Ana Cristina A. da Silva, Edinardo F. F. Matias, Janaína E. Rocha,
Ana Carolina Justino de Araújo, Thiago S. de Freitas, Fábia F. Campina, Maria do S. Costa,
Luiz E. Silva, Wanderlei do Amaral, Beatriz Helena L. N. S. Maia, Aurea P. Ferriani,
Camila F. Bezerra, Marcello Iriti* and Henrique D. M. Coutinho*

Gas chromatography coupled to mass spectrometry (GC-MS) characterization and evaluation of antibacterial bioactivities of the essential oils from *Piper arboreum* Aubl., *Piper aduncum* L. e *Piper gaudichaudianum* Kunth

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Abstract: The objective of this study was to determine the chemical profile and to evaluate the antibacterial activity of the essential oils of *Piper* species and modulation of the antibiotic activity, using the microdilution method to determine the minimum inhibitory concentration. The chemical components were characterized by gas chromatography coupled to mass spectrometry, which revealed β-copaen-4-α-ol (31.38%), spathulenol (25.92%), and germacrene B (21.53%) as major constituents of the essential oils of *Piper arboreum*, *Piper aduncum*, *and Piper gaudichaudianum*, respectively. The essential oils analyzed in this study did not present a clinically relevant activity against standard and multiresistant *Escherichia coli*. However, in the case of multiresistant *Staphylococcus aureus*, there was a significant activity, corroborating with reports in the literature, where Gram-positive bacteria are more susceptible to antimicrobial activity. The essential oils modulated the effect of the antibiotics norfloxacin and gentamicin, having on the latter greater modulating effect; however, for erythromycin, no statistically significant effect was observed. In conclusion, the results obtained in this study demonstrated that the essential oils of the analyzed *Piper* species present an inhibitory effect against *S. aureus* and modulate antibiotic activity, most of which presents synergistic activity.

Keywords: antibacterial activity; essential oil; modulation; Piperaceae.

***Corresponding authors: Marcello Iriti**, Departement of Agricultural and Environmental Sciences, Milan State University, via G. Celoria 20133, Milan, Italy. E-mail: marcello.iriti@unimi.it, and **Henrique D. M. Coutinho**, Laboratório de Microbiologia e Biologia Molecular—LMBM, Universidade Regional do Cariri—URCA, Av. Cel. Antônio Luiz, 1161, Pimenta, CEP 63105–000, Crato, CE, Brazil. E-mail address: hdmcoutinho@urca.br
Ana Cristina A. da Silva, Edinardo F. F. Matias, Janaína E. Rocha, Ana Carolina Justino de Araújo, Thiago S. de Freitas, Fábia F. Campina and Maria do S. Costa: Regional University of Cariri (URCA), Crato, CE, Brazil, E-mail: ana.cristina.erivanda@gmail.com (A.C.A. da Silva), effm_biologia@hotmail.com (E.F.F. Matias), janainaesmeraldo@gmail.com (J.E. Rocha), thiagocrato@hotmail.com (T.S. de Freitas), fabiacampina@gmail.com (F.F. Campina), corrinha_live@yahoo.com.br (M.S. Costa)
Luiz E. Silva, Wanderlei do Amaral, Beatriz Helena L. N. S. Maia and Aurea P. Ferriani: Federal University of Paraná (UFPR), Curitiba, PR, Brazil, E-mail: luiz_everson@yahoo.de (L.E. Silva), wdoamaral@hotmail.com (W. Amaral), bhsalesmaia@gmail.com (B.H.L.N.S. Maia), aurea.portes@hotmail.com (A.P. Ferriani)
Camila F. Bezerra: Federal University of Pernambuco (UFPE), Recife, PE, Brazil, E-mail: camilawasidi@gmail.com

Introduction

Many medicinal plants have a large amount of bioactive compounds, such as phenolic compounds, terpenoids, nitrogen compounds, vitamins, and several other secondary metabolites, and since the beginning of mankind, they have been used for therapeutic purposes [1–3]. Studies show that several phytochemicals present in medicinal plants have anti-inflammatory, antitumoral, antibacterial, or viral action [4].

