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Antibacterial and antioxidant potential of *Spondias tuberosa* Arruda (Anacardiaceae) extracts

Potencial antibacteriano e antioxidante de extratos de *Spondias tuberosa* Arruda (Anacardiaceae)

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Abstract

Antimicrobial resistance and the consequent inefficiency of antibiotics are the main problems faced by medicine. In view of this, numerous researches have been aimed at looking for new agents with antibacterial activity, among them natural products. Thus, this study aims to evaluate the antioxidant activity of aqueous and hydroalcoholic extracts of the leaves and roots of *Spondias tuberosa* by Thin Layer Chromatography (TLC), as well as to verify the antibacterial action of the extracts alone and in combination with commercial antibiotics to evaluate its potential in action of change of antibiotics. The extracts showed polar and nonpolar phenolic substances with antioxidant action. The Minimum Inhibitory Concentration (MIC) of the hydroalcoholic extracts of the leaves and roots was 1,024 µg/mL compared to the *Staphylococcus aureus* 25923 strain, whereas with the other strains, the products showed an MIC ≥ 2,048 µg/mL. The effect of combining extracts with amikacin, ampicillin and norfloxacin against the multidrug-resistant bacteria *Escherichia coli* 06, *Staphylococcus aureus* 10 and *Pseudomonas aeruginosa* 24 resulted in synergistic effects with aminoglycoside alone, achieving up to 75 % reduction in the MIC of the antibiotic. In view of the results obtained, it can be concluded that the extracts of *S. tuberosa* presented polar and nonpolar phenolic substances, in the antibacterial activity it can positively modify the effect of the aminoglycoside antibiotic against multi-resistant bacteria, but future studies are necessary to discover the mechanism of action of such an effect.

Keywords: Antibiotics; Resistance; Umbu; Bacteria.

Resumo

A resistência antimicrobiana e a consequente ineficiência de antibióticos são os principais problemas enfrentados pela medicina. Diante disto, inúmeras pesquisas têm sido destinadas a procurar novos agentes com atividade antibacteriana, dentre eles os produtos naturais. Dessa forma, esse estudo tem como objetivo avaliar a atividade antioxidante dos extratos aquosos e hidroalcoólicos das folhas e raízes de *S. tuberosa* Arruda (umbu) por Cromatografia em Camada Delgada (CCD), bem como verificar a ação antibacteriana dos extratos isoladamente e em combinação com antibióticos comerciais para avaliar seu potencial na ação de modificação de antibióticos. Os extratos apresentaram substâncias fenólicas polares e apolares com ação antioxidante. A Concentração Inibitória Mínima (CIM) dos extratos hidroalcoólicos das folhas e raízes foi 1024 µg/mL frente a cepa *Staphylococcus aureus* 25923, já com as demais cepas os produtos demonstraram uma CIM ≥ 2.048 µg/mL. O efeito da combinação dos extratos com amicacina, ampicilina e norfloxacina frente às bactérias multirresistentes

Escherichia coli 06, *Staphylococcus aureus* 10 e *Pseudomonas aeruginosa* 24 resultou em efeitos sinérgicos apenas com o aminoglicosídeo, alcançando até 75 % de redução da CIM do antibiótico. Diante dos resultados obtidos, pode-se concluir que os extratos de *S. tuberosa* apresentaram substâncias fenólicas polares e apolares, na atividade antibacteriana pode modificar positivamente o efeito do antibiótico aminoglicosídeo contra bactérias multirresistentes, mas estudos futuros é preciso para descobrir o mecanismo de ação de tal efeito.

Palavras-chave: Antibióticos; Resistência; Umbu; Bactérias.

