Archives of Microbiology (2021) 203:4727–4736
https://doi.org/10.1007/s00203-021-02393-5

SHORT COMMUNICATION

Chemical composition and potentiating action of Norfloxacin mediated by the essential oil of *Piper caldense* C.D.C. against *Staphylococcus aureus* strains overexpressing efflux pump genes

Antonio Linkoln Alves Borges Leal[1,2] · Camila Fonseca Bezerra[3] · Camila Confortin[10] · Luiz Everson da Silva[10] · Emanuelle Machado Marinho[5] · Márcia Machado Marinho[6] · Mayron Alves Vasconcelos[7] · Terezinha Gonçalves da Silva[8] · Emmanuel Silva Marinho[9] · Alexandre Magno Rodrigues Teixeira[1] · Henrique Douglas Melo Coutinho[1] · Humberto Medeiros Barreto[2] · Hélcio Silva dos Santos[1,4]

Received: 7 November 2020 / Revised: 14 May 2021 / Accepted: 20 May 2021 / Published online: 29 May 2021

Abstract

Infectious diseases caused by multidrug-resistant microorganisms has increased in the last years. *Piper* species have been reported as a natural source of phytochemicals that can help in combating fungal and bacterial infections. This study had as objectives characterize the chemical composition of the essential oil from *Piper caldense* (EOPC), evaluate its potential antimicrobial activity, and investigate the synergistic effect with Norfloxacin against multidrug-resistant *S. aureus* overproducing efflux pumps, as well as, verify the EOPC ability to inhibit the *Candida albicans* filamentation. EOPC was extracted by hydrodistillation, and the chemical constituents were identified by gas chromatography, allowing the identification of 24 compounds (91.9%) classified as hydrocarbon sesquiterpenes (49.6%) and oxygenated sesquiterpenes (39.5%). Antimicrobial tests were performed using a 96-well plate microdilution method against *C. albicans* ATCC 10231, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 standard strains, as well as against multidrug-resistant strains *S. aureus* SA1199B (overexpressing *norA* gene), *S. aureus* K2068 (overexpressing *mepA* gene) and *S. aureus* K4100 (overexpressing *qacC* gene). The oil showed activity against *C. albicans* ATCC 10231 ($\geq$ 512 µg/mL) and was able to inhibit hyphae formation, an important mechanism of virulence of *C. albicans*. On the other hand, EOPC was inactive against all bacterial strains tested ($\leq$ 1,024 µg mL). However, when combined with Norfloxacin at subinhibitory concentration EOPC reduced the Norfloxacin and Ethidium bromide MIC values against *S. aureus* strains SA1199B, K2068 and K4100. These results indicate that EOPC is a source of phytochemicals acting as NorA, MepA and QacC inhibitors.

Keywords Essential oil · *Piper caldense* · Antibacterial · Efflux pump inhibitors · Antifungal · Fungal dimorphism

Communicated by Erko Stackebrandt.

✉ Hélcio Silva dos Santos
helciodossantos@gmail.com

[1] Department of Biological Chemistry, Regional University of Cariri, Crato, CE, Brazil

[2] Department of Microbiology and Parasitolog, Federal University of Piaui, Teresina, PI, Brazil

[3] Department of Pharmaceutical Sciences, Federal University of Pernambuco, Recife, PE, Brazil

[4] Center for Exact Sciences and Technology, Vale do Acarau State University, Sobral, CE, Brazil

[5] Department of Analytical Chemistry and Physical Chemistry, Federal University of Ceará, Fortaleza, CE, Brazil

[6] Faculty of Education, Science and Letters of Iguatu, State University of Ceará, Iguatu, CE, Brazil

[7] Minas Gerais State University, Divinópolis Unit, MG, Brazil

[8] Department of Antibiotics, Drug Planning and Synthesis Laboratory-LPSF, Federal University of Pernambuco, Cidade Universitária, Recife, PE, Brazil

[9] Group of Theoretical Chemistry and Electrochemistry, State University of Ceará, Campus FAFIDAM, Limoeiro do Norte, Fortaleza, CE, Brazil

[10] Postgraduate Programme in Sustainable Territorial, Federal University of Paraná, Curitiba, PR, Brazil

Introduction

Infectious diseases caused by multidrug-resistant microorganisms has become a global public health concern (Guschin et al. 2015). Multidrug-resistant bacteria have been isolated with high frequencies from community or hospital-acquired infections (Klein et al. 2020; Xiang et al. 2020), as well as, from infections of veterinary importance (Lima et al. 2012). On the other hand, infections caused by resistant fungi are more and more prevalent in hospitals, being known as a sleeping giant, for never having completely solved the problem of ineffective antifungals (Veríssimo et al. 2016). In fact, antimicrobial resistance it was predicted to reach pandemic proportions by 2050, accounting for 10 million deaths annually (Sugden et al. 2016).

Antibiotic resistance can acquired by a mutation that generate a change in a target protein with lower for affinity for antibiotic or through acquisition of resistance placed in plasmids, transposons or phage DNA that often involves inactivation, antibiotic degradation or extrusion by active efflux pumps (Santos et al. 2007). Efflux pumps are transmembrane proteins that become both bacteria and fungi resistant, because they are able to extrude antibiotics and biocide reducing their intracellular concentrations using ATP or proton gradient as energy source (Du et al. 2018).

Traditionally used antibiotics have been losing their effectiveness because of increased microbial resistance (Mayers et al. 2009). Epidemiological studies warn of the importance of further research for discovery of new drugs or compounds with therapeutic properties (Balouiri et al. 2016). Another important point, to combat infections caused by resistant microorganisms, is the discover of compound acting as efflux pump inhibitors to provide new ways to reverse bacterial and fungal resistance (Rezende-Júnior et al. 2020). In this way, alternative forms such as in vitro methods in antimicrobial tests to assess the potential of extracts, essential oils, and isolated compounds have been intensified, revealing potential antimicrobial agents or even enhancing the activity of antibiotics used in clinical practice (Balouiri et al. 2016; Coutinho et al. 2008, 2010; Matias et al. 2013; Costa et al. 2017; Siebra et al. 2018). The association of natural products as well as the use of these combined with antimicrobials, has been widely discussed in the scientific literature, given the ability to provide antimicrobial efficacy in previously ineffective doses due to the antimicrobial resistance presented by some strains (Guimaraes et al. 2010; Silva et al. 2014; Tulgar et al. 2018).

