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Research Article

Anti-Quorum Sensing Activity of Argentinean Honey and Effect of pH on Main Flavonoids

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Abstract: In this study, the anti-QS activity of regional floral honey from the southeast region of Argentina was investigated using the bacterial model *Chromobacterium violaceum*. In addition, the anti-QS activity of quercetin, myricetin and luteolin was evaluated in an acidic medium. Two of the tested honey samples had the capacity to exert a notable anti-QS effect on *C. violaceum*. The effect of quercetin on the anti-QS activity at pH 5.8 was higher than myricetin and luteolin. The results obtained from the identification of quercetin, myricetin and luteolin of the honey extracts by High-Performance Liquid Chromatography (HPLC) showed that quercetin was the main flavonoid followed by myricetin and luteolin. It was observed that honey with high values of quercetin and high value of free acidity, exerted a greater effect on the inhibition of QS. Thus, we can conclude that an umbral value of pH seems to be necessary to increase the anti-QS activity of quercetin.

Keywords: Acidity, anti-QS activity, cell-to-cell communication, *Chromobacterium violaceum*, floral honey, main flavonoids

INTRODUCTION

The current quest for new antimicrobials is nowadays aimed at discovering non-toxic inhibitors of Quorum Sensing (QS) from natural sources which can be used to avoid the growth of bacteria. QS has been shown to modulate the expression of genes involved in processes related to survival, virulence and pathogenicity of many spoilage bacteria. Quorum Sensing is a signal system of bacteria to determine their population density through the synthesis, release and capture of autoinducers (Bassler, 1999; Brackman *et al*., 2009). This cell to cell signaling system is mediated by chemical signal molecules. Gram-negative bacteria use a QS system mediated by diffusible molecules of N-Acyl-Homoserine Lactone (AHLs) (Gram *et al*., 2002). In the past few years, studies have demonstrated that many plants produce anti-QS substances. Vasavi *et al*. (2014) showed anti-QS activity *Centella asiatica* against *Chromobacterium violaceum* and *Pseudomonas aeruginosa*. Vattem *et al*. (2007) demonstrated that various phytochemical extracts which inhibited QS also inhibited swarming of pathogenic bacteria, known to be modulated by QS. Traditionally honey has been considered to have antibacterial activity related to factors such as pH, sugar contents, hydroxide peroxide (H_2O_2) and the presence of phytochemicals, mainly phenolic compounds including phenolic acid, flavonoids, etc. (Mavric *et al*., 2008). Truchado *et al*. (2009) had reported that phenolic compounds of monofloral honey contributed to the non-peroxide anti-QS activity. Indeed, the action of flavonoids like quercetin, naringenin, etc., on the modulation of bacterial QS, biofilm generation and virulence factor has been reported (Vikram *et al*., 2010). Flavonoids like myricetin, kaempferol and quercetin can affect the expression of specific genes and then decrease the synthesis of QS molecules (Vandeputte *et al*., 2011). Therefore, the inhibition of QS of spoilage potential microorganism by honey is considered as a mechanism to avoid spoilage. Argentina is the third largest producer of honey and Buenos Aires is the largest honey-producing province in Argentina accounting for more than 50% of Argentina's honey production. Even though there are studies focused on antimicrobial activity of Argentinean honey (Fangio *et al*., 2010; Isla *et al*., 2011), there is no information about the anti-QS activity of regional honeys. In addition, the relationship between acidity and the anti-QS activity of major flavonoids was studied.

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Table 1: Floral origin, date of harvest and color of honey samples

Honey sample	Month/Year of harvest	Type of honey, main plant sources	Color (mm Pfund)
HC1	November 2015	Multifloral (eucalyptus, llanten, clover, thistle)	Extra white (17^{b})
HC2	November 2015	Multifloral (eucalyptus, thistle)	Extra clear amber (38.2^{c})
HC3	January 2015	Multifloral (eucalyptus, thistle)	White (32.2^{c})
HC4	March 2015	Multifloral (clover, thistle, dandelion)	Water white (1^{a})
HC5	December 2015	Multifloral (clover, thistle)	Clear amber (53.4^{d})

^{a,b,c,d}: The subscripts letters along the column indicate significant difference ($p<0.05$)

MATERIALS AND METHODS

Honey samples: Five floral honey samples collected during 2015 flowering season were used. Honey samples came from individual apiaries located at the southeast of Buenos Aires province (38° 0' S, 57° 33' O) in Argentina. Samples were stored at 4°C in the dark until their use. Details of the honey samples used in the assays are described in Table 1.

