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Original article

Antioxidant, anti-inflammatory, and antimicrobial activity of a fluid extract and anthocyanidins of *Vaccinium floribundum* Kunth (Mortiño) berries from Cotopaxi

Actividad antioxidante, antiinflamatoria y antimicrobiana de un extracto fluido y de antocianidinas de bayas de *Vaccinium floribundum* Kunth (Mortiño) de Cotopaxi

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Abstract

Vaccinium floribundum Kunth (QUPS Herbarium accession number 4803), commonly known as mortiño, is characterized by its high concentrations of bioactive compounds, particularly polyphenolic compounds, which play a significant role in its biological activity. Here, we aimed to investigate the role of anthocyanins after identifying and quantifying them using differential pH, thin-layer chromatography (TLC), and high-performance liquid chromatography (HPLC) on a fluid extract. Its biological activity was evaluated with antioxidant assays (ABTS and DPPH), which yielded antioxidant activity values of 833.47 µg/mL (ABTS) and 1418.01 µg/mL (DPPH). The anti-inflammatory activity, evaluated using membrane stability (MS) assays under thermal stress, showed maximum inhibition percentages of 32.31% and 34.26% for the anthocyanidins (cyanidin and delphinidin) at 1 µg/mL, with 92.56% inhibition in a 1000 µg/mL extract. Similarly, an additive effect was observed from the anthocyanins and flavonoids in the extract. Furthermore, antimicrobial evaluations using antibiograms and microplate dilution methods against urinary tract infection (UTI) pathogens demonstrated significant inhibition of *Staphylococcus saprophyticus* (50% extract, MIC = 3125 µg/mL, 55.29% inhibition), *Pseudomonas aeruginosa* (50% extract, MIC = 12500 µg/mL, 71.45% inhibition), *Escherichia coli* (50% extract, MIC = 12500 µg/mL, 35.04% inhibition), and *Candida tropicalis* (100% extract, MIC = 6250 µg/mL, 12.88% inhibition). Our findings suggest that the mortiño extract contains substantial levels of anthocyanins, with cyanidin being the predominant anthocyanin. Both anthocyanins and anthocyanidins exhibited significant antioxidant, anti-inflammatory, and antimicrobial activity, supporting the potential therapeutic value of mortiño in addressing oxidative stress, inflammation, and microbial infections.

Keywords: *Vaccinium floribundum;* antimicrobial activity; anti-inflammatory activity; antioxidant activity; urinary tract infections (UTIs); anthocyanidins.

Resumen

Vaccinium floribundum Kunth (número de registro en el Herbario QUPS 4803), comúnmente conocida como mortiño, se caracteriza por sus altas concentraciones de compuestos bioactivos en compuestos polifenólicos, los cuales desempeñan un papel importante en su actividad biológica. Nuestro objetivo fue investigar el papel de las antocianinas en lo concerniente a sus propiedades biológicas después de identificarlas y cuantificarlas mediante pH diferencial, cromatografía en capa fina (TLC) y cromatografía líquida de alta resolución (HPLC) en un extracto fluido de mortiño. Las actividades biológicas se evaluaron mediante ensayos antioxidantes (ABTS y DPPH), que arrojaron valores de actividad antioxidante de 833,47 µg/mL (ABTS) y 1418,01 µg/mL (DPPH). La actividad antiinflamatoria evaluada en ensayos de estabilidad de membrana (MS) bajo estrés térmico mostraron porcentajes máximos de inhibición de 32,31 % y 34,26 % para las antocianidinas (cianidina y

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delfinidina) en 1 µg/mL, con 92,56 % de inhibición para el extracto de 1000 µg/mL, así como un efecto aditivo de las antocianinas y flavonoides. Además, las evaluaciones antimicrobianas mediante antibiogramas y métodos de dilución en microplaca contra patógenos causantes de infecciones del tracto urinario (ITU) demostraron una inhibición significativa de *S. saprophyticus* (extracto al 50 %, CMI = 3125 µg/mL, 55,29 % de inhibición), *P. aeruginosa* (extracto al 50 %, CMI = 12500 µg/mL, 71,45 % de inhibición), *E. coli* (extracto al 50 %, CMI = 12500 µg/mL, 35,04 % de inhibición) y *C. tropicalis* (extracto al 100 %, CMI = 6250 µg/mL, 12,88 % de inhibición). Estos hallazgos sugieren que el extracto de mortiño contiene niveles sustanciales de antocianinas, siendo la cianidina la predominante en el estudio. Tanto las antocianinas como las antocianidinas exhibieron importantes actividades antioxidantes, antiinflamatorias y antimicrobianas, lo que respalda el valor terapéutico potencial del mortiño para abordar el estrés oxidativo, la inflamación y las infecciones microbianas.