The Piperaceae family encompasses 12 genera with about 1100 species. The genus *Piper* widely distributed in subtropical regions is known for its aromatic herbs [5]. Many species of this genus produce essential oils which contain monoterpenes (germacrene A, α-pinene), sesquiterpenes (germacrene B, germacrene D, α-humulene, β-copaen-4-α-ol) , phenylpropanoids (humulene epoxide

II, muurola-4,10(14)-dien-1-β-ol), aldehydes (cinnamaldehyde), ketones, and long chain alcohols [6].

The species *Piper arboreum* Aubl., popularly known as "pau-de-angola", "jaborandi", chilli pepper, has antifungal, trypanocidal, antibacterial, and antioxidant activities [7,8]. *Piper aduncum* L., popularly known as "jaborandi do mato", monkey pepper, "jaboti" [9] herb possesses antifungal [10], antiprotozoal [11], and insecticide activities [12]. *Piper gaudichaudianum* Kunth is popularly known as "pariparoba" or "jaborandi" [13], its leaves are used in folk medicine to relieve toothache. Other studies report biological activities such as fungicide [14], insecticidal, anti-inflammatory, larvicidal, and analgesic effects [15,16].

Bacterial resistance is considered one of the most important public health problems [17]. It is characterized by mechanisms by which bacteria decrease the action of antibiotic agents and may present in a natural or acquired form [18].

Due to the great difficulty found in the treatment of infections caused by multiresistant bacteria, the need for new antimicrobial substances that are effective in the fight against microorganisms is well known. Based on this premise, this work aims to evaluate the antimicrobial and modulating effects of the essential oils of *P. arboreum*, *P. aduncum* and *P. gaudichaudianum*.

Material and methods

Collection and identification of plant material

The fresh leaves of the plants were collected (summer of 2017) in the Biological Reserve of Bom Jesus Biological Reserve, at Vale do Ribeira, Guaraqueçaba—PR, Brazil. A *voucher specimen* of each plant was deposited in the Botanical Museum of Curitiba and in the Herbarium of the Faculdades Integradas Espírita with numbers of 396412 (*P. arboreum*), 396411 (*P. aduncum*), and 396403 (*P. gaudichaudianum*). This research was registered with an SISGEN N. A216E5A and an SISBIO N. 49770-1.

Extraction and chemical characterization of the essential oils

The extraction of the essential oils was performed by the hydrodistillation method using the Clevenger-type apparatus. Fresh leaves of each plant (50 g) were crushed and placed in a glass flask with 1.0 L of distilled water, remaining in the boil for 4.5 h for extraction [19]. The leaves were dried with an electric dryer (FANEM—Mod. 320 SE) with air circulation at 40°C for 24 h. To determine the essential oil content in dry basis, the total mass of each essential oil obtained was considered in relation to the amount of the dry mass of the botanical material used in the extraction. After extraction, the samples were collected with a precision pipette and conditioned in a freezer until the analysis. Each oil was named as follow: essential oil of *P. arboreum* Aubl. (EOPar), essential oil of *P. aduncum* L. (EOPad), and essential oil of *P. gaudichaudianum* Kunth (EOPg).

The chemical constituents of the essential oils were identified by gas chromatography coupled to mass spectrometry (GC-MS). The essential oils were diluted to a concentration of 1% in dichloromethane and 1.0 µL of the solution was injected with a 1:20 flow split into a chromatograph (Agilent 6890—Palo Alto, CA) coupled to a mass selective detector (Agilent 5973N—Palo Alto, CA). The injector was maintained at 250°C, and the constituents were isolated in an HP-5MS capillary column (5%-phenyl–95%-dimethylpolysiloxane, 30 m × 0.25 mm × 0.25 µm) using helium as carrier gas (1.0 mL min^{-1}). The oven temperature was programmed from 60 to 240°C at a rate of 3°C min^{-1}. The mass detector was operated in electronic ionization mode (70 eV), at a rate of 3.15 s^{-1} sweeps, and a mass band of 40 to 450 u. The transfer line was maintained at 260°C, with ion source at 230°C and analyzer (quadrupole) at 150°C.

For quantification, the diluted samples were injected into a chromatograph (Agilent 7890A—Palo Alto, CA) equipped with a flame ionization detector (FID), operated at 280°C. The same column and analytical conditions described above were employed, except for the carrier gas, which was hydrogen, at a flow rate of 1.5 mL min^{-1}. The percentage composition was obtained by the electronic integration of the FID signal by dividing the area of each component by the total area (area%).

