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Antibiofilm potential of *Psidium guajava* and *Passiflora edulis* pulp extracts against *Staphylococcus aureus*, cytotoxicity, and interference on the activity of antimicrobial drugs

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Abstract

Background: Pathogenic strains of *Staphylococcus aureus* can cause several diseases including septicemia and endocarditis, in spite of being a commensal species of the human microbiota. The current drug resistance of *S. aureus* raises the need for new antimicrobials, and natural products represent a feasible source for prospection of such compounds, due to features including the diversity of structures and mechanisms of action. Here, we provide evidence of the antimicrobial activity of methanolic of *Psidium guajava* and *Passiflora edulis* pulps against planktonic cells and biofilms of clinical isolates of *S. aureus*.

Results: The extracts were effective against the strains in concentrations up to 7.81 and 250 μg/mL for planktonic cells and biofilms, respectively. Antagonistic interactions of the extracts to antimicrobial drugs were observed. The pulps caused no cytotoxic effects on BGM cells. GC-MS analysis found relevant molecules, and UPLC analysis suggested the presence of flavonoids. To the best of our knowledge, this is the first antibiofilm evidence of such extracts.

Conclusion: The extracts seem to be safe and effective enough for more studies aiming at exploring isolated antimicrobial molecules using in vivo models for the treatment of staphylococcal diseases.

Keywords: *Staphylococcus aureus*, Fruit pulp, Psidium guajava, Passiflora edulis

Background

Staphylococcus aureus is a commensal bacterium that can be found in the skin and in nasal mucosa, and around 30% of the human population is colonized with this species [1]. *S. aureus* is also an important pathogen associated to diseases such as infective endocarditis, osteomielitis, septicemia, infections related to devices such as catheters and stents, and skin/soft tissues infections like abscesses, pleuropulmonary infection, and purulent cellulitis [2–4]. The growing resistance of *S. aureus* to the currently available antimicrobial drugs is a critical issue for treatment of infectious diseases caused by this species: as the strains develop varied mechanisms of drug resistance such as biofilm formation, production of drug degrading enzymes, and expression of efflux pumps, the effectiveness of antimicrobials drugs is hampered [2, 5]. In this context, natural products of vegetable origin are important sources of antimicrobial compounds. Most of the studies on herbal-based antimicrobials provide evidence of lack of resistance mechanisms, and compared to the development of synthetic

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drugs, the costs on the development of phytoformulations are considerably lower.

Psidium guajava is common in tropical and subtropical countries, and the fruit (guava) is used for artisanal and industrial manufacturing of jellies, sweets, powder fibers, and juice [6, 7]. Extracts of different parts of *P. guajava* present antibacterial and antifungal activities and are popularly used for epilepsy, convulsions, diarrhea, stomachache, constipation, wound healing, and diabetes [8–11]. Guava juice is specially appreciated, and pulps are prepared in order to allow the consumption of the juice throughout the year, avoiding seasonal influences on the availability of fruit [11, 12]. However, the biological potentials of the pulp are poorly investigated.

Passiflora species are also present in tropical and subtropical countries, and more than 500 species of the plant are known [13, 14]. In Brazil, *Passiflora edulis* has an important economic role. The pulp is largely used for juice preparation, the dry powder of the epicarp is used for diabetes and lipid metabolism disorders, and leaf extract is used for the treatment of sleep and gastric disorders [15–19]. The leaf and stem bark extracts are effective against microbial species such as *Bacillus subtilis*, *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [20, 21]. The biological potentials of the pulp are poorly investigated as well as for guava.

In spite of the benefits of plants to human health, data on pharmacological potential of edible vegetable parts are often scarce. With the growing resistance of *S. aureus* to the available antimicrobial drugs, an urgent need for new therapeutic options is raised. Here we show that the methanolic extracts (80% v/v) of *P. guajava* and *P. edulis* fruit juice pulps are effective against planktonic cells and biofilms of clinical isolates of *S. aureus*. We conducted ultra-performance liquid chromatography (UPLC) and gas chromatography coupled to mass spectrometry (GC-MS) analyses of the extracts and investigated the effects of the joint use of the extracts and clinically relevant antimicrobial drugs, and observed mostly antagonistic responses. Moreover, we conducted toxicity tests against BGM cells. Our data open doors for isolating and exploring antimicrobial molecules of *P. guajava* and *P. edulis* pulp extracts for the treatment of staphylococcal diseases.

