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Original Research

UPLC-QTOF-MS/MS analysis and antibacterial activity of the *Manilkara zapota* (L.) P. Royen against *Escherichia coli* and other MDR bacteria

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Abstract: With the spread of bacterial resistance against clinically used antibiotics, natural plant-derived products are being studied as new sources of antibacterial molecules. *Manilkara zapota* is a common plant species in the American continent that is used as a food source. Studies show the *M. zapota* extract is rich in phenolic substances that can serve as basic molecules for the pharmaceutical industry. An extract from fresh *M. zapota* leaves was produced and tested to identify the compounds present, as well as its direct antibacterial and clinical antibiotic modulatory activities. To analyze the results, a new statistical methodology based on the Shannon-Wiener index was tested, capable of correcting distortions in heterogeneous environments. The Hydroethanolic Extract of *Manilkara zapota* leaves (HEMzL) presented a wide variety of phenolic products, as well as tannins, in the UPLC analysis. The extract showed direct antibacterial activity against the standard *Staphylococcus aureus* strain, however, it either acted antagonistically when associated with the tested antibiotics, or it did not present statistical significance when compared to the control. This demonstrates a need to be cautious when associating natural products with antibiotics for clinical use, as a hindrance to infectious treatments may occur. As for the statistical analysis mechanism tested, this proved to be effective, reducing false negatives at low antibiotic concentrations and false positives at high concentrations in the microdilution plate.

Key words: Plant extract; Phenolic compounds; Antimicrobial activity; Antagonism; Statistical model.

Introduction

Bacterial resistance to clinically used antibiotics and its dissemination across the globe has become a worldwide concern (1). Several mechanisms are involved with the emergence and dissemination of resistance, thus making its control difficult (2). In this sense, naturally derived products, such as plant extracts, are important sources in the search for molecules with antibacterial activities (3).

In recent studies, a compound derived from the seeds of *Cullen corylifolium* L. presenting a wide range of possible clinical applications, including antibacterial therapies (4). In a review paper on lesions of the oral mucosa, several plant extracts, including *Matricaria recutita* L. and *Aloe vera* (L.) Burm.f., presented compounds capable of acting on certain bacterial strains (5). In addition, plant extracts and substances derived from these extracts are capable of presenting antibiotic adjuvant activities, increasing their antibacterial potential, when added in subinhibitory doses (6, 7, 8, 9, 10).

For these reasons, the study of plants used in traditional medicine is necessary for the search for new biomolecules. *Manilkara zapota* (L.) P Royen is a plant present in tropical regions, originating in Mexico, whose fruits are used in food (11), with recognized medicinal properties (12). Its anti-cancer (13), anti-hyperglycemic (14), antioxidant (15), anti-inflammatory (16, 17), antifungal (18) and antibacterial effects (19) are among its previously cited properties in the literature.

In this article, the Hydroethanolic Extract of *Manilkara zapota* leaves (HEMzL) was analyzed in terms of its composition and its direct antibacterial effects, as well as its antibiotic modifying activity. A new statistical analysis model for the interaction of the extract with the clinically used antibiotics is also proposed herein.

Materials and Methods

Botanical material

The collection of the leaves of *Manilkara zapota* was carried out in a private property in the urban area of

the municipality of Crato-CE, according to coordinates 7º14'04"S, 39º24'13" W, by the author of this article. An excicata of the specimen was deposited at the Herbarium Caririense Dárdano de Andrade Lima - HCDAL of the Regional University of Cariri - URCA, under number 13484.

To obtain the extract, fresh leaves were partially crushed and filled with 70% ethyl alcohol, in a proportion of 10 mL of alcohol for each gram of fresh leaf, at room temperature for 72 h (20). The filtered liquid was then placed in a ratio of 1:1 by volume of water/alcohol. This final solution was disposed in the Mini-spray dryer MSDi 1.0 equipment (Labmaq do Brasil), using a 1.2 mm spray nozzle, for concentration and lyophilization of the extract.

Extract and antibiotics for clinical use

In the preparation of the extract, as well as the antibiotics for clinical use (cephalexin, ciprofloxacin and levofloxacin), 10 mg of lyophil were weighed and diluted in 0.5 ml of Dimethylsulfoxide (DMSO), with subsequent dilution in distilled water, until obtaining a concentration of 1024 µg/mL for all compounds.

