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Antibacterial enhancement of antibiotic activity by *Enterolobium contortisiliquum* (Vell.) Morong

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ABSTRACT

Objective: To identify the main chemical classes of compounds from aqueous extract of *Enterolobium contortisiliquum* (*E. contortisiliquum*) seed bark and to evaluate its antibacterial activity, as well as its potential to increase the activity of antibiotics against strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli.*
Methods: Different classes of compounds in the aqueous extract of *E. contortisiliquum* were evaluated based on the visual changes in the coloration and the formation of precipitate after the addition of specific reagents. The antibacterial activity of the extract and its potential to increase of antibiotic activity of antibiotics drugs, gentamicin and norfloxacin was determined by using the microdilution method.
Results: Our results demonstrated that the following secondary metabolites were presented in *E. contortisiliquum* seed bark: flavones, flavonols, xanthones, flavononols, chalcones, aurones, flavones and catechins. The extract itself had very low antibacterial activity against all bacterial strains tested (MIC $\geq$ 1 024 μg/mL), but there was an increase in the antibiotic activity of gentamicin and norfloxacin when combined in the subinhibitory concentration (*i.e.*, MIC/8).
Conclusions: Our data suggests that *E. contortisiliquum* seed bark may be an alternative source for new drugs with the potential to increase antibiotic activity against different strains of bacteria.

1. Introduction

Bacterial resistance is associated with the emergence of antibiotic-resistant bacterial strains that can grow even at concentrations higher than recommended [1]. This phenomenon can be explained at least, in part, by the indiscriminate use of antibiotic, and thus requires alternatives to potentiate the effect of antibiotics on resistant bacteria [2]. In this context, some natural products from plant origin that exhibit antimicrobial activity have considerably attracted the scientific community because they contain a variety of chemical compounds that may or may not modify/potentiate the effects of antibiotics drugs [3].

The genus *Enterolobium* has about thirteen species with Neotropical distribution [4,5] in which the most represented species in Brazil are *Enterolobium contortisiliquum (E. contortisiliquum)*, *Enterolobium timbouva*, *Enterolobium gummiferum* and *Enterolobium schomburgkii* [6]. Of particular interest is *E. contortisiliquum* (Vell.) Morong (Family, Fabaceae), popularly known in Brazil as 'tamboril'

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or ‘orelha de negro’ for its fruit with a flattened shape and black color, is a native plant of the atlantic forest biome that can reach (25–30) m in height [7,8]. Essential oil from the seeds of *E. contortisiliquum* has shown antibacterial activity against different strains of gram-negative and gram-positive bacteria [9].

Staphylococcus aureus (*S. aureus*) is gram-positive bacteria found in the skin and mucosa of animals, and it is known to cause wound infection, endocarditis, skin infection and pneumonia among other infections. Numerous studies indicate that it is the major cause of community-acquired infections that can lead to high rate of morbidity and mortality [10]. Among the gram-negative bacteria, *Escherichia coli* (*E. coli*) is the most important one to human infectious and it is known to produce enterotoxins involved in diarrheal diseases [11,12]. Another gram-negative bacteria that deserve attention is *Pseudomonas aeruginosa* (*P. aeruginosa*), an important human opportunistic pathogen, responsible for causing predominantly mortality in patients with cystic diseases. These infections are difficult to control, mainly due to the bacteria's natural resistance to antibiotics, which can lead to death [13]. In this sense, more attention has been paid on plant-derived products such as flavonoids and terpenes not only for their antibiotic action, but specifically for their capacity to modify the action of standards antibiotic drugs when combined together [1].

Because of increased cases of bacterial resistance to conventional antibiotics drugs, studies have been carried out with the purpose to evaluate the antibacterial effects of natural products such as herbal products against infectious microorganisms, and to investigate the mechanism(s) of action resulting from the association of antibiotics and plant extracts [14]. Such preliminary study is of utmost importance since it represents a prerequisite for the development of new drugs.

In this context, the objective of this study was to identify the main chemical classes of compounds from *E. contortisiliquum* seed bark extract and to evaluate its antibacterial activity, as well as its potential to increase the activity of antibiotics against *S. aureus*, *P. aeruginosa* and *E. coli*.

2. Materials and methods

2.1. Plant collection and identification

The botanical material of *E. contortisiliquum* was collected at 2:00 p.m., September 21, 2015, at the Pedro Felício Cavalcanti Exposition Park, in the city of Crato, CE, Brazil. The geographical coordinate were: 07° 14′ 20.9″ south latitude and 39° 24′ 48.1″ West longitude of Greenwich. The plant species was identified by Prof. Dr. Maria Arlene Pessoa da Silva and a voucher specimen was deposited in the Caririense Dárdano de Andrade-Lima Herbarium of the Regional University of Cariri-URCA under the number # 12.239.

2.2. Preparation of extract

The extract was prepared by using the seed pericarp, which was previously grounded and degreased in soxhlet apparatus using hexane as the extractive solvent. Thereafter, the pericarp pie was dried at room temperature and 300 g of this was used to prepare aqueous extract. The obtained aqueous extract was lyophilized (LIOTOP L101 model) to yield 3.67%, which was saved in freezer and used for the experiments.