Methods

Pulp samples and preparation of the methanolic extract

P. guajava and *P. edulis* industrialized liquid pulps used in this study belong to a brand widely commercialized in Brazil (probably the most commercialized concerning fruit pulps) and were purchased at a local market in plastic bottles. They were disposed in environmental temperature and protected from sunlight. The manufacturer describes at the label that the pulps were pasteurized and are free of sucrose. The labels indicated the presence of food additives like BHT (2,6-bis(1,1-dimethylethyl)-4-methylphenol), ascorbic acid, and colorants. The pulps were freeze-dried prior to the preparation of the extracts, which were prepared in environmental temperature with 80% methanol/water solution through magnetic stirring at maximum speed for 48 h. After the extraction, the content was centrifuged (5000*g*, 20min, 4 °C) and the supernatant was freeze-dried. The final product was then weighed and stored at 4 °C until used.

Polyphenol detection by ultra-proficiency liquid chromatography

The polyphenol content of the methanolic extracts of the pulps was analyzed using a C18 column (Waters) in an UPLC system coupled to a diode array (UPLC/DAD) (Acquity H-Class Bio, Waters, Germany), following a method previously described by our group [22]. The column temperature was set at 35 °C. A linear gradient mode was programmed as follows: 20% A and 80% B at start, then 30% A and 70% B at 4 min, 70% A and 30% B at 6 min, falling to 100% A and 0% B at 8 min, and finally, 20% A and 80% B at 10 min. Polyphenols were detected at 280 nm.

Detection of free carbohydrates and volatile components by gas chromatography-mass spectrometry

Free carbohydrates and volatile molecules of the pulps were analyzed following a GC-MS method in positive mode previously described by our group [23]. Using HPLC grade dichloromethane, helium as carrier gas, and DB-5 column, we proceeded to the analysis in duplicate. Mass spectra and retention times obtained from each sample were matched to NIST library data for interpretation.

Bacterial strains

Clinical *S. aureus* isolates from indwelling catheters of hemodialysis patients were from the Clinical Laboratory of the Pitágoras College. We used VITEK 2 system (version R04.02, bioMérieux, Marcy-l'Étoile, France) gram-positive identification cards to confirm the identity of 10 isolates according to the manufacturer's instructions.

Minimal inhibitory concentration assay

The minimal inhibitory concentration (MIC) of the extracts was determined in untreated sterile 96-well polystyrene microtiter plates using Mueller Hinton broth, in a final concentration of the bacterial inoculum equal to 0.5 McFarland scale and final concentrations of the extracts ranging from 1 mg/mL to 7.8 μg/mL. Plates were incubated overnight at 35 ± 2 °C, and resazurine 0.1% solution was used to observe the antimicrobial effect.

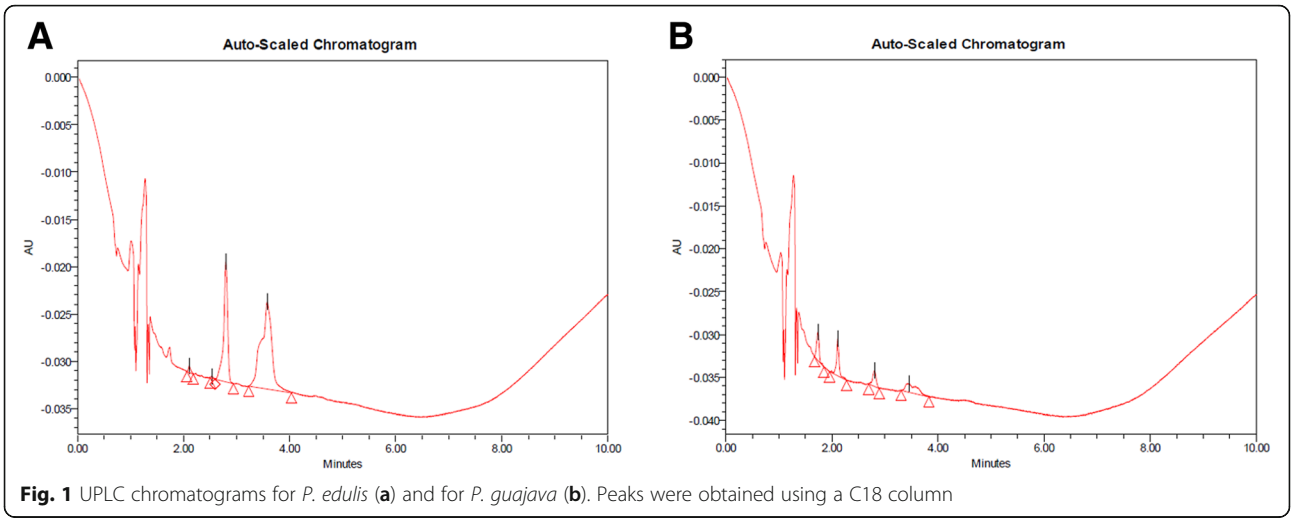

Fig. 1 UPLC chromatograms for *P. edulis* (**a**) and for *P. guajava* (**b**). Peaks were obtained using a C18 column

