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Evaluation of the antibacterial and inhibitory activity of NorA and MepA efflux pumps from *Staphylococcus aureus* by diosgenin

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ABSTRACT

The increase in bacterial resistance to available antibiotics has driven several researchers to search for new agents with therapeutic properties. Diosgenin is a naturally occurring steroidal saponin that has demonstrated several pharmacological properties. In the present study, we report the antimicrobial activity of diosgenin against the standard and multidrug-resistant bacteria of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in addition to the efflux pump inhibitory activity against *Staphylococcus aureus* strains carrying NorA and MepA pumps. For this purpose, the broth microdilution method was used, from which the value of the Minimum Inhibitory Concentration (MIC) was obtained, and this was associated with subinhibitory concentration (MIC/8) with antibiotic of clinical use and ethidium bromide for strains carrier by efflux pump. Diosgenin showed antimicrobial activity for standard *S. aureus* bacteria and potentiating activity in association with gentamicin and ampicillin for *P. aeruginosa* multidrug-resistant bacteria, it also showed potentiation in association with norfloxacin against the *E. coli* strain and gentamicin against the *S. aureus* strain. Antimicrobial activity against efflux pump-bearing strains revealed that saponin did not interfere with the efflux pump mechanism or intervened antagonistically. Thus, saponin has shown to be very promising against bacterial resistance in association with aminoglycoside, fluoroquinolones and beta-lactam, however additional studies should be carried out to better elucidate the mechanism of action of diosgenin.

1. Introduction

The indiscriminate use of antibiotics as well as misuse has resulted in the selection of multidrug-resistant bacteria, making infections more difficult to fight or even impossible [1]. Infections caused by multidrug-resistant bacteria represent the death of 700,000 people per year worldwide, and this estimate may increase until 2050 [2].

Among the mechanisms of bacterial resistance to antibiotics are the enzymatic inactivation of the drug through degradation or chemical modification, alteration of the site of action by mutation, and reduction of the intracellular concentration of the antibiotic through alteration of the membrane permeability and/or decrease of the inflow or outflow increase through efflux pumps [3,4].

Efflux pumps are families of membrane transport proteins capable of extrusion of the antibiotic or toxic substrate into the extracellular environment, which can be expressed in both Gram-positive and Gram-negative bacteria [5].

Natural products of plant origin have been investigated in an attempt to combat pathogenic microorganisms [6]. Saponins are an important class of secondary metabolites found in several plant species, it is a type

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of high molecular weight carbohydrate, formed by a polycyclic part of aglycone combined with one or more side chains of sugar molecules [7]. They have different chemical structures due to the presence of different sugars in different locations and orientations. In addition, it presents a range of biological activities related in the literature [8–13].

Diosgenin is a naturally occurring type of steroidal saponin, it was extracted from the species *Dioscorea cayennensis* Lam, however, it can be found in other species such as *Trigonella foenum-graecum* L. and *Dioscorea nipponica* [8,14,15]. Diosgenin is a sapogenin that spirostan is substituted by a hydroxy group at the 3β position, has an R-configuration at position 25 and contains a double bond at the 5–6 position [16]. They have a range of pharmacological activity including anti-inflammatory [17], antitumor [18], antithrombotic [19], antioxidant [20,21] and antibacterial activity [8]. In addition, diosgenin has been widely used in the composition of several pharmaceutical formulations, improving the efficacy and bioavailability of drugs [22].

The present study aimed to investigate the antibacterial activity of diosgenin against strains of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. As well as the investigation of the efflux pump inhibitory activity on strains of *Staphylococcus aureus*, SA-1199B carrying the norA resistance gene, which expresses the NorA efflux protein and the SA-K2068 strain carrying the MepA efflux pump.

