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Laurel extracts inhibit *Quorum sensing*, virulence factors and biofilm of foodborne pathogens

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ABSTRACT

Antimicrobial, antibiofilm, anti-*Quorum sensing* (QS) and virulence factors inhibitory capacity of different polarity *Laurus nobilis* extracts against several pathogenic microorganisms were studied. Some extracts exhibited antibiotic effect against *Staphylococcus aureus* multidrug-resistant strains. However, all extracts (100 μg/mL) inhibited to some extent the biofilm of most bacteria tested (until 40% for Gram-negative and 76% for Gram-positive). Hexane (HE) and chloroform extract (CE) were potent inhibitors of *S. aureus* biofilm and the microscopies further confirmed an important reduction in adherent cells. Polystyrene surfaces coated with these extracts showed a decrease in bacterial adhesion with the resulting inhibition of *S. aureus* biofilm formation and biofilm activity. Moreover, they also interfere with the coagulase and hemolysin activities of this bacterium. With respect to Gram-negative bacteria, these extracts showed anti-QS activity against *Chromobacterium violaceum* and inhibited biofilm formation, swarming motility, pyocyanin production, and elastase activity of *Pseudomonas aeruginosa*. Chemical analysis of HE and CE revealed that the fatty acids were the main compounds, particularly linolenic, oleic and hexadecanoic acids followed by the oxygenated monoterpenes. Non-toxic laurel extracts offer a natural alternative to control contamination and/or spoilage of food, as well as infectious diseases, by attacking the biofilm and the virulence of Gram-positive and Gram-negative bacteria.

1. Introduction

Laurus nobilis L. commonly known as bay laurel, belonging to the Lauraceae family is one of the most widely used culinary spices in many countries. It is an aromatic evergreen tree used in foods, drugs, and cosmetics. In the food industry, it is utilized to flavor meat products, soups, fishes, stews, sauce, pickles, and sausages. It is used in traditional medicine to treat dermatitis, rheumatism, earaches, indigestion, sprains, diabetes, migraine (Zeković, Lepojević, & Mujić, 2009). It has been reported to possess wound healing, neuroprotective, antioxidant, anti-ulcerogenic, analgesic, anticonvulsant, antimutagenic, antiviral, anticholinergic, antibacterial, antifungal, and immune-modulating capacities (Chahal, Kaur, Bhardwaj, Singla, & Kaur, 2017; Sırıken, Yavuz, & Güler, 2018). Due to its antimicrobial activity, bay laurel could be used in the food industry as a food preservative.

Food spoilage with pathogenic microorganisms is a major concern for food manufacturers, as it causes significant economic losses. In addition, foodborne illnesses are an important public health problem worldwide (Quinlan, 2013). The tolerance of foodborne pathogens to several environmental stressors (heat, cold, salt, and acidic conditions) and its capacity to form biofilms on biotic or abiotic surfaces are main factors for these bacteria to remain into the processing environment and cross-contaminate food contact surfaces, equipment, floors, drains between others (Alejo-Armijo et al., 2017). In biofilm formation, *Quorum sensing* (QS) allows a phenotypic change in the bacteria, so sessile biofilm bacteria show increased resistance to many biocides, disinfectants, and antibiotics (Amaral et al., 2015). Also, QS can regulate the virulence factors expression, such as swarming, pyocyanin production, and elastase, coagulase, and hemolysin enzymes.

The use of natural preservatives in foods has been widely accepted by

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consumers, who demand natural and healthier products, free of synthetic additives (da Silveira et al., 2014). In addition, consumers are accustomed to the use of herbs and spices to provide flavor and aroma to the food. Thereby, many studies have been focused on different extracts and essential oils of aromatic plants, such as rosemary, oregano, thyme, mint, and clove, which are considered to promote human health and to have a huge potential as food preservatives (Galié, García-Gutiérrez, Miguélez, Villar, & Lombó, 2018; Ramos et al., 2012).

Although the antimicrobial properties of *L. nobilis* were demonstrated in planktonic cells (Chahal et al., 2017), there is still a paucity of studies on the effect of different polarity laurel extracts on biofilm and even less on the virulence factors mediated by QS. Therefore, the evaluation of *L. nobilis* extracts' antipathogenic activity is undoubtedly a field to explore.

The objectives of the present study were: (1) to perform toxicity assays of different polarity bay laurel extracts (2) to find the Minimum Inhibitory Concentration (MIC) of extracts against 12 selected foodborne pathogenic bacteria; (3) to evaluate the antibiofilm and anti-QS activities; (4) to verify extracts ability to protect a surface against bacterial biofilm formation; (5) to determine the chemical composition of the most active extracts.

2. Materials and methods

2.1. Chemicals

Violet crystal was from Cicarelli (Santa Fe, Argentina). LIVE/DEAD BacLight Bacterial Viability Kit was obtained from Life Technologies (California, USA). Ciprofloxacin, vancomycin, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), N-hexanoyl-homoserine-lactone and elastin-congo red were purchased from Sigma-Aldrich (MO, USA). Other chemicals were of analytical grade quality. All culture media were from Britania (Buenos Aires, Argentina).

2.2. Plant material and extracts preparation

The commercial spice bay laurel was provided by Saborigal S.A. (Buenos Aires, Argentina).

Ground bay leaves (250 g) were successively extracted by maceration during 24 h at room temperature with solvents of increasing polarity, n-hexane, chloroform, ethyl acetate and, methanol (thrice) (5%, w/v). Total extracts with only methanol were also prepared. Each extract was concentrated to dryness by a rotary evaporator (Büchi, R-300) at less than 40 °C, obtaining n-hexane (HE), chloroform (CE), ethyl acetate (EAE), methanol (ME) and total methanol (TME) extracts. The dry extracts were stored at 4 °C until use (two months). The yields of extraction were determined using the following equation: yield (%) = (weight of extract/weight of dried plant material) x 100. For the biological assays, each extract was dissolved with dimethyl sulfoxide (DMSO, Sigma-Aldrich) to obtain stock solutions.