Palabras clave: *Vaccinium floribundum;* actividad antimicrobiana; actividad antiinflamatoria; actividad antioxidante; infecciones del tracto urinario (UTIs); antocianidinas.

Introduction

Anthocyanins are glycosylated derivatives of anthocyanidins; they are phenolic compounds with essential biological functions in plants, including UV protection, osmotic regulation, and pollinator attraction. They also act as sun filters, photoprotective agents, providers of post-harvest benefits, and defense mechanisms (**Liu *et al.***, 2018; **Riaz *et al.,*** 2016). These compounds typically accumulate in young tissues and sun-exposed parts, such as fruits, where they protect against photoinhibition and photobleaching (**Esquivel-Alvarado *et al.***, 2020). Anthocyanins and anthocyanidins (aglycones) share a core chemical structure, which is the flavylium or 2-phenylbenzopyrylium cation that contains the oxonium group or charged oxygen (2,6,9). The six most common anthocyanidin derivatives are cyanidin (Cy), delphinidin (Df), celargonidin (Pg), petunidin (Pt), peonidin (Pn), and malvidin (Mv), which collectively represent approximately 90% of identified anthocyanins (**Riaz *et al.***, 2016; **Yuzhi Jiao**, 2012; **Cerrato *et al.***, 2022). Their biosynthesis occurs via the phenylpropanoid metabolic pathway (a mixed process involving the shikimic and acetic acids pathway) mediated by multiple enzymes, in which chalcones are synthesized. Chalcones are then converted into dihydroflavonols, which are subsequently reduced to form leucoanthocyanidins that are later transformed into anthocyanidins. These last compounds are derivatized to stabilize them and produce anthocyanins (**Zhang *et al.***, 2013). Additionally, their biosynthesis is controlled by the regulatory complex MYB-bHLH-WD40 (MBW), which encodes structural and regulatory genes responsible for the enzyme production of the metabolic pathway and transcription factors, respectively (**Liu *et al.***, 2018).

Vaccinium floribundum Kunth, commonly known as mortiño, Andean berry, wild berry, or Andean blueberry, exhibits high concentrations of biologically active compounds, particularly anthocyanins. Its fruit is rich in anthocyanins, flavonols, phenolic acids, and proanthocyanins (PCAs), with anthocyanins and flavonols being the most predominant compounds (**Martău *et al.***, 2023). The species typically grows at an average altitude of 3,600 meters above sea level (MASL), thriving in moors and mountain ranges along the Andes. Taxonomically, it belongs to the order Ericales, family *Ericaceae,* and genus *Vaccinium*. Its scientific name is *Vaccinium floribundum* Kunth, with the synonym *Vaccinium mortinia* (**Howell**, 2020). *Vaccinium* genus fruits, including *V. floribundum*, are notable for their antioxidant properties, which are attributed to their high content of bioactive compounds. This characteristic is shared with other *Vaccinium* species whose anthocyanins are the compounds associated with significant biological activities (**Esquivel-Alvarado *et al.***, 2020; **Howell,** 2020; **Pérez *et al.***, 2021; **Re *et al.***, 1999). Anthocyanins are water-soluble active ingredients that can be degraded at high temperatures and basic pH, for which a suitable extraction method can be percolation, since it is a mild process allowing for the obtention of unstable molecules (**Re *et al.***, 1999). It is a commonly employed technique that uses methanol as a solvent and a strong acid (e.g., HCl) as an acidifying agent. However, the solvent can be substituted by ethanol, given the toxicity of methanol, and the strong

acids by soft ones or no acid, given the hydrolysis of anthocyanins. Rotary evaporation of the extract needs temperatures between 30 and 60°C (**Re *et al.***, 1999) and is a common extraction method for various preparations in the Chinese pharmacopeia since it is easy, but it uses large amounts of solvent, and the extraction time is long. In Ecuador, it is part of phytopharmacology, and it is a common method for obtaining raw extracts with bioactive metabolites from medicinal plants.