2. Material and methods

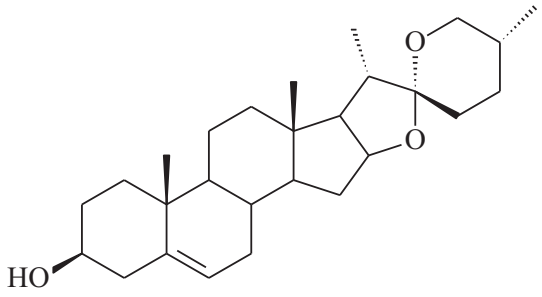
2.1. Substance and drugs used

The antibiotics ciprofloxacin, norfloxacin, gentamicin and ampicillin; ethidium bromide (EtBr), carbonylcyanide 3-chlorophenylhydrazone (CCCP), chlorpromazine (CPZ) were obtained from Sigma Aldrich Co. Ltd. Diosgenin (Fig. 1) was obtained from Sigma Aldrich Co. Ltd. being extracted from the species *Dioscorea cayennensis* Lam, it has a molecular weight of 414.62 g/mol. The drugs and diosgenin were dissolved in 10 % dimethylsulfoxide (DMSO) and then in sterile water until obtaining a concentration of 1024 μg/mL. CCCP was dissolved in a methanol/water solution in a 1:3 ratio. Ethidium bromide solutions were dissolved in sterile distilled water and stored at −20 °C, protected from light.

2.2. Bacterial strains

The standard microorganisms used in the tests: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, and the multidrug resistant strains: *Escherichia coli* 06, *Staphylococcus aureus* 10, *Pseudomonas aeruginosa* 24 were obtained from the Laboratory of Microbiology and Molecular Biology - LMBM from the Regional University of Cariri – URCA.

The *Staphylococcus aureus* strains carrying efflux pumps: SA-1199B which overexpresses the NorA efflux protein and the SA-K2068 strain which expresses the MepA protein. Both strains are capable of extrusion of fluoroquinolones. The bacteria carrying efflux pumps were kindly provided by Prof. S. Gibbons (University of London, Malet St, Bloomsbury, London WC1E 7HU, UK), being maintained on blood agar (Laboratories Difco Ltda., São Paulo-SP, Brazil) and, before the experiments, all bacterial strains were cultivated by 24 h at 37 °C on Brain Heart Infusion-Agar (BHI-Agar, Acumedia Manufacturers Inc.).



$C_{27}H_{42}O_3$

Fig. 1. Diosgenin.

2.3. Culture mediums

The following culture media were used to perform the microbiological tests: Brain Heart Infusion Agar-BHI-Agar (Acumedia Manufacturers Inc.). Prepared according to the manufacturer and Brain Heart Infusion-BHI (Acumedia Manufacturers Inc.) prepared at a concentration of 10 %.

2.4. Determination of the Minimum Inhibitory Concentration

It was determined by the broth microdilution method with CLSI modifications [23]. For the procedures, the inoculum was prepared from the 24-hour growth cultures grown in Petri dishes on BHI-Agar. One elevation of each strain was suspended in 0,9 % saline and its turbidity compared to the McFarland scale (1×10^5 CFU/mL) at 0.5.

Eppendorfs® tubes with a volume of 1.5 mL of solution were prepared, 10 % of this solution corresponded to the bacterial inoculum and the remainder of the solution was completed with a 10 % BHI culture medium (1350 μL). Subsequently, 100 μL were added to a microdilution plate. Then, microdilution was carried out with the substances in a serial proportion of 1:1 until the penultimate well, the last well being used to control microbial growth. The concentrations of substances ranged from 0,5 to 512 μg/mL. The plates were taken to the microbial growth oven for 24 h at 37 °C.

To read the MIC, 20 μL of resazurin solution (7-hydroxy-10-oxidofenoxazin-10-ium-3-one) at a concentration of 400 μg/mL was used, observing the color change after the oxidation-reduction reaction in the room temperature within 2 h. The color change from blue to pink is interpreted as the occurrence of bacterial growth [24,25]. The procedures were performed in triplicate.

2.5. Modifying effect of diosgenin on the activity of clinically used antibiotics

To verify whether diosgenin would modify the action of antibiotics against multidrug-resistant bacteria, the method proposed by Coutinho et al. [26] was used. The compounds used were evaluated in sub-inhibitory concentration (MIC/8) so that there was no inhibition of bacterial growth by direct action.

