Article

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Antimicrobial Activity of Berries Anthocyanin Extracts against Phytopathogenic Bacteria

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Abstract. Anthocyanins are compounds that give fruits a color ranging from red to purple, and berries are among those with the highest anthocyanin content. Anthocyanins are known to have antimicrobial activity against various pathogens, but their effectiveness against phytopathogenic bacteria has not yet been investigated. In this research, the antimicrobial effect of anthocyanin extracts from blueberry, raspberry, strawberry, and blackberry fruits against *Pseudomonas syringae*, *Pseudomonas aeruginosa* and *Clavibacter michiganensis* subsp. *michiganensis* was evaluated. Anthocyanin extract of each berry was obtained and characterized by high-performance thin layer chromatography, high performance liquid chromatography and by the differential pH assay. The extracts were confronted against phytopathogenic bacteria *in vitro* by the broth microdilution technique, evaluating their minimum inhibitory concentration (MIC) and their minimum bactericidal concentration (MBC). Likewise, the content of phenolic compounds, flavonoids and antioxidant capacity were determined. According to the results, the content of anthocyanin, total phenols and flavonoids in the extracts ranged from 48 to 963 mg eq C3G/100 g DW, 13 to 25 mg GAE/g DW and 0.1 to 0.5 mg QE/g DW, respectively. The extract with the highest antioxidant capacity was from blueberries. A 6.5 % MIC value of extract was observed for all berry extracts against *P. aeruginosa* and *C. michiganensis*. The smaller MBC value (12.5 % of extract) was observed for the strawberry and blackberry extracts against all the studied microorganisms. In general, anthocyanin extracts from all studied berries demonstrated antimicrobial effect against phytopathogenic bacteria, which opens an option for a more environmentally friendly control of these microorganisms.
Keywords: Anthocyanins; control; *Pseudomonas syringae*; *Clavibacter michiganensis*; *Pseudomonas aeruginosa.*

Resumen. Las antocianinas son compuestos que dan a las frutas un color que va del rojo al morado, y las bayas se encuentran entre las que mayor contenido de antocianinas tienen. Se sabe que las antocianinas tienen actividad antimicrobiana contra varios patógenos, pero aún no se ha investigado su eficacia contra bacterias fitopatógenas. En esta investigación se evaluó el efecto antimicrobiano de los extractos antociánicos de frutos de arándano, frambuesa, fresa y zarzamora contra *Pseudomonas syringae*, *Pseudomonas aeruginosa* y *Clavibacter michiganensis* subsp. *michiganensis*. Se obtuvo extracto antociánico de cada frutilla y se caracterizó mediante HPTLC, HPLC y por el ensayo de pH diferencial. Los extractos fueron confrontados *in vitro* contra las bacterias por microdilución en caldo, evaluándose su concentración mínima inhibidora y su

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concentración mínima bactericida. Asimismo, se determinó el contenido de compuestos fenólicos, flavonoides y capacidad antioxidante. Según los resultados, el contenido de antocianinas, fenoles totales y flavonoides en los extractos osciló entre 48 y 963 mg eq C3G/100 g PS, 13 a 25 mg GAE/g PS y 0.1 a 0.5 mg QE/g PS, respectivamente. El extracto con mayor capacidad antioxidante fue el de arándano. Se observó la CMI de 6.5 % en todos los extractos de frutillas contra *P. aeruginosa* y *C. michiganensis*. El menor valor de CMB (12,5 %) se observó en los extractos de fresa y zarzamora frente a todos los microorganismos estudiados. En general, los extractos antociánicos de frutillas demostraron efecto antimicrobiano contra las bacterias fitopatógenas, lo que abre una opción para un control de estos microorganismos más amigable con el medio ambiente.
Palabras clave: Antocianinas; control, *Pseudomonas syringae*; *Clavibacter michiganensis*; *Pseudomonas aeruginosa.*

Introduction

Plant pathogen control is an important issue regarding food security, since bacteria, fungi, viruses, and nematodes, among others, reduce crop yield. Tomato (*Solanum lycopersicum*) is the second most produced vegetable in the world; however, diseases caused by bacterial pathogens affect tomato foliage, reducing its production and leading to complete crop losses [1]. *Pseudomonas syringae*, *Pseudomonas aeruginosa* and *Clavibacter michiganensis* subsp. *michiganensis* are pathogens responsible for severe tomato disease [2]. *P. syringae* is the cause of the disease called "bacterial speck", characterized by isolated and watery green spots in leaves and fruits that turn brown with yellowish halos as the disease progresses and watery and white lesions in the roots [3,4]. *P. aeruginosa* is a phytopathogen that causes watery, white injuries on the roots of plants, causing them to rot [4]. *C. michiganensis* subsp. *michiganensis* causes the disease “bacterial canker”, which is characterized by wilting of the leaves, canker on the stem and peculiar bird’s eye spots on the fruits [5].