Resumen

La resistencia a los antimicrobianos y la consiguiente ineficacia de los antibióticos son los principales problemas a los que se enfrenta la medicina. Ante esto, numerosas investigaciones se han dirigido a buscar nuevos agentes con actividad antibacteriana, entre ellos productos naturales. Así, este estudio tiene como objetivo evaluar la actividad antioxidante de extractos acuosos e hidroalcohólicos de las hojas y raíces de *Spondias tuberosa* mediante Cromatografía de Capa Fina (CCF), así como verificar la acción antibacteriana de los extractos solos y en combinación con antibióticos comerciales para evaluar su potencial en la acción del cambio de antibióticos. Los extractos mostraron sustancias fenólicas polares y apolares con acción antioxidante. La Concentración Mínima Inhibitoria (CMI) de los extractos hidroalcohólicos de hojas y raíces fue de 1.024 µg / mL en comparación con la cepa *Staphylococcus aureus* 25923, mientras que con las otras cepas, los productos mostraron una CMI ≥ 2.048 µg / mL. El efecto de combinar extractos con amikacina, ampicilina y norfloxacina contra la bacteria multirresistente *Escherichia coli* 06, *Staphylococcus aureus* 10 y *Pseudomonas aeruginosa* 24 resultó en efectos sinérgicos con el aminoglucósido solo, logrando reducción de hasta un 75 % en la CMI del antibiótico. A la vista de los resultados obtenido, se puede concluir que los extractos de *S. tuberosa* presentó sustancias fenólicas polares y apolares, en el actividad antibacteriana puede modificar positivamente el efecto del antibiótico aminoglucósido contra bacterias multirresistentes, pero el futuro son necesarios estudios para descubrir el mecanismo de acción de tal efecto.

Palabras clave: Antibióticos; Resistencia; Umbu; Bacterias.

1. Introduction

Antimicrobial resistance represents a serious threat to global public health and food security, affecting anyone, of any age, in any country, being responsible for a longer stay in hospitals, high medical costs and increased mortality (Iriti; Vitalini & Varoni, 2020).

This mechanism is mainly due to the inappropriate use of antibiotics, which leads to the selection of resistant microorganisms. This resistance can be developed, acquired or even transported through the bacterial gene recombination, being increasingly virulent (Lima et al., 2019).

Such mechanisms mainly affect nosocomial microorganisms, such as the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus* (Santos et al., 2019).

In this context, natural products have a wide source of active substances, containing complex mixtures of several distinct constituents that can be synergistically active once administered. In addition, plant extracts and phytochemicals may be important in therapy helping to improve and the effectiveness of conventional antimicrobials, reducing their adverse effects and reversing resistance to numerous drugs (Aboody & Mickymaray, 2020; Bezerra et al., 2019; Costa et al., 2020).

In this case, the use of natural products based on their therapeutic use appears as a viable alternative, since they are culturally accepted, have a low cost and high availability (Bezerra et al., 2017). Thus, ethnopharmacological studies assist in the selection of plants with possible active principles, as they are based on popular knowledge and the medicinal use of plants in traditional practices (Albuquerque & Hazanaki, 2006). Such traditional uses and knowledge are present in several regions of the world, and one that can stand out is the Northeast of Brazil, due to its history, since before 1988 the local populations had no access to a public health system. As a result, communities used plants from the Caatinga, a type of seasonally dry tropical forest that has high biological diversity, to treat diseases (Magalhães et al., 2019).

Among the plants used by Northeasterners, *Spondias tuberosa* Arruda (Anacardiaceae) stands out, a tree popularly known as “umbu” and “umbuzeiro”, with great ecological, social, food and economic importance (Lins-Neto et al., 2010). Its leaves and roots are used by rural communities to treat infections, nausea, conjunctivitis, digestive problems, diarrhea, diabetes, inflammatory conditions and menstrual disorders (Albuquerque et al., 2007; Siqueira et al., 2016; Cordeiro et al., 2018). Such medicinal uses are linked to the chemical heterogeneity of

this species, as such vegetative organs have xanthones, steroids, flavonoids, alkaloids, phenols and triterpenoids (Santos et al., 2019). Which may be responsible for their biological and pharmacological activities, such as antibacterials (Silva et al., 2012), anticancer (Zeraik et al., 2016), antivirals (Silva et al., 2011), anti-inflammatory (Siqueira, 2015) and antioxidants (Almeida; Albuquerque & Castro, 2011).

Based on these premises, the hypothesis is raised that the species *S. tuberosa* has biological activity against pathogenic microorganisms and has antioxidant action. Thus, this work sought to evaluate the antioxidant activity of the aqueous and hydroalcoholic extracts of the leaves and roots of *S. tuberosa* Arruda (umbu), as well as to verify the antibacterial action of the extracts alone and in combination with conventional antibiotics to evaluate their potential in the action of antibiotics change.