In several studies, chromatography techniques have shown that *V. floribundum* (mortiño) phytochemical profile reveals the abundance of anthocyanins and flavonols as key components of its composition. Among the anthocyanins, cyanidin (Cy), delphinidin (Df), peonidin (Pn), and pelargonidin (Pg) have been identified, with cyanidin derivatives being the most predominant. Mortiño's antimicrobial activity against urinary tract infections (UTIs) caused by fungi (*Candida* spp.: *C. albicans, C. glabrata, C. auris, C. parapsilosis, C. tropicalis, C. kefir, C. lusitanae, C. guilhermondi*, and *C. dubliniensis*), Gram-positive (*Enterococcus* spp., *S. saprophyticus, S. aureus*, Group B *Streptococcus*), and Gram-negative bacteria (*E. coli, Klebsiella pneumoniae, Proteus mirabilis, P. aeruginosa*) (**Ortega**, 2018) was demonstrated by **Barba-Ostria *et al.*** (2024) in their study: *V. floribundum* extract exhibited minimum inhibitory concentrations (MIC) ranging from 2.1–2.5 mg/mL for Gram-positive bacteria, 12–18 mg/mL for Gram-negative bacteria, and 100–180 mg/mL for yeasts. The extract was most effective against Gram-positive bacteria, showing susceptibility across all strains studied (**Pérez *et al.***, 2021). Another study evaluated the ability of the extract to inhibit biofilm formation, demonstrating its inhibitory effects on *S. aureus, E. faecalis, L. monocytogenes, C. tropicalis*, and *B. cepacia*. The minimum biofilm inhibitory concentrations (MBIC50) were found to be between 0.5 and 5 mg/mL for the first four strains and 20 mg/mL for *B. cepacian* (**Brand-Williams *et al.***, 1995). Focusing on pathogens associated with UTIs, previous studies have highlighted the role of cranberry, a related species from the *Vaccinium* genus, in preventing them (**Alarcón-Barrera *et al.***, 2018).

There is no in-depth information regarding the potential anti-inflammatory and antimicrobial activity (i.e., antiadhesion activity) on uropathogens of pure anthocyanidins, the more reactive form of anthocyanins, and of pure anthocyanin mortiño extract, which has cyanidin derivatives as the main anthocyanin. In this context, we aimed to determine the presence of anthocyanins (identification and quantification) in the hydroethanolic extract of *V. floribundum* (mortiño), to evaluate its anti-inflammatory activity and its antimicrobial activity on uropathogens, as well as the antioxidant properties of mortiño anthocyanin-rich extract, and compare it to pure anthocyanin.

Materials and methods

Vegetal material

Plant material was collected in the province of Cotopaxi, Sigchos canton, at Mercado 24 de Mayo, located at 00° 42' 03''S and 78°53'14'W. Before obtaining the *V. floribundum* ethanolic extract, the fruits were cleaned to remove impurities, including leaves, stems, and damaged or unripe fruits. The fruits were then washed three times with distilled water to eliminate surface impurities and subsequently disinfected using 1% sodium hypochlorite ($NaClO_3$) (**Howell**, 2020).

Extraction

The fruit (3000 g) was left in the solvent for 24 hours at room temperature. The following day, the first filtration was performed, yielding 85% of the required final volume. Solvent was added again for another 24 hours to extract the second fraction. The resulting material also had to be filtered. The total volume obtained over the two days was concentrated in a rotary evaporator (Rotavapor, IKA RV) at 40°C until 15% of the required final volume was obtained. The two fractions were combined to produce the final fluid extract with a concentration of 1 g FF/mL. The extract was stored in amber glass bottles and refrigerated at 4°C for subsequent analysis. The extraction yield amounted to 36.67%.

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. 2026
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Anthocyanins hydrolysis

The extract sample was pretreated through hydrolysis to facilitate the identification of anthocyanins, as the available standards were in the form of cyanidin chloride (CyCl) (Sigma-Aldrich) and delphinidin chloride (DfCl) (Sigma-Aldrich). The procedure followed a slightly modified version of the protocols described by **Pinho *et al*.** (2011) and **Filip *et al.*** (2012). For acidification, 50 mL of pure extract was combined with 5 mL of 6 M hydrochloric acid (HCl) (Fischer Scientific, 37%), and heated at 60°C for 30 minutes.