Sanitation practices are not sufficient to control outbreaks, and there are reports that antibacterial synthetic products promote resistance in pathogens and damage the environment as well as human health [6]. Natural extracts represent an alternative since their isolated compounds display not only antimicrobial activity but are also environmentally friendly [7]. In this sense, anthocyanins are phenolic secondary metabolites, from the flavonoid family, which give color, from red to purple, to different parts of the plant [8]. Among the fruits that have a high quantity of anthocyanins are berries such as blueberry (*Vaccinium myrtillus*), raspberry (*Rubus idaeus*), strawberry (*Fragaria ananassa*), and blackberry (*Rubus ulmifolius*) [9].

Anthocyanins are known to have human health benefits due to their antioxidant and anticancer properties, among others [10]. There are reports of the antimicrobial activity of anthocyanin extracts from berries; for example, blueberry and aronia (*Aronia melanocarpa*) anthocyanin extracts exhibited *in vitro* antimicrobial activity against the enteropathogenic bacteria *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus faecalis* being the blueberry extract the most effective [11]. In another study, anthocyanins from strawberries, cherries (*Prunus avium*), pomegranate (*Punica granatum*), grapes (*Vitis vinifera*), flame of the forest flowers (*Canna indica*), hibiscus flowers (*Hibiscus rosa-sinensis*), and guava leaves (*Psidium guajava*) showed *in vitro* antimicrobial activity against *S. aureus, E. coli*, and *Klebsiella pneumoniae* [12].

The antifungal effect of anthocyanins on phytopathogenic fungi has been studied, anthocyanin extracts from pomace of different grape varieties, successfully inhibited the growth of *Botrytis cinerea* [13]. Similarly, the *in vitro* inhibitory effect of anthocyanin extracts from grape varieties was investigated against fruit rot-causing fungi, including *B. cinerea, Mucor racemosus, Sordaria macrospora, Phoma herbarum, Phoma* sp., *Didymella* sp., *Aureobasidium pullulans*, and *Colletotrichum* sp., achieving up to 50 % growth inhibition [14]. On the other hand, anthocyanins from various berries tested *in vitro* against *Aspergillus flavus, Aspergillus niger, Penicillium* sp., and *Rhizopus* sp. inhibited the growth of all tested fungi, being the raspberry extract the one which displayed the most effective effect [15].

Despite the great advances in the research of anthocyanins as antimicrobial compounds, there is still no knowledge about their activity against phytopathogenic bacteria. Therefore, the objective of this research was to evaluate the *in vitro* antimicrobial activity of blueberry, raspberry, strawberry, and blackberry anthocyanin extracts against *P. syringae*, *P. aeruginosa and C. michiganensis* subsp. *michiganensis.*

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Experimental

Anthocyanin extracts

The extracts were obtained following the methodology of previous reports with some modifications [16, 17]. Four berries in maturity stage 5 (full maturity) according to the Mexican standard NMX-FF-132-SCFI-2018 were collected from a greenhouse located in the Tangancícuaro region in Michoacán, Mexico (9°53′20″N 102°12′18″ W): blueberry var. 'Biloxi', raspberry var. 'Adelita', strawberry var. 'Sayulita', and blackberry var. 'Tupi'. Berries were freeze-dried in a freeze dryer (Labconco, Kansas City, MO, USA) at 0.06 mbar for 72 h. Then, 1 g of grounded lyophilized fruit was added to 20 mL of ethanol- 1N HCl (85/15 v/v) and the pH was adjusted to 1 with a potentiometer (Hanna, Rhode Island, USA). Subsequently, the mixture was sonicated for 30 min in a sonicator (ULTRAsonik Denstply, Neytech, CA, USA) at 55±5 Hz at room temperature (22±1 °C), and centrifuged in a centrifuge (Centurion Scientific, Stoughton, UK) at 6000 rpm for 20 min, the supernatant was recovered. The procedure was performed until the supernatant was clear. Finally, each extract was concentrated to 5 mL with a rotary evaporator (Rotary evaporator r-200, BUCHI, Zurich, Switzerland). Samples were stored in a freezer (TorRey, Monterrey, Mexico) at -20 °C until use.