UPLC-QTOF-MS / MS analysis

Samples of the extract of Manilkara zapota were filtered (PTFE, pore of 0.2 µm and diameter of 13 mm, Millipore Millex) and injected in the UPLC system (Waters Co., Milford, MA, USA). The instrumental UPLC analysis was performed in a Waters Acquity UPLC system, coupled to the Q-TOF Premier mass spectrometer (Waters MS Technologies, Manchester, United Kingdom) with electrospray ionization interface (ESI) in negative ionization mode. MS data were collected for m / z values with a range of 110 - 1180 Da. Mass and molecular formula were obtained with MassLynx 4.1 software (Waters MS Technologies).

Bacterial strains

Multidrug-resistant strains (*Staphylococcus aureus* 10, *Pseudomonas aeruginosa* 03 and *Escherichia coli* 06) and standards (*Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* 9027 and *Escherichia coli* 25922) grown at the Laboratory of Microbiology and Molecular Biology (LMBM) of the Universidade Regional do Cariri (URCA) used in the test.

Each strain is resistant to at least 2 of the 3 antibiotics used in this test (*Staphylococcus aureus* 10 is resistant to cephalexin, ciprofloxacin and levofloxacin; *Pseudomonas aeruginosa* 03 and *Escherichia coli* 06 are resistant to ciprofloxacin and levofloxacin), according to the resistance profile.

Direct antibacterial activity test

For this test, we used the methodology proposed by Javadpour and collaborators (21) (1996), adapted for broth microdilution tests. The bacterial inoculants were made from cultures in HIA (Heart Infusion Agar), with growth of 24 hours. One trawl of each strain was placed in 0.9% saline solution in test tubes, with turbidity measured according to the McFarland 0.5 scale (1 x 10^8 CFU/mL). Eppendorfs® tubes were filled with a solution composed of BHI (Brain Heart Infusion Broth) and 10% bacterial inoculum. This solution was used in the test. This procedure was performed for each bacterium and in triplicate.

After being disposed on the 96-well plates, the solution was microdiluted by HEMzL in a 1:1 ratio. Plaque concentrations ranged from 512 µg/mL to 8 µg/mL. The reading of the Minimum Inhibitory Concentration (MIC) was performed by colorimetric method, with the Resazurin reagent, after 24 hours of incubation at 37 ºC in a bacteriological oven.

Antibiotic action modification test

In this test, we prepared the eppendorfs® as described above, however the HEMzL in sub-inhibitory concentration (MIC/8) was added to the final solution, using the methodology described by Coutinho and collaborators (2008) (22). In this step, microdilution was performed with antibiotics for clinical use. Antibiotic controls for each bacterium were also prepared. In this step, the plates were used in the numerical sense, with concentrations ranging from 512 µg/mL to 0.5 µg/mL.

To evaluate the mechanism of action of HEMzL, a control was also performed with the standard efflux pump inhibitor (EPIS) chlorpromazine (CPMZ), in subinhibitory operations.

Statistical analysis

On the microdilution plate, we analyzed 11 different concentration points of the substance, so that there are numerous unanalyzed points between one analysis point and another. These unanalyzed points vary across the plate, with a greater number of unanalyzed points at higher concentrations, and lower at lower concentrationsIn order to solve this problem of scale between the different MIC points analyzed in the microdilution plate, the MIC values obtained in each triplicate were transformed into their corresponding logarithms, making the intervals regular. These values were submitted to an arithmetic mean and the result was returned in terms of natural number, that is, in terms of tested concentration. This value corresponds to the geometric mean of the concentrations and was used as a central data.

The diversity of unread MIC points between the intervals applied in the test also affects the standard deviation between the points represented, since it has a higher limit at higher concentrations and a lower limit at lower concentrations, not being standardized during the analysis. To solve this distortion, we use an idea used in biodiversity measures to correct distortions in sampling for calculations of species richness and diversity between different environments, proposed in the Shannon-Wiener index (23), whose formula, to correct the heterogeneity of two areas, multiplies the value of the proportion of the number of individuals of a species, by its value in terms of logarithm, thus being able to evaluate two heterogeneous areas.

Using this proposition, the values of the standard deviations found in each scale of MIC points were multiplied by their calculated value as a logarithmic scale, generating a corrected standard deviation, proportional to the number of MIC points not read in each variation, generating the uniformity of data necessary for the evaluation of each point of heterogeneous MIC in the microdilution plate, and in this way, avoiding false-positive errors at higher concentrations and false-negative

at lower concentrations.