Identification of anthocyanins by thin-layer chromatography (TLC)

Anthocyanins were identified following the protocol by **Poole** (2023). To confirm the presence of anthocyanins in the pure (Ep) and hydrolyzed extract (Ea), we performed a TLC with a mobile phase comprising ethyl acetate (AcEt), formic acid (HCOOH), glacial acetic acid (CH_3COOH), and distilled water (H_2O d.) in a 10:1.1:1.1:2.6 ratio. The samples were compared with two cyanidin chloride and delphinidin chloride standards at a concentration of 0.1 mg/mL using a high-performance TLC F254 silica gel glass plate (10 × 10 cm) (Merck) and a Camag Linomat 5 applicator. Subsequently, the TLC was visualized with a Camag UV lamp at 365 nm and 254 nm, and we measured the retention factors (Rf) for each sample and standard.

Anthocyanins quantification by pH differential method

We quantified total monomeric anthocyanins (TAC) using the AOAC official method (**AOAC**, 2005). Potassium chloride (KCl, 0.025 M, pH 1.0) and sodium acetate (AcONa, 0.4 M, pH 4.5) were used as buffers. The extract was then diluted in the buffer for analysis with a Jasco V730 UV-Vis spectrophotometer. Absorbance of each dilution was measured at 520 nm and 700 nm in triplicate. TAC was calculated using the following equations: cyanidin-3-glucoside (C3G) equivalents per liter of extract (mg C3G Eq/L Ep) (Equation 1) and C3G equivalents per gram of fresh fruit (mg C3G Eq/g FF) (Equation 2) (**Chandra Singh *et al*.**, 2022):

$$\text{TAC 1}(mg\ C3G\ Eq) = (A \times MW \times DF \times 103) / (\mathcal{E} \times 1) \quad (1)$$

$$\text{TAC 2}\ (mg\ C3G\ Eq / g\ FF) = CTA1 \times V \times M \quad (2)$$

(MW: molecular weight, 449.2 g/mol, FD: dilution factor, V: volume of the sample obtained from the extract (L), Ɛ: molar absorptivity coefficient of C3G, 26 900 1/M*cm, M: weight of the sample used for extract (g), I: length of the distance traveled; Absorbance: $A = (A520nm - A700nm)pH\ 1.0 - (A520nm - A700nm)pH\ 4.5$, where A520nm pH 1.0 and A700nm pH 1.0: Absorbance of the sample diluted in KCl buffer pH 1 at 520 nm and 700 nm, respectively; and A520nm pH 4.5 and A700nm pH 4.5: Absorbance of the sample diluted in NaOAc buffer pH 4.5 at 520 nm and 700 nm, respectively).

Anthocyanins quantification and identification using high-performance liquid chromatography

To confirm anthocyanins quantification and identification, analyses were conducted using an Ultimate 3000 HPLC system equipped with a Hypertensil GOLD™ C-18 column (150 × 4.6 mm), an autosampler, a quaternary pump, a column compartment, and a diode array detector (DAD). The samples analyzed included the pure (Ep) and hydrolyzed (Ea) extracts, and the cyanidin chloride (CyCl) standard at 1000 ppm (2 mg/mL). All samples were filtered using 0.45 µm filters and placed in amber HPLC vials to prevent degradation. The analysis was performed using a reversed-phase C-18 column under isocratic conditions with a mobile phase of type I water (H_2O, 70%) and acetonitrile (ACN, analytical grade, 30%). The total run time was 10 minutes, with a flow rate of 1 mL/min and an injection volume of 5 µL. Anthocyanins were detected at an absorbance wavelength of 535 nm, which was also used for their quantification. Total anthocyanins were quantified using the internal standard method with cyanidin chloride (CyCl, 2 mg/mL) as the standard, and the following formula (Equation 3):

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$$FR = ((Ax \times Cs)) / ((Cx \times As) \rightarrow Cx = ((Ax \times Cs)) / ((FR \times As)) \quad (3)$$

(*FR*: response factor, 1.36, *Ax*: sample relative area, *As*: standard relative area, *Cx*: sample concentration, *Cs*: standard concentration).