Detection and quantification of Anthocyanins by HPTLC

The detection and quantification of anthocyanins were carried out using the high-performance thin layer chromatography (HPTLC) technique in a CAMAG® automated equipment with VisionCats 2.1 software [18]. 20x10 cm silica plates G 60 F_{254} (Merck, Missouri, USA) were activated at 120 °C in a TLC plate heater (CAMAG, Muttenz, Switzerland) for 15 min. After cooling to room temperature, 2 µL of each extract was applied in triplicate to the plate obtaining a band of 8 mm in length with a syringe of 25 µL using the automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland) at a rate of 150 nL/s. Cyanidin 3-glucoside (C3G), pelargonidin 3-glucoside (P3G) and delphinidin 3-rutinoside (D3R) (Sigma-Aldrich, Missouri, USA) were used as reference anthocyanins, which were diluted with acidified methanol to 1.15 % (1N HCl) (methanol: Meyer, CDMX, Mexico; HCl: Fermont, Monterrey, Mexico) and adjusted to a concentration of 1.1 mg/mL. The applied concentrations were 2.2, 3.3, 4.4, 5.5, and 6.6 µg/mL for C3G; 1.1, 2.75, 5.5, 11, and 16.5 µg/mL for P3G; 0.55, 1.1, 1.65, 2.2, and 2.75 µg/mL for D3R. HPTLC development was carried out with a mixture of ethyl acetate, acetic acid, formic acid (Baker Analyzed, Madrid, Spain) and bidistilled water (produced by the reverse osmosis water purification system MILLPORE, model ELIX) (100:11:11:27, v/v/v/v) with Automated Developing Chamber 2 (ADC 2, CAMAG, Muttenz, Switzerland). The humidity was controlled at 47±2 % using a saturated solution of potassium thiocyanate (Sigma-Aldrich, Missouri, USA) (223 g/100 g of water) for 5 min. The migration distance was 60 mm from the bottom edge of the plate. At the end of the development, the plate was automatically dried with cold air for 5 min. Finally, the plate was derivatized using a 1 % ethanolic solution of 2-aminoethyl diphenyl borate (Merck, Missouri, USA) by vertical immersion at a speed of 5 cm/s through the device immersion (CAMAG, Muttenz, Switzerland); after that, the plate was dried at 40 °C for 3 min in the TLC plate heater. Images were recorded with the Visualizer documentation system equipped with a high-resolution 12-bit CCD digital camera (CAMAG, Muttenz, Switzerland).

The standard curves for C3G ($Y= 7.751*10^{-8}x + 1.215x*10^{-2}$, $R^2= 99.72$ %), P3G ($Y= -1.382*10^{-14}x^2 + 1.616*10^{-7}x + 1.379*10^{-2}$, $R^2= 99.07$ %), and D3R ($Y= -4.436*10^{-15}x^2 + 4.179*10^{-8}x + 1.776*10^{-2}$, $R^2= 98.52$ %) were obtained and the content of anthocyanins in the extracts was expressed as mg of the major anthocyanin equivalents/g of dry weight.

Quantification of total anthocyanins by the differential pH spectrophotometric method

Differential pH method was followed with some modifications to quantify the total anthocyanin content in the extracts [19]. Briefly, potassium chloride buffer (pH 1, 0.025 M) (Baker Analyzed, Madrid, Spain) and sodium acetate buffer (pH 4.5, 0.4 M) (Baker Analyzed, Madrid, Spain) were prepared. Subsequently, each extract was mixed with each buffer separately in a 1:15 ratio (v/v, buffer/extract). The absorbance of each sample was measured at the wavelength of C3G maximum absorbance (λmax= 535 nm) and at 700 nm in a spectrophotometer (Molecular Devices Co., Sunnyvale, USA). Equation 1 was used to determine the total anthocyanin content (TAC):

$$TAC\ (mg\ eqC3G/100\ g\ DW) = (A)(MW)(DF)(1000)/(\varepsilon \times 1) \quad \text{Eq 1}$$

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where: A= Absorbance of the diluted sample (Aλmax-A700 nm) pH 1 – (Aλmax-A700 nm) pH 4.5; MW= Molecular weight C3G (449.2 g/mol); DF= Dilution Factor (DF= Total volume of dilution/added volume of sample); ε= C3G molar absorbance (29,600). Three independent trials were performed in triplicate (n=9). Results were expressed as mg equivalents of cyanidin 3-glucoside per 100 g of dry weight (mg eqC3G/100 g DW).

Identification of phenolic compounds by HPLC

The high-performance liquid chromatography (HPLC) technique was used to identify the phenolic compounds in the studied extracts [20]. Extracts were filtered with 13-mm nylon acrodiscs with a 0.45 µm pore size (Waters, Massachusetts, USA) and analyzed on an Agilent 1100 Series HPLC system (Agilent Technologies, CA, USA) with a DAD detector under the following conditions: Zorbax Eclipse XDB-C18 Guard Column (4.6 mm x 12.5 mm, 5 µm, 400 mbar working pressure limit) and Zorbax Eclipse XDB-C18 Column (4.6 × 250 mm, 5 µm, 10 µL injection volume and velocity flow rate set to 0.750 mL/min). The mobile phase consisted of two diluents: eluent A (acidified water with formic acid at a ratio of 1:9) (Baker Analyzed, Madrid, Spain) and eluent B (acetonitrile) (Baker Analyzed, Madrid, Spain). The gradient used was 0 min [A:B 100/0], 15 min [A:B 75/25], 25 min [A:B 50/50], 26 min [A:B 25/75], 27 min [A:B 0/100], and four minutes under isocratic elution conditions. The compound identification was made based on the comparison of the chromatograms at λ = 240, 280 and 360 nm and retention times of the extracts run under the same conditions. The area under the curve of the samples was interpolated into standard linear equations.

Total phenolic content

The total phenolic content was determined following the UV-Visible spectroscopic technique at λ=700 nm in a spectrophotometer (PowerWave HT, Biotek Instruments, Vermont, USA) [21]. A calibration curve (A700= 3.5191 [gallic acid] + 0.1631, R^2= 0.9857) was made with 10 concentrations of gallic acid (Sigma-Aldrich, Missouri, USA) (0-0.8 mg/mL). The results were reported as milligrams of gallic acid per gram of dry weight (mg GAE/g DW). Assays were performed in triplicate.