2.3. Qualitative chemical prospecting

The chemical tests were performed for the presence or absence of secondary metabolites in the aqueous extract of *E. contortisiliquum* seed bark. The method described by Matos [15] was used to detect the presence or absence of tannins, phenols, flavonoids and alkaloids. The assays were based on visual observation of color changes and the formation of precipitate after addition of specific reagents.

2.4. Microbial strains

The microorganisms used in the assays were obtained from the Laboratory of Microbiology and Molecular Biology of the Regional University of Cariri. The standard strains of *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 25923 and *E. coli* ATCC 25922, and the multiresistant strains of *S. aureus* 10, *P. aeruginosa* 24 and *E. coli* 06 were used.

2.5. Antibiotics and extract preparation

The antibiotics norfloxacin and gentamycin were diluted to a concentration of 1 024 μg/mL which was used for the assay. However, the *E. contortisiliquum* seed bark extract was prepared by diluting 10 mg of the extract into 9 765 mL directly into distilled water.

2.6. Inoculums preparation and determination of minimal inhibitory concentration (MIC)

The antibacterial effect of the extract was performed by using microdilution method as recommended by NCCLS M7-A6 [16]. Briefly, bacterial cultures were seeded in Petri dishes containing heart infusion agar and incubated at 37 °C for growth, for 24 h. After this period, a trawl of each microbial culture was carried out and diluted in test tubes in triplicate. After this procedure, the culture medium was diluted and put into three tubes, and then, the turbidity of the medium was tested by using a solution of 0.5 McFarland as control. The eppendorfs, prepared in triplicate for each bacteria contained 1 350 μL 10% brain and heart infusion and 150 μL of the inoculum (corresponding to 10% of the total solution) for the MIC. A total of 100 μL culture medium containing different bacterial strains was distributed in 96-well plates and then subjected to serial dilution using 100 μL of extract per column, with final concentrations varying from 512 μg/mL for the first well to 0.5 μg/mL for the last well. The plates were then incubated for 24 h at 37 °C. The antibacterial activity was determined by addition of 20 μL of resazurin (0.01% in distilled water) to the medium after the incubation period. The colour developed by resazurin was measured 1 h after its addition using microplate reader [17].

2.7. Potentiation of antibiotic activity

To evaluate the capability of the extract to potentiate the effects of antibiotics drugs against the tested strains, the method proposed by Coutinho *et al*. was used [18]. In eppendorfs, it was added with 188 μL extract at the sub-inhibitory concentration (*i.e.*, MIC/8), 150 μL of the bacterial inocula (corresponding to 10% of the solution) and 1 162 μL of 10% brain and heart infusion was placed in eppendorf. The control group follows the same procedure, but, without addition of the extract. A 100 µL of this solution mixture was placed in each well and 100 µL of the antibiotic drugs was added.

2.8. Statistical analysis

The results were expressed as mean ± standard error of 3 independent experiments. They were analysed by using GraphPad Prisma version 5.0. Statistical analysis was performed by using two-way ANOVA followed by Bonferroni posttest and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical prospecting

The qualitative chemical characterization of *E. contortisiliquum* seed bark aqueous extract revealed the presence of the following classes of secondary metabolites: flavones, flavonols, xanthones, flavononols, chalcones, aurons, flavones and catechins. However, the following secondary metabolites were absents: phenols, tannin pyrogallates, condensed tannins, anthocyanins and anthocyanidines.

3.2. MIC

The MIC value of *E. contortisiliquum* seed bark against the multiresistant strains of *S. aureus* (ATCC 6538 and SA10), *P. aeruginosa* (ATCC 25923 and PA24) and *E. coli* (ATCC 25922 and EC06) was value ≥1 024 µg/mL. Based on the fact that the MIC value was higher than 1 000 µg/mL, it was not clinically relevant, suggesting that the extract did not show antibacterial activity.

3.3. Increase of antibiotic activity by aqueous extract of E. contortisiliquum seed bark

Although the MIC value obtained for the *E. contortisiliquum* seed bark against the bacterial strains was not relevant in a clinical point of view, its combination at the sub-inhibitory concentration with the antibiotics drugs presented a synergistic action. Table 1 showed that *E. contortisiliquum* seed bark significantly potentiated the action of norfloxacin against *S. aureus*, *P. aeruginosa* and *E. coli* in comparison to norfloxacin alone ($P < 0.05$).

When *E. contortisiliquum* seed bark at the sub-inhibitory concentration was combined with the antibiotic gentamicin against the three bacterial strains, it was possible to observe a dramatic reduction of the bacterial growth, especially for *S. aureus* and *E. coli* (Table 1). However, *E. contortisiliquum* seed bark did not present any significant increase in antibiotic activity of gentamicina against *P. aeruginosa.* These results indicate the potential of *E. contortisiliquum* seed bark to synergistically increase antibiotic activity of norfloxacin and gentamicin against these bacterial strains.