Antioxidant activity

We evaluated the antioxidant activity of mortiño using two well-established methods: the ABTS assay (2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (**Re *et al.,*** 1999; **Rumpf *et al.***, 2023) and the DPPH assay (2,2-diphenyl-1-picrylhydrazyl) (**Alarcón-Barrera *et al.***, 2018; **Howell**, 2020; **Xiao *et al.***, 2020).

The ABTS assay followed the procedure described by **Brand-Williams *et al.*** (1995), which involves generating the cationic radical ABTS•+. To prepare it, we used a 2.45 mM potassium persulfate ($K_2S_2O_8$) solution to activate a 7 mM ABTS solution. Once the cationic radical was formed, the ABTS•+ solution was diluted in phosphate-buffered saline (PBS, pH 7.4, prepared in advance) to achieve an absorbance of 0.70 ± 0.02 at 734 nm.

After obtaining the appropriately diluted ABTS•+ solution, we conducted the antioxidant test. We mixed 990 µL of diluted ABTS•+ with 10 µL of each sample, including dilutions of the pure extract (Ep), ascorbic acid (AA, as the standard), or the blank (PBS). The mixture was shaken and allowed to react for 6 minutes, after which its absorbance was measured at 734 nm (λ = 734 nm). Ep and AA dilutions were prepared to determine their high minimum inhibitory concentration (IC50). The IC50 values were calculated using Equation (4) (**Meneses & Moncayo**, 2022; **Musuña & Varela**, 2020):

$$\% ((Ai - Af)) / (Ai) \times 100) \quad (4),$$

where Ai is ABTS•+ absorbance with the blank (PBS), and Af is ABTS•+ absorbance with the antioxidant after 6 minutes.

To calculate the ABTS antioxidant capacity, we used the average inhibition percentage of the pure extract (Ep) at 0.1 µg/mL. Using the linear estimation equation derived from the ascorbic acid (AA) standard curve (Equation 5), the equivalent concentration (Ceq) was determined. Subsequently, the equivalent concentration (Ceq) was used to compute the ABTS antioxidant capacity, expressed as milligrams of ascorbic acid equivalents per gram of fresh fruit (mg AA Eq/g FW), according to Equation 6 (**Meneses & Moncayo**, 2022; **Musuña & Varela**, 2020).

$$Equivalent\ Concentration = Ceq((ug\ AA\ Eq) / (mL\ of\ Extract) = /\mathrm{m} \quad (5)$$

$$ABTS\ Value\ (mg\ \ Eq / (g\ FW) = ((Ceq \times DF \times V)) / ((M \times 1000))) \quad (6)$$

(DF: dilution factor, V: volume of sample obtained from the extract (L), M: weight of the sample used for the extract (g), %I: Inhibition percentage of the sample (Ep 0.1 g/mL), m: slope of the standard line (AA), b: intercept of the standard line (AA).

The DPPH method followed the procedure described by **Brand-Williams *et al.*** (1995). First, the DPPH radical (DPPH•) was prepared. Then, a 0.120 mM DPPH solution was prepared to achieve an absorbance of approximately 1.0 at 517 nm (λ = 517 nm) and stored in an amber glass bottle for 24 hours. After, 1000 µL of DPPH• solution were mixed with 100 µL of each sample, including dilutions of the pure extract (Ep), ascorbic acid (AA, as a standard), and a blank (methanol, MeOH). The mixture was shaken and allowed to react for 30 minutes before measuring the absorbance at 517 nm. Ep and AA dilutions were prepared to determine their IC50 values. The IC50 was calculated using Equation 7:

$$\%I = ((Ai - Af)) / (Ai) \times 100) \quad (7),$$

where Ai: absorbance of DPPH• with the blank (MeOH) and Af: absorbance of DPPH• with the antioxidant after 30 minutes.

To determine the DPPH test value, we used the average inhibition percentage of the pure extract (Ep) at 0.1 µg/mL. Using the logarithmic estimation equation derived from the standard Equation 8, we calculated the equivalent concentration (Ceq). Subsequently, we

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. 2026
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used it to estimate the DPPH antioxidant capacity, expressed as milligrams of ascorbic acid equivalents per gram of fresh fruit (FW) (mg AA Eq/g FW), using Equation 9 (**Meneses & Moncayo**, 2022; **Musuña & Varela**, 2020).

$$Equivalent\ Concentration = Cep\ ((ug\ AA\ Eq)/(mL\ of\ Extract) = ((\%I - b))/m) \quad (8)$$

$$DPH\ Value\ \ ((mg\ AA\ Eq)/(g\ FW) = ((Ceq \times DF \times Eq))/((M \times 1000)) \quad (9)$$

(FD: dilution factor, V: volume of the sample obtained from the extract (L), M: weight of the sample used for the extract (g), %I: percentage of inhibition of the sample (Ep 0.1 g/mL), m: slope of the standard line (AA), b: intercept of the standard line (AA).