Flavonoid content

The flavonoid content was determined using the UV-Visible spectroscopic method at λ = 425 nm in a spectrophotometer (PowerWave HT, Biotek Instruments, Vermont, USA) [22]. A calibration curve of quercetin (Sigma-Aldrich, Missouri, USA) (A425= 1.1549 [quercetin]+0.0447, R^2= 0.9899) with 8 concentrations (0-0-7 mg/mL) was made. The total content was reported as milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW). Assays were performed in triplicate.

Antioxidant capacity by ABTS, DPPH and HPTLC-DPPH assays

The antioxidant capacity of the extracts was obtained by the ABTS, DPPH and HPTLC-DPPH assays. For the ABTS method [23], an ABTS solution was prepared by dissolving 360 mg ABTS (Sigma-Aldrich, Missouri, USA) in 100 mL of distilled water. Consecutively, 100 mL of 2.45 mM potassium persulfate (Meyer, CDMX, Mexico) were added, and the mixture was refrigerated (4 °C) for 24 h in absolute darkness. After this time, its absorbance was adjusted to 0.7 with distilled water, and measured at λ =735 nm in a spectrophotometer (PowerWave HT, Biotek Instruments, Vermont, USA). 20 µL of each anthocyanin extract were placed in a microplate, and 280 µL of ABTS were added. After 15 min in absolute darkness, the absorbance was measured. A calibration curve of Trolox (Sigma-Aldrich, Missouri, USA) (µM TE= -5.9424 [A735] + 1.283, R^2= 0.992) was made with 5 concentrations (0-0.15 µM Trolox). The antioxidant capacity was reported as micromolar of Trolox per gram of dry weight (µM TE/g DW), and assays were performed in triplicate.

For DPPH method [24], 20 µL of extract were placed in a microplate. Consecutively, 200 µL of 150 µM DPPH methanolic solution (methanol: Meyer, CDMX, Mexico; DPPH: Sigma-Aldrich, Missouri, USA) were added. The plate was left at rest for 30 min in absolute darkness. Subsequently, the absorbance was measured at λ = 515 nm in a spectrophotometer (PowerWave HT, Biotek Instruments, Vermont, USA). A calibration curve of Trolox (µM TE= -3.0715 [A515 nm] + 0.5233, R^2= 0.9067) with 5 concentrations (0-0.15 mg/mL) was made. The antioxidant capacity was reported in micromolar of Trolox per gram of dry weight (µM TE/g DW). The assay was performed in triplicate.

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For HPTLC-DPPH method [25], 40 mg of DPPH were dissolved in 200 mL of methanol (Meyer, CDMX, Mexico). Plaque development was previously described in the section of quantification and identification of anthocyanins by HPTLC. Subsequently, it was derivatized with DPPH (Sigma-Aldrich, Missouri, USA) in the immersion device (CAMAG, Muttenz, Switzerland) vertically at a speed of 5 cm/s for 1 s. Then, the plate was dried at room temperature in total darkness for 30 min. Finally, the image of the plate was documented in the TLC viewer (CAMAG, Muttenz, Switzerland) and processed in CAMAG® version 2.4 VisionCATS software.

Antimicrobial activity

Assays of the antimicrobial activity of the extracts against *P. syringae*, *P. aeruginosa* and *C. michiganensis* subsp. *michiganensis* were performed. For this, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by the standard broth microdilution method [26].

100 µL of each extract were placed in a microplate and diluted 8 times serially in each well with 50 µL of sterile deionized water to obtain 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 % of extract. Consecutively, 100 µL of sterile King B broth (KB) (Pronadisa, Madrid, Spain) for *P. syringae*, Mueller-Hinton (MH) (BD Bioxon, State of Mexico, Mexico) for *P. aeruginosa,* and Luria Bertani (LB) (Pronadisa, Madrid, Spain) for *C. michiganensis* subsp. *michiganensis* were added. Finally, 20 µL of the respective bacterial aliquot to be evaluated adjusted to a density of $1x10^8$ CFU/mL were added. As a positive control, 20 µL of the antibiotic ciprofloxacin (Ciproflox®, Altia, CDMX, Mexico) at a concentration of 50 mg/mL, and as a negative control, 150 µL of broth were placed with 20 µL of the respective bacterial inoculum. Subsequently, the plates were incubated for 24 h at 24°C for *P. syringae* and at 37 °C for *P. aeruginosa* and *C. michiganensis* subsp. *michiganensis*. After the time elapsed, 100 µL of MTT tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich, Missouri, USA) was applied to each well of the plate and incubated for 1 h at the respective temperature of each bacterium. After that, the MIC was registered.

To obtain the MBC, 50 µL were taken from the wells without bacterial growth and placed in Petri dishes with the respective medium of each bacterium, spreading them throughout the dish with sterile glass beads, and incubated at their respective temperature for 24 h. Finally, it was observed whether there was bacterial growth in petri plates, and the results were recorded.