The values obtained as central data and standard deviation were analyzed using a two-way ANOVA test, followed by a *post hoc* Bonferroni test (where $p < 0.05$ will be considered significant) using the GraphPad Prisma 5.0 statistical program.

Results and discussion

UPLC-QTOF-MS/MS analysis

With the data obtained through mass spectrometry, comparisons were made between the fragments and the specialized literature, looking for patterns of compounds, initially between extracts of plants of the same species, later within the genus and finally, comparing with analyzes of extracts of plants of the *Manilkara zapota* family. 10 compounds were identified from the 17 peaks present in the chromatogram of figure 1. These compounds are identified in Table 1, with their specifications determined.

The compounds found were mainly phenolic derivatives from the catechin (compounds 5, 6, 7 and 9) and flavone (10, 11, 12, 13 and 14) groups, both of which belong to the large flavonoid group. Compound 8, which belongs to the tannin class, was the exception.

Flavonoids are compounds with anti-inflammatory and anti-diabetogenic activity, which act by modifying cellular insulin resistance (24) and/or reducing carbohydrate absorption, where compounds from this class are used in their isolated form, as well as in plant extracts (25). Flavonoids have also been shown to be effective against inflammatory bowel disease (26) by inducing natural mucosal protection mechanisms (27). Additionally, flavonoids present antioxidant activity, combating oxidative stress (28), they decrease the probability of developing chronic-degenerative diseases arising from several causes (29, 30), present analgesic activity (31) and function as immune modulating agents (32).

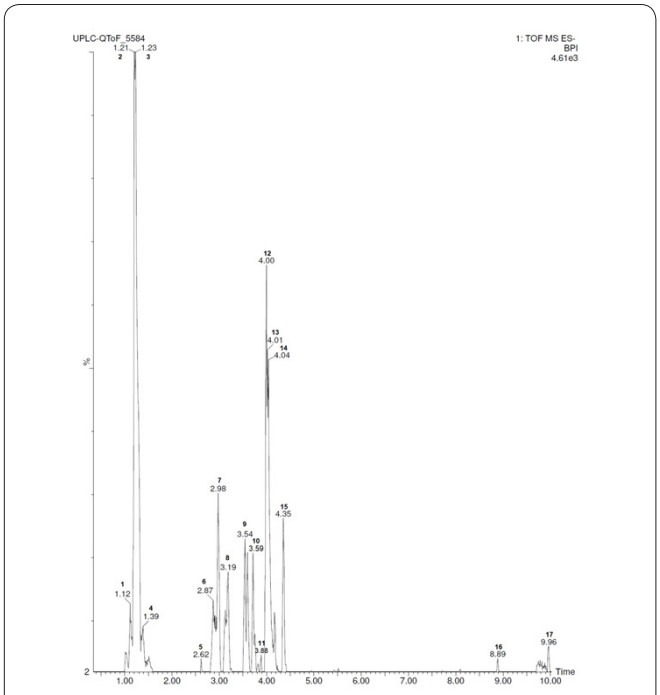

Figure 1. Chromatogram of the Hydroethanolic Extract of *Manilkara zapota* leaves (HEMzL).

Similarly, to flavonoids, tannins are phenolic compounds (33) presenting antioxidant (34), neuroprotective (35), anti-inflammatory and cardioprotective (36) as well as antibacterial activities (37).

The presence of phenolic compounds has been re-

Table 1. Characterization and Identification of compounds present in the hydroethanolic extract of *Manilkara zapota* leaves.