Anti-inflammatory activity

We used the membrane stabilization (MS) assay to evaluate the anti-inflammatory activity in two anthocyanidins (Df and Cy), a flavonol (quercetin, Qc), a commercial anti-inflammatory (diclofenac sodium, Ds), and the pure extract (Ep). For the *in vitro* analysis, we used each of the compounds (Df, Cy, Qc, Ds, Ep) to measure their capacity to inhibit human red blood cells (HRBC) hemolysis induced by heat. For this, we collected intravenously fresh whole blood (5 mL) in heparinized tubes to prevent coagulation. A volume of 3 mL of blood was transferred to a Falcon tube and centrifuged at 3000 rpm for 5 minutes at room temperature using an Eppendorf 5430R refrigerated centrifuge. The red blood cell pellet was resuspended three times in an equal volume of normal saline solution (0.9% m/v saline solution, SS) to wash and remove turbidity. The volume obtained from the dissolved red blood pellet was measured and reconstituted as a 40% v/v suspension with isotonic buffer solution (sodium phosphate buffer, PBS pH 7.4). This reconstitution was used for the analysis, while the corresponding Qc, Ds, Df, Cy, and Ep dilutions were made from stock solutions (0.1 mg/mL) (**Qamar *et al.***, 2021; **Xiao *et al.***, 2020).

Antimicrobial activity

Reactivation of strains. To prepare viable strains for antibiogram and minimum inhibitory concentration (MIC) tests in microplates, we cultured the microorganisms from a cryobank using beads. We used the following bacterial strains and a yeast strain: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 9027, *S. saprophyticus* ATCC 15305, and *C. tropicalis* ATCC 13803. A bead from each strain was extracted and streaked onto Petri dishes containing the appropriate culture medium for each microorganism. For *E. coli, P. aeruginosa*, and *S. saprophyticus*, tryptic soy agar (TSA) was used, and the plates were incubated at 37°C for 18–24 hours. For *C. tropicalis*, Sabouraud dextrose agar (SDA) was used, with incubation at 25°C for 48 hours (**Sadilova *et al.***, 2007).

The antimicrobial activity assays required standardization of the inoculum to a 0.5 McFarland scale after the incubation period.

Antibiogram by agar diffusion method: Kirby-Bauer antibiotic testing

We inoculated 200 µL of each microbial strain on a plate containing Mueller-Hinton Agar (MHA) medium. We inoculated four discs for each microorganism: one containing an antibiotic, one blank disc, one impregnated with 50% blueberry extract, and another one with 100% blueberry extract. The antibiotics used were the following: streptomycin for *E. coli*, cefepime for *P. aeruginosa*, nitrofurantoin for *S. saprophyticus*, and fluconazole for *C. tropicalis*. Petri dishes were incubated under the conditions described in the section "Reactivation of strains". After the incubation period, we assessed the results by measuring the inhibition halos around each disc. The measurements were recorded and analyzed using Equation 10:

$$\%I = ((Dd - Dh))/(Dd) \times 100) \quad (10)$$

(Dd: diameter of disc (6 mm) and Dh: diameter of inhibition halo). If no inhibition halo was observed, the result was reported as 6 mm, which corresponds to the diameter of the disc itself.

Minimum inhibitory concentration (MIC)

To assess the MIC, we used the method described by **Meneses & Moncayo** (2022), with some slight modifications. Two microplates were prepared for each microorganism (*E. coli, P. aeruginosa, S. saprophyticus*, and *C. tropicalis*). The controls included a blank consisting of Mueller-Hinton Broth (MHB) alone, a negative control with MHB and inoculum, and a positive control with MHB, inoculum, and the appropriate antibiotic. MICs were determined using the microtiter dilution method with serial double dilutions of the extracts (ranging from 50000 to 195.32 µg/mL), anthocyanidins (ranging from 10 to 0.078 µg/mL), and antibiotics (ranging from 10 to 0.078 µg/mL). The inoculum for each dilution was standardized to a 0.5 McFarland scale.