2.3. Biological activity

2.3.1. Foodborne bacterial strains

The pathogenic bacteria studied were: *Staphylococcus aureus* ATCC 6538, *S. aureus* ATCC 25904, *S. aureus* ATCC 29213, *S. aureus* ATCC 43300 (methicillin-resistant, MRSA), *S. aureus* ATCC 33591 (MRSA), *S. aureus* ATCC 33592 (MRSA), *S. aureus* ATCC 700698 (MRSA), *S. aureus* ATCC 700699 (vancomycin intermediate-resistant, VISA), *Pseudomonas aeruginosa* PAO1, *P. aeruginosa* PA14, *P. aeruginosa* ATCC 27853, *P. aeruginosa* LVP 60 (water isolation), *Escherichia coli* ATCC 35218, *E. coli* ATCC 700728, *E. coli* LHICA S58, *Salmonella enterica* ATCC 14028, *S. enterica* CECT 5326, and *S. enterica* CECT 4396.

2.3.2. Antibacterial assay

The minimal inhibitory concentration (MIC) of the extracts was determined by the serial agar dilution method according to the guideline M07-A8 of the Clinical and Laboratory Standard Institute (CLSI, 2009).

2.3.3. Antibiofilm activity

2.3.3.1. Bacterial growth. Bacterial suspensions were prepared in Tryptic Soy Broth (TSB), Luria Bertani (LB) or Brain Heart Infusion (BHI). Microtiter plate wells were filled with bacterial suspension (10^7 CFU/mL) and the extract solution (final concentrations 10 and 100 μg/mL). Vehicle controls were prepared with DMSO and each bacterial culture. Vancomycin (8 μg/mL) was employed as antibiotic control. The microplates were incubated at 37 °C for 24 h and the growth was detected using a microplate reader (Multiskan Go, Thermo) at 560 nm.

2.3.3.2. Biofilm development. Biofilms were developed according to O'Toole & Kolter (1998) with slight modifications. The supernatants of bacterial cultures prepared as described previously (see 2.3.3.1), was discarded after 24 h incubation and the biofilm fixed to the polystyrene was gently rinsed and stained with 0.1% w/v crystal violet. The absorbance was determined at 580 nm.

2.3.3.3. Biofilm metabolic activity measurement. The metabolic activity of cells in biofilm was analyzed using MTT as previously described (Luciardi, Blázquez, Alberto, Cartagena, & Arena, 2019). Biofilms were grown statically during as 24 h at 37 °C, after that the supernatant was discarded and replaced with 200 μL of extracts solutions in PBS and incubated again 24 h at 37 °C. Then the microplate was revealed with the MTT solution (0.25 mg/mL). The absorbance was determined at 570 nm.

2.3.4. Biofilm microscopy

2.3.4.1. Scanning electron microscopy (SEM). Biofilms of *S. aureus* formed in presence of the most active extracts and their respective controls were visualized by scanning electron microscopy (Molina et al., 2020). Positive controls with 2% DMSO and negative controls with 8 μg/mL vancomycin were included in all assays.

2.3.4.2. Confocal laser scanning microscopy (CLSM). Biofilm architecture in the presence and absence of bioactive extracts was visualized by confocal microscopy following a previously developed method (Molina et al., 2020). Biofilms formed on glass-bottom dishes containing the extracts were washed to remove unbound cells. Bacterial biofilms were stained with the fluorescent LIVE/DEAD BacLight Biofilm Viability kit that distinguishes between dead (red) and lives (green) bacteria. Ambiguous colors like yellow or orange have sometimes been observed in bacterial cells stained with the LIVE/DEAD kit. Yellow cells are considered viable, while orange cells are considered damaged (Boulos, Prevost, Barbeau, Coallier, & Desjardins, 1999). Images were acquired with an Olympus FV 1000 fluorescence microscope using the FV10-ASW software. The quantification of the fluorescence was determined using the ImageJ software (National Institutes of Health). Positive controls with 2% DMSO and negative controls with 8 μg/mL vancomycin were included in all assays.

2.3.5. Surface coating test

2.3.5.1. Impregnation of a polystyrene surface. Fragments (1.6 cm × 1.6 cm) were covered with two extracts solutions (10 and 100 μg/mL) according to Molina et al. (2020). The impregnated fragments were tested as is and after being subjected to a 24 h wash with distilled water. A spectral scan between 200 and 1000 nm using a Spectrophotometer Genesys 50 (Thermo Scientist) was carried out.

Table 1
Antimicrobial activity (MIC values, μg/mL) of laurel extracts.

Bacteria	Hexanic	Chloroformic	Ethyl acetate	Methanolic	Total methanolic	Ciprofloxacin
Gram negative						
Escherichia coli						
ATCC 35218	R	R	R	R	R	2
ATCC 700728	R	R	R	R	R	2
LHICA S58	R	R	R	R	R	0.125
Salmonella enterica						
ATCC 14028	R	R	R	R	R	2
CECT 5326	R	R	R	R	R	2
CECT 4396	R	R	R	R	R	2
Pseudomonas aeruginosa						
PA01	250	R	R	R	R	0.25
PA14	R	R	R	R	R	2
ATCC 27853	250	R	R	R	R	0.25
LVP 60	250	R	R	R	R	0.25
Gram positive						
Staphylococcus aureus						
ATCC 6538	500	250	125	R	500	0.25
ATCC 29213	500	500	500	R	500	0.25
ATCC 33592 MRSA	500	500	500	R	500	0.25
ATCC 43300 MRSA	500	500	500	R	500	0.25
ATCC 25904	500	500	500	R	R	0.25
ATCC 33591 MRSA	500	500	500	R	R	0.25
ATCC 700698 MRSA	500	500	500	R	R	>4
ATCC 700699 VISA	1000	1000	500	R	R	>4

R: resistant until 1000 μg/mL.

2.3.5.2. Biofilm biomass measurement and metabolic activity of cells in the biofilm formed on covered surfaces. Covered fragments (washed and not washed) were inoculated with *S. aureus* ATCC 6538 for 24 h at 37 °C as previously described in 2.3.3.1. After incubation, the fragments were rinsed with PBS to remove the unbound cells. Then, the biofilm formed in the fragments was evidenced with violet crystal (see 2.3.3.2) and MTT (see 2.3.3.3).

2.3.6. Swarming motility

The swarming motility was examined as was described previously (Ha, Kuchma, & O'Toole, 2014). Briefly, LB agar (0.5%) was cooled at a temperature between 45 and 50 °C and then supplemented (DMSO 2% or extracts at 100 μg/mL) before being poured into Petri dishes. Gelled plates were inoculated at their center with 2 μL cultures of *E. coli* ATCC 35218, *E. coli* LHICA S58, *S. enterica* ATCC 14028, *S. enterica* CECT 5326, *P. aeruginosa* PAO1, and *P. aeruginosa* LVP 60. Bacteria spreading from the inoculation spot were measured after 24 h at 37 °C. The diameter of swarming motility was measured and compared with the positive control (DMSO 2%).

