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In vitro e *in silico* evaluation of the inhibition of *Staphylococcus aureus* efflux pumps by caffeic and gallic acid

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ABSTRACT

Staphylococcus aureus has been reported as one of the most difficult to treat. In the search for new treatment alternatives, isolated plant substances such as phenolic compounds, have demonstrated the ability to reverse bacterial resistance. The present study aims to evaluate the inhibitory action of caffeic acid and gallic acid on efflux pumps from *S. aureus* resistant strains. The broth microdilution assay was carried out to obtain the MICs of caffeic acid and gallic acid while the efflux pump inhibition test was assessed through the reduction of the minimum inhibitory concentration of the antibiotic and ethidium bromide. In addition, in silico theoretical parameters were analyzed to determine the theoretical efficacy of the compound and its free energy of interaction. In the results, the inhibition concentration of the two compounds did not certify clinical relevance with 1024 μg/mL for all strains. In the efflux pump inhibition effect, caffeic acid inhibited the MrsA pumps of the strain RN-4220 and NorA of the strain 1199B. Caffeic acid showed greater efficacy in the docking model, in agreement with the demonstrated experimental efficacy. Isolated compounds can be indicated as efficient options in the inhibition of resistance mechanisms.

1. Introduction

Bacterial infections have been increasing worldwide, being responsible for the increase in mortality rates and the increase in public health costs [1,2]. Among the microorganisms that cause death, a group of the most studied are bacteria from the genus *Staphylococcus*. *Staphylococcus aureus* is one of the most difficult microorganisms to treat because it has a high capacity for host colonization, as well as for the manifestation of virulence factors and the development of resistance to a great variety of drugs [3,4]. This is a Gram-positive bacterium associated with skin, wound and soft tissue infections, in addition to being pointed to as the cause of endocarditis and infections linked to medical device implants, such as valves and catheters [5]. In addition, the number of *S. aureus* strains present in clinical isolates resistant to multiple drugs is increasing [6].

Bacteria become resistant to drugs by obtaining resistance genes generally contained in plasmids and transposons, and by mutations that produce changes in the active site of antibiotics, leading to the emergence of resistance mechanisms [7,8]. These mechanisms include changes in membrane permeability, inactivation by enzymes, target alterations and active efflux [9,10].

Active efflux is caused by transmembrane proteins capable of actively expelling or exchanging toxic compounds out of the bacterial cell, enabling the survival of the micro-organism, denominated as efflux pumps [11]. There are several types of pumps distributed among five different families according to their characteristics. Among them, three of the most studied are NorA, TetK and MrsA. The NorA pump is responsible for the efflux of various drugs such as fluoroquinolones, quinolones, verapamil and omeprazole, as well as dyes such as acridine and ethidium bromide, belonging to the Major Facilitator Superfamily (MFS) [12–16]. TetK, a member of the MFS family, acts on reducing tetracycline concentration from the intracellular medium [17]. The

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MrsA pump is part of the RND family, is ATP-dependent and causes the efflux of erythromycin [18].

Many studies are being carried out in order to find new substances capable of reversing the bacterial resistance promoted by efflux pumps. Several compounds isolated from plants, as well as extracts and essential oils, have shown good results in relation to the inhibition of this mechanism [19,20]. Among these substances, phenolic compounds have been widely reported as promising alternatives in the search for new treatment sources [9,21].

Phenolic compounds can contribute satisfactorily as a new complementary treatment source, since they are able to modify bacterial performance, impairing their locomotion, surface adhesion, biofilm formation and formation of virulence determinants [22–30]. Phenolic compounds also present a variety of properties such as anti-allergic, antiviral, anticancer and anti-inflammatory properties, as well as acting in the elimination of free radicals and metal chelators [31,32]. Considered as one of the groups with the greatest diversity of secondary metabolites, phenolic compounds contain polyphenol categories such as flavonoids, tannins, lignins, phenolic acids, quinones, flavones, flavonols and coumarins [32,33].

In the search for the reversion of bacterial resistance, recent studies with phenolic compounds have shown satisfactory advances. Diniz-Silva indicated in their work the antibiotic modifying activity of quercetin in relation to norfloxacin in *S. aureus* carrying the NorA pump [34]. Lima demonstrated the synergistic effect of the combination of gentamicin and norfloxacin with pyrogallol against *S. aureus* strains [35]. Tintino verified the inhibitory action of tannic acid on the NorA efflux pump in the 1199B strain of the aforementioned bacterium [36].

Phenolic acids can often be identified in foods [37]. Caffeic acid (3,4-dihydroxycinnamic acid) is a compound derived from hydroxycinnamic acid, considered to be one of the most commonly found phenolic acids, and can be represented in the ester form [38,39]. It has several biological activities such as antioxidant, antibacterial and fungicide [40,41].