Honey physicochemical parameters: Moisture was determined with an Abbe refractometer. Liquid honey was placed directly on the prism and solid honey was first dissolved in a water bath (≤ 40°C). Readings were made at room temperature. Moisture content values were obtained from Chataway's Table (Bianchi, 1984). Free acidity was expressed as milliequivalents of NaOH/Kg of honey and was determined by acid-base titration. Honey samples (10 g each) were diluted in 75 mL of CO_2-free distilled water and titrated with a solution of NaOH (0.1 N) stirring constantly until reaching a pH of 8.5 using a pH-meter (HANNA model HI 9321) (Bianchi, 1984). The pH of honey samples was determined according to Iurlina and Fritz (2005).

The color was measured by the spectrophotometric method (Bath and Singh, 1999) with some modifications. Five grams of honey was dissolved in distilled water and filtered and the absorbance was read at 635 nm using an UV-vis Scanning spectrophotometer model Agilent 8453. Measures were correlated with Pfund's value scale (Ferreira *et al*., 2009).

Total phenolic content was determined using the Folin-Ciocalteu method (Singleton *et al*., 1999) with some modifications. 100 mL of the honey solution was mixed with the Folin-Ciocalteu reagent (2N) (100 µL), vortexed and after 5 min of repose, Na_2CO_3 (300 µL) was added and incubated in a water bath (40°C) for 30 min. Measurements were made at 765 nm. Results were obtained by extrapolation using a calibration curve (0 to 0.8 mg/mL) and expressed as milligrams of gallic acid equivalents in 100 g of honey (mg GAE/100 g).

Standards: Flavonoid aglycones used were quercetin dehydrate (minimum 98%), luteolin (99%) from Sigma (USA) and myricetin (≥95%) from Fluka (Switzerland). A stock solution was prepared in methanol HPLC grade (1 mg/mL) and stored at -20°C.

Flavonoid extraction: The flavonoid extraction was made using an adsorption resin Amberlite XAD-4 (Fluka Chemie; pore size 9 nm, particle size 0.3-1.2 mm) (Iurlina *et al*., 2009). For each honey sample, 50 g was dissolved with five parts of water pH 2 (adjusted with concentrated HCl), filtered and then put onto the resin column (40×1.2 cm). The column was washed with acid water pH 2 (100 mL) and subsequently washed twice with water pH 7 (200 mL). The whole phenolic fraction was then eluted with methanol (100 mL) and concentrated under reduced pressure (40°C) using a rotary evaporator model Büchi 461. The phenolic compounds were extracted with ethyl ether (5 mL×3) and the solvent was removed by flushing with nitrogen. The dried residue was redissolved in 1 mL of methanol (HPLC grade), membrane filtered (0.2 µm) and analyzed by HPLC. Extracts were stored at -20°C until their use.

HPLC analysis of honey flavonoids: A SHIMADZU model Prominence Analytical HPLC (diode array detector) system was used. The analysis of flavonoid extracts was carried out using a reversed-phase column end-capped (150×4.60 mm) Gemini 3 µm C18 110 Å (Phenomenex, USA). Elution was performed at a solvent flow rate of 1 mL/min using a gradient of methanol (solvent A) and water:formic acid, 19:1 (v/v) (solvent B) (Iurlina *et al*., 2009). The chromatograms were recorded at 340 nm. The identification of chromatographic peaks was based on the retention time compared with standard solutions, quercetin (11.5 min), myricetin (24.8 min) and luteolin (25.5 min). The concentration of quercetin, myricetin and luteolin were calculated using the external standard method based on peak area. The calibration curves of each flavonoid were prepared from each stock solution diluted with methanol to obtain working solutions from 0.01 to 0.1 µg/µL. The concentrations were correlated with the measured area.