2.3.7. Elastase B activity and pyocyanin quantification

Each bay leaf extract at 100 μg/mL was added to LB broth containing a culture of *P. aeruginosa* PAO1 ($OD_{560} = 0.08$) and was incubated at 37 °C overnight. In the supernatants, elastase activity was assessed using an elastin-congo red reagent (Rudrappa & Bais, 2008) and the pyocyanin concentration was determined after extractions with chloroform and 0.2 M HCl (Musthafa, Sivamaruthi, Pandian, & Ravi, 2012).

2.3.8. Coagulase and hemolysin inhibition assays

S. aureus ATCC 6538 cultures ($OD_{560} = 0.08$) were grown for 24 h at 37 °C and 150 rpm in presence and absence of bay leaf extracts (100 μg/mL). For coagulase, the Bae, Kim, Kim, and Paik (2019) method with slight modifications was used. Tubes containing human plasma were inoculated with bacterial cultures; the mixtures were incubated during 4 h at 37 °C and observed each 30 min. For hemolysin assay, cultures were added to dilute human red blood cells in PBS buffer. Mixtures were incubated at 37 °C for 1 h at 150 rpm. Optical density was measured at 450 nm (Larzábal et al., 2010).

2.3.9. Anti-QS assays

The *Chromobacterium violaceum* QS system was used for this assay. QS in the wild type strain *C. violaceum* CECT 494 is known to control violacein production (a purple pigment) in response to the synthesis of N-acyl-L-homoserine lactones (AHLs) autoinducer molecules such as C6-acyl homoserine lactones and C4-acyl homoserine lactones. In the case of *C. violaceum* CV026 and VIR07 mutant strains, exogenous autoinducers (short-chain AHLs and long-chain AHLs, respectively) must be added for the violacein production. The well diffusion method was employed to detect the anti-QS activity of coriander extracts (Galván et al., 2018). In this test, bacterial growth inhibition results in a clear halo around the well, while a positive result of QS inhibition is exhibit by a turbid halo harboring pigmentless bacterial cells.

2.4. Toxicity assay with Galleria mellonella model

Groups of ten larvae in the final stage weighing 220–260 mg were used. Larvae were exposed at concentrations until 500 mg/kg of the extracts. Solvent control (PBS with 2% DMSO) and death control (DMSO) were also assayed. Larvae were observed daily up to 5 days and were evaluated according to survival. The larvae were considered dead when they did not show any movement in response to touch (Silva et al., 2017). Experiments were performed by triplicate.

2.5. Gas-chromatography-mass spectrometry analysis

GC-MS analysis was carried out using a mass spectrometer (5977A Agilent) and a gas chromatograph (Agilent 7890B) apparatus, equipped with a capillary column (95 dimethylpolysiloxane - 5% diphenyl), Agilent HP-5MS (30 m long and 0.25 mm i.d. with 0.25 μm film thickness). The column temperature program was 60 °C during 5 min, with 3 °C/min increases up to 180 °C, then 20 °C/min increases up to 280 °C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the m/z 30–650 range with an ionizing voltage of 70 eV. The resulting individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using co-chromatographed standard hydrocarbons relative to C_8–C_{32} *n*-alkanes, and mass spectra with reference samples or

Table 2
Effect of laurel extracts on biofilm formation (violet crystal method).

Bacteria	Biofilm formation (%)										
	HE		CE		EAE		ME		TME		Control
	Concentration (μg/mL)										
	10	100	10	100	10	100	10	100	10	100	
Escherichia coli											
ATCC 35218	95 ± 7^{ab}	80 ± 9^{bcd}	100 ± 6^{a}	74 ± 10^{cd}	99 ± 7^{a}	80 ± 7^{bcd}	86 ± 11^{abc}	62 ± 11^{d}	81 ± 10^{abc}	80 ± 9^{bcd}	100 ± 8^{a}
LHICA S58	75 ± 2^{bc}	78 ± 4^{bc}	80 ± 7^{bc}	70 ± 4^{bc}	84 ± 11^{b}	83 ± 10^{b}	74 ± 10^{bc}	67 ± 12^{c}	74 ± 8^{bc}	77 ± 11^{bc}	100 ± 7^{a}
Salmonella enterica											
ATCC 14028	100 ± 4^{a}	82 ± 2^{c}	99 ± 2^{a}	97 ± 8^{ab}	100 ± 8^{a}	79 ± 2^{c}	100 ± 2^{a}	99 ± 4^{a}	85 ± 9^{bc}	82 ± 6^{c}	100 ± 5^{a}
CECT 5326	87 ± 3^{bc}	62 ± 3^{d}	100 ± 2^{a}	100 ± 3^{a}	86 ± 4^{bc}	90 ± 5^{abc}	80 ± 4^{c}	80 ± 7^{c}	95 ± 7^{ab}	93 ± 3^{ab}	100 ± 6^{a}
Pseudomonas aeruginosa											
PAO1	99 ± 1^{ab}	63 ± 2^{e}	100 ± 1^{a}	78 ± 3^{d}	95 ± 1^{ab}	85 ± 3^{c}	99 ± 3^{ab}	100 ± 4^{a}	97 ± 1^{ab}	94 ± 3^{b}	100 ± 3^{a}
LVP 60	98 ± 2^{a}	60 ± 3^{d}	95 ± 4^{a}	69 ± 2^{c}	99 ± 6^{a}	81 ± 4^{b}	100 ± 3^{a}	100 ± 4^{a}	100 ± 2^{a}	100 ± 3^{a}	100 ± 3^{a}
Staphylococcus aureus											
ATCC 6538	98 ± 4^{a}	31 ± 6^{cd}	100 ± 4^{a}	24 ± 9^{d}	99 ± 10^{a}	25 ± 13^{d}	100 ± 3^{a}	80 ± 8^{b}	100 ± 3^{a}	41 ± 4^{c}	100 ± 8^{a}
ATCC 25904	68 ± 5^{bcde}	45 ± 9^{f}	80 ± 7^{bc}	63 ± 9^{de}	80 ± 7^{b}	51 ± 8^{ef}	75 ± 8^{bcd}	63 ± 10^{cde}	68 ± 6^{bcde}	60 ± 10^{de}	100 ± 9^{a}

Hexanic (HE), Chloroformic (CE), Ethyl acetate (EAE), Methanolic (ME) and Total methanolic (TME) extracts. Values (mean ± SD, n = 4) in the same row followed by a different letter are significantly different (Tukey test, $p \leq 0.05$).

with data already available in the NIST 11 mass spectral library and in the literature (Adams, 2007).