The inhibition of the production of virulence factors by pathogenic microorganisms is also an approach that can be useful in the treatment of infectious diseases. *Candida* species, produce infections ranging from skin and mucous infections, to deep and generalized infections, they make use of alternative mechanisms to invade the host organism and develop virulence factors, where they colonize significantly, leaving the responses to aggression, weakened (Egue et al. 2018). *C. albicans* is the species responsible for about 50–70% of the cases of invasive candidiasis (Castro et al. 2016). *C. albicans* is a yeast species capable of producing hyphae or pseudo-hyphae able to invade host tissues leading to candidiasis (Fernandes et al. 2020; Sellam and Whiteway 2016). The ability to alter structural morphology from yeast to filamentous cells, is known as one of the main mechanisms of fungal virulence (Hou et al. 2011; Sánchez-Martínez and Pérez-Martín 2001).

Natural products with antimicrobial properties represent an alternative to the use of traditional antimicrobial agents. *Piper caldense* C.D.C. (Piperaceae) is a shrub popularly known as "pimenta d'agua" that has been used in some Brazilian regions as a sedative agent (Cardozo Júnior and Chaves 2003). This study aimed to characterize the chemical composition of the essential oil from the leaves of *Piper caldense* (EOPC), as well as, its antimicrobial activity against bacteria and yeast strains. The ability of the EOPC to inhibit cell dimorphism in *C. albicans* was also assessed. Futhermore, the effect of the EOPC on the activity of Norfloxacin against multidrug-resistant *S. aureus* SA1199B, *S. aureus* K2068 and *S. aureus* K4100 strains was assessed to evaluate its potential as inhibitor of the NorA, MepA or QacC efflux pumps.

Materials and methods

Botanical material

Plant material was collected in the dense lowland rainforest, Antonina, PR 2015/2106, Brazil, under coordinates 25° 17′ 51.9″ S and 48° 40′ 51.4″ W at 14 m elevation, during the spring. Material collection was carried out under license from the Environmental Institute of the Paraná State, number 284/11, and an exsiccata was deposited at the Herbarium of the Municipal Botanical Museum (MBM) in Curitiba, PR, under No. 267636. Fresh leaves of *P. caldense* were used to extract the oil without any indication of contamination by parasites.

Extraction of the essential oil

The oil was prepared by hydrodistillation using a Clevenger graduated device. The proportion of fresh leaves was 100 g/L of distilled water, following the methodology described by Wasicky (1963). An electric dryer model—FANEM 320 SE was used, with air circulation at 40° C for

24 h to dry the leaves. Following to determine the moisture content at the time of extraction, samples of 20 g were collected in triplicates, submitted to drying in an electric dryer model FANEM 320 SE with air circulation at 65 °C until reaching constant weight. After extraction, the samples were collected with a precision pipette and placed in a freezer where they remained until the time of the chromatographic analysis.

Strains and chemicals

Evaluation of the intrinsic antimicrobial activity of the EOPC was performed against standard microbial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231, as well as, against multidrug-resistant strains *S. aureus* SA1199B overexpressing the *norA* gene (Kaatz et al. 1993), *S. aureus* K2068 overexpressing the *mepA* gene (Kaatz et al. 2005) and *S. aureus* K4100 overexpressing the *qacC* gene (Littlejohn et al. 1991). Assays for evaluation of the modulating effect on drug resistance were performed only with drug-resistant *S. aureus* SA1199B, K2068 and K4100 strains. Assays for evaluation of the ability to inhibit the cellular dimorphism were conducted against *C. albicans* ATCC 10231 strain. Bacterial and yeast strains were maintained on Brain Heart Infusion Agar (BHIA, Himedia, India) slants at 4 °C, and prior to assay the cells were grown overnight at 37 °C in Brain Heart Infusion (BHI, Himedia, India). Subsequently, an aliquot of each microbial culture was removed and diluted in 3 mL of 0.9% saline, and the turbidity adjusted to the McFarland 0.5 scale.

Norfloxacin (Nor), Ethidium Bromide (EtBr) and Chlorpromazine (CPZ) were obtained from Sigma Chemical Corp., St. Louis. With the exception of Nor that was dissolved in a mixture of 1 M NaOH and sterile distilled water (1:9 proportion), EtBr and CPZ were dissolved in sterile water.

Determination of the minimum inhibitory concentration (MIC).

Initially, the essential oil was diluted in dimethylsulfoxide (DMSO) at a concentration of 10,000 µg/mL. Subsequently, this solution was diluted in sterile distilled water to a concentration of 1024 µg/mL. The minimum inhibitory concentration of EOPC against bacteria and fungi strains was determined using the 96-well plate microdilution technique. Initially, 100 µL of essential oil was diluted serially in BHI 10% medium in concentrations that ranged from 1024 to 8 µg/mL. Subsequently, 100 µL of the microbial suspension (bacteria or fungi) in BHI 10% was added. For the control of microbial growth, BHI 10% was composed and microbial suspension in the absence of essential oil (Leal et al. 2019a) The plates were incubated at 37 °C for 24 h, and the MIC was identified by adding 20 µL of sodium resazurin to each well and after 1 h visually checking the presence or absence of colorimetric change.

Fungal viability was achieved by adding a sterilized stem to each well that did not show fungal growth and subculturing in an Sabouraud Dextrose Agar (SDA, Himedia, India) petri dish. The plates were incubated at 37 °C for 24 h and, subsequently, the development or suppression of fungal colonies (Morais-Braga et al. 2016) was verified.

Modulating effect of the antibiotic resistance

To evaluate if the EOPC was able to modulate antibiotic resistance in *S. aureus* strains overexpressing specific efflux pump genes, antibiotic MICs were determined in the presence or absence of subinhibitory concentrations of EOPC (1/8 or 1/4 MICs). Antibiotic concentrations ranged from 0.125 to 128 µg/mL. The microtiter plates were incubated at 37 °C for 24 h and readings were performed with resazurin as described above. To verify whether the modulation of drug resistance occurred due to inhibition of the efflux pump, the modulation assay was performed by substituting antibiotics for EtBr, which is a known substrate of efflux pumps (Markham et al. 1999), used herein as inhibition of the pump. Control trials were also performed, replacing each natural product with CPZ, which is a known efflux pump inhibitor (Neyfakh et al. 1993).