Peak no.	Rt Min	$[M-H]^-$ Observed	$[M-H]^-$ Calculated	Product Ions (MS/MS)	Empirical Formula	Ppm (error)	Putative Name	Referências
1	1.12	317.0465	317.0450	318.0506, 317.0462215.0344	$C_{19}H_9O_5$	4.7	N.i	-
2	1.21	191.0538	191.0556	192.0409, 191.0536, 127.0375	$C_7H_{11}O_6$	-9.4	N.i	-
3	1.23	191.0528	191.0556	192.0409, 191.0536, 127.0375	$C_7H_{11}O_6$	-14.7	N.i	-
4	1.39	191.0516	191.0556	192.0409, 191.0536, 127.0375	$C_7H_{11}O_6$	9.9	N.i	-
5	2.62	305.0624	305.0661	305, 261, 247, 221, 219, 179, 165, 137, 125	$C_{15}H_{14}O_7$	-12.1	Gallocatechin	(49, 50)
6	2.87	305.0594	305.0603	305, 287, 261, 247, 219, 179, 165, 151, 137, 125	$C_{22}H_9O_2$	-3.0	Epigallocatechin	(49)
7	2.98	441.0973	441.0974	305, 287, 261, 247, 219, 179, 165, 151, 137, 125	$C_{26}H_{17}O_7$	-0.2	Epicatechin 3-O-gallate	(50)
8	3.19	354.0908	354.0814	353.0775, 355.0796	$C_{16}H_{18}O_9$	-11.0	Chlorogenic acid	(51)
9	3.54	289.0612	289.0653	289, 271, 245, 231, 205, 179, 161, 137, 125	$C_{15}H_{14}O_6$	-14.2	Epicatechin	STD
10	3.59	317.0266	317.0297	317, 179, 151, 125	$C_{15}H_{10}O8$	-9.8	Myricetin	STD
11	3.88	449.1377	449.1389	317, 179, 151, 125	$C_{20}H_{18}O_{12}$	-2.7	myricetin-O-pentoside	(52)
12	4.00	463.0861	463.0877	463, 359, 337, 317, 316, 169	$C_{21}H_{20}O_{12}$	-3.5	Myricetin 3-O-α-rhamnopyranoside	(49)
13	4.01	464.0971	464.3800	301.38	$C_{21}H_{20}O_{12}$	-3.0	quercetin-3-O-galactoside (hyperoside)	(52)
14	4.04	615.2481	615.2500	615, 463, 317, 297, 179	$C_{25}H_{43}O_{17}$	-3.1	Myricetin O-deoxyhexosylgallate	(49)
15	4.35	477.0791	477.0775	301.0288, 477.0811, 448.0838	$C_{17}H_{19}O_{14}$	3.6	N.i	-
16	8.89	397.1302	397.1287	277.2156, 397.1336, 415.1541	$C_{22}H_{21}O_7$	3.8	N.i	-
17	9.96	277.2130	277.2168	253.0910, 277.2142, 311.1672	$C_{18}H_{29}O_2$	-13.7	N.i	-

*N.i - No identified; STD - corresponde a Standa

ported in the composition of extracts from various *Manilkara zapota* parts in several clinical studies using this plant, associating these compounds to the extract's activities (38, 39, 40, 41).

In a review study, the main phytochemical components present in the elaborated extracts were phenolic compounds, represented by gallic acid, catechin, quercetin, kaempferol, chlorogenic acid, p-hydroxybenzoic acid, ellagic acid, ferrulic acid, trans-cinnamic acid (42). Of these, 3 were present in one or more of a chemical form in HEMzL.

Among the compounds indicated in table 1, Epigallocatechin (43), Epicatechin (44, 45), Epicatechin 3-O-gallate (46) and Myricetin (47, 48), presented important antioxidant activities that have been previously reported in the literature.

Regarding the antibacterial effects, Epigallocatechin presented antibacterial effects against bacteria representative of the oral microbiota, including being used for the development of silver nanoparticles (53, 54).

Epicatechin showed antibacterial activity *in vitro* against strains that promote infections in the oral mucosa and dental biofilms, in disk diffusion tests against *Enterococcus faecalis* and *Streptococcus mutans* (55). It also showed antibacterial activity against *Helicobacter pilory* strains in broth and diffusion disc microdilution tests, with probable protection over the gastric mucosa (56).

Furthermore, myricetin derivatives showed reasonable antibacterial activity against pathogenic plant strains (57, 58), thus corroborating the statement that compounds derived from plants can serve as efficient substances for the production of substances capable of acting against bacterial resistance.

Direct antimicrobial activity

The Minimum Inhibitory Concentration (MIC) of HEMzL for each strain tested is indicated in the table below (Table 2).

The minimum inhibitory concentration (MIC) was ≥ 1024 µg/mL for almost all strains, with the exception of the standard *Staphylococcus aureus* strain that presented a MIC = 512 µg/mL. Studies that sought to establish a dose limit for *in vitro* antibacterial tests state that concentrations greater than 1000 µg/mL are of no importance in clinical practice, as the substance concentration needed in human plasma for an *in vivo* effect would be impractical (59). As for the inhibitory concentration presented against the *S. aureus* strain, although clinically relevant, its activity is not promising since extracts with important effects have MICs below 100 µg/mL (60).