2. Material and Methods

2.1 Botanical Material Collection

The leaves and roots of *Spondias tuberosa* were collected under the consent of the Biodiversity Authorization and Information System - SISBIO with number 64293-1, in the Lameiro community (07º15'03.1" south latitude and 39º23'48.3" west longitude Greenwich), in the municipality of Crato, Ceará, Brazil, were collected in June 2018 from 7 individuals, from 8:00 am to 9:30 am. Of the material collected, a copy was deposited at the Herbário Caririense Dárdano de Andrade Lima (HCDAL) of the Regional University of Cariri - URCA with a voucher of #13,728, being identified by M.ª Ana Cleide Alcântara Morais Mendonça.

2.2 Preparation of Extracts

The aqueous extract was acquired through an infusion process, in which the leaves and roots were crushed to increase their contact surface. Subsequently, 1 L of distilled water at 100 ºC was added to each 132.2 g of plant part, after which the container was closed, where it was kept at rest for 15 minutes, for later filtration. While the hydroalcoholic extract (tincture) was prepared by maceration in 70 % ethanol in a proportion of 500 g of fresh leaves and 400 g of dried roots for each 2.652 L of ethanol (70 %). The mixture was stored in a dark place for 72 h (Matos, 2002 - with modifications).

The drying of the extracts was performed using the spray drying technique (spray drying) using the *Mini-spray dryer* MSDi 1.0 equipment (Labmaq do Brasil), using a 1.2 mm spray nozzle, under the following operational conditions: a) flow control: 500 mL/h; b) inlet temperature: 130 ± 2 ºC; c) outlet temperature: 74 ± 2 ºC; d) atomization air flow: 45 L/min; e) blower flow: 1.95 m^3/min. The spray drying process consists of changing a product that is in a liquid to a solid state in powder form, through its passage in a heated medium, in a continuous operation (Masters, 1991).

2.3 Antioxidant Activity by Thin Layer Chromatography (TLC)

The trial was carried out based on methodologies proposed by Formagio et al., (2014) & Hidalgo, Nunomura & Nunomura (2016), with adaptations. The plant extracts were analyzed, in triplicate, by TLC using quercetin and gallic acid as positive comparison standards (1 mg/mL in methanol). As an adaptation of the method, chromatographic plates were prepared, using glass plates (10 x 5 x 0.3 cm) as support, and as a stationary phase the mixture of plaster and corn starch (1:1) (Collins, 2010; Pereira, 2010). 20 µL of each sample were applied to the plates, together with the positive controls and the elution system: chloroform/ethanol (9:1) was used. After drying at room temperature (30 ± 2 ºC), the plates were nebulized, separately, with the specific developers: a) Developer of phenolic compounds: diluted solution of ferric chloride ($FeCl_3$, 2 %), resulting in a dark blue color; b) Developer for antioxidant compounds: 0.5 % solution of the DPPH radical (2,2-diphenyl-1-picryl-hydrazil) in methanol, resulting in the appearance of yellowish spots under a purple background; c) Developer of antioxidant compounds with reducing potential: mixture (1: 1) of aqueous solutions of $K_3[Fe(CN)_6]$ (1 %) and $FeCl_3$ (0.1 %), resulting in dark blue stains (Prussian blue).

2.4 Bacterial Strains and Inoculum Preparation

The strains used in the tests were the standard strains of bacteria *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, and multi-resistant strains *Escherichia coli* 06, *Staphylococcus aureus* 10 and *Pseudomonas aeruginosa* 24 (Table 1). The bacteria were grown on solid slopes on Heart Infusion Agar and maintained at 37 °C for 24 h. The inoculants were derived from this solid medium using test

tubes containing standardized sterile saline according to the McFarland 0.5 scale corresponding to 10^8 CFU (Colony Forming Units) (NCCLS, 2003).