Strain and culture conditions: *C. violaceum* wild-type strain ATCC (American Type Culture Collection) 12472 (Malbrán Institute, Argentina) was used for the anti-QS assays. Bacterial strain was cultured aerobically in Luria-Bertani (LB) broth supplemented with 1.5% (w/v) of sodium chloride (NaCl). Cell counts were made on LB agar (1.2%) supplemented with 1.5% (w/v) of NaCl and incubated at 30°C for 24 h.

Agar-well diffusion assay: The agar-well diffusion assay was performed to determine the pigment inhibition of honey samples to obtain a qualitative

Table 2: Physicochemical characterization of honey

Honey sample	Moisture (%)	pH	Free acidity (meq NaOH/Kg of honey)	Total phenolic compounds (GAE*/100 g of honey)
HC1	20± 0.81[a]	3.85± 0.07[a]	32.19± 0.27[b,c]	14.42± 0.1[c]
HC2	17± 0.82[a]	3.92± 0.04[a]	33.06± 0.04[b,c]	8.92± 0. 06[b]
HC3	17.8± 0.61[a]	4.03± 0.02[a]	24.36± 0.4[b]	7.61± 0.04[a,b]
HC4	17.6± 0.83[a]	4.44± 0.1[a]	16.53± 0.06[a]	7.36± 0.08[a]
HC5	16.8± 0.62[a]	4.5± 0.1[a]	17.4± 0.32[a]	10.73± 0.17[b]

* Equivalents of Gallic Acid; Values are expressed as arithmetic mean± standard deviation (n = 3); [a,b,c]: The subscripts letters along the column indicate significant difference ($p<0.05$)

screening. LB agar (1.2%) plates were made by adding approximately 10^5 CFU/mL of an overnight culture of *C. violaceum*. Wells were filled with 20 µL of each honey concentration (1, 5, 10, 20, 30, 40 and 66% w/v, respectively). DMSO was used as negative control and H_2O_2 as positive inhibitory control. Plates were incubated for 24 h at 30°C to corroborate the inhibition of pigment production around the well. Concentrations of honey with bactericidal effect were not considered for subsequent assays. The experiment was carried out three times and was three replicates per honey concentration.

QS Inhibition assay: Flask incubation assays were performed to quantify the pigment production of *C. violaceum* against honey samples. Bacteria (10^5 CFU/mL) was grown in LB+ broth supplemented with 1, 2, 3, 4, 5, 8 and 10% (w/v) of honey and incubated at 30°C for 24 h in aerobic conditions. The quantification of violacein was carried out according to Choo *et al*. (2006) with some modifications. Two milliliters of the culture was centrifuged at 9000 rpm for 15 min to precipitate the insoluble violacein. Then the pellet was solubilized in Dimethyl sulfoxide (2 mL), vortexed to homogenize violacein and centrifuged at 9000 rpm for 15 min to remove cells. The absorbance of violacein supernatant was measured at 585 nm. The anti-QS effect was determined by measuring violacein and cell counts after 24 h of incubation.

Major flavonoid anti-QS activity: The anti-QS activity of myricetin, quercetin and luteolin was evaluated at pH 5.8 and 6.7 (pH<5 inhibits *C. violaceum* growth). The culture pH was adjusted to 5.8 with buffer 0.07M (KH_2PO_4/K_2HPO_4). A stock solution of each flavonoid was prepared in methanol (500 µg/mL). One hundred milliliters of LB broth was inoculated with 10^5 CFU/mL of an overnight culture of *C. violaceum* and spiked with different concentrations of individual flavonoids 11, 22, 59, 110, 185 and 370 µg, these flavonoid concentrations are usually found in floral honey from Buenos Aires province (Iurlina *et al*., 2009). The control sample consisted of incubating the microorganism in LB broth without adding the bioactive. Flasks were incubated at 30°C for 24 h.