In a review article, the antibacterial activity of myricetin isolated on several Gram-positive and Gram-negative strains was reported, varying its mechanism of action, from interactions with the bacterial membrane to acting on DNA (61). Epigallocatechin, another component of HEMzL, showed antibacterial activity against methicillin-resistant strains of *S. aureus*, as well as the ability to inhibit biofilm formation by these bacteria (62). Notably, the maintenance of the effects on the Gram-Positive strain of this test or the loss of the ability to act on Gram-negative bacteria results from numerous interactions of the substances present in the extract.

This HEMzL effect over the standard *S. aureus* strain shows a trend in plant extracts, previously reported in the literature, to have a greater antibacterial effect against Gram-positive bacteria, especially *S. aureus* (63,64,65).

Extracts from *Manilkara zapota* leaves, rich in tannins, extracted using various solvents, demonstrated differential antibacterial activities according to the solvent used, with the best action being obtained by the methanolic extract, acting against all tested bacteria (Gram-positive and Gram-negative), and the worst by the aqueous extract, which was inert (66).

A study using *Bauhinia kockiana* extracts, rich in flavonoids and tannins, demonstrated antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In this study, the extract's mode of action was also verified, notably the stability of the plasma membrane until its rupture. An interesting observation was that the presence of flavonoids in the extract increased the MIC compared to the extract that contained only tannins, demonstrating a loss of effectiveness (67).

Table 2. Minimum Inhibitory Concentration Values (µg/mL).

Substances	Tested Bacteria					
	S.A.	E.C.	P.A.	S.A.	E.C.	P.A.
	25923	25922	9027	10	06	03
HEMzL	512	≥1024	≥1024	≥1024	≥1024	≥1024

S.A., *Staphylococcus aureus;* P.A., *Pseudomonas aeruginosa;* E.C., *Escherichia coli;*

Table 3. Table for calculating the values used in the modulatory activity of HEMzL in *Staphylococcus aureus*.

Subs	value 1	value 2	value 3	Log 1	Log 2	Log 3	Mean	Dv	Dv Log
E+Ce	1024	1024	1024	3,01030	3,01030	3,01030	1024	0	0
Ce	1024	1024	1024	3,01030	3,01030	3,01030	1024	0	0
E+Ci	512	512	512	2,70927	2,70927	2,70927	512	0	0
Ci	64	64	64	1,80618	1,80618	1,80618	64	0	0
E+Le	16	8	8	1,20412	0,90309	0,90309	10,07937	4,61880	0,17379
Le	2	4	4	0,30103	0,60206	0,60206	3,174802	1,15470	0,17379

Subs = Substance; Value = value of each test triplicate; Log = Logarithm of the value of each test triplicate; Mean = Geometric mean; Dv = Deviation from the values of natural numbers; Dv Log = Deviation of logarithm values from natural numbers; E = Hydroethanolic extract of *Manilkara zapota* leaves; Ce = Cephalexin; Ci = Ciprofloxacin; Le = Levofloxacin.

Superimposing the results from the studies above with those presented in this study, we can infer that the mixture between flavonoids and tannins in the HEMzL composition may have hindered its antibacterial effect. Moreover, the antioxidant activity of the flavonoids that were present in the HEMzL composition may impose an obstacle to antibacterial activity, since several studies point to a pro-oxidant effect of antibiotic products as being responsible for their action (68, 69, 70).

Calculation of the values used for the modulatory activity

In Table 3, the values found in the modulatory activity test against the *Staphylococcus aures* 10 strain are shown in order to clarify the statistical analysis, with respect to the standard deviation and mean.

The triplicate values calculated based on the broth microdilution method in 96-well plates always fall halfway between one MIC analysis point and another, creating a proportion problem between these points, with different MIC points not being analyzed, as shown in figure 2. The range of unanalyzed points is elevated at higher MIC concentrations and decreases as MIC values become smaller, with the calculated mean and standard deviation encompassing this distortion in the analysis.

For data parameterization and to avoid distortions, the means and deviations are calculated using their logarithmic values, making the intervals between one analysis point and another equal. The exponentiation was thus performed with base 10 (Value = $10^{\text{mean Log}}$). Taking the E+Le value as an example, we have: $10^{[(\text{Log16} + \text{Log8} + \text{Log8})/3]}$ as the mean of the triplicates. This value corresponds to the geometric mean of the MIC's natural values.