The identification of the chemical constituents was performed by comparing their mass spectra with those of spectral libraries [20,21] and by their linear retention indexes, calculated from the injection of a homologous series of hydrocarbons (C_7–C_{26}) and compared with data from the literature [22].

Antibiotics, culture media, and microorganisms

The liquid antibiotics gentamicin and amikacin were obtained from LaborClin, Brazil. Heart Infusion Agar (HIA) and Brain Heart Infusion (BHI) culture media were acquired from HIMEDIA. The microorganisms used in the tests were provided by the Laboratory of Microbiology and Molecular Biology of the Regional University of Cariri. The following standard and resistant bacterial strains were used throughout this study: *Escherichia coli* ATCC 25922, *E. coli* 06, *Staphylococcus aureus* ATCC 25923, and *S. aureus* 10.

Preparation of test solutions

The test solutions were prepared using 10 mg of each oil diluted in 0.5 mL of dimethyl sulfoxide. Each solution was diluted to a final concentration of 1024 µg/mL. The solutions of the oils at this concentration were used in the antibacterial and modulation tests. The antibiotics used in the tests were also prepared at an initial concentration of 1024 µg/mL.

Determination of the minimum inhibitory concentration (MIC) by direct contact

Bacterial samples were seeded in Petri dishes containing HIA and placed in an oven at 37°C for 24 h to grow. After this period, the

samples were collected and diluted in test tubes in triplicate. Then, the turbidity of the solution was determined according to the McFarland scale.

To evaluate the antibacterial activity, 100 µL of the inoculum solution was added to each well of the microdilution plate. Then, the treatments were performed using 100 µL of each oil per column at final concentrations ranging from 512 to 0.5 µg/mL. Of note, all treatments were performed in triplicate. The plates were taken to an oven at 37°C for 24 h. Then, each well was added with 20 µL of resazurin (a colorimetric indicator) and 1 h later the MIC was determined by ocular observation [23,24].

The antibiotic activity modulation assay was performed using amikacin and gentamicin according to the method described by [25]. Briefly, test tubes were added with 150 µL of the bacterial suspension in a solution containing 10% BHI medium and the essential oils at subinhibitory concentrations (MIC/8). Control tubes were prepared using 150 µL of the bacterial suspension in a solution containing 10% BHI medium. Then, 100 µL of these solutions were transferred to corresponding wells in the plate and 100 µL of the antibiotic were added to the first well and serially diluted. The treatments were performed in triplicate, and the MICs were determined as described above.

Statistical analysis

The results were expressed as mean ± standard deviation and differences were evaluated through analysis of variance followed by Bonferroni posttests using GraphPad Prism 6.0 software. Results with $p < 0.05$ were considered as statistically significant.

Results

Chemical composition

The relative amounts of the individual components were calculated based on the GC peak area (FID response), which those having a percentage above 10% were considered as major components.

The essential oil of *P. arboreum* had a yield of 0.23%. A total of 15 components were identified, representing 83.58% of the composition (Table 1), of which, 5.57% are monoterpenes oxygenated, 44.57% are sesquiterpenes oxygenated, 33.43% are sesquiterpenes nonoxygenated, having as main constituents β-copaen-4-α-ol: 31.38%, muurola-4,10-(14)-diene-1-β-ol: 17.32%.

The volatile oil of *P. aduncum* L. was isolated in 0.35% and presented high concentration of sesquiterpenes nonoxygenated (45.45%) and sesquiterpenes oxygenated (44.52%; Table 1), with spathulenol as the major compound (25.92%). In addition, β-macrocarpene (9.52%), α-humulene (5.33%) aromadendrene (5.61), and β-copaen-4-α-ol (5.45%), were also found in considerable yield.

Distillation of the essential oil of *P. gaudichaudianum* presented a yield of 0.56%. Twenty-two components were identified, representing 87.04% of the composition (Table 1), of these 3.96% are monoterpenes hydrocarbon, 67.26% sesquiterpenes nonoxygenated and 15.82% sesquiterpenes oxygenated, having as main constituent germacrene B: 21.53%.