Statistical analysis: Analyses were carried out in triplicate and the data were expressed as means ± standard deviations (SD), which were calculated using Excel (Microsoft Office, Version 2010). Statistical evaluations were based on an ANOVA test ($p = 0.05$) using the Microcal Origin 5.0 software program.

RESULTS AND DISCUSSION

Physicochemical parameters of honey: The results of moisture, free acidity, pH and total phenolic content of honey samples are presented in Table 2. Honey samples from the middle-east region of Argentina arising from the Buenos Aires province correspond, usually, to light-colored honey regardless of their mixed or monofloral origin (Iurlina *et al*., 2009). The color of the honey samples ranged from water white to clear amber and the average value according to Pfund method was 28.36±18.11 mm (Table 1). The province of Buenos Aires is situated in the zone called 'the wet Pampa' that is an extended plain tempered region with rain levels between 500 and 1000 mm/year. Clover, lotus, eucalypts, sunflower and thistle are part of the vegetation of apicultural interest (Cabrera, 1976). Similar results of pH, free acidity and humidity were obtained by Malacalza *et al*. (2005) and Acquarone *et al*. (2007) who studied honey coming from Buenos Aires province. Free acidity presented significant differences between honey samples, HC1, HC2 and HC3 had the highest values, around 29.87 milliequivalents of NaOH/kg of honey, meanwhile, HC4 and HC5 presented the lowest values, around 16.97 milliequivalents of NaOH/kg of honey. The pH and moisture values showed no significant differences ($p>0.05$).

In our study, the total phenolic content of the samples ranged from 7.36 to 14.42 mg GAE/100 g. Popova *et al*. (2007) found a correlation between the concentration of total phenolic in the propolis and its antimicrobial properties. In addition, the phenolic content of honey samples has also been associated with antimicrobial activity (Küçük *et al*., 2007).

HPLC analysis of quercetin, myricetin and luteolin in honey samples: Table 3 shows average values of quercetin, myricetin and luteolin of the five honey samples. The three flavonoids were present in all honey samples with the exception of luteolin in sample HC2. Quercetin was the main flavonoid found in the samples studied. Samples HC1, HC3 and HC4 had the highest values; between 1410 and 1930 µg/100 g of honey. On the other hand, myricetin values

Table 3: Quercetin, myricetin and luteolin content of honey samples

	Flavonoids (µg/100 g of honey)		
Honey sample	Quercetin	Myricetin	Luteolin
HC1	1840± 14.38^{c}	210± 16.99^{d}	30± 4.11^{b}
HC2	220± 10^{a}	6.6± 0.29^{a}	ND
HC3	1820± 4.49^{c}	24± 3.56^{b}	19± 1.25^{a}
HC4	1930± 10.49^{c}	18.3± 1.43^{b}	45± 3.86^{c}
HC5	1410± 11.86^{b}	96± 6.02^{c}	17± 3.39^{a}

ND: Not detected; Values are expressed as arithmetic mean± standard deviation (n = 3); ^{a,b,c,d}: The subscripts letters along the column indicate significant difference ($p<0.05$)

varied between 6.6 and 210 µg/100 g of honey with significant differences ($p<0.05$) and luteolin content varied between 17 and 45 µg/100 g of honey with significant differences ($p<0.05$). In accordance with the floral origin of our samples, Martos *et al*. (2000) studied European *Eucalyptus* honey and found a common and characteristic flavonoid composition, where myricetin, tricetin, quercetin, luteolin and kaempferol are quite constant in their concentration and relative amounts.