2.6. Data analysis

Data are presented as mean ± SD from at least three independent experiments. The statistical significance of differences between mean values was evaluated by Tukey's test. A value of $p \leq 0.05$ was considered significant.

3. Results

3.1. Extract yields

Extract yields of *Laurus nobilis* leaves prepared by the maceration method using different solvents were determined. The highest yields were 10.6 and 9.1%, for TME and ME, respectively followed by HE, CE, and EAE (1.5, 1.4, and 1%, respectively).

3.2. In vivo toxicity assay in Galleria mellonella larvae

Extracts solutions administered to larvae hemocoel, at concentrations up to 500 mg/kg did not result in death or visible damage, indicating that the extracts were not toxic in this *in vivo* model. While in the death control all larvae died on the second day of incubation (data not shown).

3.3. Extract effects on bacterial growth

In general, the extracts were unable to inhibit the growth of Gram (−) strains except for the HE that presented a MIC of 250 μg/mL against three of the four strains of *P. aeruginosa* tested.

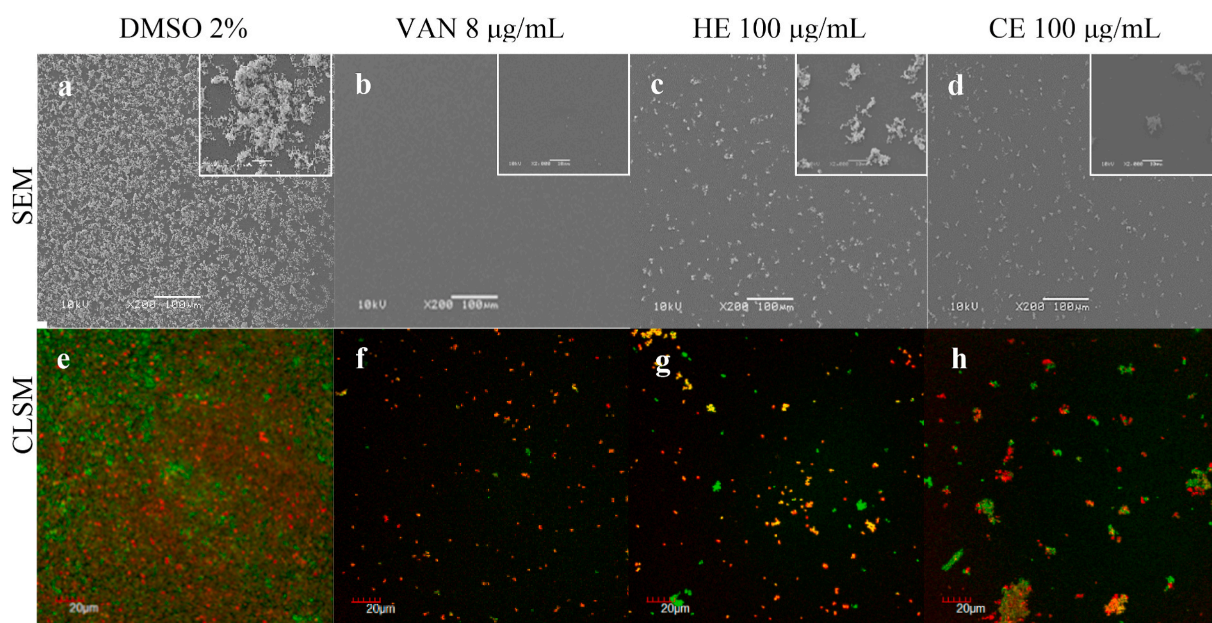

Fig. 1. *S. aureus* ATCC 6538 biofilms formed in presence and absence to laurel extracts. Scanning electron microscopy (SEM) images: Control with DMSO 2% shows biofilm formation after 24 h of incubation (a). Treatments: Vancomycin (VAN) 8 μg/mL (b), Hexanic (HE) 100 μg/mL (c) and Chloroformic (CE) 100 μg/mL (d) extracts. Scale bars: 100 μm (inserts in the images represent 10 μm). Confocal laser scanning microscopy (CLSM) images: Control with DMSO 2% shows biofilm formation after 24 h of incubation (e). Treatments: Vancomycin (VAN) 8 μg/mL (f), Hexanic (HE) 100 μg/mL (g) and Chloroformic (CE) 100 μg/mL (h) extracts. Scale bars: 20 μm.