For the procedures, Eppendorf® tubes were prepared with a volume of 1500 μL of the solution, 10 % corresponding to the bacterial inoculum (150 μL) and the MIC/8 of saponin. The control solution was prepared in Eppendorf® tubes containing 1350 μL of BHI and 150 μL of the bacterial suspension. Immediately afterwards, the plates were filled with 100 μL of the solution and soon after, microdilution was carried out with 100 μL of the antibiotics in a 1:1 ratio until the penultimate well; the last well was used to control bacterial growth. All procedures were performed in triplicate and the reading was performed using resazurin.

2.6. Evaluation of efflux pump inhibition by reducing the MIC of antibiotics and ethidium bromide (EtBr)

To verify if diosgenin acts as a potential inhibitor of NorA (SA-1199B) and MepA (SA-K2068) efflux pump, a comparative study was performed on the effects of standard efflux pump inhibitors, evaluating the ability of both to decrease MIC of EtBr and the antibiotics norfloxacin for NorA and ciprofloxacin for MepA. Standard inhibitors CCCP and CPZ were used to provide pump expression for each strain tested.

Efflux pump inhibition was tested using a sub-inhibitory concentration (MIC/8) of the inhibitors and diosgenin. In the experiments, 10 % of the bacterial inoculum, MIC/8 of the standard inhibitors and diosgenin were added, completed with BHI in Eppendorfs®. These were then transferred to 96-well microdilution plates, to which 100 μL of antibiotic or EtBr were added in serial dilutions (1:1) ranging from 0,5 to 512 μg/mL, where the last well was used for the control of microbial growth. Controls were performed using the MIC of antibiotics and EtBr alone. The plates were incubated at 37 °C for 24 h and bacterial growth was evaluated with resazurin [23,27].

2.7. Statistical analysis

The microbiological assays were performed in triplicate, and the results were expressed as a geometric mean. Statistical analysis was performed using two-way ANOVA, followed by *post hoc* Bonferroni, using the GraphPad Prism® software version 6.0.

3. Results

3.1. Minimum Inhibitory Concentration

After performing the antimicrobial activity tests, the determination of the MIC was obtained through a geometric mean. In Table 1 we can see the result of the MIC of diosgenin for standard and multidrug-resistant bacteria, where the MIC for most of the bacteria tested was ≥1024 μg/mL, except for the bacterium *S. aureus* ATCC 25923 which showed antibacterial activity with the MIC of 406 μg/mL.

3.2. Modifying effect of diosgenin on the activity of clinically used antibiotics

The association of diosgenin with clinical antibiotics (Fig. 2) showed potentiating activity in association with gentamicin and ampicillin for *P. aeruginosa* 24, reducing the MIC of the antibiotic from 40 μg/mL to 25 μg/mL and 203 μg/mL to 128 μg/mL, respectively.

In addition, it also showed an additive effect against *E. coli* 06 in association with norfloxacin with the reduction of MIC from 322 μg/mL to 128 μg/mL and *S. aureus* 10 when combined with gentamicin reduced the MIC of the antibiotic 25 μg/mL to 8 μg/mL.

However, when associated with ampicillin, it increased the MIC of the antibiotic in *S. aureus* 10 and *E. coli* 06 from 256 μg/mL to 512 μg/mL and 128 μg/mL to 512 μg/mL respectively. Similarly, when combined with gentamicin for *E. coli* 06 with an increase in MIC from 40 μg/mL to 80 μg/mL and *P. aeruginosa* 24 when combined with norfloxacin with an increase from 50 μg/mL to 64 μg/mL.

3.3. Efflux pump inhibition by reducing the MIC of antibiotics and ethidium bromide (EtBr)

In Table 2, we can see the MIC result for *Staphylococcus aureus* strains carrying an efflux pump, where diosgenin showed a higher MIC than standard inhibitors for efflux pump (CCCP, CPZ and EtBr).