When calculating the standard deviation using the logarithms from the natural numbers and then reversing them through exponentiation, first a flattening of this standard deviation occurs. Secondly, higher MIC points will show the same standard deviations from lower MIC points (see "Dv Log" in table 1 for levofloxacin data), which can lead to false positives at higher concentrations and false negatives at lower concentrations. Using the same example for the antibiotic Levofloxacin, its standard deviation would be $10^{0.17379}$, that is, 1.49, for both the control and for its association with the extract, despite the variation in association occurring at higher MIC points.

We propose in this article a method to correct these two presented problems. Standard deviations were calculated by multiplying the standard deviations as a function of the natural numbers over the standard deviation as a function of the logarithms. This corrective factor is applied in ecology areas by the Shannon-Wiener index to adjust the heterogeneity of two areas in order to compare them (71,72,73). Thus, the standard deviation used (SD) = Deviation X Dv Log.

Thus, in the E+Le example, we have: SD = 4.61880 x 0.17379.: SD = 0.8027. For the Levofloxacin control, we have: SD = 1.155470 x 0.17379.: SD = 0.2006.

Through this correction, a standard deviation representative of the unanalyzed MIC point intervals was obtained by the method, elongating the deviations at higher concentrations, thus excluding false positives, and distinguishing the interval deviations on the plate, differentiating them at lower concentrations, thus avoiding false negatives.

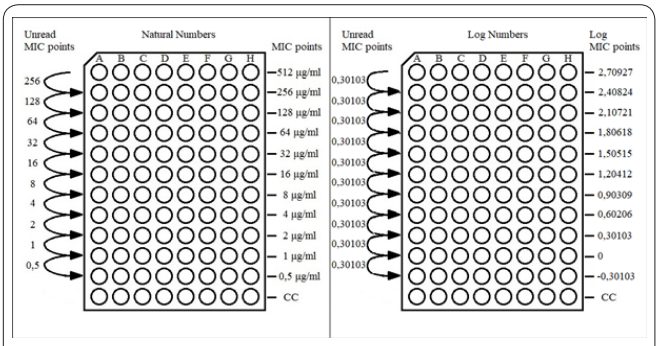

Figure 2. Number of unread points at each CIM interval.

Antibiotic activity modulatory action

In the bacterial resistance reversal tests, two antibiotics with intracellular action, Ciprofloxacin and Levofloxacin, and one with action on the bacterial wall, Cephalexin, were used. Chlorpromazine (CPMZ) was used as a standard efflux pump inhibitor in an attempt to establish a potential mechanism of action.

Efflux pumps (EP) are transmembrane proteins capable of extruding various compounds with biocidal action to the cell (74). In bacteria, EPs act in various mechanisms associated with pathogenicity, as well as in resistance to antibiotics (75).

Efflux pump inhibition by the standard inhibitor CPMZ can be seen in figure 3. A reduction in the ciprofloxacin antibiotic MIC was observed against the three tested strains when this was associated with CPMZ. This demonstrates an active efflux pump mechanism in the resistance of these strains against the antibiotic ciprofloxacin. As for the antibiotic Levofloxacin, the presence of an EP mechanism was not observed in any of the tested strains.

The presence of EPs against ciprofloxacin has previously been reported in studies with *Escherichia coli* (76), *Pseudomonas aeruginosa* (77, 78) and *S. aureus* (79), corroborating with the present study.

As for the HEMzL action, this was inefficient in terms of its ability to reverse bacterial resistance. The extract antagonized the effect of the antibiotic in five of the six scenarios shown in the graph, increasing by 3 to 128 times the amount of antibiotic needed for a biocidal effect. As for the antibiotic Cephalexin, a total lack of interaction, be it synergistic or antagonistic, was noted for all tested strains.

The *Murraya paniculata* leaf extract, whose composition is rich in flavonoid compounds, obtained a similar effect to that found in the present article with respect to a direct antibacterial activity, with an action against a standard *S. aureus* strain being observed. However, in terms of a modulatory activity, the *M. paniculata* extract presented synergism for both groups of bacteria, Gram-positive and Gram-negative, for 3 of the 4 antibiotics tested (80). Qualitative and quantitative differences in the compounds present in the extracts, as well as the class of antibiotics used, may explain the divergent results.