Effect of *P. caldense* essential oil on *C. albicans* micromorphology

The tests were performed according to Sidrim and Rocha (2010), with some modifications. In this test, the effect of the EOPC on the suppression of the filament of *C. albicans* was verified. Depleted Potato Dextrose Agar (PDA) was placed inside of Falcon tubes and, then the EOPC was added varying the concentration according to MIC as follow: MIC (512 µg/mL), MIC 1/2 (256 µg/mL) and MIC 1/4 (128 µg/mL). Then, PDA was immediately poured in a slide. Petri dishes with a blade were inoculated by a calibrated loop (1 µg), where two parallel streaks were made in the medium already solidified and then covered by a sterile microscopic sheet. After incubation at 24 h (37 °C), the culture was visualized by optical microscopy. A control without the EOPC was made for the growth of yeasts whose hyphae emission was stimulated by the impoverishment of the medium, once stress caused by starvation leads to hyphae emission.

Statistical analysis

Data were analyzed using the statistical program GraphPad Prism 6.0. Differences between treatment with Norfloxacin and treatment with Norfloxacin combined with EOPC (or Chlorpromazine) were analyzed using a two-way ANOVA test, using the geometric mean of the triplicates as the central data and standard deviation. Then, a post hoc Bonferroni test was performed and p values < 0.05 were considered significant.

Table 1 Relative percentage of essential oil components from fresh samples of native species of *Piper caldense* collected in the lowland rainforest, Antonina, PR—Brazil

Compounds	IR[a]	IR[b]	%
α-Pinene	939	932	0.8
Camphene	952	946	2.1
α-Copaene	1375	1374	2.8
trans-Caryophyllene	1417	1417	2.6
β-Copaene	1427	1430	0.9
Trans-α-bergamotene	1435	1432	2.7
γ-Muurolene	1475	1478	6.0
α-Amorfene	1479	1483	3.3
β-Selinene	1483	1489	3.2
Valencene	1492	1496	6.7
α Muurolene	1498	1500	4.2
δ-Cadinene	1512	1522	13.9
Humulene epoxide II	1605	1608	1.0
α-Calacorene	1541	1544	1.2
β-Calacorene	1564	1564	1.9
Spathulenol	1575	1577	9.1
Caryophyllene oxide	1579	1582	11.9
Rosifoliol	1597	1600	1.3
1,10 di-epi-Cubebol	1611	1618	1.6
1-epi-Cubenol	1625	1627	3.4
epi-α-Muurolol	1639	1640	3.1
α-Muurolol	1644	1644	1.8
α-Cadinol	1652	1652	4.2
Cadalene	1672	1675	2.3
Total identified (%)			91.9

[a]I.R = Calculated Retention Index

[b]I.R = Literature Retention Index

Results and discussion

Chemical composition

The content of the EOPC was 14.74% and 24 constituents (91.9%) were identified (Table 1). Chemical compounds were separated into groups, according to their chemical classification as hydrocarbon sesquiterpenes (49.6%), oxygenated sesquiterpenes (39.5%) and hydrocarbon monoterpenes (2.9%). Oxygenated monoterpenes were not found in the EOPC. Caryophyllene oxide (11.9%) was the major constituent found, followed by δ-Cadinene (9.6%) and Spathulenol (9.1%) (Fig. 1). Chemical composition of the EOPC used in the present study (from Antonina, PR at 14 m elevation) was different to that verified by the EOPC from Piraquara, PR (528 m elevation), with nine different compounds as follow: trans-Caryophyllene, β-Copaene, Trans-α-bergamotene, α-Amorfene, β-Selinene and Valencene (Bezerra et al. 2020). Variations in the chemical composition of the EOPC are probably associated with different edaphomatic and rainfall conditions in the region where the specimen was found (Viana et al. 2019). Seasonality, circadian rhythm, temperature and brightness, as well as the stage of development and nutritional factors, are all likely to influence the chemical composition of essential oils.

Evaluation of the antimicrobial activity

MIC values above 1000 µg/mL for plant extracts have been considered as clinically irrelevant, because it may be impracticable to extrapolate the dose from that giving activity in vitro, to that which would be required in equivalence for the size of a human adult (Houghton et al. 2007). Using this value as a cutoff point, the EOPC did not show antimicrobial activity against bacterial strains tested (Table 2). Similar results were verified for other *Piper* species, such as, *P. rivinoides* (Leal et al. 2019b) and *Piper cernuum* (Leal et al. 2019a) that also did not present activity against both

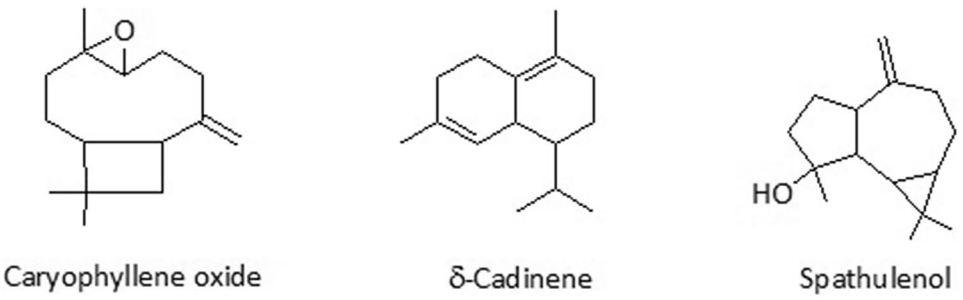

Fig. 1 Majority constituents of essential oil of *Piper caldense*

Table 2 Minimum Inhibitory Concentration (MIC) values obtained for *P. caldense* essential oil (EOPC) against microbial strains tested

Strains	MIC (µg/mL)	Effect
Staphylococcus aureus SA1199B	≥ 1024	Inactive[a]
Staphylococcus aureus K2068	≥ 1024	Inactive
Staphylococcus aureus K4100	≥ 1024	Inactive
Staphylococcus aureus ATCC 25923	≥ 1024	Inactive
Escherichia coli ATCC 25922	≥ 1024	Inactive
Candida albicans ATCC 10231	512	Active

[a]MIC values upper than 1000 µg/mL were considered inactives (Houghton et al. 2007)

S. aureus and *E. coli*. However, a previous studies found a diferente result where the EOPC showed activity against Gram-negative *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, as well as, against Gram-positive species *S. aureus* and *Bacillus subtilis* (Rocha et al. 2016).