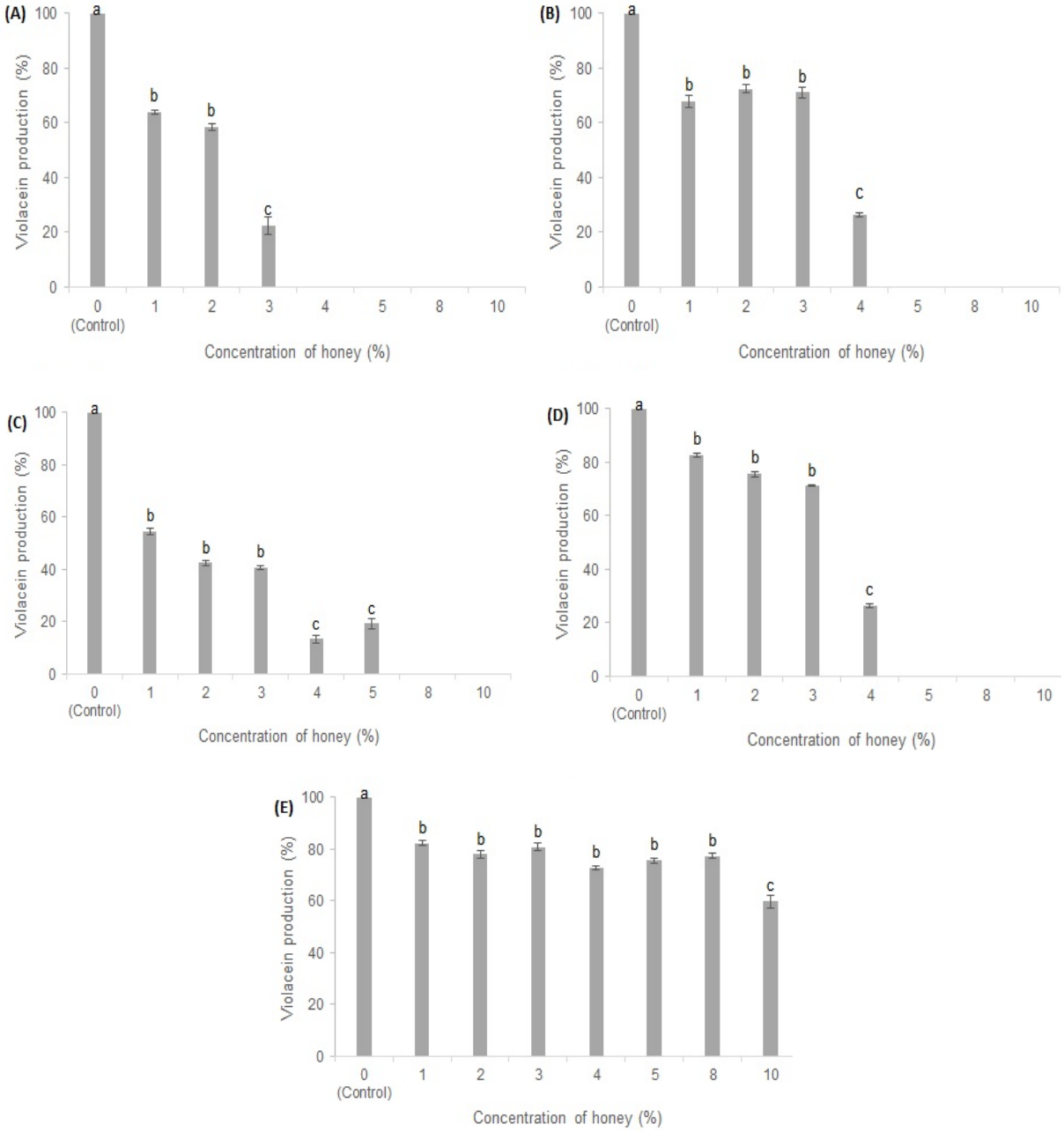

Fig. 1: Inhibition of violacein production by honey using *Chromobacterium violaceum* as indicator strain. HC1 (A), HC2 (B), HC3 (C), HC4 (D) and HC5 (E). The absorbance of measured violacein was transformed in percentage with the untreated (control) set as 100%. Data are represented as mean ± standard deviation, n = 3. Different letters in the columns are significantly different ($p<0.05$)