The MIC was defined as the lowest concentration of the extracts that visibly inhibited bacterial growth, and the MBC was defined as the concentration of the extracts that completely killed the bacteria. All assays were performed in triplicate with three replicates for each dilution and control.

Statistical analysis

An ANOVA analysis of variance with the Tukey test ($p < 0.05$) was performed on the obtained results using the SAS program in version 2.0.

Results and discussion

Detection and quantification of anthocyanins

Fig. 1 shows the chromatogram obtained by HPTLC for the extracts of berries and the reference anthocyanins. According to the results, the reference anthocyanins (tracks 13-17) have Rf= 0.44, 0.33, and 0.05, for P3G, C3G, and D3R, respectively. For blueberry samples (tracks 1-3), the most intense band corresponded to C3G (Rf 0.33). Meanwhile, for raspberry extracts (tracks 4-6), the band associated with D3R (Rf 0.05) was predominant. On the other hand, for the strawberry samples (tracks 7-9) the band with the highest intensity was observed at Rf 0.41, corresponding to P3G. Finally, for the blackberry samples (10-12), C3G showed the most intense band (Rf 0.33).

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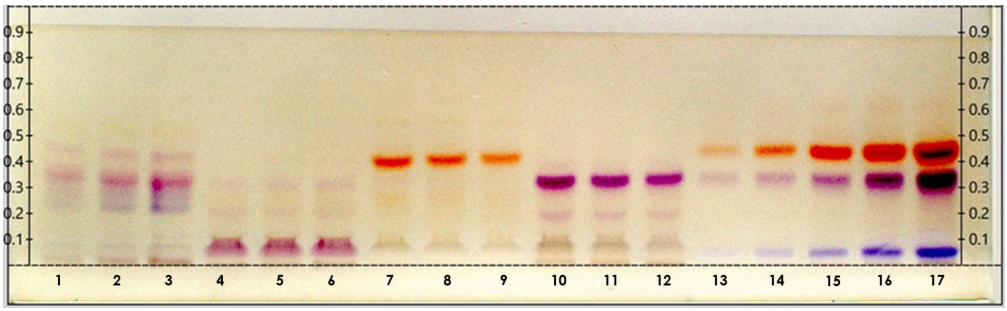

Fig. 1. HPTLC chromatogram of extracts and standard anthocyanins. Blueberry (tracks 1-3), raspberry (tracks 4-6), strawberry (tracks 7-9), and blackberry (tracks 10-12). Standard anthocyanins (tracks 13-17) in different concentrations: C3G (2.2, 3.3, 4.4, 5.5 and 6.6 µg/mL) Rf 0.33, P3G (1.1, 2.75, 5.5, 11 and 16.5 µg/mL) Rf 0.44 and D3R (0.55, 1.1, 1.65, 2.2 and 2.75 µg/mL) Rf 0.05.

The contents of the major anthocyanins for each extract obtained by HPTLC and the total anthocyanin content by differential pH method are shown in Table 1. C3G is the main anthocyanin in blueberry and blackberry. Meanwhile, D3R predominated in raspberry, and the P3G in strawberry. On the other hand, the content of total anthocyanins obtained by differential pH showed that there was a significant difference in the content of anthocyanin, with the blueberry extract having the highest content.

Table 1. Total anthocyanin content in extracts of berries determined by HPTLC and Differential pH method.

Berry	HPTLC	Differential pH (mg eq C3G/100 g DW)
Blueberry	140.7 µg/mL C3G	936.33±10.51[a]
Raspberry	745.9 µg/mL D3R	59.61±2.04[c]
Strawberry	190.8 µg/mL P3G	48.68±2.58[d]
Blackberry	537.8 µg/mL C3G	203.25±5.54[b]

Values represent the mean ± standard deviation of assays performed in triplicate. Different letters in the column indicate significant differences ($p \leq 0.05$) using Tukey's test.

Identification of phenolic compounds by HPLC

Table 2 shows the content of the identified phenolic compounds in the studied extracts by HPLC. Seven phenolic compounds were identified and quantified in blueberry (ellagic acid, catechin, rutin, chlorogenic acid, P-coumaric acid, ferulic acid, and quercetin), six standards were identified in raspberry (ellagic acid, epicatechin, synaptic acid, hydroxyphenylacetic acid, P-hydroxybenzoic acid, and rutin), and eight compounds were identified in strawberry and blackberry (ellagic acid, catechin, epicatechin, synaptic acid, gallic acid, P-hydroxybenzoic, rutin, and chlorogenic acid). Interestingly, ellagic acid and rutin were present in all the studied berries. However, the major compounds in blueberry were catechin and quercetin (both with 51.75 µg/g), the latter was found only in this fruit. On the other hand, the main compounds in strawberry extract were catechin and epicatechin (24.32 and 22.7 µg/g, respectively). Likewise, in the raspberry and blackberry extracts, epicatechin showed the highest amount (52.8 and 91.47 µg/g, respectively).