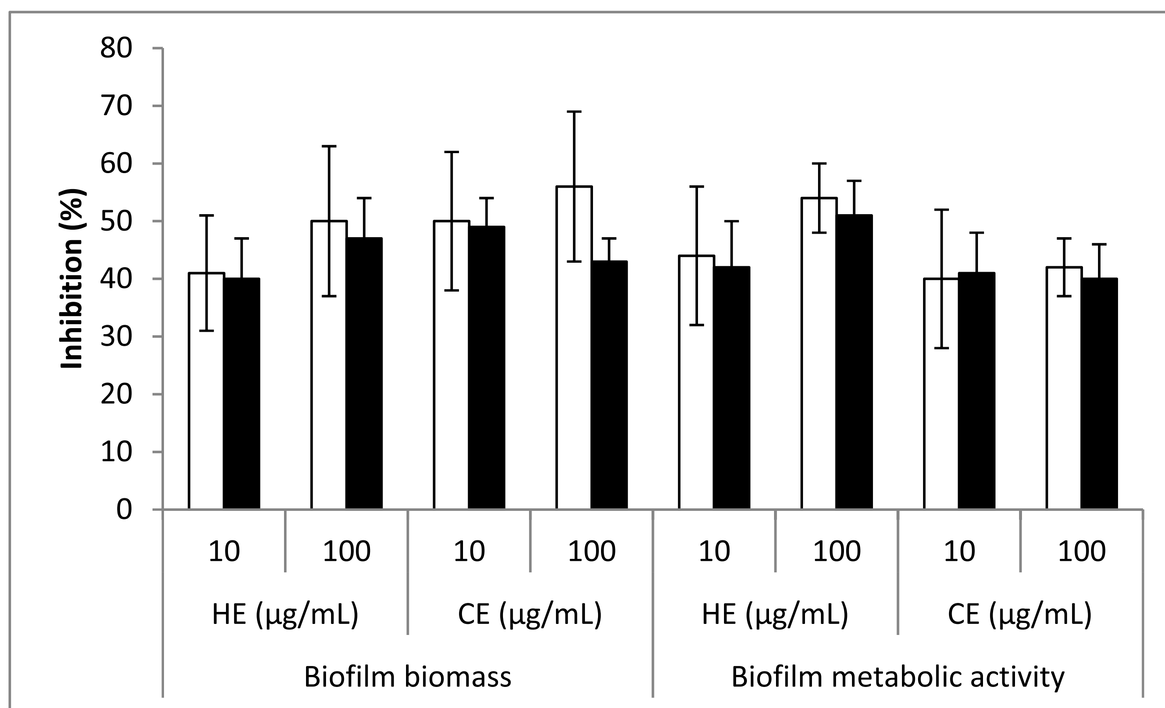

Fig. 2. *S. aureus* ATCC 6538 biofilm inhibition on polystyrene surfaces coated with 10 and 100 µg/mL Hexanic (HE) and Chloroformic (CE) laurel extracts, washed (■) and unwashed (□). Results are expressed as means ± standard deviation (n = 3).

Most *S. aureus* strains were sensitive to the extracts except for ME. The lowest MIC corresponded to the EAE for the *S. aureus* ATCC 6538 strain (125 µg/mL), followed by CE (250 µg/mL) (Table 1). It is important to emphasize that the growth of all the MRSA strains was inhibited with HE, CE and EAE (MIC 500 µg/mL). As well, MICs of 1000 µg/mL (HE and CE) and 500 µg/mL (EAE) were obtained for the VISA strain tested.

Concerning the assay carried out at microplates, the growth inhibition of all Gram (−) bacteria tested was less than 40% by all bay leaf extracts (100 µg/mL), being null for *E. coli* strains ATCC 35218, *S. enterica* CECT 5326 and for both strains of *P. aeruginosa*. While for *S. aureus* ATCC 6538 strain the growth was strongly inhibited by 100 µg/mL of the EAE (76%), which is consistent with the MIC results obtained.

3.4. Biofilm formation inhibition

All extracts, at 100 µg/mL, affected the biofilm formation of both *E. coli* strains with inhibitions below 40%. While the biofilm of *S. enterica* strains was poorly inhibited by the extracts, except for 100 µg/mL HE against CECT 5326 strain (38%). Similarly, low or no activity on the decrease of biofilm formation of both *P. aeruginosa* strains was observed, except by HE and CE (22–40%) at 100 µg/mL. In contrast, the biofilm production of the *S. aureus* strains was strongly inhibited by the extracts at a concentration of 100 µg/mL, up to 76% and 55% by the strains ATCC 6538 and ATCC 25904, respectively. The HE (100 µg/mL) was the only extract able of inhibiting the biofilm formation of all strains tested. Likewise, the CE and EAE extracts also showed significant inhibition of biofilm production of the species *E. coli, P. aeruginosa*, and *S. aureus* (Table 2).

Because the best biofilm inhibitory activity was obtained against

Table 3
Effect of laurel extracts on a 24 h preformed biofilm metabolic activity (MTT method).

Bacteria	Biofilm metabolic activity (%)										
	HE		CE		EAE		ME		TME		Control
	Concentration (µg/mL)										
	10	100	10	100	10	100	10	100	10	100	
Escherichia coli											
ATCC 35218	93±9^{ab}	67±6^{c}	96±9^{a}	73±9^{c}	98±4^{a}	77±9^{c}	99±4^{a}	75±6^{c}	97±7^{a}	80±5^{bc}	100±4^{a}
LHICA S58	78±6^{bc}	82±5^{b}	72±6^{bc}	74±4^{bc}	70±5^{c}	71±4^{bc}	70±1^{c}	74±4^{bc}	77±6^{bc}	78±5^{bc}	100±4^{a}
Salmonella enterica											
ATCC 14028	98±4^{a}	70±6^{b}	100±1^{a}	74±3^{b}	91±7^{a}	97±3^{a}	99±5^{a}	94±4^{a}	68±6^{b}	53±8^{c}	100±5^{a}
CECT 5326	80±4^{b}	74±3^{bc}	100±3^{a}	79±7^{b}	100±3^{a}	99±6^{a}	100±6^{a}	100±4^{a}	69±5^{bc}	60±5^{c}	100 ± 12^{a}
Pseudomonas aeruginosa											
PAO1	72±3^{bc}	75±9^{bc}	79±3^{bc}	86±5^{ab}	73±6^{bc}	65±8^{c}	86±8^{b}	84±9^{b}	76±9^{bc}	70±9^{bc}	100 ± 10^{a}
LVP 60	91 ± 10^{a}	92 ± 12^{a}	91±3^{a}	94±9^{a}	92±8^{a}	91 ± 13^{a}	94±6^{a}	92 ± 10^{a}	92±6^{a}	91 ± 11^{a}	100±4^{a}
Staphylococcus aureus											
ATCC 6538	90±3de	84±1^{ef}	90±1^{d}	79±5^{f}	97±2^{abc}	100±1^{ab}	91±4^{cd}	98±2^{ab}	100±3^{ab}	94±3^{bcd}	100±1^{a}
ATCC 25904	87±3^{a}	89±7^{a}	100±3^{a}	92 ± 11^{a}	96±8^{a}	91 ± 11^{a}	98±5^{a}	86±3^{a}	100±3^{a}	98±3^{a}	100±7^{a}

Hexanic (HE), Chloroformic (CE), Ethyl acetate (EAE), Methanolic (ME) and Total methanolic (TME) extracts. Values (mean ± SD, n = 4) in the same row followed by a different letter are significantly different (Tukey test, $p \leq 0.05$).

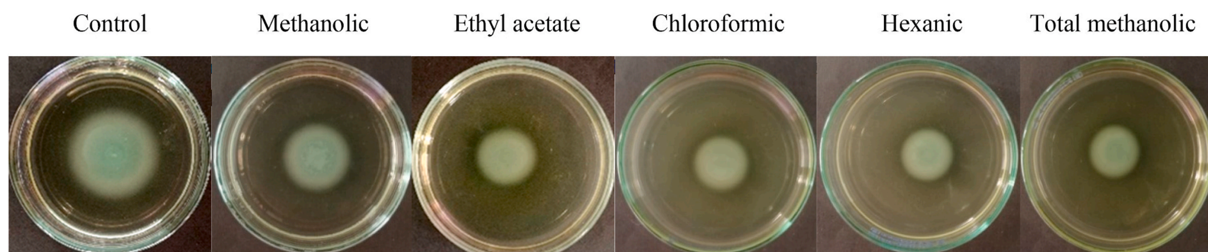

Fig. 3. *Pseudomonas aeruginosa* PAO1 swarming motility on presence of 100 μg/mL laurel extracts. Control (DMSO 2%).