MIC was the lowest concentration in which no color modification from blue (resazurine) to pink (resofurine) was observed in all strains. The extracts were used as negative controls [24]. This assay was conducted in triplicate.

Minimum bactericidal concentration assay

The minimum bactericidal concentration (MBC) of the extracts was determined in triplicate by spotting 10 μL of each well of MIC plates in Mueller-Hinton agar (Difco). Extracts were inoculated as negative controls. Agar plates were incubated overnight at 35 ± 2 °C, and bacterial growth was observed. MBC was established as the lowest concentration that yielded no bacterial growth on the plates [24].

Minimal biofilm eradication concentration assay

Before minimal biofilm eradication concentration (MBEC) test, biofilm formation was induced overnight using a bacterial inoculum equal to 0.5 McFarland scale in sterile non-treated polystyrene plates using BHI media [23]. The MBEC was then assessed in triplicate as described [24]. Aliquots of 100 μL of each extract concentration were added in triplicate for each biofilm, and plates were then incubated overnight at 35 ± 2 °C. Resazurine stain was used as described in MIC for MBEC [24].

Interference of the pulp extract on the activity of antimicrobial drugs

The interference of the extracts on antimicrobial drugs was assessed in duplicate as described in previous works of our group [22–25]. Antimicrobial disks (ciprofloxacin 5 μg, erythromycin 15 μg, and gentamycin 10 μg, all from Laborclin) were used as for antimicrobial susceptibility assays. Following, briefly, 10 μL of the extracts in their MBC concentration was dispensed in each disk. Plates were incubated overnight at 35 ± 2 °C, and the inhibition zone mean diameter was compared with control disks (without addition of the extract). Synergism was considered if the inhibition zone mean diameter was at least 2 mm larger than the control, and antagonism was considered if the inhibition zone mean diameter was at least 2 mm shorter than the control, both with statistical significance. If the inhibition zone mean diameters were larger or shorter than the control but no statistically significant difference was detected, data was described as tendency of synergism or antagonism [22].

Cytotoxicity test

The potential cytotoxic effect of the pulp extracts was tested in duplicate using BGM cells (Sigma), as previously described [22]. Cell suspensions were prepared in RPMI 1640 media supplemented with glutamine (0.3 mg/L), penicillin (200 IU/mL), streptomycin (100 μg/mL), and fetal bovine serum (10%), to reach an estimated counting of 1×10^4 cells in 180 μL. Plates were then incubated overnight at 37 °C in humidified conditions, to allow cells to reach the logarithmic growth phase. The stock solution of each extracts was prepared and diluted in PBS. Cells were treated with 20 μL of each extract as in MIC assays. Plates were incubated overnight, and cell viability was assessed by resazurine staining (0.1 g/L) using a fluorimetric microplate reader (λ_{ex}570 nm, λ_{em}590 nm). Untreated cells (extract-free RPMI media) were used as the control group. Readings were also taken from the RPMI medium with the extract to remove possible interferences on fluorescence readings.

Statistics

Normality of data was assessed through the Shapiro-Wilk test. Data were analyzed using ANOVA followed