Table 1 - Resistant profile of the Strains

Bacteria	Origin	Resistance Profile
Staphylococcus aureus 10	Rectal swab	Amc, Amox, Amp, Asb, Azi, Ca, Cef, Cf, Cip, Cla, Clin, Eri, Lev, Mox, Oxa, Pen
Pseudomonas aeruginosa 24	Nasal discharge	Ami, Cip, Cpm, Ctz, Imi, Lev, Mer, Ptz
Escherichia coli 06	Urine Culture	Asb, Ca, Cef, Cfo, Cmp, Cro

Subtitle: Amc: Amoxicillin + Clavulanic acid, Ami: Amikacin, Amox: Amoxicillin, Amp: Ampicillin, Asb: Ampicillin + Sulbactam, Azi: Azithromycin, Ca: Cefadroxil; Cef: Cephalexin, Cfo: Cefoxitin, Cip: Ciprofloxacin, Cla: Clarithromycin, Clin: Clindamycin, Cmp: Cefepime, Cro: Ceftriaxone, Ctz: Ceftazidime, Eri: Erythromycin, Imi: Imipenem, Lev: Levofloxacin, Mer: Meropenem, Mox: Moxifloxacin, Oxa: Oxacillin, Pen: Penicillin and Ptz: Piperacillin. Source: The Author.

2.5 Culture Mediums

In the tests for antibacterial evaluation, the solid medium Heart Infusion Agar (HIA), which was prepared according to the manufacturer's instructions and the liquid medium Brain Heart Infusion (BHI) at 10 %. The media were solubilized with distilled water and sterilized in an autoclave at 121 °C for 15 minutes.

2.6 Preparation of Extracts and Drugs

Was weighed 20 mg of extract and diluted in 1 mL of dimethylsulphoxide (DMSO, Merck, Darmstadt, Germany), then diluted in 8,765 µL of distilled water to obtain a concentration of 2,048 µg/mL and reduce the concentration DMSO, which was used in the tests. The antibiotics amikacin (aminoglycoside), norfloxacin (fluoroquinolone) and ampicillin (beta-lactam) (SIGMA Chemical Co., St. Louis, USA) were used at a concentration of 1,024 µg/mL in the experiments.

2.7 Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the extracts was determined using the broth microdilution technique, using 96-well plates and in triplicate. Eppendorfs® were prepared, each containing approximately 1,350 µL of 10 % BHI and 150 µL of the bacterial suspension (corresponding

to 10 % of the solution). The plate was filled in the numerical direction by adding 100 µL of this solution to each well, and then serial microdilution was performed in a 1:1 ratio, varying in concentrations from 1,024 to 0.5 µg/mL. The plates were taken to the incubator for 24 hours at 37 ºC (Javadpour et al., 1996). The determination of bacterial MIC was made using the addition of 20 µL of resazurin in each well and observation after 1 hour. The MIC was verified through the color change caused by resazurin, where the change from blue to pink indicates bacterial growth and the unchanged blue color indicates growth inhibition, verifying the lowest concentration of the product capable of inhibiting bacterial growth. In all tests, the last wells were not microdiluted, as they are used to control bacterial growth.

2.8 Evaluation of the Antibiotic Action Modifying Effect

To verify whether the extracts would modify the action of antibiotics against the tested resistant strains, the method proposed by Coutinho et al., (2008). The extracts were tested in sub-inhibitory concentration (MIC/8). Eppendorfs® containing 10 % BHI, 150 µL of the bacterial suspension and the volume corresponding to the MIC/8 of the tested extracts were prepared. For the control of antibiotics, eppendorfs® tubes were prepared with 1.5 mL of solution containing 1,350 µL BHI (10 %) and 150 µL of microorganism suspension. The plate was filled in the numerical direction by adding 100 µL of this solution to each well. Then, 100 µL of the antibiotics were added, proceeding in series microdilution, in a proportion of 1:1 until the penultimate well. The plates were taken to the incubator for 24 hours at 37 ºC. Antibiotic concentrations ranged from 512 µg/mL to 0.5 µg/mL. All tests were performed in triplicate and the reading was done through the colorimetric variation of resazurin as previously described.