Determination of minimum inhibitory concentration

In microdilution tests, EOPar showed MICs = 512 µg/mL against standard (ATCC 6538) *S. aureus*, 128 µg/mL against multiresistant *S. aureus*, and ≥ 1024 µg/mL against standard (ATCC 25922) and resistant *E. coli*. The EOPad had MIC = 512 µg/mL against standard *S. aureus*, 16 µg/mL against multi-resistant *S. aureus*, ≥ 1024 µg/mL against standard *E. coli* and 813 µg/mL against resistant *E. coli*. The EOPg had MIC = 813 µg/mL against standard *S. aureus* and ≥ 1024 µg/mL against resistant *S. aureus* and standard and resistant *E. coli* (Table 2).

Modulation of antibiotic activity

In the evaluation of the modulating effect, the combination of the EOPar (Figure 1) with the antibiotics norfloxacin and gentamicin caused decrease of the MIC against *S. aureus* 10, indicating synergism in the association of these treatments, whereas for *E. coli* 06 only synergism was observed in the combination with gentamicin, norfloxacin did not show a significant effect (Figure 2).

The combination of *P. aduncum* essential oil with norfloxacin resulted in increased MIC, gentamicin, and decreased MIC indicating respectively antagonism and synergism against *S. aureus* 10 and *E. coli* 06 (Figures 3 and 4).

The association of the EOPg with the drugs norfloxacin and gentamicin presented a synergistic effect against *S. aureus* 10 causing a decrease in MIC (Figure 5) and an antagonistic effect against *E. coli* 06, provoking an increase in MIC (Figure 6). The antibiotic erythromycin did not present significant results against any of the strains used in this study.

Discussion

Essential oils are substances derived from medicinal herbs that are widely used in the pharmaceutical, food,

Table 1: Essential oil composition of leaves of *Piper arboreum*, *Piper aduncum*, and *Piper gaudichaudianum*.

Compounds	EOPar RI (%)	EOPad RI (%)	EOPg RI (%)
Monoterpenes hydrocarbon			
α-pinene	-	-	935 (2.19)
Monoterpenes oxygenated			
Khusimone	1600 (2.48)	-	-
Sesquiterpenes oxygenated			
(E)-Caryophyllene	1426 (3.61)	1426 (2.46)	1426 (3.75)
δ-cadinene	1531 (1.02)	-	-
α-amorphene	1488 (2.09)	-	-
δ-selinene	1493 (1.03)	(9.52)	1498 (1.65)
4,5-di-epi-Aristolochene	-	-	1470 (1.27)
Allo-aromadendrene	-	(5.61)	1467 (2.47)
Amorpha-4,7(11)-diene	-	-	1477 (2.86)
Aromadendrene	-	1445 (1.44)	
Bicyclogermacrene	-	-	1504 (2.57)
Cubebol	-	1522 (3.28)	
α-calacorene	-	-	1551 (3.5)
β-calacorene	-	-	1572 (1.98)
δ-cadinene	-	1530 (4.07)	1530 (9.39)
α-copaene	-	-	1381 (4.36)
4-epi-cis-dihidroagarofuran	-	-	1508 (2.99)
β-elemene	-	-	1438 (6.1)
γ-elemene	-	-	1397 (5.24)
(E,E)-α-farnesene	-	-	1513 (1.92)
Germacrene B	1561 (2.44)	-	1566 (21.53)
Germacrene D	-	1493 (3.13)	1487 (1.2)
Heptan-2-one-6-methyl-6-(3-methylphenyl)	1645 (1.63)	-	-
α-humulene	-	1460 (5.33)	1460 (3.67)
β-macrocarpene	-	1499 (9.52)	-
α-murolene	-	1506 (1.09)	-
Sesquiterpenes non-oxygenatedrowhead			
β-copaen-4-α ol	1593 (31.38)	1588 (5.45)	1587 (2.06)
Cedrol	-	1604 (2.12)	-
Caryophyllene oxide	-	-	1592 (1.96)
Ledol	1612	(2.66)	-
Cubenol	1637	(2.20)	-
Eudesm-7(11)-em-4-ol	1690 (1.03)	-	-
7-acetoxy-elema-1,3-dien-8-8-ol	1792 (2.06)	-	-
Germacra-4(15),5,10(14)-trien-1-α-ol	1683 (1.5)	(1.05)	-
Intermedeol	1666 (1.75)	-	-
(E)-nerolidol	-	-	1569 (3.26)
Pogostol	-	1650 (3.15)	-
Muurola-4,10(14)-dien-1-β-ol	1640 (17.32)	(1.97)	-
β-himachalene oxide	1619 (6.16)	-	-
Spathulenol	1587 (8.08)	(25.92)	-