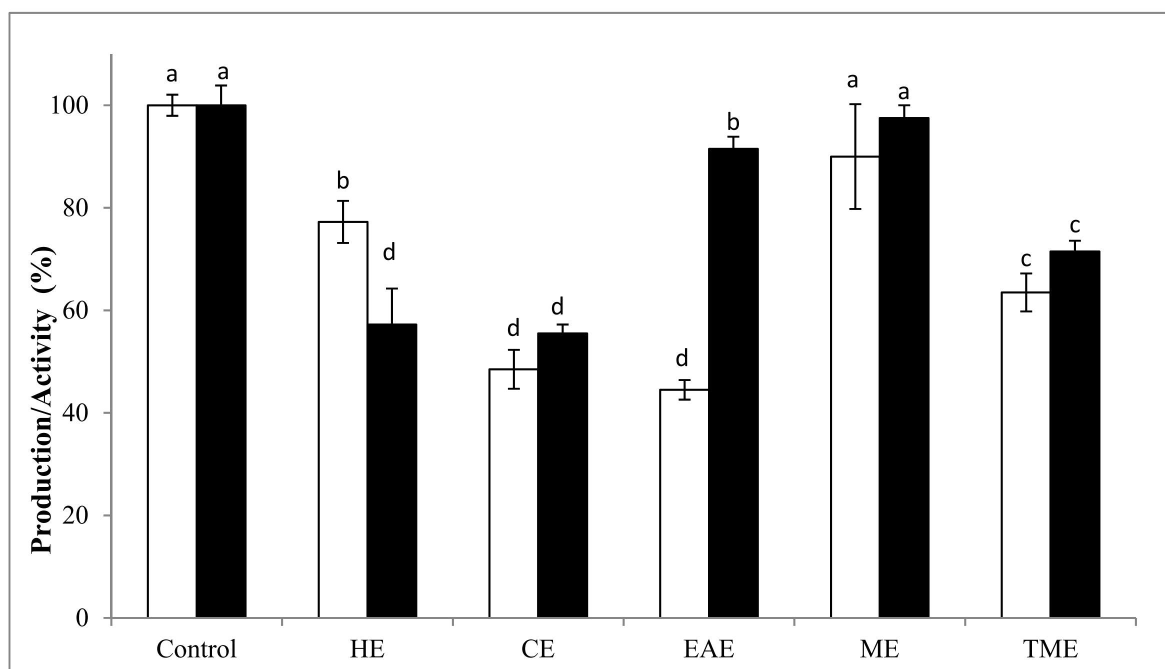

Fig. 4. Effect of 100 μg/mL Hexanic (HE), Chloroformic (CE), Ethyl acetate (EAE), Methanolic (ME) and Total methanolic (TME) laurel extracts on *Pseudomonas aeruginosa* PAO1 pyocianin production (□) and elastase activity (■). Results are expressed as means ± standard deviation (n = 3). Different letter are significantly different (Tukey test, $p \leq 0.05$).

S. aureus strain ATCC 6538 with HE and CE, they were selected for microscopic studies. SEM and CLSM images of the treatments with both extracts exhibited not only a strong reduction in adherent cells (Fig. 1c and d), but also several of these cells were dead (Fig. 1g and h), compared with the untreated controls (Fig. 1a and e). In fact, total (green and red) arbitrary fluorescence units (AFU) decreased from 102 (control) to 22 (HE) and 31 (CE). Additionally, CLSM images shown that the antibiofilm effect of extracts was similar to vancomycin (antibiotic control) (Fig. 1f), with 16 AFU.

To evaluate that HE and CE could protect a surface against biofilm formation, polystyrene fragments were covered with 10 and 100 μg/mL of those extracts, and the biofilm biomass and metabolic activity were measured. It was observed a decrease in *S. aureus* biofilm biomass and activity between 40 and 60%, without significant difference among different treatments, indicating that the bacterial adhesion was less favorable when the surfaces were coated with the extracts. It is important to highlight that the protective effect of extracts remained after 24 h washing (Fig. 2).

3.5. Biofilm metabolic activity inhibition upon preformed biofilms

The metabolic activity of the Gram (+) strains biofilm was practically not inhibited by the extracts, while better inhibitions were obtained with Gram (−) strains, although all were less than 50% and no extract affected the biofilm of the *P. aeruginosa* LVP 60, water isolated strain. It is important to emphasize the inhibition of the biofilm metabolic activity of *S. enterica* strains by TME (40–47%) at 100 μg/mL. Again, HE (100 μg/mL) was the most effective in inhibiting the metabolic activity from six of the eight strains tested, followed by CE and TME (Table 3).

3.6. *P. aeruginosa* virulence factors inhibition

Swarming agar containing bay leaf extracts were inoculated with the mobile bacteria *P. aeruginosa* PAO1 and the average radios of bacterial motility were determined (Fig. 3). A significant inhibitory effect ($p < 0.05$) on the motility of *P. aeruginosa* PAO1 was observed with all the bay leaf extracts, being higher than 30% for EAE, HE, CE, and TME. The inhibitory effects of 100 μg/mL bay leaf extracts on pyocyanin production and elastolytic activity are shown in Fig. 4. The pyocyanin reduction was 23, 35, 50, and 54% for HE, TME, CE, and EAE, respectively, while the elastase activity decrease was 29, 43, and 45% for TME, HE, and CE, respectively.

3.7. *S. aureus* virulence factors inhibition

Coagulase activity of *S. aureus* was inhibited by 100 μg/mL bay leaf extracts based on the increased time required for human plasma

Table 4
Anti-coagulant and anti-hemolytic activities of laurel extracts.

Sample	Coagulation time (min)	Hemolysis inhibition (%)
Control	30[a]	0±1[a]
HE	90[b]	22±1[d]
CE	90[b]	60±1[d]
EAE	90[b]	6±1[b]
ME	90[b]	12±1[c]
TME	90[b]	20±2[d]

Hexanic (HE), Chloroformic (CE), Ethyl acetate (EAE), Methanolic (ME) and Total methanolic (TME) extracts. Values (mean ± SD, n = 3) in the same row followed by a different letter are significantly different (Tukey test, $p \leq 0.05$).

coagulation (Table 4). Bay leaf extracts produced a 60 min lag in coagulation time compared to control groups not exposed to extracts. While, in the hemolytic activity assay the best inhibition was found with TME, HE, and CE (20, 22, and 60%, respectively).