The tannin gallic acid (3,4,5-trihydroxybenzoic acid) is a water-soluble polyphenol found in pomegranate peel, in processed beverages such as red wine and in various types of vegetables [42–44]. Considered the primordial initiator of ellagitannins and gallotannins [45,46]. It is widely mentioned for its antiviral, antibacterial, anticancer and antioxidant properties [47,35]. In this context, the objective of this work was to evaluate the inhibitory action of caffeic acid and gallic acid on efflux pumps from *Staphylococcus aureus* resistant strains and demonstrate this mechanism through in silico studies.

2. Materials and methods

2.1. Substances

Caffeic acid, gallic acid and ethidium bromide (EtBr), as well as the antibiotics tetracycline, norfloxacin and erythromycin, were obtained from SIGMA Chemical Co., St. Louis, U.S.A.

2.2. Strains utilized

We used the following *S. aureus* strains in the tests: RN4220 carrying the MrsA efflux pump; IS-58, which possesses the TetK pump; 1199B with resistance to hydrophilic fluoroquinolones via the NorA efflux protein and the 1199 strain which is considered wild-type. Bacteria were kept on blood agar for testing of their type (Laboratories Difco Ltda., Brazil) and then transferred and maintained in two stocks, one in glycerol at −80 °C and the other in *Heart Infusion Agar slants* (HIA, Difco) at 4 °C.

2.3. Culture medium

During the assays we used the following culture media: *Heart Infusion Agar* (HIA, Difco laboratorises Ltd.), *Brain Heart Infusion* (BHI, difco Laboratories Ltda.), glycerol and blood agar.

2.4. Preparation of the substances

Each antibiotic was used for a particular bacterial efflux pump: erythromycin for the MrsA pump; tetracycline for the TetK pump; norfloxacin for the NorA pump and the wild-type strain. All antibiotics, caffeic acid, gallic acid and EtBr were prepared according to the CLSI guidelines [48]. Substances were diluted in DMSO with 10 mg/mL and then diluted in water, decreasing the concentration to 1024 μg/mL. EtBr was diluted only in water.

2.5. Preparation and standardization of inocula

Inoculations from the stocks were prepared for all assays using the standard method determined by CLSI [48]. Bacteria were cultured again on solid *Heart Infusion Agar slants* and maintained at 37 °C for 24 h. Deriving from this solid medium, the inocula were made using test tubes containing sterile saline solution which were standardized according to the McFarland 0.5 scale corresponding to 10^6 CFU (Colony Forming Unit).

2.6. Minimum inhibitory concentration (MIC) assays

All microbiological procedures as well as the readings were performed following the CLSI guidelines with some adaptations [48]. In the minimum inhibitory concentration assays with caffeic and gallic acid, ethidium bromide and the antibiotics, the distribution media were prepared in *eppendorfs* using 900 μL of BHI liquid culture medium and 100 μL of the inoculum. Subsequently, the solutions contained in the *eppendorfs* were transferred to a 96-well microdilution plate, in a horizontal fashion. 100 μL were placed in each well, making up 10 wells. Subsequently, serial microdilution of the substances was performed, where 100 μL of each substance was microdiluted up to the penultimate well (1:1), where the last well is intended for growth control. The concentrations are varied, from 512 μg/mL to 1.0 μg/mL. The reading was performed after 24 h by visualization of the color change of the medium, as indicated by the addition of 20 μL of Resazurin (7-hydroxy-3H-phenoxazine-3-one 10-oxide). The color change of the medium from blue to red points to the presence of bacterial growth, while the presence of the blue color indicates the absence of growth. All experiments were prepared in triplicates.

2.7. Efflux pump inhibition assays by the reduction of the MIC of ethidium bromide

The analysis of the effect of decreasing the MIC of EtBr was performed in triplicates. Firstly, the distribution media of the test as well as that of the control were prepared in *eppendorfs*. In the test 150 μL of the inoculum and the substances at the sub-inhibitory concentration (MIC/8) were added and the volume of the *eppendorfs* were supplemented with liquid medium up to 1.5 mL. The control contains the same amount of inoculum as the test and 1350 μL of the medium. Subsequently, the prepared solution was transferred to 96-well microdilution plates, the distribution was performed vertically by the addition of 100 μL of the *eppendorfs* content in each well. Then, the microdilution of EtBr was performed with 100 μL added to this medium until the penultimate cavity (1:1). The last cavity did not receive any of the substances under evaluation, because it is considered as the growth control. Concentration ranges start from 1024 μg/mL to 0.5 μg/mL. After 24 h, the plates were read by observing the color change of the medium, determined by the addition of 20 μL Resazurin. The reading has as an indicative the color change of the medium from blue to red pointing to the presence of bacterial growth and the persistence in blue, the nonexistence of growth. The decreased MIC of ethidium bromide or