EOPC tested in the present showed a weak antifungal activity against *C. albicans* ATCC 10231 (Table 2). This antifungal activity could be related with the presence of some constituents such as Caryophyllene oxide, a compound described as showing antifungal activity against *C. albicans* and dermatophyte fungi (Ghavam et al. 2020; Schmidt et al. 2010; Yang et al. 1999). However, the Caryophyllene oxide content found in the oil tested in the present study was lower (11.9%) than that verified for the EOPC from Piraquara, PR (13.9%), which did not show activity against *C. albicans* INCQ40006 and *C. albicans* URM4387 (Bezerra et al. 2020). Thus, we cannot rule out that other oil constituents acting isolatelly or synergistically could be related with the antifungal activity showed by the EOPC tested in the present study. As an example, Spathulenol, a sesquiterpene reported to be between major volatile components of the essential oils of several aromatic Piperaceae species, has some biological activities, including antimicrobial activity (Dib et al. 2017). Coming from the cyclization of nerolidine pyrophosphate, δ-cadinene presents a cadinane skeleton, which the precursor is the farnesyl pyrophosphate (RuizReyes and Suarez 2015). Among the activities reported in the literature for δ-cadinene can be mentioned the antitumor activity (Wright and Sutherland 2007), antimicrobial activity (Skaltsa et al. 2003). Thus, the antifungal activity observed in our approach could be associated to the levels of the compounds related to the local environments and conditions to which the assayed plant was exposed, acting possibly by a synergistic effect.

However studies with essential oils with species of *Piper cernuum* and *P. caldense* made by Duarte et al. (Duarte et al. 2016) and Constantin et al. (Costantin et al. 2001) present compounds that present several properties, among them antifungal, antibacterial and even capable of potentiating antibiotic effect. However, Costantin et al. (2001) who used the essential oil of *P. cernnum* and the compound Eugenol, demonstrated antifungal activity on *C. albicans*. The oil presented an inhibition halo of 12.2 ± 0.6 mm in diameter, and the Eugenol isolate 28.9 ± 1.3 mm.

Evaluation of the modulating effect of the antibiotic resistance

EOPC potentiated the activity of Norfloxacin against *S. aureus* SA1199B overproducing NorA, as well as, against *S. aureus* K2068 overproducing MepA at subinhibitory concentrations. MIC values for Norfloxacin against SA1199B decreased eightfold in the presence of the oil from 64 to 8 µg/mL (Fig. 2A). For K2068 strain, MIC values for Norfloxacin also were reduced eightfold when the EOPC was added to the growth medium from 16 to 2 µg/mL (Fig. 3A). These results indicate that EOPC contains phytochemicals able to modulate the Norfloxacin resistance, probably by inhibition of the NorA and MepA efflux pumps.

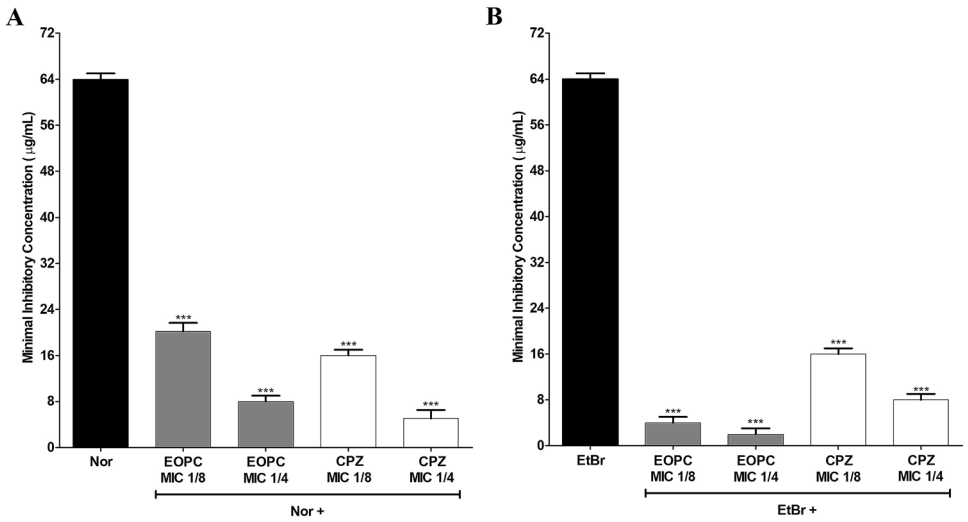

Fig. 2 MIC values of Norfloxacin (Nor) (**A**) and Ethidium Bromide (EtBr) (**B**) against *S. aureus* SA1199B (*norA*) in absence or presence of the essential oil from the leaves of *P. caldense* (EOPC) or Chlorpromazine (CPZ). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$)

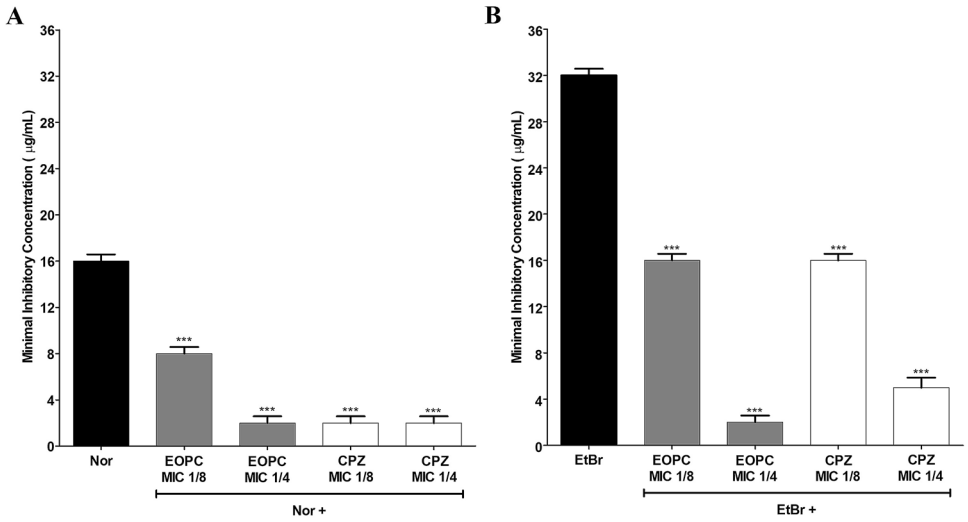

Fig. 3 MIC values of Norfloxacin (Nor) (**A**) and Ethidium Bromide (EtBr) (**B**) against *S. aureus* K2068 (*mepA*) in absence or presence of the essential oil from the leaves of *P. caldense* (EOPC) or Chlorpromazine (CPZ). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$)

To verify if the modulating effect is related with efflux pump inhibition, assays were performed replacing Norfloxacin by EtBr. As the only known mechanism of bacterial resistance to EtBr is mediated by efflux pumps (Markham et al. 1999), this DNA-intercalating dye have been used to evaluate the potential efflux pump inhibitory activity of natural products (Silva et al. 2021a), isolated phytochemicals (Silva et al. 2021b) and synthetic compounds (Faillace et al. 2021; Costa et al. 2016). Results showed that EOPC was also able to modulate the resistance to EtBr in both SA1199B (Fig. 2B) and K2068 (Fig. 3B) strains. These results indicate the presence of compounds acting as NorA and MepA inhibitors which could be used against Norfloxacin-resistant *S. aureus* strains overproducers of efflux pumps, leading to a higher antibiotic accumulation in the bacterial cell.