3.8. *C. violaceum* bioassay

Except ME, all extracts showed anti-QS activity (colorless opaque circle around the wells) against the three strains used, being the inhibition very clear for the mutants CV026 and VIR07. For all the biosensor strains, DMSO did not show any inhibition of violacein production. (Fig. 5).

3.9. *Chemical composition of the bioactive extracts*

In the HE, linolenic acid (31.7%), vitamin E (20.3%), hexadecanoic acid (11.3%), α-terpinyl acetate (6.1%) and methyl eugenol (3.9%), were the main compounds, while a large amount of oleic acid (18.6%) followed by 1,8-cineole (5.2%), α-terpinyl acetate (4.5%), hexadecanoic acid (4.4%), methyl eugenol (3.4%), linalool (3.3%) and linolenic acid (3.0%) were found in the CE (Table 5, Figs. S1 and S2 supplementary material).

4. Discussion

The present study focused on the antimicrobial, antibiofilm, anti-*QS* and virulence factors inhibitory capacity of different polarity bay leaf extracts against pathogenic microorganisms. In addition*, in vivo G. mellonella* assay showed that the extracts did not display toxicity.

From the results of this work, it is emphasized that many laurel extracts were effective in inhibiting the development of multidrug-resistant strains of *S. aureus* in planktonic state, such as methicillin resistant and vancomycin intermediate-resistant. However, 90% of bacteria exist in the biofilm state where resistance to antibiotics and disinfectants is greater than the planktonic state. At the sub-MIC concentration (100 μg/mL), all the extracts inhibited partially the biofilm of at least five to the eight strains tested, with HE being the one with the greatest spectrum of action to attenuate the biofilm formation of all of them. Likewise, biofilm formation in food factories represents a relevant public health problem because pathogens can cause food intoxications (*S. aureus)*, gastroenteritis (*E. coli, S. enterica*) and systemic diseases (*E. coli* O157:H7) (Galié et al., 2018). The results obtained show the potential of laurel as antibiofilm agent, especially against *S. aureus,* one of the major food spoilage and food poisoning bacteria (Miao et al., 2017). HE and CE were potent inhibitors of *S. aureus* biofilm biomass and the microscopies further confirmed these results an also revealed a lethal effect on the cells present in the sparse biofilm formed.

Biofilms develops quickly in food industry environments. For this reason, finding substances capable of inhibiting the stage of initial adhesion is crucial. In this work, the interference of 10 and 100 μg/mL of the most active extracts with the bacterial adhesion was demonstrated by coating polystyrene surfaces. The results indicate that both laurel extracts have preventive action on *S. aureus* biofilm formation. Additionally, they were also able to interfere with coagulase and hemolysin activities, two key virulence factors of *S. aureus* implicated in the pathogeneses of infections, that contribute to biofilm formation (Lee, Kim, Park, & Lee, 2017).

It has been demonstrated that many natural agents can attenuate the bacterial virulence by interfering with bacterial QS (Ćirić et al., 2019). The effect of laurel extracts on *P. aeruginosa* PAO1 was further evaluated by performing assays for QS-controlled virulence factors. HE and CE

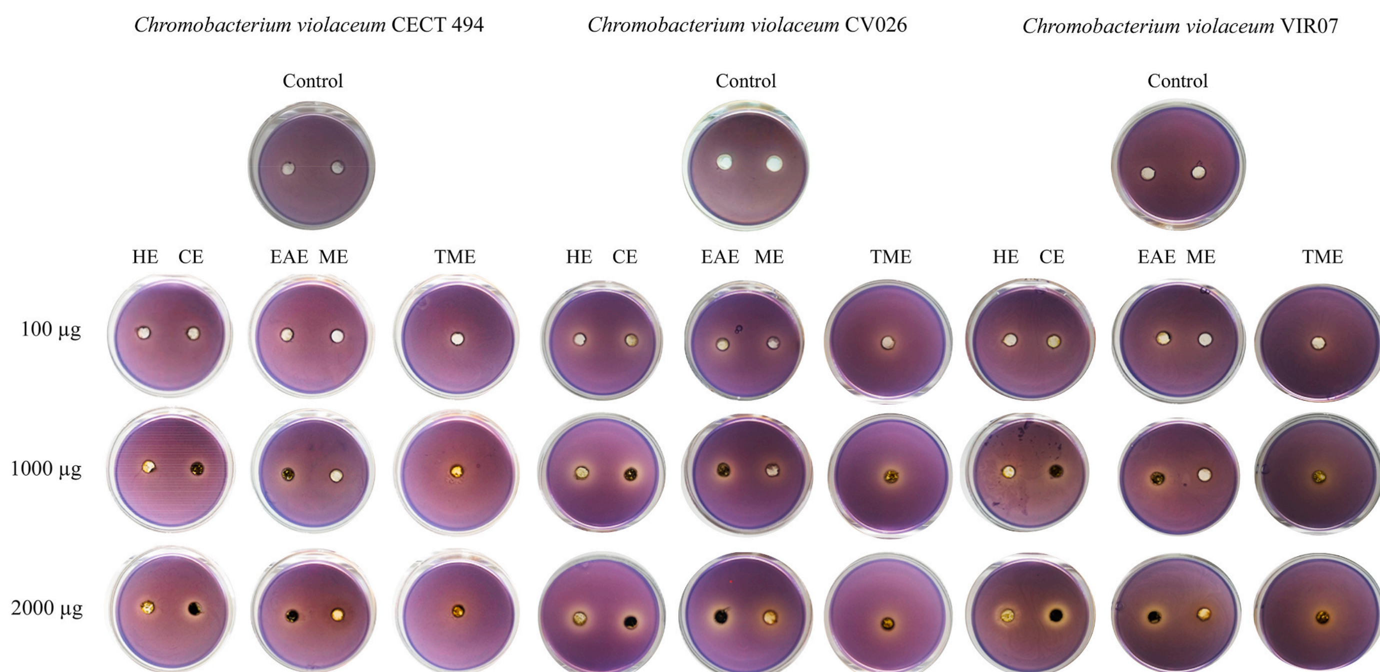

Fig. 5. Effect of Hexanic (HE), Chloroformic (CE), Ethyl acetate (EAE), Methanolic (ME) and Total methanolic (TME) laurel extracts on violacein production of *Chromobacterium violaceum* CECT 494, CV026 and VIR07. Control (DMSO).