Anti-QS activity of honey: The agar-well diffusion assay was performed with *C. violaceum* to determine the pigment inhibition of honey samples. Different concentrations were used to obtain a qualitative screening of the sensibility of the bacteria. No differences were observed in the production of violacein between honey samples when were tested at concentrations below 10% (w/v). Concentrations higher than 10% (w/v) of honey were needed to produce complete inhibition in *C. violaceum* growth. Therefore, the flask-incubation assay to quantify the inhibition of violacein production by honey was carried out using concentrations from 1% to 10% (w/v). Figure 1 shows the percentage of violacein production at different concentrations of honey. Honey samples HC1 and HC3 showed the highest anti-QS activity (Fig. 1A and 1C), while HC2 and HC4 showed the lowest inhibitory activity (Fig. 1B and D) and HC5 did not show anti-QS activity at the concentrations assayed. Truchado *et al.* (2009) found that unifloral honeys produced a significant drop in violacein production, even at the lowest tested concentration (0.1 g/mL) indicating its anti-QS activity. The inhibition produced in our studies was considerably higher as previously reported by Truchado *et al.* (2009). Honey HC1 at 3% (w/v) reduced violacein production by more than 70% (Fig. 1A) and HC3 honey at 2% (w/v) reduced violacein production more than 50% (Fig. 1C). Considering the results obtained, the Minimum Quorum Sensing Inhibitory Concentration (MQSIC) of the honey samples was estimated (Table 4). The MQSIC is designated as the effective concentration of bioactive at witch 50% of the QS activity was reduced. Estimation was developed by linear regression after the logarithmic transformation of the violacein production data for different concentration of each honey (data shown in Fig. 1). The MQSIC of honey samples was lower to the concentration at which bacterial growth (*C. violaceum*) showed significant differences compared with control cells. As it was explained before, HC1 and HC3 honey samples were those with the lowest MQSIC (Table 4) demonstrating their high anti-QS capacity at low concentration, 3.16% to HC1 and 3.23% to HC3. Samples HC1 and HC3 were characterized by high content of quercetin 1840 µg/100 g and 1820 µg/100 g of honey, respectively. In addition, free acidity of HC1 and HC3 showed values above 24 milliequivalents of NaOH/kg of honey. On the other hand, HC2 presented low content of quercetin (220 µg/100 g of honey) and high acidity (around 32 milliequivalents of NaOH/kg of honey). Opposite that HC4 presented the highest content of quercetin (1940 µg/100 g of honey) but the lowest value of free acidity (16.53 milliequivalents of NaOH/kg of honey). We hypothesized that the anti-quorum sensing activity observed is related to the synergistic effect of quercetin and free acidity. Quercetin is a naturally occurring flavonoid which belongs to the group of phenolic compounds. The profiles of phenolic compounds in honey differ according to the floral origins (Campos *et al*., 1990).

Table 4: Minimum quorum sensing inhibitory concentrations (MQSICs) of honey samples against C*hromobacterium violaceum*

Honey sample	MQSIC (%)	R^2
HC1	3.16 (3.08-3.24)	0.84
HC2	3.8 (3.72-3.88)	0.82
HC3	3.23 (3.15-3.31)	0.87
HC4	4.16 (4.08-4.24)	0.91
HC5	11.9 (11.82-11.98)	0.70

MQSIC are presented as the mean value (95% confidence interval) and regression coefficient is included

Previous studies have linked the anti-QS activity of honey and propolis to flavonoids (Truchado *et al*., 2009; Alvarez *et al*., 2012). In addition, Borges *et al.* (2014) evaluated the quorum quenching activity of isolated phenolic compounds in the concentration of 1000 µg/mL and found that gallic acid, ferulic acid and caffeic acid reduced violacein production more than 59%.

Antimicrobial activity of honey: To evaluate whether the inhibition of violacein production is due to the reduction in the microbial growth or the AHL inhibition, the antimicrobial activity of honey was analyzed using *C. violaceum* as indicator (Fig. 2). None of the tested honey samples showed antimicrobial activity when applied at MQSIC (data not shown); although this concentration was enough to significantly reduce the violacein production to 50% (Fig. 1). In the case of samples HC2 and HC4, the concentration at which there was a significant ($p<0.05$) antimicrobial effect on *C. violaceum* was at 4% and 5% (w/v) of honey, respectively (Fig. 3), while HC1, HC3 and HC5 did not show antimicrobial activity when applied at 10% (w/v) of honey. Possibly, honey concentrations higher than those tested are needed to produce a significant reduction in *C. violaceum* growth.