To verify if the antibacterial activity of diosgenin is related to the resistance mechanism through efflux pumps, it was evaluated against the strains of *S. aureus* carrying NorA and MepA efflux pumps. According to Fig. 3, we can see that the standard inhibitors CCCP and CPZ showed the presence of the NorA pump for *S. aureus* 1199B for norfloxacin, and Fig. 4 showed the presence of the MepA pump for *S. aureus* K2068 for ciprofloxacin with a decrease in MIC when associated with inhibitors. CCCP is an inhibitor that acts by inhibiting the driving force that drives the efflux protein, while CPZ is an inhibitor that acts by competition with the efflux protein site, thus using the two inhibitors we can infer the mechanism responsible for the inhibition of the pump of efflux.

When evaluating diosgenin in association with norfloxacin for *S. aureus* 1199B (Fig. 3), we noticed an increase in MIC from 128 to 812.8 μg/mL. When we analyzed diosgenin in association with ethidium bromide for *S. aureus* 1199B, there was no change, as well as *S. aureus* K2068 in association with ciprofloxacin and ethidium bromide (Fig. 4).

Table 1
Determination of diosgenin Minimum Inhibitory Concentration (μg/mL) against standard and multidrug-resistant bacteria.

Bacterium	S.A ATCC 25923	P.A ATCC 9027	E.C ATCC 25922	S.A 10	P.A 24	E.C 06
Diosgenin	406	≥1024	≥1024	≥1024	≥1024	≥1024

Legend: S.A - *Staphylococcus aureus*, P.A - *Pseudomonas aeruginosa*, E.C - *Escherichia coli*.

4. Discussion

The lower MIC presented by *S. aureus* ATCC 25923 is indicative of relevant intrinsic antibacterial activity of diosgenin. The biological activity of saponins has been attributed to amphipathic characteristics, which facilitate the formation of complexes with cell membrane components such as proteins, steroids and phospholipids, which leads to the formation of pores and, consequently, an increase in membrane permeability [10].

In a study by Masood-ur-Rahman et al. [28] using triazole analogues of diosgenin, they were shown to be inactive against bacteria, including the species *S. aureus* ATCC 25923. However, diosgenin showed antibacterial activity against other bacterial species such as *Porphyromonas gingivalis* and *Prevotella intermedia* both in planktonic forms as in biofilms [8].

Diosgenin showed potentiating activity in association with aminoglycoside (gentamicin) for both gram-positive bacteria (*S. aureus*) and gram-negative bacteria (*P. aeruginosa*). In addition, it also demonstrated synergistic activity in association with norfloxacin for *E. coli* and *P. aeruginosa* in association with ampicillin.

The synergy of drugs with plant products has been pointed out as a new way to overcome the mechanisms of resistance, producing favorable effects in the treatment of infectious diseases [29].

The increase in bacterial cell membrane permeability by diosgenin can be attributed to the detergent action of saponins, which contributes to the increased influx of antibiotics, by increasing the permeability of the cell membrane [30,31].

However, the association of diosgenin with ampicillin belonging to the β-lactam class, with a mechanism of action in the cell wall synthesis [32] presented an antagonistic effect increasing the MIC of the antibiotic for two tested strains *S. aureus* 10 and *E. coli* 06. Furthermore, it also had an antagonistic effect against *E. coli* 06 in association with gentamicin and *P. aeruginosa* 24 in association with norfloxacin.

In a study by Monte et al. [33] the antagonistic effect was also observed in association with saponin and the antibiotics tetracycline and ciprofloxacin for *S. aureus* CECT 976.

The antagonistic effect can be explained by the binding at the site of action that the antibiotic would occupy or even due to a possible mechanism of chelation of antibiotics, which reduces the spectrum of action of antibiotics [34,35].

The increase in norfloxacin MIC when associated with diosgenin for *S. aureus* 1199B corroborates the work of Tintino et al. [36] using ergosterol (steroid) where its association with norfloxacin for *S. aureus* 1199B was antagonistic, this effect was attributed to the interaction of steroids with the antibiotic, due to the possible chelating effect that prevented the influx of norfloxacin into the bacterial cell.