Table 1 Volatile compounds identified in *P. guajava* extract

RT	A%	Identity	m/z
2.079	7.08	L-5-Propylthiomethylhydantoin	188
2.144	5.54	Ethanediamide	88
2.23	1.63	L-Alanine, methyl ester	103
2.364	8.35	Sulfur tetrafluoride	108
2.513	11.81	Pentanoic acid, 3-methyl-4-oxo-	130
2.691	0.24	Silane, diethoxydimethyl-	148
2.765	1.46	Isobutyl acetate	116
3.075	0.06	2-Ethoxytetrahydrofuran	116
3.144	0.01	Furfural	96
3.222	0.05	2-Azetidinone, 1-phenyl-	147
3.341	0.02	Ethylbenzene	106
3.556	0.04	5,5-Dimethyl-1,3-dioxane-2-ethanol,tert-butyldimethylsilyl ether	274
3.72	0.01	Butyrolactone	86
4.516	0.01	1,2-Propanediol, diacetate	90
4.787	0.17	Pentanoic acid, 4-oxo-, ethyl ester	144
4.832	0.08	Propanedioic acid, diethyl ester	160
4.985	6.64	1,4-Butanediol, diacetate	174
5.086	0.01	1,2,4-Triazine-3,5(2H,4H)-dione, 6-benzoylthio-	249
5.508	0.05	Hepta-2,4-dienoic acid, methyl ester	140
5.652	0.05	Butanedioic acid, diethyl ester	174
5.687	0.04	2(1H)-Pyridinone, 5-methyl-	109
5.726	0.06	Benzene, 1-(chloromethyl)-2-fluoro-	144
5.798	0.19	Benzoic acid	122
6.079	0.21	Benzaldehyde, 2,4-dimethyl-	134
6.118	0.48	5-Hydroxymethylfurfural	126
6.322	0.08	1,5-Diacetoxypentane	188
6.433	0.35	Butanedioic acid, hydroxy-, diethyl ester, (.+/-.)-	190
6.612	0.37	Benzaldehyde, 4-propyl-	148
6.847	0.43	5-Acetoxymethyl-2-furaldehyde	168
7.175	0.01	1,3-Propanediol, 2,2-dimethyl-, diacetate	188
7.412	0.02	Acetic acid, cyclohexyl ester	142
12.435	0.11	Pentadecane	212
22.254	0.06	Hexadecanoic acid, ethyl ester	284
23.631	0.01	7-Octadecenoic acid, methyl ester	296
24.296	0.01	7-Methyl-Z-tetradecen-1-ol acetate	268
24.398	0.01	7-Tetradecenal, (Z)-	210

by the Tukey test. The significance level was set at $p < 0.05$, and highly significant values were set as $p < 0.01$. All analyses were carried out using Bioestat 5.0 Statistical Package for Windows.

Results

Chemical analyses

UPLC analysis confirmed the presence of phenolic compounds in the sample, as peaks were detected at 280 nm (Fig. 1). GC-MS analysis indicated lack of free carbohydrates in the pulps (data not shown). However, relevant volatile compounds were found for both extracts, including alcohol derivatives of glycerol, preservants, chelating agents, fatty acids, and aldehydes (Tables 1 and 2).

Table 2 Volatile compounds identified in *P. edulis* extract

RT	A%	Identity	m/z
2.091	9.47	*N*-Methoxydiacetamide	131
2.181	2.46	Iminodiacetic acid	133
2.241	3.71	Formic acid, propyl ester	88
2.57	3.52	Silane, dimethoxydimethyl-	120
2.747	5.17	Isobutyl acetate	116
3.003	2.37	Propanoic acid, 2-hydroxy-, ethyl ester, (S)-	118
3.21	0.31	Fucose, cyclic ethylene mercaptal	240
3.241	0.53	1,2-Ethanediol, monoacetate	104
3.538	0.17	(-)-O-Acetylmalic anhydride	158
3.576	0.55	Oxime-, methoxy-phenyl-_	151
3.847	0.5	2-(2-Hydroxyethoxy)ethyl acetate	148
4.676	0.24	Pentanoic acid, 4-oxo-	116
5.279	0.18	Silicic acid, diethyl bis(trimethylsilyl) ester	296
5.492	0.19	Hepta-2,4-dienoic acid, methyl ester	140
5.661	1.04	Benzoic acid	122
6.163	0.23	1,2,3-Propanetriol, 1-acetate	134
6.252	0.09	Benzeneacetic acid, ethyl ester	164
6.408	0.17	Butanedioic acid, hydroxy-, diethyl ester, (.+/-.)-	190
6.586	0.47	Benzaldehyde, 4-propyl-	148
6.685	0.1	Butanoic acid, 3-hydroxy-	104
6.827	0.16	5-Acetoxymethyl-2-furaldehyde	168
16.706	0.03	Octane, 5-ethyl-2-methyl-	156
18.836	0.03	Tetradecanoic acid, ethyl ester	256
18.954	0.02	Undecane, 3,8-dimethyl-	184
19.142	0.02	3-Acetoxydodecane	228
21.775	0.12	n-Hexadecanoic acid	256
22.259	0.09	Hexadecanoic acid, ethyl ester	284
23.628	0.02	9-Octadecenoic acid (Z)-, methyl ester	296
24.318	0.07	Octadecanoic acid	284
24.837	0.02	1-Acetoxynonadecane	326
26.702	0.01	Tetracosane	338
27.58	0.01	Tricosane	324