NorA is a proton motive-dependent efflux pump belongs to Major Facilitator Superfamily (MFS), meanwhile MepA is a member of the Multidrug and Toxin Extrusion (MATE) superfamily, which is dependent of H^+ or Na^+ gradient to extrude Norfloxacin or EtBr (Schindler et al. 2014). To guarantee the integrity and functioning of NorA, there must be a continuous proton-driving force, integrity of DNA zones responsible for the synthesis of membrane proteins and balance in the permeability of substances inside the cell (Thai et al. 2015). The modulating effect verified for EOPC could be provoked by interaction of their hydrophobic monoterpenes and sesquiterpenes constituents, leading to plasma membrane damage and dissipation of the proton gradient (Souza et al. 2013; Ahmad et al. 2013), resulting in the NorA and MepA inhibition. As a consequence, the intracellular concentrations of Norfloxacin becomes enough to inactivate their intracellular targets increasing its antibacterial effect against the *S. aureus* strains tested. Thus, the EOPC could be used in combination to Norfloxacin aiming to enhancing the effectiveness of this antibiotic against multidrug-resistent *S. aureus* overproducers of NorA or MepA.

Similar results were found for *S. aureus* K4100 overexpressing the *qacC* gene (Littlejohn et al. 1991) once the EOPC potentiate the activity of EtBr, indicating that the oil is able to inhibit QacC. MIC values for EtBr against K4100 decreased eightfold in the presence of the oil from 32 to 4 µg/mL (Fig. 4). QacC is a proton motive-dependent efflux pump that confer resistance to EtBr and quaternary ammonium compounds (QACs), as Benzalkonium Chloride (Schindler et al. 2014). Biocide agents such as QACs are widely used

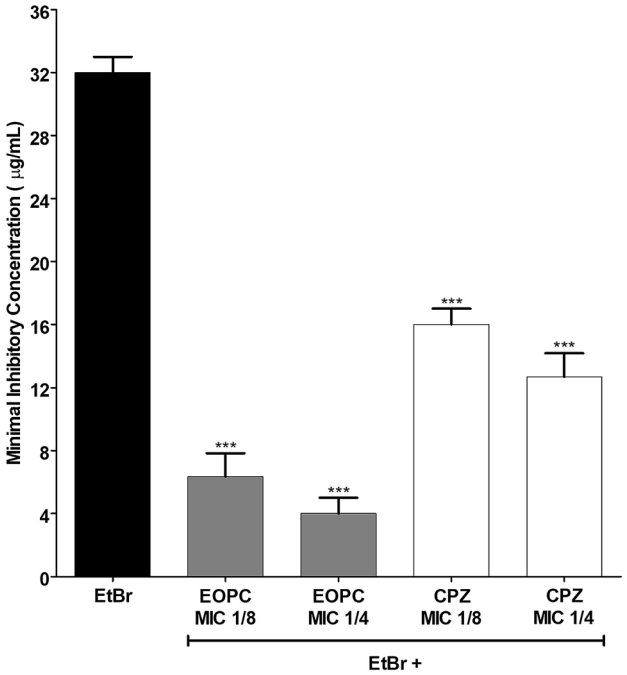

Fig. 4 MIC values of Ethidium Bromide (EtBr) against *S. aureus* K4100 (*qacC*) in absence or presence of the essential oil from the leaves of *P. caldense* (EOPC) or Chlorpromazine (CPZ). Each result represents the geometric mean of three simultaneous experiments. (***) Statistically significant values ($p < 0.0001$)

in the disinfection of surfaces mainly in hospitals, outpatient clinics, dentist offices, as well as, veterinary clinics, being including effectiveness in the SARS-CoV inactivation (Kamp et al. 2020). This intensive use can contribute for emergence of QAC-resistant *S. aureus* strains overproducers of QacC (Schindler et al. 2014). Thus, the EOPC could be used in association with QACs aiming to eliminate these multidrug strains present in surfaces preventing outbreaks of *S. aureus* infections in hospital environments.

Inhibition of the cellular dimorphism in *C. albicans*

Assays for evaluation of morphological transition of *C. albicans* were performed in the presence and absence of subinhibitory concentrations of EOPC. The fungus grown in the absence of oil showed a clear morphological transition, being possible to observe the presence of blastospores and hyphae (Fig. 5). On the other hand, EOPC showed potential to inhibit fungal dimorphism at 512 µg/mL (MIC) and at subinhibitory concentration 256 µg/mL (MIC 1/2). However, at 128 µg/mL, it was possible to observe the presence of pseudo-hyphae. Thus, besides antifungal activity, EOPC demonstrating a clear inhibition of the morphological transition in *C. albicans* ATCC 10231. Results found for EOPC tested in the present study were better than those found by Bezerra et al. (2020) once they verified inhibition of hyphae projection just at 2048 µg/mL.

Hyphae projection by fungi such as *C. albicans* is an adaptation process to environmental changes such as temperature, pH or nutrient depletion, and filamentous forms generally promote invasive diseases and hinder the action of antifungals (Wightman et al. 2004; Barbedo and Sgarbi 2010; Veses and Gow 2014). Development of pseudo-hyphae by *C. albicans* increases its ability to adhere to the host tissues, increasing its contact surface, leading to a higher propagation and developing of virulence factors (Giolo and Svidzinski 2010). Under stress, *C. albicans* change from yeast form to hyphae, that it is closely interconnected with its pathogenicity, given that filamentous fungi present better adherence to the tissue since at the end of hyphae, there are enzymes that, when secreted, can degrade proteins, lipids