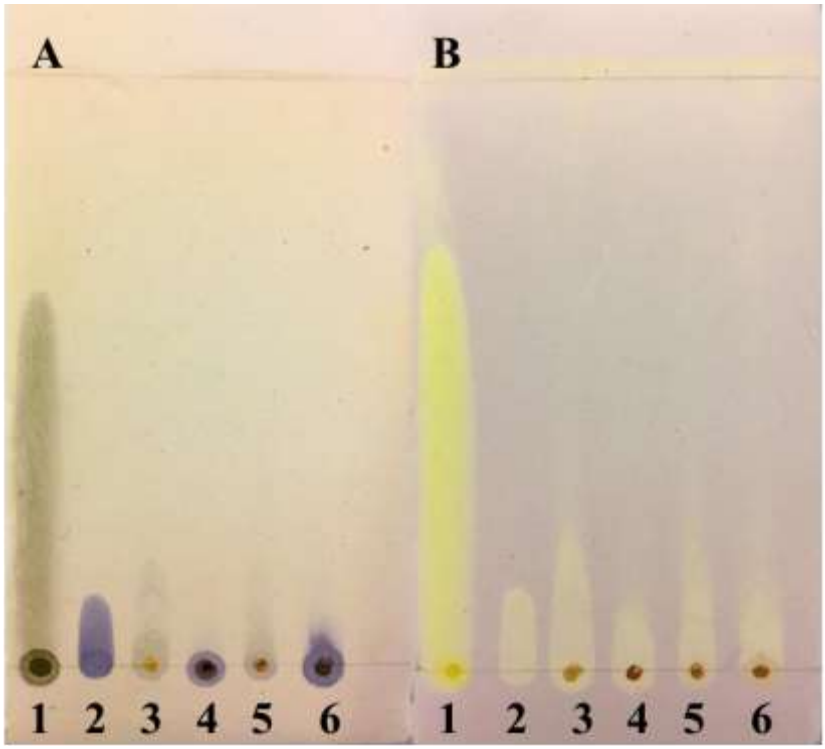
2.9 Statistical Analysis

The test results were expressed as the geometric mean. A one-way analysis of variance (ANOVA-One-Way) followed by Bonferroni's post-hoc test (where $p < 0.05$ and $p < 0.0001$ are considered significant and $p > 0.05$ not significant), was applied using the GraphPad software Prism 6.0.

3 Results and Discussion

Thin layer chromatography of the extracts demonstrated the presence of phenolic substances with varying polarities, the most polar being located at the bottom of the chromatoplate (Figure 1A), as they showed dark blue color when reacted with FeCl3, as well as the positive quercetin controls (Spot 1 in Figure 1A) and gallic acid (Spot 2 in Figure 1A). The yellow-colored bands in Figure 1B indicate DPPH radical scavenging sites, as well as the positive controls quercetin (Spot 1 in Figure 1B) and gallic acid (Spot 2 in Figure 1B). Analyzing the two plates of the two figures, it can be seen that the studied extracts presented polar and nonpolar phenolic substances with antioxidant action.

Figure 1 - Plaster-starch chromatoplates (1:1) from aqueous extract of leaves (AELST) and roots (AERST), hydroalcoholic extract of leaves (HELST) and roots (HERST) and of *Spondias tuberosa.*



A: $FeCl_3$: detect phenols, **B:** DPPH: detect antioxidants. 1: Quercetin; 2: gallic acid; 3: AELST; 4: AERST; 5: HELST and 6: HERST; Elution: chloroform/ethanol (9:1). Source: The Author.

The results presented are indicative of the presence of phenolic compounds with antioxidant action, because as pointed out by Formagio et al., (2014) & Hidalgo, Nunomura & Nunomura (2016), plant extracts that have blue colored substances revealed with $FeCl_3$ and yellow colors revealed with DPPH in chromatoplates are phenolic substances and with antioxidant action. This antioxidant effect was also found by Uchôa et al., (2015), who, when evaluating the effect on the reduction of DPPH free radicals, demonstrated that the methanol extract from the leaves is capable of reducing the amount of radicals in up to 50 % in concentrations of 500 µg/mL. In addition to the leaves, the barks of the species have constituents with antioxidant action (Araújo et al., 2012).

The assessment of the intrinsic antibacterial activity of the extracts showed that the MIC of the HELST and HERST was 1,024 µg/mL compared to the *S. aureus* 25923 strain, whereas with the other strains, the products showed an MIC of ≥ 2,048 µg/mL. These results indicate that the extracts of *S. tuberosa* used do not have antibacterial activity in concentrations of clinical relevance, considering that this concentration, in plasma values, can trigger toxic effects in the human organism (Houghton et al., 2007).

