Netzahualcoyonol from *Salacia multiflora* (Lam.) DC. (Celastraceae) roots as a bioactive compound against gram-positive pathogens

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ABSTRACT

The expression of virulence factors, such as biofilm formation, in association with the acquisition of resistance to multiple drugs, has evidenced the need for new and effective antimicrobial agents against *Staphylococcus aureus*. The evaluation of the pharmacological properties of plant-derived compounds is a promising alternative to the development of new antimicrobials. In this study, we aimed to evaluate the antibacterial, antibiofilm, and the synergistic and cytotoxic effects of netzahualcoyonol isolated from *Salacia multiflora* (Lam.) DC. roots. Netzahualcoyonol presented bacteriostatic (1.56–25.0 μg/mL) and bactericidal (25.0–400.0 μg/mL) effects against Gram-positive bacteria, disrupted the biofilm of *S. aureus*, and presented a synergistic effect after its combination with β-lactams and aminoglycosides. The low cytotoxicity of netzahualcoyonol (Selectivity Index (SI) for *S. aureus* (2.56), *S. saprophyticus* (20.56), and *Bacillus subtilis* (1.28)) suggests a good security profile. Taken together, these results show that netzahualcoyonol is promising for the development of a new effective antibacterial agent.
1. Introduction

*Staphylococcus aureus* is a Gram-positive opportunistic pathogen commonly found in the skin and mucous membranes. Approximately 30% of the population and up to 80% of health-care workers, hospitalised patients, and immunocompromised individuals are colonised by this bacterium (Tong et al. 2015; Taylor and Unakal 2021). *S. aureus* is considered the major human pathogen that can cause a wide range of infections, including septicemia, infective endocarditis, pneumonia, osteomyelitis, cutaneous, osteoarticular, and central nervous system infections (Ghalehnoo 2015). The high pathogenicity of *S. aureus* is associated with its ability to develop several virulence factors that enable the bacterium to successfully invade the host and establish infection (Tong et al. 2015). Of these, the formation of biofilms is considered a bacterial protection in which antimicrobial agents and the host immune system become ineffective. For instance, biofilms are up to 1,000 times more resistant to antimicrobial agents than planktonic cells (Crouzet et al. 2014). This resistance is attributed to the presence of (i) extracellular polymeric matrix, (ii) slow-growing cells, (iii) persisted cells, and (iv) horizontal gene transfer, among other factors (Archer et al. 2011).

Besides the development of several virulence factors, *S. aureus* can acquire resistance against numerous classes of antibiotics, which increases the clinical threat posed by this pathogen. For instance, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most successful modern pathogens and is currently responsible for 10-fold more infections than all multidrug-resistant (MDR) Gram-negative bacteria together (Lima et al. 2021). Only in the USA, more than 100,000 cases of invasive MRSA infections were reported in 2017, and, of these, 19,832 progressed to death, which is similar to the number of deaths caused by HIV/AIDS, tuberculosis, and homicides combined (Boucher and Corey 2008; Kourtis et al. 2019).

To overcome the critical scenario created by multidrug-resistant *S. aureus*, the development of new antimicrobial agents against this pathogen, as well as other bacterial species became a health priority. In this context, an approach to the development of new antibiotics is the investigation of the pharmacological properties of...
plant-derived compounds. Plants produce a variety of secondary metabolites that protect them against invaders and thus have presented several biological effects. Among the plants used in traditional medicine, Salacia multiflora (Lam.) DC. (Synonym of Salacia crassifolia (Mart. ex. Schult.) G. Don) (Celastraceae) is native to the Cerrado region of Brazil and is used to treat several diseases, including gastric ulcers, malaria, chronic cough, and headache (Rodrigues et al. 2015). Several terpenes isolated from plants of the Celastraceae family, such as pristimerin (da Cruz Nizer et al., 2021), 3,4-seco-friedelan-3-oic acid and 3β-hydroxy-olean-9(11):12-diene (Rodrigues et al. 2015), and 3-oxo-11α-hydroxy-olean-12-ene-30-oic acid (Mokoka et al. 2013) have shown promising antimicrobial activity. In this context, netzahualcoyonal (C30H38O6) (Figure S1) is a quinone methide triterpenoid commonly isolated from Celastraceae species; however, its antibacterial effect has been poorly elucidated. Thus, we aimed to investigate the antibacterial activity of netzahualcoyonal, its cytotoxicity, and antibiofilm effect. Furthermore, we also evaluated the effect of the combination of netzahualcoyonal with clinically available β-lactams, aminoglycoside, and tetracycline against S. aureus.