Table 3 Susceptibility of *S. aureus* isolates to the extracts and their interference on antimicrobial drugs

	Antimicrobial parameter (in μg/mL)		
	MIC	MBC	MBEC
P. guajava	31.25	62.5	250
P. edulis	15.62	125	250

Results are referent to all tested strains

Table 4 Interference of the extracts on antimicrobial drugs

Isolate	Drug disk inhibition zone (alone or combined to extracts) in mm								
	Ery	Ery+PE	Ery+PG	Cip	Cip+PE	Cip+PG	Gen	Gen+PE	Gen+PG
S. aureus 1	0	0	0	18.5	14	13$^{\clubsuit}$	15	17†	16.5
S. aureus 2	0	0	0	18.5	20.5†	16$^{\clubsuit}$	14.5	14	13.5
S. aureus 3	0	12$^{\blacklozenge}$	0	23.5	19.5$^{\Delta}$	18$^{\clubsuit}$	0	0	0
S. aureus 4	0	10$^{\blacklozenge}$	0	20.5	18$^{\Delta}$	17$^{\clubsuit}$	15.5	16.5	12.5$^{\Delta}$
S. aureus 5	0	13.5$^{\blacklozenge}$	0	21	18$^{\Delta}$	15.5$^{\clubsuit}$	12.5	18†	15†
S. aureus 6	0	14$^{\blacklozenge}$	0	20	18$^{\Delta}$	17$^{\clubsuit}$	0	16†	15†
S. aureus 7	0	11$^{\blacklozenge}$	0	21	16$^{\Delta}$	14.5$^{\clubsuit}$	14.5	12.5$^{\Delta}$	14
S. aureus 8	0	15$^{\blacklozenge}$	0	32	20$^{\Delta}$	18$^{\clubsuit}$	29	15.5$^{\Delta}$	14.5$^{\Delta}$
S. aureus 9	0	15$^{\blacklozenge}$	0	25.5	20$^{\Delta}$	17.5$^{\clubsuit}$	19	16.5$^{\Delta}$	15.5$^{\Delta}$
S. aureus 10	0	0	0	23.5	20$^{\Delta}$	14$^{\clubsuit}$	15	16.5	10.5$^{\Delta}$

Values without any symbol are indifferent when comparing to the control.
+PE addition of *P. edulis* extract at the MBC, *+PG* addition of *P. guajava* extract at the MBC, *Ery* erythromycin, *Cip* ciprofloxacin, *Gen* gentamicin
$^{\blacklozenge}$Statistically significant synergism
†Tendency of synergism
$^{\clubsuit}$Statistically significant antagonism
$^{\Delta}$Tendency of antagonism

Antimicrobial activity of the pulps

The antimicrobial and antibiofilm potentials of *P. guajava* and *P. edulis* pulp extracts were investigated using overnight cultures of clinical isolates of *S. aureus*, as well as overnight-formed biofilms formed in 96-wells plates. The MIC value for *P. edulis* extract was lower than *P. guajava* extract; however, the MBC value for *P. guajava* extract (two times higher than the MIC value) was lower than *P. edulis* extract (eight times higher than the MIC value). For both extracts, this observation suggests their bacteriostatic effect [26]. Interestingly, the MBEC value of the extracts was the same (Table 3).

Interference of the extracts on the activity of antimicrobial drugs

The pulp extracts were prepared in their MBC concentration for this assay. The combination of *P. guajava* and erythromycin resulted in a significant synergic effect for seven out of 10 isolates ($p < 0.01$), whereas it resulted in significant antagonism ($p < 0.01$) when combined to ciprofloxacin, and some antagonism tendency when combined to gentamicin (Table 4). Conversely, *P. edulis* extract has not altered the susceptibility profile of the isolates when combined to erythromycin. When combined to ciprofloxacin, an antagonism tendency was observed ($p < 0.05$), and tendencies of synergism and antagonism were detected for some isolates when the pulp extract was combined to gentamicin.

Cytotoxicity

Cell toxicity was not detected for BGM cell lines, and there was no significant difference of the untreated control and the treatments with *P. guajava* ($p = 0.058$) and *P. edulis* ($p = 0.06$) extracts. However, there was a discrete tendency on the average readings to be lower than the control.