After incubation, bacterial growth was assessed using 1% triphenyl tetrazolium chloride (TTC) as a color indicator, followed by an additional incubation period. The additional incubation for *E. coli* lasted 30 minutes; for *P. aeruginosa,* 15 minutes; for *S. saprophyticus,* 30 minutes, and for *C. tropicalis*, one hour. Absorbance was measured at 420 nm using a Biotek Epoch plate reader (**Cerrato *et al.***, 2022). The inhibition was expressed using Equation 11:

$$\%I = ((Ai - Af)/(Ai) \times 100) \quad (11)$$

(Ai: absorbance of MHB and inoculum, and Af: absorbance of MBH with the extract or anthocyanidin + inoculum).

Statistical analysis

Data for the thin-layer chromatography (TLC) retention factor (Rf), the total anthocyanin content (TAC), the antioxidant activity (ABTS and DPPH assays), the IC_{50} values, and the percentage inhibition (% inhibition) in antimicrobial assays were expressed as mean ± standard deviation (SD). All experiments were performed in triplicate (n = 3), and each value represents independent experimental replicates.

The anti-inflammatory assay had a completely randomized design. The effects of treatment (cyanidin, delphinidin, quercetin, and diclofenac) and the concentration (0.05–1 µg/mL), as well as their interaction, were evaluated using a two-way analysis of variance (ANOVA). Additionally, the effect of extract concentration was analyzed separately using a one-way ANOVA. When significant differences were detected, Tukey's *post hoc* test was applied for multiple comparisons. Statistical significance was established at $p < 0.05$. Normality and homogeneity of variances were verified before analysis. All statistical analyses were performed using R Studio software (Posit Team RStudio.Version 2024), and graphs were generated using Microsoft Excel.

In the experimental design for the anti-inflammatory activity, we used ANOVA 1-way (one independent variable) and 2-way (two factors), as always in anti-inflammatory tests, to compare mean values across multiple treatment groups simultaneously. One-way ANOVA tests for significant differences between treatments, while the 2-way test evaluates both individual factor effects and their interaction. In our case, 2-way ANOVA was applied because we had two factors (concentration and treatment), but also to evaluate the interaction between these two variables. On the other hand, 1-way ANOVA was applied because we only evaluated the effect of the extract concentration to prevent the hemolysis of red blood cells (**Kim**, 2014; **Qamar *et al.,*** 2021).

Results and discussion

Anthocyanins identification by thin-layer chromatography (TLC)

In the anthocyanins identification by TLC, the best results were obtained using the hydrolyzed extract (Ea) obtained under UV light at 254 nm. The analysis revealed the presence of cyanidin (Cy), with an Rf value of 6.8, while delphinidin (Df, Rf 6.2) was not detectable using this method. The mean Rf value for the extract was determined at 6.93 ±

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. 2026
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0.06, confirming the presence of Cy (**Figure 1**). Retention factor (Rf) differences among anthocyanins are influenced by their molecular structure, particularly the substituents on the C-ring. These variations depend on the number and type of hydroxyl and methoxy groups present. Here, Df, which has a higher number of hydroxyl groups, traveled a shorter distance compared to Cy.

High-performance liquid chromatography (HPLC-DAD)

We detected a primary peak corresponding to anthocyanins with retention times of 1.667 minutes for Ep and 1.737 minutes for Ea, with maximum absorbances of 276–280 nm and 526–530 nm, which are characteristic of anthocyanins. We also observed two smaller peaks and, notably, a peak in Ea at 2.127 minutes with the same retention time (2.110 minutes) and absorbance maximums (278 nm and 536 nm) as the cyanidin standard, indicating the presence of Cy as a product of hydrolysis (**Baenas *et al.***, 2020; **Howell**, 2020). These results (**Figure 2**) confirm the presence of cyanidin in the sample, suggesting that this anthocyanin predominated.