RI = retention index; EOPar = *Piper arboreum* Aubl.; EOPad = *Piper aduncum* L.; EOPg = *Piper gaudichaudianum* Kunth.

cosmetics, sanitary, and perfumery industries. The chemical composition of these oils is a mixture of several components in different concentrations, most of which are characterized by the presence of two or three components in high concentrations (20–70%) and other components in a lower concentration. The major compounds determine the biological properties of essential oils [26].

The chemical composition of essential oils differs between plants of the same species. The EOPg had as main constituent germacrene B (21.53%). This result is a disagreement with previous studies. According to Péres et al. [27], the main compounds of *P. gaudichaudianum* essential oil were (E)-nerolidol (22.4%) and α - humulene (16.5%).

Table 2: Minimum inhibitory concentration (MIC) of the essential oils (µg/mL).

Essential oil	Bacteria			
	S. A. ATCC 6538	E. C. ATCC 25922	S. A. 10	E. C. 06
EOPar	512	≥1024	128	≥1024
EOPad	512	≥1024	16	813
EOPg	≥1024	≥1024	813	≥1024

ATCC = standard strain; S. A. = *Staphylococcus aureus*; E. C. = *Escherichia coli*; EOPar = essential oil of *Piper arboretum* Aubl.; EOPad = essential oil of *Piper aduncum* L.; EOPg = essential oil of *Piper gaudichaudianum* Kunth.

The main components found in the EOPad also differ with results obtained in previous studies for this species. Almeida et al. [28] identified dillapiole (76%) as the main component of the EOPad, diverging with the result found in this study; however, it resembles the result found by Schindler and Heinzmann [29] who identified β-copaen-4-α-ol among the major constituents in the EOPg, a species that is part of the same genus of *P. aduncum*.

The component identified in this study in the highest concentration of *P. arboreum* essential oil, β-copaen-4-α-ol (31.38%) diverges with that found by Silva et al. [30], which identified bicyclogermacrene (28.7%) as the major component, in this same study β-copaen-4-α-ol was also identified, being characterized as the second component with the highest concentration.

The chemical composition of the essential oils of the species used in this study differs from the data found in the literature. Chemical variability may result from environmental and/or ecological selection pressure, characterizing a chemical adjustment to prevailing environmental conditions [31].

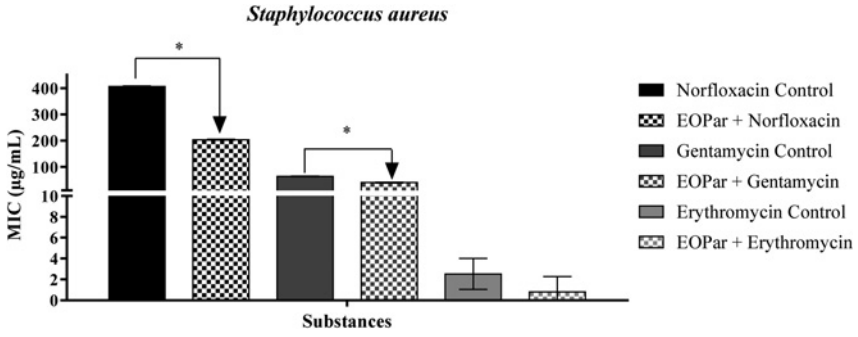

Figure 1: Modulatory effect of essential oil from *Piper arboreum* (EOPar) in the antibiotic activity of norfloxacin, gentamycin, and erytromycin against strains of *Staphylococcus aureus* 10.