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Table 2. Phenolic compounds determined by HPLC in extracts of berries.

Identified compound	Berry (µg/g)			
	Blueberry	**Raspberry**	**Strawberry**	**Blackberry**
Ellagic acid	13.03	16.71	16.45	16.49
Catechin	51.75	ND	22.78	1.91
Epicatechin	ND	52.8	24.32	91.47
Synaptic acid	ND	0.84	0.23	1.29
Gallic acid	ND	ND	2.01	0.34
Hydroxyphenylacetic acid	ND	1.51	ND	ND
P-Hydroxybenzoic acid	ND	0.44	0.58	0.97
Rutin	17.96	6.39	4.25	5.12
Chlorogenic acid	35.94	ND	3.59	0.38
P-coumaric acid	1.56	ND	ND	ND
Ferulic acid	13.03	ND	ND	ND
Quercetin	51.75	ND	ND	ND
Cyanidin	2852.63	669.08	184.82	7037.49
Pelargonidin	0.35	0.02	0.07	0.038

ND= No detected.

Total phenolic and flavonoid content

The contents of the total phenolic and flavonoid compounds in the anthocyanin extracts are show in Table 3. According to the results, the berry that presented the highest total phenolics content was blackberry (25.14 mg GAE/g DW), and the extract with the lowest content was the raspberry (13.92 mg GAE/g DW). On the other hand, the highest content of flavonoids was found in blueberries (0.58 mg QE/g DW) which had 5 times more than the raspberry (0.1 mg QE/g DW). The strawberries and blackberries had similar amounts of flavonoids (0.36 and 0.3 mg QE/g DW, respectively).

Table 3. Total phenolics and flavonoids contents in extracts of berries.

Berry	**Total phenolics (mg GAE/g DW)**	**Flavonoids (mg QE/g DW)**
Blueberry	23.63 ± 0.23^{b}	0.58 ± 0.007^{a}
Raspberry	13.92 ± 0.05^{d}	0.1 ± 0.02^{d}
Strawberry	21.39 ± 0.11^{c}	0.36 ± 0.016^{b}
Blackberry	25.14 ± 0.09^{a}	0.3 ± 0.009^{c}

Values represent the mean ± standard deviation of assays performed in triplicate. Different letters in the column indicate significant differences ($p\leq0.05$) using Tukey's test.

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Antioxidant capacity by ABTS, DPPH and HPTLC-DPPH assays

Table 4 shows the antioxidant capacity of the extracts obtained by the ABTS and DPPH methods. According to the results, the berry that had the highest antioxidant capacity was blueberry (ABTS: 3.93 µM TE/g DW; DPPH: 6.38 µM TE/g DW), followed by blackberry (ABTS: 2.74 µM TE/g DW; DPPH: 4.38 µM TE/g DW). The strawberry and raspberry had similar antioxidant activity with the ABTS assay (1.21 and 1.42 µM TE/g DW, respectively), but the strawberry extract was superior than the raspberry extract with DPPH assay (2.73 and 1.77 µM TE/g DW respectively).

Table 4. Antioxidant capacity of extracts determined by the ABTS and DPPH assays.

Berry	ABTS (µM TE/g DW)	DPPH (µM TE/g DW)
Blueberry	3.93±0.3[a]	6.381±0.007[a]
Raspberry	1.42±0.1[c]	1.777±0.02[d]
Strawberry	1.21±0.1[c]	2.732±0.01[c]
Blackberry	2.74±0.1[b]	4.386±0.009[b]

Values represent the mean ± standard deviation of assays performed in triplicate. Different letters in the column indicate significant differences ($p \leq 0.05$) using Tukey's test.

The chromatogram obtained by HPTLC with DPPH derivatization (Fig. 2) displayed for the reference anthocyanins (tracks 13-17) and all the studied anthocyanin extracts (tracks 1-12) characteristic bands associated with antioxidant capacity. In addition, bands are also observed in the anthocyanin extracts of the berries studied with Rf different from those of the reference anthocyanins.

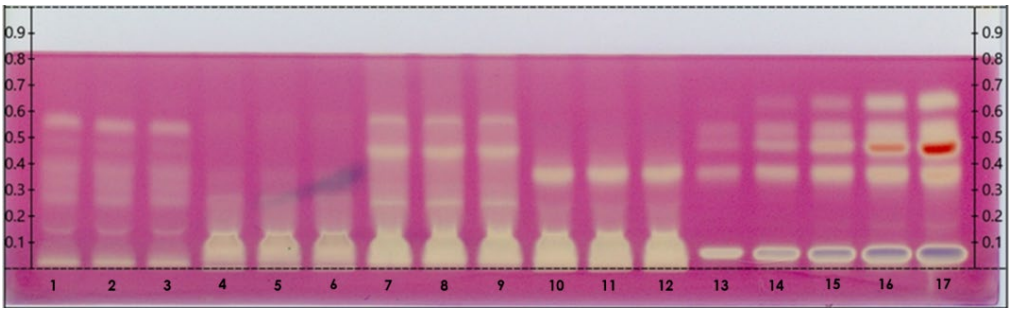

Fig. 2. HPTLC-DPPH chromatogram of the antioxidant capacity of anthocyanin extracts of berries. Blueberry (trakcs 1-3), raspberry (tracks 4-6), strawberry (tracks 7-9), and blackberry (tracks 10-12). Standard anthocyanins (tracks 13-17) in different concentrations: C3G (2.2, 3.3, 4.4, 5.5 and 6.6 µg/mL) Rf 0.33, P3G (1.1, 2.75, 5.5, 11 and 16.5 µg/mL) Rf 0.44 and D3R (0.55, 1.1, 1.65, 2.2 and 2.75 µg/mL) Rf 0.05.