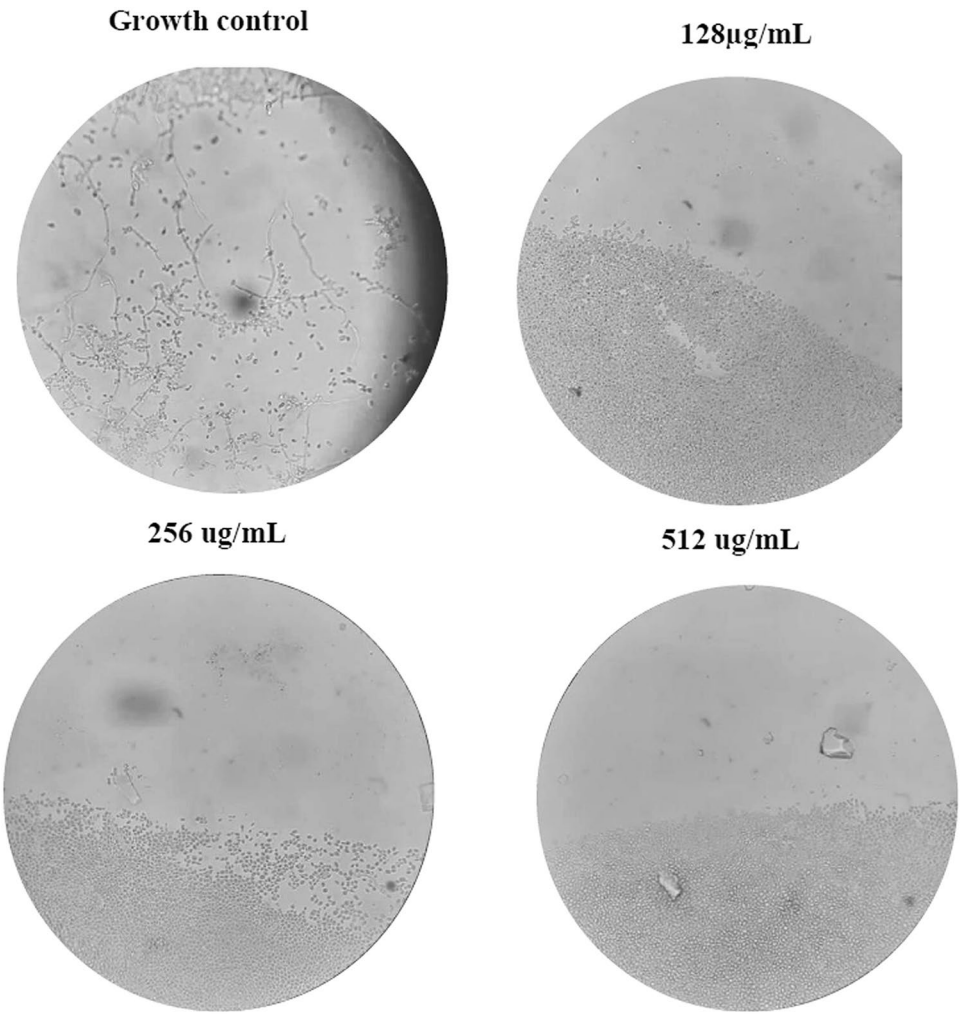

Fig. 5 Morphological transition of *C. albincans* in presence of subinhibitory concentrations of *Piper caldense* C.D.C. essential oil; Micrography was performed through 40X objective optical microscopy; photographic image captured in digital camera with 4X zoom and resized for better computer resolution. Growth control (**A**); P.caldense essential oil at 128 µg/mL (**B**); 256 µg/mL (**C**) and 512 µg/mL

and other components of host cells, facilitating the invasion process (Costa et al. 2011).

The emission of hyphae is considerably relevant in what concerns the mechanism of candidiasis virulence since the pathogenicity of the fungus is due to the fact that it presents dimorphism, this appears as an aptitude method in the development of superficial and systemic infections that hyphae spread, promoting the pathogenicity of the infectious form (Morais-Braga et al. 2016).

Although there are several antifungals available on the market, the high frequency of resistant strains becomes worrying and borderline in several clinical conditions. Thus, the EOPC tested in the present study could be applied as an agent able to inhibit the conversion of commensal yeast cells of *C. albicans* in filamentous and pathogenic forms, in the treatment or prevention of candidiasis. It is important to note that cell differentiation is regulated by intracellular signaling pathways, including cAMP-PKA and MAPK pathways (Han et al. 2011). Regarding the inhibition of the filamentous form of fungi, it is suggested that inhibition of the Efg1 transcription factor associated with the PKA pathway, which stimulates the transcription of genes that participate in the formation of hyphae (Stoldt et al. 1997; Nobile et al. 2006).

Conclusion

EOPC showed antifungal action and it was able to inhibit the hyphae projection by *C. albicans* ATCC 10231, indicating a promise use in the treatment of candidiasis. In addition, the essential oil potentiating the activity of Norfloxacin decreasing its MIC values for resistant strains overexpressing NorA or MepA efflux pumps. Futhermore, EOPC promoted a significant reduction of the MIC values for EtBr indicating that the modulating effect could be related to its ability to cause inhibition of this efflux pumps. These results point to a potential of the EOPC as an adjuvant of Norfloxacin in the treatment of infections caused by *S. aureus* strains overproducing NorA or MepA efflux pumps, a protein overexpressed in *S. aureus* SA1199B. Besides NorA and MepA, EOPC was also to act as a QacC efflux pump inhibitor compound indicating a potential technological application in association with quaternary ammonia compounds for elimination of QacC overproducing *S. aureus* strains in both human and veterinary clinical environments.

Acknowledgements This study was supported by the Coordination for the Improvement of Higher Education Personnel—Brazil (CAPES), by the National Council for Scientific and Technological Development—Brazil (CNPq), by the Cearense Foundation for Scientific and Technological Development Support—Brazil (FUNCAP) and by the Piauí Research Foundation (FAPEPI).

Funding This work was supported by the National Council for Scientific and Technological Development—Brazil (CNPq) under Grant (no. 305719/2018-1) and by the Piauí Research Foundation (FAPEPI) under Grant (no. 050/2019).

Availability of data and materials All materials and data are stored at Department of Biological Chemistry, Regional University of Cariri, Ceará. Brazil, and may be shared upon request directed to the corresponding author.

Declarations

Conflict of interest The authors declare no conflict of interest.

Consent for publication No individual data are presented in this manuscript.