When we analyzed diosgenin in association with ethidium bromide for *S. aureus* 1199B, it showed indifference, as well as *S. aureus* K2068 in association with ciprofloxacin and ethidium bromide. Thus, we report that saponin did not interfere with the MepA efflux pump mechanism. However, the antibacterial activity of diosgenin may be related to other mechanisms, such as changes in membrane permeability or through

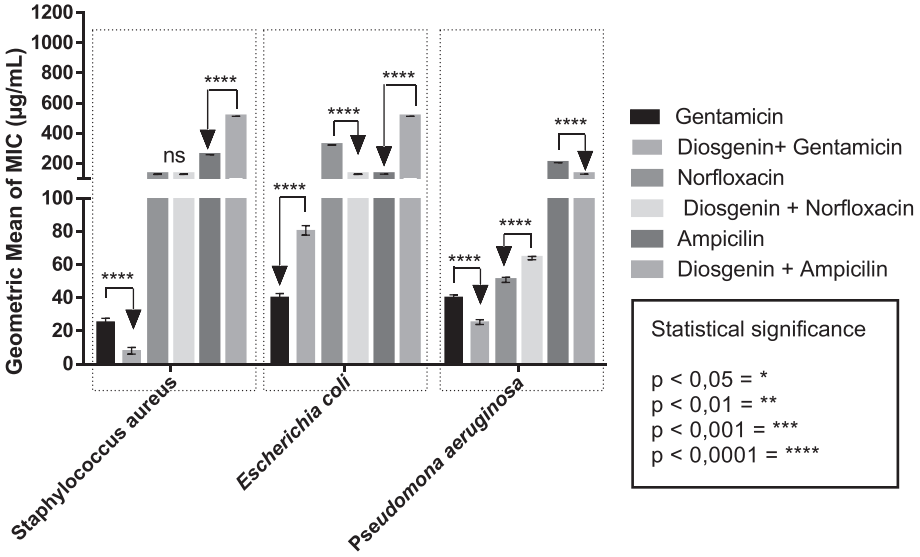


Fig. 2. Modifying effect of the antibiotic action of diosgenin on gram-positive and gram-negative strains combined with the antibiotics: gentamicin, norfloxacin and ampicillin.

Table 2
Determination of diosgenin Minimum Inhibitory Concentration (µg/mL) against *Staphylococcus aureus* strains carrying NorA and MepA efflux pump.

Substances	SA-1199B	SA-K2068
CCCP	16	8
CPZ	512	812
EtBr	256	256
Diosgenin	1024	1024

Legend: SA - *Staphylococcus aureus*, CCCP-carbonylcyanide 3-chlorophenylhydrazone, CPZ-chlorpromazine, EtBr-ethidium bromide.

other mechanisms specific to the antibiotic.

Several plant compounds and their derivatives have been applied as inhibitors for efflux pumps, such as saponin-rich plant extracts of *Crinum asiaticum* demonstrated an inhibitory effect on efflux pumps for *Mycobacterium smegmatis* and *Mycobacterium aurum* [37]. Saponin-rich extract of *Acacia macrostachya* showed inhibitory effects for efflux pumps for *S. aureus* and *E. coli* bacteria [38].

Other isolated triterpenes demonstrated activity for bacteria carrying efflux pumps such as α, β-amyrin with inhibition of the NorA pump in *S. aureus* 1199B [39]. As well as maslinic acid, lupeol and friedelin which have been reported to have antibacterial activity for both gram-positive and gram-negative bacteria [40]. However, triterpenes do not have the same chemical structure and polarity as saponins, which shows that they do not interact in the same way with the structures of bacterial cells.

Overcoming the drug efflux mechanism by using new efflux pump inhibitors is one of the strategies to combat antibiotic resistance mechanisms, reducing the rates of resistance development [41].