Discussion

Here we described the antimicrobial and antibiofilm activities of the methanolic extracts obtained from industrially manufactured *P. guajava* and *P. edulis* pulps against clinical isolates of *S. aureus*, to the best of our knowledge, for the first time. The MIC of *P. guajava* extract was of 31.25 μg/mL, and the MIC of *P. edulis* extract was of 15.62 μg/mL. Our results are consistent with the observation of others that leaf extracts of both plants present antimicrobial activity against relevant pathogenic species including *S. aureus*, *Salmonella* spp., *Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, and *Candida albicans* in concentrations around 100 μg/mL [20, 21, 27–30]. A recent study described that the methanolic extract of *P. edulis* pulp prepared with the seeds was effective against an ATCC strain of *S. aureus* at 4 mg/mL [31], which is 256-fold higher than the MIC value described in the present study. A possible explanation for this difference to our seed-free extract is that, beyond eventual variations of cultivation parameters (which influence on the phytomolecules of the extracts), the seeds may have released harmless molecules that worked as carbon and nitrogen sources necessary for bacterial growth.

Most of the known bacteria live as biofilms, especially when causing diseases [24, 25]. In this study, overnight-grown biofilms of clinical isolates of *S. aureus* were eradicated with the pulp extracts at the concentration of 250 μg/mL, considerably higher than the MIC values. Biofilms can be more than 1000-fold resistant to antimicrobial compounds when compared to planktonic

cells, due to physical and chemical protective effects of the extracellular polymeric substances surrounding the sessile cells [25, 32]. The antibiofilm potential of *P. guajava* leaf extract was also described [33].

L-5-Propylthiomethylhydantoin was found by GC-MS in *P. guajava* pulp (Table 1), and pentanoic acid was found in *P. edulis* pulp (Table 2). Both were described to be bacteriostatic molecules [34, 35]. UPLC analysis confirmed the presence of phenolic compounds in the sample (Fig. 1a, b). Taken together, these results help to explain the antimicrobial activity of the pulps. More analyses are being conducted to identify the polyphenols found in this study. Unexpectedly, the results obtained by GC-MS analysis indicated lack of carbohydrates in the pulps: only fucose was found in *P. edulis* extract (Table 2). The presence of carbohydrates naturally present in fruit such as fructose was expected. Soluble sugar content varies considerably within and among species depending on the age, maturity, and environmental conditions [36].

Here, we evaluated the toxicity of the extracts by using BGM cells as a model of normal mammalian cells. Toxicity against BGM cells was not observed for both pulps. Compounds that lack toxicity in such in vitro models, but are effective against microorganisms, can be safe for investigations towards the clinical use [22, 32].

Conclusion

The methanolic extract of the pulps presented antimicrobial activity in an in vitro model against *S. aureus* strains, and this potential is possibly related to the polyphenols and other molecules such as L-5-propylthiomethylhydantoin, found using UPLC and GC-MS. *P. guajava* combined to erythromycin resulted in significant synergic effect, but also resulted in significant antagonism when combined to ciprofloxacin. Antagonism tendency was observed for *P. edulis* extract combination to ciprofloxacin. Given the lacking toxicity of the extracts, in spite of the small number of strains used in this study, our data open doors for further studies with isolated molecules from the extracts using in vivo models for pharmacotherapeutic purposes, both isolated and combined to antimicrobial drugs.

Abbreviations

UPLC: Ultra performance liquid chromatography; GC-MS: Gas chromatography coupled to mass spectrometry; UPLC/DAD: Ultra-performance liquid chromatography coupled to a diode array; HPLC: High-performance liquid chromatography; MIC: Minimal inhibitory concentration; MBC: Minimal bactericidal concentration; MBEC: Minimal biofilm eradication concentration; +PE: Addition of *P. edulis* extract at the minimal bactericidal concentration; +PG: Addition of *P. guajava* extract at the minimal bactericidal concentration; Ery: Erythromycin; Cip: Ciprofloxacin; Gen: Gentamicin

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Not applicable.

Plant authentication

Plant authentication is provided by the manufacturer of the industrialized pulps used in this study.

Authors' contributions

All authors have contributed to this research work and read and approved the final version of the manuscript. RMDS, GC, MVDS: preparation of the extract, antimicrobial activity tests, and phytochemical analyzes. RMDS, GC: drafted the primary version of the manuscript. IPC: conducted cytotoxicity studies and drafted the manuscript. MVDS: conception and design of the study, statistical analysis, drafted the manuscript, and revised its final version.

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Availability of data and materials

All data and material are available upon request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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