References

Ahmad A, Khan A, Manzoor N (2013) Reversal of efflux mediated antifungal resistance underlies synergistic activity of two monoterpenes with fluconazole. Eur J Pharm Sci 48:80–86

Balouiri M, Sadiki M, Ibnsouda SK (2016) Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal 6:71–79

Barbedo LS, Sgarbi DBG (2010) Candidíase. DST-J Brasileiro De Doenças Sex Transm 22:22–38

Bezerra JWAB, Rodrigues FC, Da Cruz RP et al (2020) Antibiotic Potential and Chemical Composition of the Essential Oil of *Piper caldense* C. DC. (Piperaceae). Appl Sci 10:631

Cardozo Júnior EL, Chaves MCO (2003) Caldensin, a new natural n-Methylaristolactam from *Piper caldense*. Pharm Biol 41:216–218

Castro IMN, Vasconcelos Junior AA, Cunha FA et al (2016) Activity comparison of imidazole and triazole antifungals aga-inst *Candida albicans*. Revista Brasileira De Anál Clín 48:216–222

Costa CR, Souza LKHe, Ataídes FS et al (2011) Molecular analysis and dimorphism of azole-susceptible and resistant *Candida albicans* isolates. Rev Soc Bras Med Trop 44:740–744

Costa LM, de Macedo EV, Oliveira FAA et al (2016) Inhibition of the NorA efflux pump of *Staphylococcus aureus* by synthetic riparins. J Appl Microbiol 121:1312–1322

Costa AR, de Lima SJ, Lima KRR et al (2017) *Rhaphiodon echinus* (Nees & Mart.) Schauer: chemical, toxicological activity and increased antibiotic activity of antifungal drug activity and antibacterial. Microb Pathog 107:280–286

Costantin MB, Sartorelli P, Limberger R et al (2001) Essential oils from *Piper cernuum* and *Piper regnellii*: antimicrobial activities and analysis by GC/MS and 13C-NMR. Planta Med 67:771–773

Coutinho HDM, Costa JGM, Siqueira-Júnior JP et al (2008) In vitro anti-staphylococcal activity of *Hyptis martiusii* Benth against methicillin-resistant *Staphylococcus aureus*: MRSA strains. Rev Bras Farmacog 18:670–675

Coutinho HDM, Costa JGM, Falcão-Silva VS et al (2010) Effect of *Momordica charantia* L. in the resistance to aminoglycosides in methicilin-resistant *Staphylococcus aureus*. Comp Immunol, Microbiol Infect Dis 33:467–471

Dib I, Fauconnier ML, Sindic M et al (2017) Chemical composition, vasorelaxant, antioxidant and antiplatelet effects of essential oil of *Artemisia campestris* L. from Oriental Morocco. BMC Comp Alt Med 17:1–15

Du D, Wang-Kan X, Neuberger A et al (2018) Multidrug efflux pumps: structure, function and regulation. Nat Rev Microbiology 16:523–539

Duarte AE, de Menezes IR, Bezerra Morais Braga MF et al (2016) Antimicrobial activity and modulatory effect of essential oil from the leaf of *Rhaphiodon echinus* (Nees & Mart) Schauer on some antimicrobial drugs. Molecules 21:743

Egue LAN, Bouatenin J-PKM, N'Guessan FK et al (2018) Virulence factors and determination of antifungal susceptibilities of *Candida* species isolated from palm wine and sorghum beer. Microb Pathog 124:5–10

Faillace MS, Leal ALAB, Alcântara FAO, Ferreira JHL, Siqueira-Júnior JP, Nogueira CES, Barreto HM, Peláez WJ (2021) Inhibition of the NorA efflux pump of *S. aureus* by (Z)-5-(4-fluorobenzylidene)-imidazolinines. Bioorg Med Chem Lett 31:127670

Fernandes L, Ribeiro H, Oliveira A et al (2020) Portuguese honeys as antimicrobial agents against *Candida* species. J Trad Compl Med 11:130–136

Ghavam M, Manca ML, Manconi M et al (2020) Chemical composition and antimicrobial activity of essential oils obtained from leaves and flowers of *Salvia hydrangea* DC. ex Benth. Sci Rep 10:15647

Giolo MP, Svidzinski TIE (2010) Fisiopatogenia, epidemiologia e diagnóstico laboratorial da candidemia. J Bras Patol Med Lab 46:225–234

Guimaraes DO, Momesso LD, Pupo MT (2010) Antibiotics: therapeutic importance and perspectives for the discovery and development of new agents. Quim Nova 33:667–679

Guschin A, Ryzhikh P, Rumyantseva T et al (2015) Treatment efficacy, treatment failures and selection of macrolide resistance in patients with high load of *Mycoplasma genitalium* during treatment of male urethritis with josamycin. BMC Infect Dis 15:40

Han T-L, Cannon RD, Villas-Bôas SG (2011) The metabolic basis of *Candida albicans* morphogenesis and quorum sensing. Fungal Genet Biol 48:747–763

Hou L, Chen Y, Ma C et al (2011) Effects of environmental factors on dimorphic transition of the jelly mushroom *Tremella fuciformis*. Cryptogam, Mycol 32:421–428

Houghton PJ, Howes MJ, Lee CC, Steventon G (2007) Uses and abuses of in vitro tests in ethnopharmacology: visualizing an elephant. J Ethnopharmacol 110:391–400

Kaatz GW, Seo SM, Ruble CA (1993) Efflux-mediated fluoroquinolone resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 37:1086–1094

Kaatz GW, McAleese F, Seo SM (2005) Multidrug resistance in *Staphylococcus aureus* due to overexpression of a novel multidrug and toxin extrusion (MATE) transport protein. Antimicrob Agents Chemother 49:1857–1864

Kamp G, Todt D, Pfaender S, Steinmann E (2020) Persistence of coronaviruses on inanimate surfaces and their inactivation with biocidal agents. J Hosp Infect 104:246–251

Klein S, Hannesen J, Zanger P et al (2020) Entry of Panton-Valentine leukocidin-positive methicillin-resistant *Staphylococcus aureus* into the hospital: prevalence and population structure in Heidelberg, Germany 2015–2018. Sci Rep 10(13243):1–7

Leal ALAB, Bezerra CF, Rocha JE et al (2019a) *Piper cernuum* Vell.: Chemical profile and antimicrobial potential evaluation. Ind Crops Prod 140:111577

Leal ALAB, Machado AJT, Bezerra CF et al (2019b) Chemical identification and antimicrobial potential of essential oil of *Piper rivinoides* kunth (BETIS-WHITE). Food Chem Toxicol 131:10559

Lima LFA, Lira AC, Coutinho HDM et al (2012) Antimicrobial resistance in staphylococci isolated from canine pyoderma. Comun Sci 3:181–185