5. Conclusion

According to the results, we can observe that diosgenin showed to be very promising with antibacterial activity against the standard species *S. aureus*. It also showed antibiotic potentiating activity in association with the aminoglycoside gentamicin for multidrug-resistant bacteria *S. aureus* and *P. aeruginosa*, in addition, it also showed additive activity in association with norfloxacin for *E. coli* and *P. aeruginosa* in association with ampicillin. The evaluation of antibacterial activity against *S. aureus* strains carrying NorA and MepA efflux pumps revealed that saponin did not interfere with the pump mechanism or intervened in an antagonistic way, suggesting that the antimicrobial activity of diosgenin is not related to the efflux pump mechanism, which may be related to other

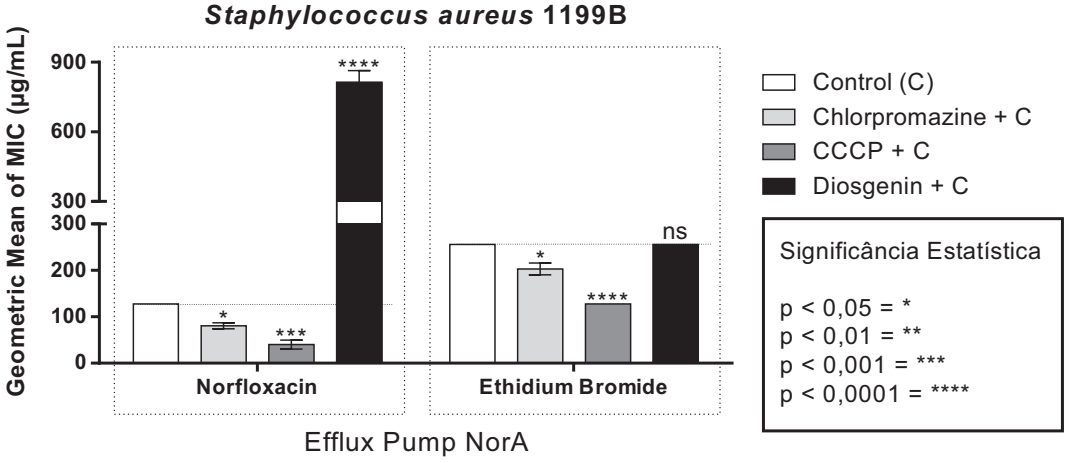


Fig. 3. Modifying effect of diosgenin antibiotic action on *Staphylococcus aureus* 1199B strain combined with norfloxacin and ethidium bromide.

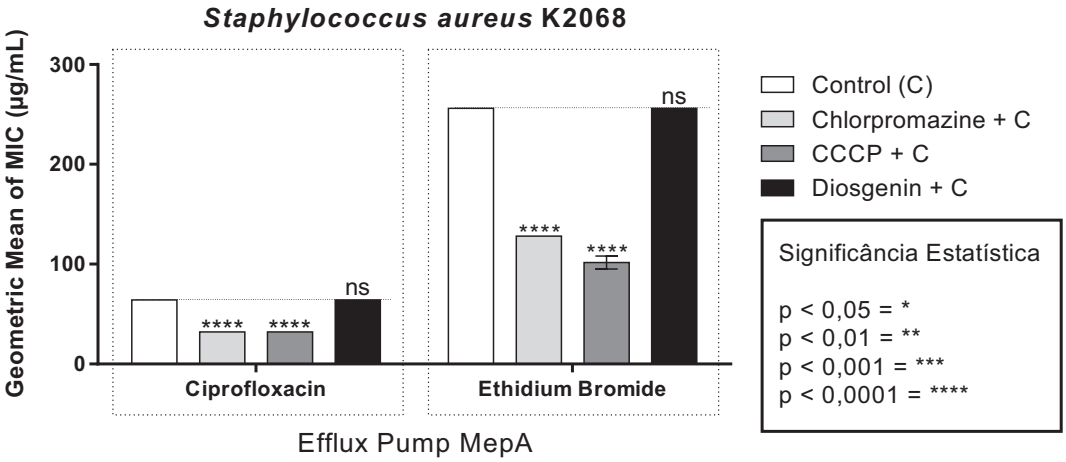


Fig. 4. Modifying effect of diosgenin antibiotic action on *Staphylococcus aureus* strain K2068 combined with ciprofloxacin and ethidium bromide.

mechanisms, such as changes in cell membrane permeability, or other mechanisms specific to antibiotics. However, further studies should be performed to better elucidate the mechanism of action of diosgenin.

Contributions

All authors contributed to the completion of the work.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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