Quercetin, myricetin and luteolin activity at pH 5.8: As it was mentioned before, a probable relation exists between the anti-QS activity, concentration of quercetin and the acidity. Therefore, the inhibition of violacein production by *C. violaceum,* due to quercetin, myricetin and luteolin in an acidified medium was also evaluated. The assays were performed employing the flavonoid solutions at pH 5.8. According to Movileanu *et al.* (2000), the bioactivity of flavonoids like quercetin is pH dependent and its polarity can change depending on the pH. On the other hand, Cushnie and Lamb (2005) proposed that flavonoids have an antibacterial effect depending on the number and distribution of hydroxyl groups in the molecule. Nevertheless, there is no information about the effect of flavonoids on the anti-QS system when the medium is acidified. Figure 3 shows the percentage of violacein production at increasing concentrations of the bioactive agents at pH 5.8 and 6.7 as control. Quercetin showed the highest anti-QS activity at pH 5.8 (Fig. 3A), while myricetin and luteolin showed the lowest inhibitory activity (Fig. 3B and 3C). Compared with control (pH 6.7), quercetin at 22 µg/100 mL produced a significant drop in

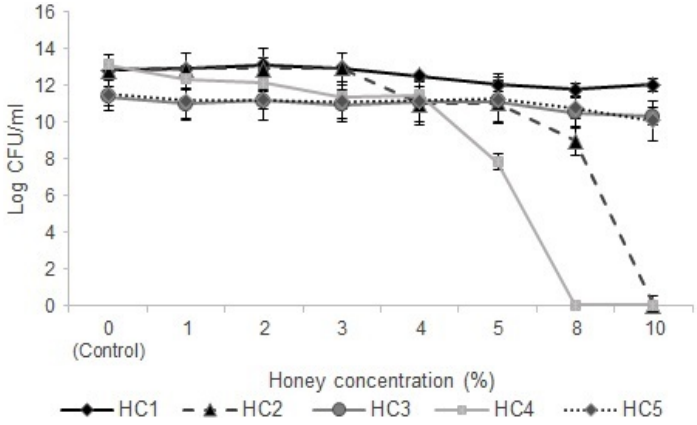

Fig. 2: Effect of increasing concentration of honey on the growth of *Chromobacterium violaceum*. Error bars represent standard deviation of three replicates

