Modulation of the resistance to norfloxacin in Staphylococcus aureus by Bauhinia forficata Link

Jonas Nascimento de Sousa\textsuperscript{a}, Aylla Beatriz Melo de Oliveira\textsuperscript{a}, Leide Maria Soares de Sousa\textsuperscript{b}, Melissa Carvalho França Rocha\textsuperscript{b}, Ana Paula de Oliveira\textsuperscript{c}, Alan Diego da Conceição Santos\textsuperscript{c}, José Pinto de Siqueira Júnior\textsuperscript{d}, Glenn William Kaatz\textsuperscript{e}, Jackson Roberto Guedes da Silva Almeida\textsuperscript{e}, João Sammy Nery de Souza\textsuperscript{f} & Humberto Medeiros Barreto\textsuperscript{a*}

\textsuperscript{a}Laboratory of Research in Microbiology, Federal University of Piauí, Teresina – PI, Brazil; \textsuperscript{b}Postgraduate Program in Pharmaceutical Sciences, Federal University of Piauí, Teresina - PI, Brazil; \textsuperscript{c}Nucleus of Studies and Research of Medicinal Plants, Federal University of the São Francisco Valley, Juazeiro – BA, Brazil; \textsuperscript{d}Laboratory of Genetics of Microorganisms, Federal University of Paraíba, (UFPB), Brazil; \textsuperscript{e}Department of Medicine, Division of Infectious Diseases, Wayne State University School of Medicine, Detroit, Michigan, USA;

Microdilution assays were performed in order to evaluate the antimicrobial activity of the ethanoic extract from the leaves of Bauhinia forficata (EEBF) against different microorganisms. The extract did not present inner antimicrobial activities against the tested strains. However, EEBF was able to modulate the norfloxacin-resistance against Staphylococcus aureus SA1199-B that overproduce the NorA efflux pump, once sub-inhibitory concentrations of EEBF reduced the minimal inhibitory concentration of the norfloxacin in 87.5%. This modulatory effect was also found when the antibiotic was replaced by ethidium bromide, suggesting that EEBF acts probably by inhibition of NorA, allowing the antibiotic accumulation intracellularly, and making the line more sensitive. These results point out the EEBF potential as a source of NorA inhibitors that could be used in combination with norfloxacin for treatment of infections caused by multidrug-resistant S. aureus.

Keywords: Bauhinia forficata link; drug resistance; efflux pump inhibitors; infectious diseases
1. Experimental

1.1. Plant material and extraction

The leaves of *Bauhinia forficata* Link were collected in the city of Santa Luz, locality of Angical, (latitude 8°54'28.3" South and longitude 44°11'3.7" Western) Piauí, Brazil, on July 23, 2018. The plant material was identified and a voucher specimen was deposited with the number 161 at the Herbarium of Bom Jesus of the Universidade Federal do Piauí – UFPI.

Leaves from *B. forficata* were oven dried at 60° C for five days. Next, 500g were ground in a knife mill and subjected to alcoholic extraction for 72h. Then, this material was filtered in simple funnel and submitted to rotation evaporation for the production of the ethanolic extract. This process was done in triplicate obtaining 30g of ethanolic extract.

1.2. Strains and chemicals

Evaluation of the intrinsic antimicrobial activity of the EEBF was performed against standard microbial strains, including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. Antimicrobial activity of the EEBF was also evaluated against drug resistant *S. aureus* 1199-B strains that overexpressed efflux pumps. Assays for evaluation of the modulating effect on drug resistance were performed only with drug resistant *S. aureus* 1199-B strains over-expressing the *norA* gene encoding the NorA efflux pump (Kaatz and Seo, 1993).

Bacterial strains were maintained on Brain Heart Infusion Agar (BHIA, Himedia, India) slants at 4 ºC, and prior to assay the cells were grown overnight at 37 ºC in Brain Heart Infusion (BHI, Himedia, India). The yeast strain was maintained on Sabouraud Dextrose Agar (SDA, Himedia, India) slants at 4 ºC and prior to assay the cells were grown for 24 h at 37 ºC in Sabouraud Dextrose Broth (SDB, Himedia, India).

1.3. Assays for evaluation of the intrinsic antimicrobial activity

Stock solutions of EEBF or CPZ were prepared in DMSO followed by dilution in sterile distilled water to a final concentration of 16.384 μg/mL. Minimal inhibitory concentrations (MICs) were determined by microdilution assay in BHI broth with bacterial suspensions of $10^5$ CFU/mL and partition fraction concentrations ranging from 8.192 a 128μg/mL. Microtiter plates were incubated at 37 ºC for 24 h, then 20 μL of
resazurin (0.01% w/v in sterile distilled water) was added to each well to detect bacterial growth by colour change from blue to pink.

1.4. Assays for evaluation of the drug-resistance modulation

To evaluate if EEBF were able to modulate antibiotic resistance in S. aureus strains overexpressing specific efflux proteins, MICs of antibiotics were determined in the presence or absence of sub-inhibitory concentrations of each natural product (1/8 or 1/4 MIC). Antibiotic concentrations ranged from 0.125 to 128 μg/mL. Microtiter plates were incubated at 37 ºC for 24 h and readings were performed with resazurin as previously described.

To verify if the drug-resistance modulation occurred due to efflux pump inhibition, the modulation assay was performed by replacing antibiotics with EtBr, which is a known substrate of efflux pumps (Markham et al., 1999), here used as an indicator of efflux pump inhibition. Control assays were also performed replacing EEBF by CPZ which is a known efflux pump inhibitor (Neyfakh, Borsch, Kaatz, 1993).

1.5. Statistical analysis

Experiments were performed in triplicate and results were normalized by calculation of geometric mean values. Error deviation and standard deviation of the geometric mean were revealed. Statistical analyses were performed using GraphPad Prism, version 7.00. Differences between treatment with antibiotics (or EtBr) alone or associated with EEBF, or CPZ were examined using one-way analysis of variance (ANOVA). The differences mentioned above were analyzed by Bonferroni posttest and p < 0.05 were considered statistically significant.
**Figure S1.** MICs obtained for norfloxacin (Nor) (A) against *S. aureus* SA1199-B (norA) in the absence or presence of the ethanoic extract from the leaves of *B. forficata* (EEBF) or chlorpromazine (CPZ). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values (*p* <0.0001).
Figure S2. MICs obtained for ethidium bromide (EtBr) against *S. aureus* SA1199-B (norA) in the absence or presence of the ethanoic extract from the leaves of *B. forficata* (EEBF) or chlorpromazine (CPZ). Each result represents the geometric mean of three simultaneous experiments. (*** Statistically significant values ($p <0.0001$).

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