Table 5
Chemical composition of Hexane (HE) and Chloroform (CE) extracts from bay leaves.

RT	RI_{Cal}	RI_{Ref}	Compound	Relative area (%)	
				HE	CE
		Monoterpene hydrocarbons		–	**0.43 ± 0.03**
10.683	1027	1029	Limonene	–	0.43 ± 0.03
		Oxygenated monoterpenes		**10.32 ± 0.47**	**14.75 ± 1.70**
10.803	1029	1031	1,8-Cineole	0.29 ± 0.03	5.21 ± 0.65
14.031	1094	1096	Linalool	1.81 ± 0.10	3.34 ± 0.32
17.784	1175	1177	Terpinen-4-ol	0.34 ± 0.01	–
18.491	1188	1188	α-Terpineol	1.76 ± 0.11	1.66 ± 0.17
25.395	1347	1349	α-Terpinyl acetate	6.12 ± 0.23	4.54 ± 0.57
		Sesquiterpene hydrocarbons		**0.27 ± 0.03**	**-**
32.299	1510	1513	γ-Cadinene	0.27 ± 0.03	–
		Oxygenated sesquiterpenes 3.38 ± 0.14			**0.10 ± 0.01**
34.75	1578	1578	Spathulenol	0.76 ± 0.06	–
34.925	1582	1583	Caryophyllene oxide	2.20 ± 0.13	–
37.698	1647	1650	β-Eudesmol	0.43 ± 0.07	–
	1735	1740	Oplopanone	–	0.10 ± 0.01
		Aromatic compounds		**4.71 ± 0.13**	**4.15 ± 0.51**
25.619	1352	1359	Eugenol	0.68 ± 0.05	0.73 ± 0.08
27.748	1401	1403	Methyl eugenol	3.87 ± 0.19	3.43 ± 0.44
33.763	1556	1557	Elemicin	0.16 ± 0.02	–
		Other compounds		**68.01 ± 1.60**	**25.90 ± 1.93**
46.555	1923	1921	Methyl hexadecanoate	0.29 ± 0.03	–
47.06	1963	1960	Hexadecanoic acid	11.27 ± 0.51	4.36 ± 0.71
48.697	2133	2133	Linolenic acid	31.71 ± 2.04	2.97 ± 0.14
48.922	2143	2142	Oleic acid	2.30 ± 0.37	18.57 ± 1.70
53.971	2901	2900	Nonacosane	2.18 ± 0.32	–
57.156	3115	3112*	Vitamin E	20.26 ± 3.94	–
			Total identified	**86.69 ± 0.83**	**45.33 ± 0.49**

Hexane (HE) and Chloroform (CE) extracts. RT: retention time (min); RI_{Cal}: retention index relative to C_8–C_{32} *n*-alkane on HP-5MS column; RI_{Ref}: retention index reported in Adams, 2007; *Kovats retention index reported in NIST Mass Spectrometry Data Center; Values are means ± standard deviation of the three samples.

inhibit biofilm formation, swarming motility, pyocyanin production, and elastase activity of *P. aeruginosa* PAO1. Since these extracts have the ability to inhibit the QS associated violacein production of *C. violaceum* strains but do not interfere with the growth of *C. violaceum* and *P. aeruginosa* PAO1, it suggests that the inhibitory effect on virulence factors is caused by disruption of QS signaling systems rather than by inhibition of bacterial growth. QS inhibitors have been reported from edible plants (Gilabert, Cartagena, Escobar, Bardón, & Arena, 2014), medicinal plants (Bouyahya, Dakka, Et-Touys, Abrini, & Bakri, 2017) and essential oils (Luciardi, Blázquez, Cartagena, Bardón, & Arena, 2016; Luciardi et al., 2019).

Chemical analysis revealed that the unsaturated fatty acids, linolenic plus oleic acids, were the main compounds in the most active extracts (HE and CE). It is interesting to note the high content of the saturated fatty acid hexadecanoic acid as well as vitamin E in the HE. A similar amount of this tocopherol was obtained also in the hexane extract (18.8%) from italian bay leaf samples with significant antioxidant activity in bovine brain (Conforti, Statti, Uzunov, & Menichin, 2006). In-line with our results, Lee et al. (2017) reported the antibiofilm and anti-hemolytic activities of unsaturated fatty acids against *S. aureus*. Biofilm formation by *S. aureus* has been reported to be inhibited *in vitro* by oleic acid (Grumezescu et al., 2011; Mirani et al., 2017) and linolenic acid (Lee et al., 2017). In regards to *P. aeruginosa*, has been informed the anti-QS, antibiofilm and anti-virulence (particularly swarming and pyocyanin) activities of linolenic acid (Chanda et al., 2017)

S. aureus antimicrobial and antibiofilm activities of essential oils extracted from *L. nobilis* were described (Merghni, Marzouki, Hentati, Aouni, & Mastouri, 2016), where oxygenated monoterpenes were the dominant components. Nevertheless, the active concentrations informed were far superior to those found in the present work. Therefore, the presence of oxygenated monoterpenes, the second predominant group of compounds identified in HE and CE could partially explain the biological effects observed.

5. Conclusions

This study demonstrated the ability of non-toxic bay leaf extracts to interfere the QS, to decrease the biofilm biomass and cell viability into a preformed biofilm and to attenuate the virulence factors production (elastase, pyocyanin, hemolysin, and coagulase). Laurel extracts offer a safe alternative to control contamination and/or spoilage of food, as well as infectious diseases, by targeting the biofilm and the virulence of the Gram (+) and Gram (−) bacteria. In addition, HE and CE could be interesting sources for the search for anti-pathogenicity compounds.

CRediT authorship contribution statement

Rocío Daniela Inés Molina: Formal analysis, Investigation, Methodology, Software, Writing - original draft. **Rodrigo Campos-Silva:** Investigation. **Myriam Anabel Díaz:** Investigation, Writing - review & editing. **Alexandre José Macedo:** Formal analysis, Funding acquisition, Supervision, Writing - review & editing. **María Amparo Blázquez:** Formal analysis, Investigation, Methodology, Writing - review & editing. **María Rosa Alberto:** Formal analysis, Methodology, Writing - original draft. **Mario Eduardo Arena:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing - original draft.

Declaration of competing interest

The authors have declared that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.109899.

Data availability

All data generated or analyzed during this study are included in this published article.

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