2. Results and discussion

Netzahualcoyonal isolated from the roots of S. multiflora presented a significant antibacterial effect against the Gram-positive bacteria tested (MIC 1.56–25.0 µg/mL) (Table S1). Setzer et al. (2001) showed that netzahualcoyonal is active against Bacillus cereus and S. aureus at concentrations of 0.3 µg/mL and 2 µg/mL, respectively. Furthermore, it also presented a bactericidal effect (MBC 25.0–400.0 µg/mL), showing that higher concentrations of netzahualcoyonal are able to eliminate the pathogen. It is a desirable characteristic of antimicrobial agents since the rapid and efficient eradication of microbes may decrease the spread of the infection and the chance of resistance acquisition (Pankey and Sabath 2004). The antimicrobial activity of terpenes has been attributed to their hydrophobic property and the presence of hydroxyl groups in the molecule, which facilitates their incorporation and binding in the cell membrane (Zomorodian et al. 2011; da Cruz Nizer et al. 2021).

On the other hand, netzahualcoyonal did not inhibit the growth of Gram-negative bacteria. The lack of action against Gram-negative bacteria may be attributed to the morphology of these cells, in which the presence of an outer membrane composed of lipopolysaccharides protects the cells against external factors, including antimicrobial agents (Lima et al. 2018). Thus, these data show that netzahualcoyonal is a narrow-spectrum antibiotic which covers Gram-positive sporulating (i.e. B. cereus) and non-sporulating (i.e. Staphylococcus spp) pathogens.

In addition to the antibacterial activity presented by netzahualcoyonal, its combination with amoxicillin, penicillin, or gentamycin produced a synergistic effect against S. aureus with FICI of 0.51, 0.31, and 0.30, respectively (Table S2). For instance, the combination of netzahualcoyonal with amoxicillin reduced in 125-fold (from 0.98 µg/mL to 0.008 µg/mL; 82% reduction) the concentration of antimicrobial required to inhibit the growth of S. aureus. The combination therapy of antibiotics is an alternative to the development of new antimicrobial agents that have been extensively used because it
increases the efficacy of the antimicrobial agents, suppresses the emergence of resistance, and decreases the toxicity of the agents since usually a lower dose of compounds is required (Tyers and Wright 2019).

The pathogenicity of microbial species is determined by the development of virulence factors, which are responsible for the colonisation and evasion of the host, establishment of the infection, and spread. Several bacterial species form biofilms as a protective strategy against environmental stressors. In this context, we evaluated the effect of netzahualcoyonol on the pre-formed biofilm of *S. aureus* ATCC 29213. This plant-derived compound disrupted the pre-formed biofilm in all concentrations tested (Figure S2). The antibiofilm activity of terpenes has been extensively studied and is often attributed to the disruption of membrane structures by these compounds (Mahizan et al. 2019).

*In vitro* cytotoxicity assays can be used to predict toxicity by a general screening of chemicals. As most antimicrobial agents are excreted in the urine and some tend to accumulate in the kidneys, inducing adverse effects, the prediction of nephrotoxicity in renal cell lines is an important indicator of clinical safety (Andrade et al. 2018). Thus, the toxicity of netzahualcoyonol against renal mammalian cells (Vero lineage) was evaluated using the MTT assay. The 50% cytotoxic concentration (CC50) of this terpene against Vero cells was 32.07 ± 12.8 μg/mL. The selectivity index (SI) values obtained were 2.56, 20.56, and 1.28 for *S. aureus*, *S. saprophyticus*, and *B. subtilis*, respectively. Considering the FIC of netzahualcoyonol after its combination with amoxicillin, penicillin, and gentamicin, we obtained SI values of 64.14, 32.32, and 133.62. According to Mokoka et al. (2013), SI values higher than 1 indicates that the compound is more toxic to the bacteria than to the mammalian cells. Thus, it is suggested that netzahualcoyonol presents low toxicity.

### 3. Experimental

See Supplementary Material. Spectral data based on FT-IR, ¹H and ¹³C and DEPT-135 NMR spectra (Figures S3 to S6 and Table S3) of netzahualcoyonol are available in Supplementary material.

### 4. Conclusion

To our knowledge, this is the first study that demonstrates the antibacterial effect of netzahualcoyonol, highlighting its activity as a narrow-spectrum antimicrobial which covers Gram-positive pathogens. This terpene has effect against the mature biofilm of *S. aureus* and enhanced the efficacy of clinically available β-lactam and aminoglycosides antibiotics. Furthermore, in our study, netzahualcoyonol presented low toxicity against Vero cells, suggesting its safety. However, future studies focusing on the *in vivo* effects of netzahualcoyonol should be performed to better characterize its pharmacological potential against infections by Gram-positive bacteria.
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Disclosure statement

No potential conflict of interest was reported by the authors.

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