The study by Cristofoli et al., (2018) also showed no significant effects against *E. coli*, *P. aeruginosa* and *S. aureus* with the aqueous extract of the leaves of *Spondias mombin* L. using the agar diffusion method. Jaiswal et al., (2019), in the same sense, corroborate the present research, since the aqueous and ethanolic extracts of the leaves of *Spondias mangifera* Willd. were not effective against Gram-positive and Gram-negative bacteria.

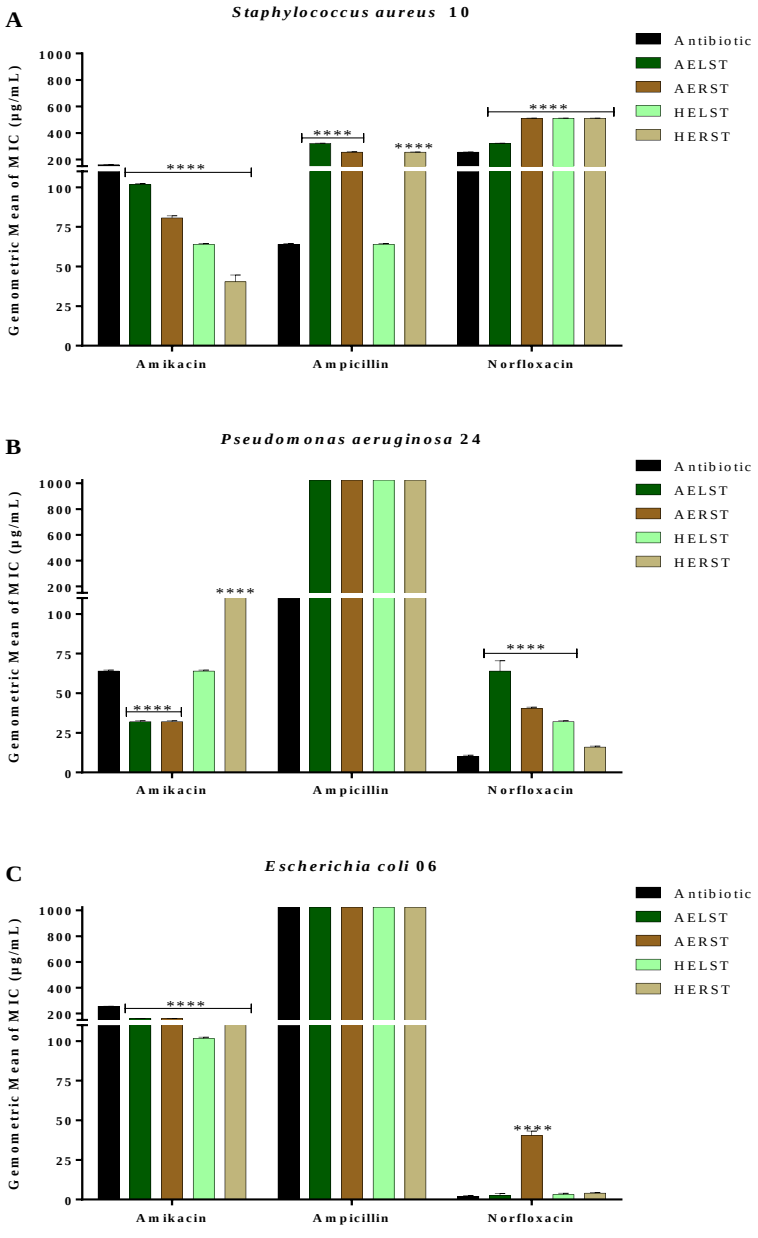
In contraposition, Silva et al., (2012) showed that the methanolic extract of the leaves of *S. tuberosa* had MIC of 125 µg/mL and zone of inhibition of 20.1 mm against *P. aeruginosa*, however, it is noteworthy that different solvents have distinct selectivity, resulting in unequal bioactivities with a same vegetable, due to the difference of the extracted components (Costa & Hoscheid, 2018).

Although the isolated extracts did not have a significant action on bacterial strains, when associated with amikacin, these products potentiated the action of antibiotics against multi-resistant nosocomial bacteria (Figure 2). These are the greatest reductions in MIC of amikacin: in *S. aureus* 10, MIC was reduced from 161.27 to 40.32 µg/mL by the HERST (Figure 2A); in *P. aeruginosa* 24 the AELST and AERST reduced the MIC from 64 to 32 µg/mL (Figure 2B); and in *E. coli* the HELST decreased the MIC from 256 to 101.59 µg/mL (Figure 2C).