The *Tarenaya spinosa* aqueous and ethanolic extracts, also rich in flavonoids, presented the same effects found in the present study for the *S. aureus* and *P. ae-*

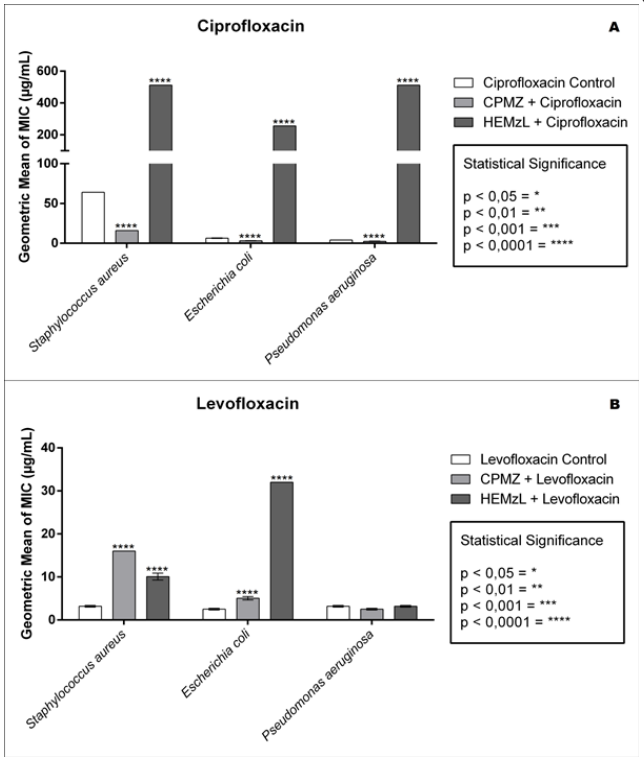

Figure 3. Modifying effect of the antibiotic action of HEMzL on Gram-positive and Gram-negative strains. Combined action of the extract with the antibiotic Ciprofloxacin (A); Combined action of the extract with the antibiotic Levofloxacin (B); CPMZ = Chlorpromazine; HEMzL = Hydroethanolic Extract of *Manilkara zapota* Leaves; * = Represents the statistical significance of a bar when compared to antibiotic control.

ruginosa strains, both with the Beta-lactam and fluoroquinolone tested. The only difference with the present results was a synergism between the *T. spinosa* extract against *E. coli*, when using Beta-lactam (81).

A similar effect was also found when the *Ximenia americana* leaf extract was associated with beta-lactams and fluoroquinolones, presenting several antagonisms against *S. aureus*, *P. aeruginosa* and *E. coli* strains, with the exception of *S. aureus* in association with the antibiotic norfloxacin (82).

Myricetin and epicatechin 3-gallate present synergisms in the literature with antibiotics against strains of *S. aureus* and *P. aeruginosa*, respectively. Myricetin was able to reduce the minimum inhibitory concentration of the antibiotic oxacillin in strains resistant to methicillin (83), while epicatechin 3-gallate reduced the MIC of the antibiotics Rifampicin and Erythromycin, making the bacteria susceptible to these antibiotics again (84).

The effective action of an antibiotic on a bacterial strain derives, in addition to the specific effect of each antibiotic (action on the primary target), from a pro-oxidative effect on the bacteria. Antibiotics, promote the production of Reactive Oxygen Species (ROS), damage the bacterial cell and cause an effect secondary to the main effect of the antibiotic (85). This pro-oxidative action has already been reported in the literature as an important route and one of the mechanisms by which several substances exert bactericidal or bacteriostatic effects (86, 87), losing or decreasing its antibacterial activity when adding antioxidant substances to the *in vitro* culture medium (88).

The antioxidant activity of plant extracts rich in phenolic compounds is commonly described in the literature, with a direct correlation between the amount of phenolic compounds and the antioxidant activity (89, 90, 91). Thus, the antagonisms of antibiotics in the presence of HEMzL may be related to its rich composition in phenolic compounds.

Given the above, we can infer that associations between different compounds with antioxidant properties in the composition of extracts can interfere with the pro-oxidant activity of an antibiotic, causing antagonisms or a lack of a synergistic interaction.

The new data analysis methodology proposed in this article achieved its goals of correcting false positives and false negatives, being representative for the proposed test.

Extracts derived from plants, whose composition is rich in flavonoids may produce undesirable effects when in association with clinically used antibiotics, interfering with their action. Since these plants are used as teas and other products by the population, their use in conjunction with medications should be cautious, so as not to disturb the effects of the drugs.

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