value is determined by the Fractional Inhibitory Concentration Index (FICI), which is calculated by summing the Fractionated Inhibitory Concentrations (FICs) of isoeugenol and gentamicin. The FIC is calculated through MIC of compound in combination/MIC of compound alone. FICI is interpreted as follows: Synergism: FICI ≤ 0.5 / Additive: 0.5 < FICI ≤ 1 / Indifference: 1 < FICI ≤ / Antagonism: FICI > 4.

RESULTS AND DISCUSSION

The MIC of gentamicin was 2 µg/mL against all clinical isolates investigated, indicating that these bacteria were sensitive to this drug (CLSI, 2018). For isoeugenol, the MIC was 512 µg/mL against all strains analyzed, and these MIC values indicates strong antibacterial activity against the strains investigated (Sartoratto *et al*., 2004). In addition, these results are close to the values found by Zhang *et al*. (2017) who observed slightly lower MIC values (312.5 µg/mL) of isoeugenol against *S. aureus*, reinforcing the strong classification character of antibacterial activity. It is believed that isoeugenol acts by causing damage to the bacterial cell membrane in a non-disruptive manner (Hyldgaard *et al*., 2015), but it is remains unknown whether this compound acts on intracellular targets as well. The MBC values for gentamicin and isoeugenol were 16 and >4096µg/mL respectively, and the MBC/MIC ratio was higher than 4 for all the test strains, indicating that both substances were bacteriostatic for investigated microorganisms (Siddiqui *et al*., 2013). Regarding the *S. aureus* SA-116 and ATCC-13150 time-kill curve (Figure 1), isoeugenol and gentamicin had a bacteriostatic effect, since there was no reduction greater than or equal to 3log10CFU/mL from the initial inoculum in MIC at all times and concentrations analyzed. Furthermore, it was observed that isoeugenol showed non-concentration dependent bacteriostatic activity, since the increase in concentration did not induce significant improvements in activity.

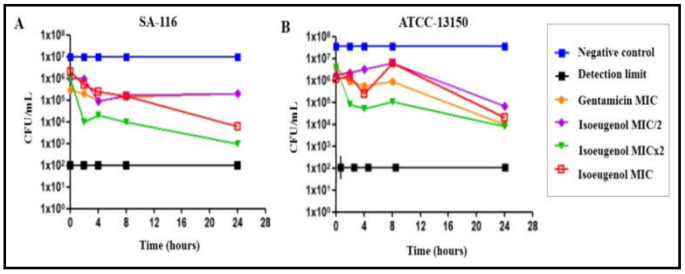

Figure 1. Time kill curve against *S. aureus* SA-116 (A) and ATCC-13150 (B)

The Fractional Inhibitory Concentration Index (FICI) can be seen below (Table 2).

Table 2. Checkerboard test association with gentamicin

S. aureus	FIC		FICI	Effect
	Isoeugenol	Gentamicin		
SA-116	0,125	0,125	0,25	Synergism
ATCC-13150	1	1	2	Additivity

Resulting from the combination of isoeugenol and gentamicin could be characterized as synergistic and additive, demonstrating an interesting finding that makes isoeugenol more attractive as a drug candidate, since the association between antimicrobials decreases the appearance of resistance phenomena, adverse effects and toxicity.

Conclusion

Isoeugenol showed bacteriostatic effect against *S. aureus* and the association between this phytoconstituent and gentamicin showed synergistic and additive effect. Faced with the need to develop new antibacterial drugs, isoeugenol is an interesting alternative to be better understood, and further studies are needed to better investigate its mechanism of antibacterial action, and verify the viability of its application in clinical practice.

Acknowledgements

The authors thank "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)" and "Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)" for financial support given to the research.

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