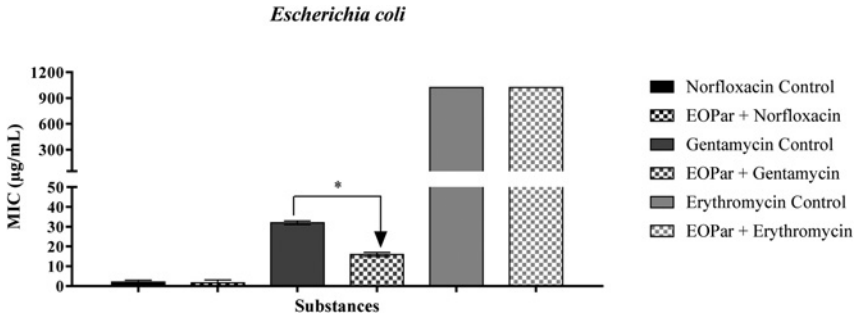

Figure 2: Modulatory effect of essential oil from *Piper arboreum* (EOPar) in the antibiotic activity of norfloxacin, gentamycin, and erytromycin against strains of *Escherichia coli* 06.

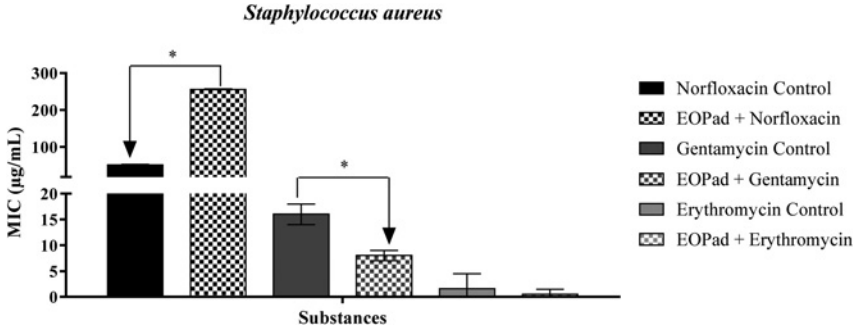

Figure 3: Modulatory effect of essential oil from *Piper aduncum* (EOPad) in the antibiotic activity of norfloxacin, gentamycin, and erytromycin against strains of *Staphylococcus aureus* 10.

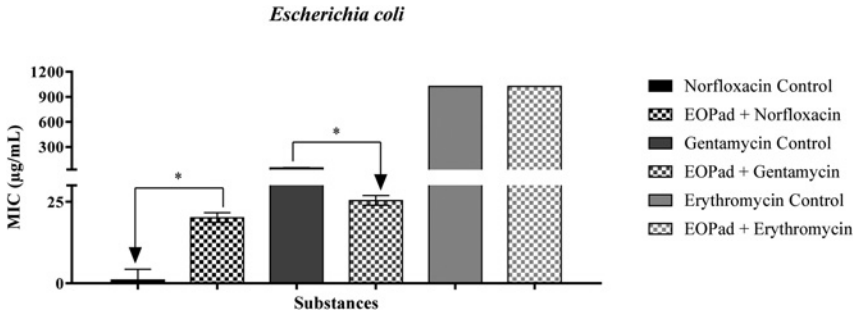

Figure 4: Modulatory effect of essential oil from *Piper aduncum* (EOPad) in the antibiotic activity of norfloxacin, gentamycin, and erytromycin against strains of *Escherichia coli* 06.

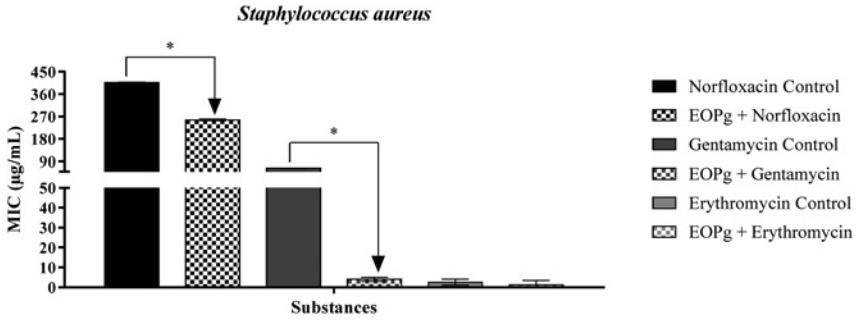

Figure 5: Modulatory effect of essential oil from *Piper gaudichaudianum* (EOPg) in the antibiotic activity of norfloxacin, gentamycin, and erytromycin against strains of *Staphylococcus aureus* 10.