In addition, the extracts also demonstrated indifferent or antagonistic effects when combined with antibiotics, especially with ampicillin and norfloxacin, not interfering or reducing their activities against the tested multidrug-resistant strains.

The antagonistic effects presented may have been due to the antioxidant substances present in the extracts, and can then be explained by the capture of these radicals by antioxidant compounds, intercepting functional oxygen and developing stable radicals, so that the action of the antibiotic is reduced (Mello & Santos, 2017; Andrade et al., 2019).

Figure 2 - Antibacterial effect of the extracts/antibiotic combination (µg/mL)



Source: Authors.

Even though this is a pioneering study in the evaluation of the modification of aminoglycosides by species of the genus *Spondias*, other research has demonstrated synergism between plant extracts of the Anacardiaceae family and this class of antibiotics: the methanolic extract of the fruits of *Rhus coriaria* L. potentiated amikacin against *E. coli* 3 (0 to 24 mm) (El-Zawahry; Reda & Azazy, 2013); the ethanolic extract of the leaves of *Myracrodruon urundeuva* Allemão reduced the MIC of gentamicin (8 to 4 µg/mL) and amikacin (64 to 16 µg/mL) against *S. aureus* 358 (Figueredo et al., 2014); the ethanolic extract and the methanolic and ethyl acetate fractions of the stem bark of *Anacardium microcarpum* L. increased the effects of amikacin and gentamicin against *P. aeruginosa* 03, *E. coli* 06 and *S. aureus* 10 (Barbosa-Filho et al ., 2015).

As present in the literature, antibiotics that inhibit protein synthesis, such as aminoglycosides, are among those that most present synergistic effects when associated with natural products to fight resistant bacteria, however, the mechanism by which this occurs has not yet been elucidated (Adwan; Abu-Shanab & Adwan, 2009; Saraiva et al., 2011; Shaeer et al., 2019).

Adegoke, Aiyegoro & Stenstrom (2016), when analyzing the interaction between the methanolic extract of *S. mombin* leaves with amoxicillin (beta-lactam) on diarrhogenic strains of *E. coli* (DEC), noticed that the extract-antibiotic combination was more effective against the *E. coli* (ETEC) enterotoxygenic strain than the isolated drug, increasing the zone of inhibition from 15.1 to 21.5 mm, and the result validated by the checkerboard and time-kill methods. For the enteroinvasive (EIEC) and enterohemorrhagic (EHEC) strains the results were indifferent.

In the same way as Temitope et al., (2017) demonstrated that the association between aqueous, ethanolic and ethyl acetate extracts from leaves, bark and stem of *S. mombin* with ofloxacin (fluoroquinolone) resulted in significant zones of inhibition against *E. coli* ATCC 25922 (up to 31 mm), *S. aureus* ATCC 29213 (up to 35 mm) and *P. aeruginosa* ATCC 25619 (up to 29 mm).

In the same way as Temitope et al., (2017) demonstrated that the association between aqueous, ethanolic and ethyl acetate extracts from leaves, bark and stem of *S. mombin* with ofloxacin (fluoroquinolone) resulted in significant zones of inhibition of *E. coli* ATCC 25922 (up to 31 mm), *S. aureus* ATCC 29213 (up to 35 mm) and *P. aeruginosa* ATCC 25619 (up to 29 mm).

The synergistic effects mentioned above with antibiotics of the beta-lactam and fluoroquinolone classes can be explained because in these studies the extracts were used in

concentrations ranging from 6,250 to 200,000 µg/mL, 24 to 781 times higher than the present study (256 µg/mL), without necessarily potentiating the drugs by inhibiting resistance, but high toxicity of the extracts themselves on microorganisms (Zhou et al, 2013; Leja et al., 2019).

4. Conclusion

In view of the results obtained, it can be concluded that the extracts of *S. tuberosa* presented polar and nonpolar phenolic substances with antioxidant action. They have no efficacy in antibacterial activity by direct action, however, they positively modify the effect of the aminoglycoside antibiotic against multi-resistant bacteria. It is necessary to elucidate the mechanism by which this synergism occurs in order to understand its pharmacological potential.

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