4. Discussion

These secondary metabolites may be directly associated with the antibacterial activity of the extract, since they are known to exhibit a variety of pharmacological activities [19] Studies on the modulating activity of natural products showed interesting results associated with classes of secondary metabolites, such as flavonoids [14]. For example, in the study of Tintino *et al.* [20], it was verified that the class of flavonoids in the extract of *Beta vulgaris* exerted antibacterial activity responsible for the increase of the drug activity of aminoglycosides. Although we did not investigate the presence of carvone and estragole in the extract, they were found to be responsible for the antimicrobial activity of the essential oil from the seeds of *E. contortisiliquum* [9]. Similarly, a recent study on unsaponifiable matter of *E. contortisiliquum* revealed α-amyrin, β-amyrin and 4-methyl-2,6-di-*tert*-butylphenol as its main components by GC–MS analysis, with antimicrobial activity [21].

Although this is the first study to investigate the antibacterial or increase of the antibiotic activity of the *E. contortisiliquum* aqueous extract in combination with antibiotics against bacterial strains, it has already been shown that the essential oil of *E. contortisiliquum* seeds showed MIC of 150 mg/mL against the Gram-positive *S. aureus* bacterium, while no effect was observed with gram-negative bacteria [9]. Since there are no studies on the antibacterial activity or the increase of the antibiotic activity of *E. contortisiliquum* aqueous extract in combination with antibiotics against bacterial strains, the results are of particular interest since *E. contortisiliquum* seed bark showed the capacity to potentiate the antibacterial effect of the tested antibiotics. However, studies performed with other plant extracts by using the same methodology in this study also presented synergism. This is the case, for example, the ethanolic extract of leaves of *Lygodium venustum* SW, which showed increase in the antibiotic activity of aminoglycosides gentamicin against *E. coli* 27 (decrease of MIC value from 1 250.00 µg/mL to 39.06 µg/mL), *S. aureus* 358 (MIC from 19.53 µg/mL to 2.44 µg/mL) [22]. Ethanolic extract of the leaves of *Lantana montevidensis* (Spreng.) Briq is in the same way. Its antibacterial activity and its ability to interfere with the antibiotic resistance to aminoglycosides were tested, demonstrating its synergistic action. The ethanolic extract of the leaves of *L. montevidensis* (Spreng.) at the concentration

Table 1

Modulatory action of norfloxacin and gentamicin by *E. contortisiliquum* seed bark against different strains of bacteria (mean ± SD) (µg/mL).

Groups	*S. aureus*	*P. aeruginosa*	*E. coli*
E.A.C.E.C + norfloxacin	128.00 ± 2.00***	203.19 ± 2.22***	645.08 ± 1.49***
Norfloxacin	203.19 ± 1.49	256.00 ± 1.00	812.75 ± 1.49
E.A.C.E.C + gentamicin	5.04 ± 2.90***	6.35 ± 1.49	16.00 ± 4.00***
Gentamicin	40.32 ± 1.49	10.08 ± 2.22	40.31 ± 1.49

$^{***}P < 0.0001$ indicates significant difference in comparison with control norfloxacin.

E.A.C.E.C: aqueous extract of *E. contortisiliquum* seed bark.

of 1 μg/mL when were combined with gentamicin against *P. aeruginosa* significantly enhanced the activity of gentamicin, when compared to gentamicin alone. This was evidenced with the reduction in the MIC value of the antibiotic (*i.e.*, gentamycin) from 156 μg/mL to 20 μg/mL [23]

A study of Cunha *et al*. on the increase in antibiotic activity of the ethanolic extract from the stem and leaves of *Costus* cf. *arabicus*, presented a 4-fold reduction in the MIC value of gentamicin (from 8 μg/mL to 2 μg/mL) when both extracts were combined with gentamicin against *E. coli* 27 [24].

Similar results were obtained by Tintino *et al*. when they analyzed the ethanolic and hexane extracts from the root of *Costus* cf. *arabicus* [12]. The authors demonstrated that the combination of ethanolic and hexane extracts together with gentamicin against *S. aureus* resulted in an improved activity of gentamicin [12]. Considering that the synergism observed with the extract can be attributed to its ability to potentiate antibiotic activity or revert to the resistance of antibiotic drugs which usually used in the treatment of infectious diseases, we can presume that these extracts can be considered as modifiers of antibiotic activity [25].

Thus, it seems that the combination of standard antibiotic drugs with natural products, especially of plant origin for the treatment of infectious diseases, may be an efficient alternative to reduce the side effects associated with antibiotics of the class of aminoglycosides [26].

The results obtained in this study showed that *E. contortisiliquum* seed bark did not exhibit antibacterial activity against *S. aureus*, *P. aeruginosa* and *E. coli*, but it enhanced the effect of antibiotics norfloxacin and gentamicina at the sub-inhibitory concentration against these bacterial strains. These results can be attributed at least in part, to the presence of secondary metabolites (flavones, flavonols, xanthones, flavononols, chalcones, aurones, flavones and catechins) identified in this extract. Further studies are required to test the constituents isolated against bacterial strains and their potential increase in antibiotic activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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