Antimicrobial activity: minimum inhibitory concentration and minimum bactericidal concentration

The antimicrobial activity of the studied anthocyanin extracts against *P syringae*, *P. aeruginosa* and *C. michiganensis* subsp. *michiganensis* is shown in Table 5. In general, all the anthocyanin extracts of berries had antimicrobial activity against the bacteria. *P. syringae* showing the same sensitivity toward the blueberry, raspberry, and strawberry anthocyanin extracts, while blackberry extract exhibited a lower MIC value. Likewise, a concentration of 6.25 % of each extract is needed to inhibit bacterial growth, except for *P. syringae*; however, 12.5 % of the extract killed *P. syringae*. *C. michiganensis* subsp. *michiganensis* was killed at 12.5 %

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of strawberry and blackberry extract. The strawberry and blackberry extracts exhibited similar antimicrobial effects against all tested microorganisms.

Table 5. Antimicrobial activity of anthocyanin extracts of berries against *P. syringae, P. aeruginosa* and *C. michiganensis* subsp. *michiganensis*.

Berry	***P. syringae***		***P. aeruginosa***		***C. michiganensis* subsp. *michiganensis***	
Extract concentration (% dilution)	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Blueberry	12.5	12.5	6.25	50	6.25	50
Raspberry	12.5	12.5	6.25	50	6.25	25
Strawberry	12.5	12.5	6.25	100	6.25	12.5
Blackberry	6.25	12.5	6.25	100	6.25	12.5

MIC= Minimum Inhibitory Concentration. MBC= Minimum Bactericidal Concentration.

Discussion

The results obtained for the identification and quantification of the main anthocyanins in berry extracts using HPTLC are similar to reported studies. For example, P3G and C3G with an Rf of 0.46 and 0.34, respectively, were reported for berry extracts [18]. Likewise, the most intense band in blueberry was assigned to the C3G (Rf 0.33) [26]. In raspberry extracts, delphinidin has been found in greater quantity [27]. The most intense band in strawberry corresponded to P3G, which is the anthocyanin with the greatest presence in these fruits [28]. Finally, in the blackberry extract the band corresponding to C3G predominated, which has been reported as the main anthocyanin in blackberries [29].

On the other hand, the total anthocyanin content obtained by differential pH method was significantly different among the studied samples. In this regard, it has been shown that the concentration of anthocyanins and their profile may vary depending on the species, variety, and cultivation area where the plant was developed [30]. The anthocyanin content of blueberry and raspberry are comparable with values of 1045 and 59.5 mg C3G/100 g DW, respectively, previously reported [31]. A total of 37.1 mg of C3G/100 g DW in strawberry extracts has been reported, which agrees with the results obtained in this research [32]. These values are in the typical range for ripe strawberries (18-60 mg C3G/100 g) [33]. Finally, the blackberry content is comparable to that obtained in other investigations, in which 200 mg C3G/100 g DW was reported [34].

The identified phenolic compounds by HPLC coincided with previous reports [35,36]. Ellagic acid and rutin were present in all the extracts. Both compounds are recognized for their antioxidant properties [37]. Its presence in blueberry, raspberry, strawberry, and blackberry fruits has been previously reported [38]. Flavonoids as quercetin and its derivatives have been reported as the ones with the highest presence in blueberries [39]; these compounds are involved in the germination of seeds, the development of pollen, and are recognized as powerful antioxidants [40]. Likewise, catechin and epicatechin were present in strawberry anthocyanin extract; these compounds interact directly with reactive oxygen species [41]. There are reports confirming the presence of these compounds in strawberry fruits in large quantities [42]. Similarly, epicatechin predominated in raspberry and blackberry extracts; this compound fulfils functions in plants, such as protection against oxidative stress, defense against pathogens and regulation of abiotic stress [43]. Epicatechin has been reported to predominate in blackberries, temperature and light have been shown to influence the amount of this compound in fruits [44].

The total phenolic content demonstrated variation in the amount of these compounds depending on the type of berry. Similar quantities to those obtained in the present research have been reported for

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blackberry (27- 32 mg GAE/g DW) [45], blueberries (27.4 mg GAE/g DW) [46], strawberry (18.41 mg GAE/g DW) [47], and raspberry (12.88 mg GAE/g DW) [48]. On the other hand, the flavonoid content results agree with the HPLC determination, where blueberry has the highest content of flavonoids, with catechin and quercetin being the major flavonoids. It has been reported that quercetin content differs depending on the cultivar or cultivation conditions of different species [49]; in addition, factors such as climatic conditions, soil composition, and berry management are responsible of differences in bioactive compounds content [50].