Littlejohn TG, DiBerardino D, Messerotti LJ, Spiers SJ, Skurray RA (1991) Structure and evolution of a family of genes encoding antiseptic and disinfectant resistance in *Staphylococcus aureus*. Gene 101:59–66

Markham PN, Westhaus E, Klyachko K et al (1999) Multiple novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. Antimicrob Agents Chemother 43:2404–2408

Matias EFF, Santos KA, Falcão-Silva VS et al (2013) Modulation of the norfloxacin resistance in *Staphylococcus aureus* by *Cordia verbenaceae* DC Modulação da resistência à norfloxacina em *Staphylococcus aureus* por *Cordia verbenaceae* DC. Indian J Med Res 137:178–182

Mayers DL, Sobel JD, Ouellette M et al (2009) Antimicrobial drug resistance c: clinical and epidemiological aspects. Springer, Dordrecht Heidelberg

Morais-Braga MFB, Carneiro JNP, Machado AJT et al (2016) High-performance liquid chromatography-diodic array detector, fungistatic, and anti-morphogenical analysis of extracts from *Psidium brownianum* Mart. ex DC. against yeasts of the genus *Candida*. Int J Food Prop 19:1837–1851

Neyfakh AA, Borsch CM, Kaatz GW (1993) Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. Antimicrob Agents Chemother 37:128–129

Nobile CJ, Andes DR, Nett JE et al (2006) Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. PLoS Pathog 2:e63

Rezende-Júnior LM, Andrade LMS, Leal ALAB (2020) Chalcones isolated from *Arrabidaea brachypoda* flowers as inhibitors of NorA and MepA multidrug efflux pumps of *Staphylococcus aureus*. Antibiotics 9(351):1–12

Rocha DS, Silva JM, Navarro DMAF et al (2016) Potential antimicrobial and chemical composition of essential oils from *Piper caldense* Tissues. J Mex Chem Soc 60:148–151

Ruiz-Reyes E, Suarez M (2015) Lactonas sesquiterpénicas. Diversidad estructural y sus actividades biológicas. Rev CENIC Cienc Biol 46:9–24

Sánchez–Martínez C, Pérez-Martín J (2001) Dimorphism in fungal pathogens: *Candida albicans* and *Ustilago maydis*—similar inputs, different outputs. Curr Opin Microbiol 4:214–221

Santos ALd, Santos DO, Freitas CCd et al (2007) *Staphylococcus aureus*: visitando uma cepa de importância hospitalar. J Bras Patol Med Lab 43:413–423

Schmidt E, Bail S, Friedl SM et al (2010) Antimicrobial activities of single aroma compounds. Nat Prod Commun 5:1934578X1000500906

Schindler BD, Frempong-Manso E, DeMarco CE et al (2014) Analyses of Multidrug Efflux Pump-Like Proteins Encoded on the *Staphylococcus aureus* Chromosome. Antimicrob Agents Chemother 59:747–748

Sellam A, Whiteway M (2016) Recent advances on *Candida albicans* biology and virulence. F1000Research, p 2582

Sidrin JJC, Rocha MFG (2010) Micologia médica à luz de autores contemporâneos. Guanabara Koogan, Rio de Janeiro

Siebra ALA, Oliveira LR, Martins AOBPB et al (2018) Potentiation of antibiotic activity by *Passiflora cincinnata* Mast. front of strains *Staphylococcus aureus* and *Escherichia coli*. Saudi J Biol Sci 25:37–43

Silva JA, Oliveira FF, Guedes ES et al (2014) Atividade antioxidante de *Piper arboreum*, *Piper dilatatum* e *Piper divaricatum*. Rev Bras Pl Med 16:700–706

Silva AKF, Reis AC, Pinheiro EEA et al (2021a) Modulation of the drug resistance by *Platonia insignis* Mart. extract, ethyl acetate fraction and Morelloflavone/Volkensiflavone (Biflavonoids) in *Staphylococcus aureus* strains overexpressing efflux pump genes. Curr Drug Metab 22:114–122

Silva PT, Xavier JC, Freitas TS et al (2021b) Synthesis, spectroscopic characterization and antibacterial evaluation by chalcones derived

of acetophenone isolated from Croton anisodontus Müll.Arg. J Mol Structure 1226:129403
Skaltsa HD, Demetzos C, Lazari D et al (2003) Essential oil analysis and antimicrobial activity of eight *Stachys* species from Greece. Phytochemistry 64:743–752
Souza EL, Oliveira CEV, Stamford TLM et al (2013) Influence of carvacrol and thymol on the physiological attributes, enterotoxin production and surface characteristics of *Staphylococcus aureus* strains isolated from foods. Brazilian J Microbiol 44:29–35
Stoldt VR, Sonneborn A, Leuker CE et al (1997) Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J 16:1982–1991
Sugden R, Kelly R, Davies S (2016) Combatting antimicrobial resistance globally. Nat Microbiol 1:16187
Thai KM, Ngo TD, Phan TV et al (2015) Virtual screening for novel *Staphylococcus aureus* NorA efflux pump inhibitors from natural products. Med Chem 11:135–155
Tulgar S, Alasehir EA, Selvi O (2018) The antimicrobial activity of ephedrine and admixture of ephedrine and propofol: an in vitro study. Rev Bras Anestesiol 68:69–74
Veríssimo C, Sabino R, Martins C et al (2016) Fungal infections in Portugal - The sleeping giant. Infecção Sepsis 2:1–9
Veses V, Gow NA (2014) Pseudohypha budding patterns of *Candida albicans*. Med Mycol 47:268–275
Viana MG, Lutterbach MTS, Silva DR et al (2019) Antimicrobial and antibiofilm activity of essential oil of *Lippia gracilis* Schauer on *Clostridium bifermentans* and fungal-containing biofilms. Austin Chem Eng 6:1066
Wasicky R (1963) Uma modificação do aparelho de Clevenger para extração de óleos essenciais. Rev Farm Bioquim 1:77–81
Wightman R, Bates S, Amornrrattanapan P et al (2004) In *Candida albicans*, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 also controls septin organization. J Cell Biol 164:581–591
Wright GD, Sutherland AD (2007) New strategies for combating multidrug-resistant bacteria. Trends Mol Med 13:260–267
Xiang Y, Li F, Dong N et al (2020) Investigation of a salmonellosis outbreak caused by multidrug resistant *Salmonella* Typhimurium in China. Front Microbiol 11:1–12
Yang D, Michel L, Chaumont JP et al (1999) Use of caryophyllene oxide as an antifungal agent in an in vitro experimental model of onychomycosis. Mycopathologia 148:79–82