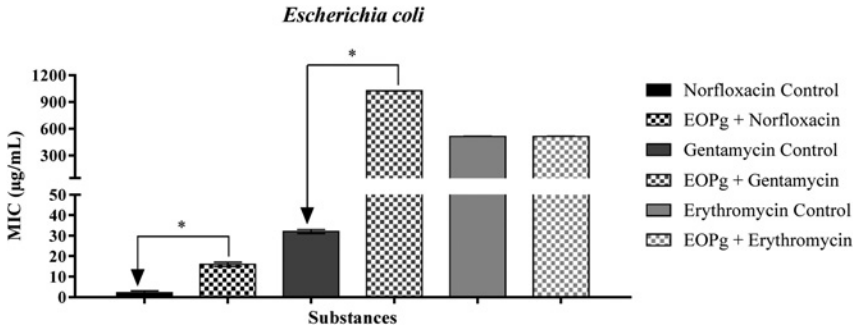

Figure 6: Modulatory effect of essential oil from *Piper gaudichaudianum* (EOPg) in the antibiotic activity of norfloxacin, gentamycin, and erytromycin against strains of *Escherichia coli* 06.

The essential oils showed low activity against *E. coli*, this result can be justified by the presence of an outer membrane in this microorganism, which makes difficult the passage of the components present in the essential oils, as well as of other antimicrobials, thus hampering their action [32,33].

Clinically relevant activity of essential oils against multidrug-resistant *S. aureus* has been observed, corroborating with reports found in the literature that indicate that Gram-positive bacteria are more susceptible to essential oils than Gram-negative bacteria [34]. This result diverges with previous study carried out with essential oils of *Piper* species, where they did not find significant activity against *S. aureus* [35].

In the combination of the compounds with antibiotics, it was possible to observe the synergism of the essential oil of *P. arboreum* against *S. aureus* and *E. coli* combined with gentamicin and norfoxacin against *S. aureus*. The efflux pumps present on the plasma membrane of *E. coli* [36] may be related to the indifferent result found in combination with norfloxacin. Synergism is the combination of drugs or natural compounds, which have the ability to act in several sites of the microbial cell, potentializing the agonist's action in the test [25].

The antagonism observed in the combination of the EOPad with norfloxacin (against *S. aureus* and *E. coli*) and EOPg (against *E. coli*) can be explained by binding of the components at binding sites for the antibiotic, or chelation of the drug causing a decrease in its action spectrum [37], mechanisms may also explain the antagonism between EOPg and gentamicin against *E. coli*. Synergism observed with gentamicin can be explained by the efficacy of aminoglycosides against Gram-negative and its ability to act in conjunction with other drugs or natural products against Gram-positive [38].

The composition of the cell wall of Gram-positive microorganisms is thicker, with more peptides, making them more susceptible to antibiotics than Gram-negative microorganisms [39]. The synergism between the EOPg and the antibiotics norfloxacin and gentamicin against the Gram-positive bacteria *S. aureus* may be due to changes in the permeability of the wall and cell membrane of the microorganism due the alteration in the lipid bilayer, which may facilitate the passage of drugs acting inside the cell, such as the aminoglycosides and norfloxacin [40].

The observed indifference in EOPar, EOPad, and EOPg modulations with erythromycin can be attributed to the resistance mechanisms that these strains have developed for this antibiotic, such as the efflux pumps present in *E. coli* [41].

Conclusion

The results obtained in this study showed that the essential oils of *P. arboreum*, *P. aduncum*, and *P. gaudichaudianum* have an antibacterial effect against *S. aureus* and interfere with the action of antibiotics. These findings become important in the search for new effective therapies for infections triggered by multiresistant bacteria.

Subtitles and abbreviations lists

ATCC	American Type Culture Collection;
E. C.	*Escherichia coli*;
EOPad	Essential Oil of *Piper aduncum*L.;
EOPar	Essential Oil of *Piper arboretum*Aubl.;
EOPg	Essential Oil of *Piper gaudichaudianum* Kunth.;
MIC	Minimum Inhibitory Concentration;
RI	Retetion Index;
S. A.	*Staphylococcus aureus;*

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