The antioxidant capacity of berries is known and has been ascribed to phenolic compounds and ascorbic acid [51]. Likewise, it has been reported antioxidant capacity by ABTS assay for cyanidins with values ranging from 0.32 to 0.5 µM of Trolox [52]. Bands associated with antioxidant capacity were observed in the chromatogram obtained by HPTLC-DPPH at the sites corresponded to the anthocyanins. It has been shown that anthocyanins represent approximately two-thirds of the antioxidant power in berries, so it is possible that the antioxidant capacity in these berries is influenced by the presence of these compounds [51]. However, bands not assigned to anthocyanins were also observed, possibly, they are other phenolic compounds and flavonoids present in the studied extracts, which are known to possess antioxidant capacity [53].

To date, there are no reports on the antimicrobial activity of berry anthocyanin extracts against phytopathogenic bacteria, but some research reports have attributed antimicrobial activity to anthocyanins against pathogens such as *S. typhy*, *S. aureus,* and *E. coli*, among others [54].

In general, all the anthocyanin extracts from the berries showed antimicrobial activity to varying extents. The effect of a bioactive compound on bacteria depends on the bacterial species itself [55]. In this sense, *P. syringae* and *P. aeruginosa* are gram-negative bacteria, while *C. michiganensis* subsp. *michiganensis* is a gram-positive bacterium; their difference is that the former has a thin peptidoglycan wall in the middle of an internal bilipid membrane and an external membrane rich in lipopolysaccharides, which is selective for the entry and exit of compounds [56]. Therefore, it may be that some compounds present in each extract penetrate the cell more easily than others [57].

Anthocyanins are known to damage bacterial walls and membranes [58]. In this regard, using Scanning Electron Microscopy (SEM), the leakage of intracellular contents from foodborne pathogenic bacteria was demonstrated when confronted with anthocyanins from Chinese wild blueberries. Additionally, upon penetrating the bacterial membrane, anthocyanins reduce the enzymatic activity of alkaline phosphatase, adenosine triphosphatase, and superoxide dismutase, inhibiting bacterial growth. A reduction in the tricarboxylic acid cycle was also shown by the decrease in formazan production as the concentration of anthocyanins increased, along with a reduction in cellular protein content due to membrane alteration and suppression of protein synthesis [59]. In addition, the difference in the antimicrobial power of an extract depends on the variation in chemical components [60]. The anthocyanin extracts of the studied berries contained different compounds, so it was expected that their activity would also be different. Furthermore, it has been reported that the strongest antimicrobial effects of anthocyanins are observed when other compounds are involved in the mixture [58]; therefore, it is likely that compounds not yet identified in some berries have an antimicrobial effect.

Although each extract contains different compounds, it was observed that between 6.25 and 12.5 % of the total concentration was sufficient to inhibit bacteria growth. Likewise, blueberry, raspberry and strawberry extracts needed the same concentration to inhibit the growth and kill P. syringae (12.5 %). It is possible that these compounds act synergistically, enhancing the antimicrobial effect until achieving an intrinsically similar efficacy with a mechanism of action that may be common or complementary, but further experiments need to be performed.

On the other hand, the strawberry and blackberry extracts exhibited similar antimicrobial effects against all the tested microorganisms. According to HPLC results, the same compounds were identified in both berries. Likewise, their antimicrobial effect could be associated with more than one family of compounds, since additive and synergistic effects of secondary metabolites have been reported [61]; furthermore, a concentration dependent antimicrobial effect has been reported when confronting strawberry extracts *in vitro* against *E. faecalis* and *Porphyromonas gingivalis* [62]. The same effect was reported when testing strawberry extracts against *Salmonella choleraesuis*, *S. flexneri* and *E. coli* [63]. In the present study, the anthocyanin extracts of berries had the highest amount of anthocyanins, phenolics and flavonoids, so these compounds may be acting synergistically against bacteria, which increase susceptibility to their presence.

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Conclusions

The anthocyanin extracts of blueberry, raspberry, strawberry, and blackberry fruits showed high contents of anthocyanins and phenolic compounds and, consequently, high antioxidant capacity. In addition, all the evaluated anthocyanin extracts showed *in vitro* antimicrobial activity against *P. syringae*, *P. aeruginosa* and *C. michiganensis* subsp. *michiganensis*, possibly because of a synergistic action of secondary metabolites, including anthocyanins, flavonoids and phenolic acids. Interestingly, the extracts not only inhibited the growth of bacteria but also had bactericidal effects at similar concentrations. To the best of our knowledge, this is the first study in which *in vitro* effectiveness of anthocyanin extracts from berries against phytopathogenic bacteria has been reported. In future research, these data may be useful to test its effect directly on infected plants with these phytopathogens or for the development of strategies for its control.

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