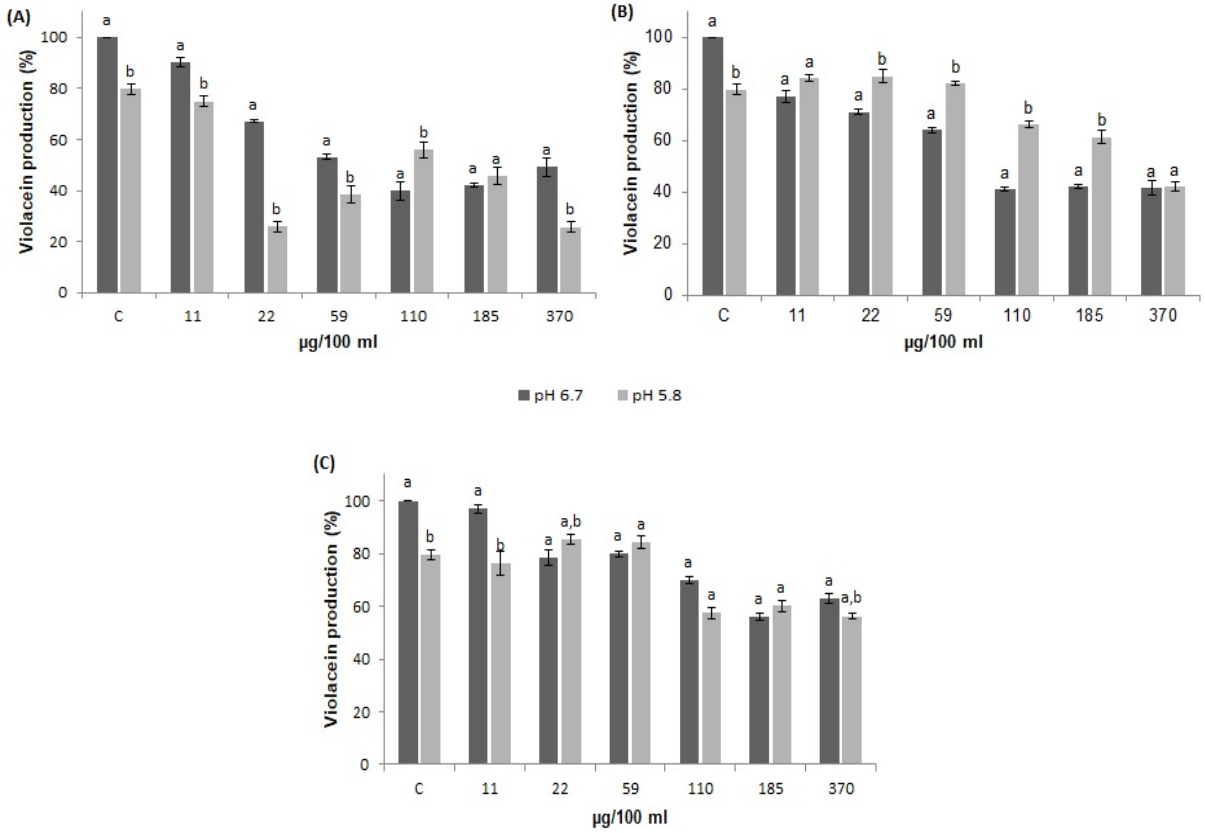

Fig. 3: Inhibition of violacein production at increasing concentrations of main flavonoids in acidified and non-acidified LB culture medium. Dark grey bars correspond to pH 6.7 and light grey bars to pH 5.8.; Quercetin (A) Myricetin (B) and Luteolin (C). The absorbance of measured violacein was transformed in percentage with the untreated (control) set as 100%

violacein production, more than 50% of the violacein synthesis was inhibited indicating strong anti-QS activity in an acidic medium. Studies carried out by Movileanu *et al*. (2000), demonstrated that the intercalation of quercetin molecules between the acyl chains of phospholipids of the lipid bilayer is significant at lower pH when the quercetin molecules are not dissociated. In this condition, molecules are completely liposoluble and they insert deeper into the hydrophobic core than at neutral or alkaline pH. Meanwhile, the inhibition of violacein by myricetin was significant at 110 µg/100 mL when pH was 6.7 (control), suggesting that the anti-QS activity of this flavonoid was not modified at pH 5.8. On the other

hand, the inhibition of violacein by luteolin was the same at pH 5.8 and 6.7. Considering the results of the anti-QS activity of the honeys tested, the free acidity of honey would modify the activity of quercetin present in honey. Therefore, the anti-QS activity of samples HC1 and HC3 was attributed to the combination of high content of quercetin and high acidity. On the other hand, the lowest anti-QS activity of sample HC2 could be attributed to the low concentration of quercetin (220 µg/100 g of honey) although the acidity of HC2 was high (33.06 milliequivalents of NaOH/Kg of honey). The same effect was observed with honey HC4 which had a high concentration of quercetin (1930 µg/100 g of honey) but presented low value of free acidity (16.53 milliequivalents of NaOH/kg of honey). Finally, the anti-QS effect of sample HC5 was not significant ($p>0.05$) because it was needed a concentration of 11.9% (w/v) of honey in order to reach 50% of inhibition of violacein production (Table 4).

CONCLUSION

In the present study, we determine a correlation between honey free acidity, quercetin concentration and the anti-QS activity. Quercetin was the dominant flavonoid in honey samples and it was considered the main cause of QS inhibition coming from honey. However, an acidity threshold value was necessary to potentiate the activity of this flavonoid. A free acidity value of 24 milliequivalents of NaOH/Kg of honey can modify the polarity of some phenolic compounds and thus determine their ability to adhere to biological membranes contributing to the anti-QS capacity. Therefore, the control of bacterial communication using honey typified on their phenolic content could be favored in an acidic medium.

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