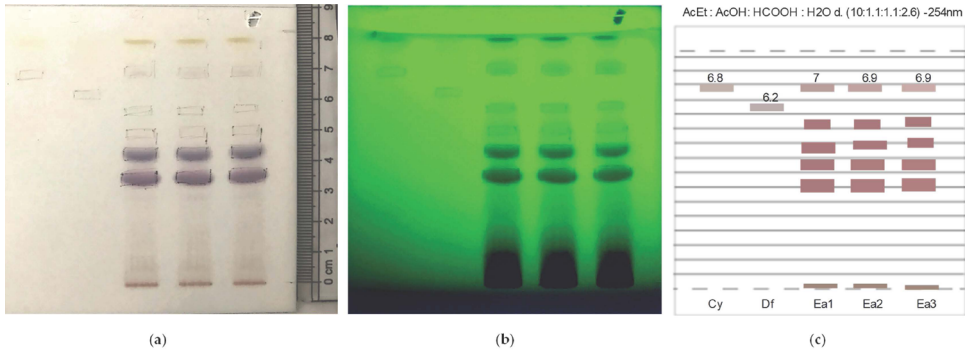

Figure 1. Identification of anthocyanins by TLC of *Vaccinium floribundum* (mortiño) acidified extract (Ea). **(a)** TLC with white light; **(b)** TLC with UV-254nm; **(c)** TLC scheme with the respective Rf. Cy: standard cyanidin; Df: standard delphinidin; Ea: acidified extract repetition; 1, 2, and 3: repetition number

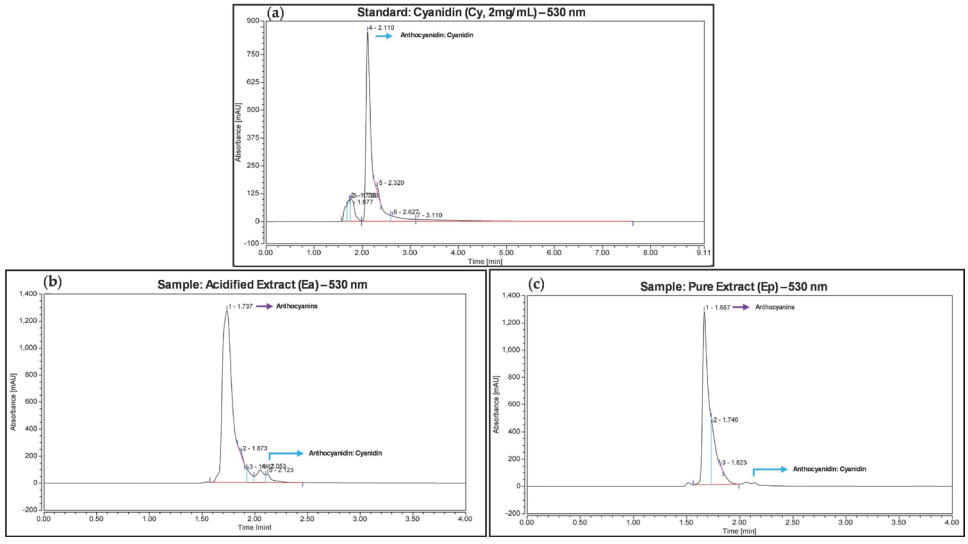

Figure 2. Chromatogram of **(a)** CyCl standard (2mg/mL), **(b)** Vaccinium floribundum (mortiño) Ea and **(c)** Ep extracts

Anthocyanins quantification by differential pH method and HPLC

Anthocyanins quantified with the differential pH method in the mortiño extract were 416 ± 3.13 mg C3G Eq/L of extract, or 0.416 mg ± 0.003 C3G Eq/mg of fresh fruit (FW). When comparing with other studies, we found that *V. floribundum* samples from Peru and Costa Rica had been used after they were freeze-dried (dry weight, DW) and extracted primarily using 70% acetone, reporting TAC values of 14.5 mg C3G Eq/g DW and 9. 17 mg C3G Eq/g DW, respectively (**Cerrato *et al.***, 2022; **Esquivel-Alvarado *et al.***, 2020). In contrast, studies using fresh fruit (FW) from Peru and Ecuador in samples macerated with alcoholic solvents at varying concentrations (20% and 60% ethanol; 20%, 60%, and 80% methanol), and extracted at different temperatures (30°C and 60°C), reported TAC values ranging from 4.19 mg to 1.79 mg C3G Eq/g FW (**Re *et al.***, 1999). When comparing TAC values, anthocyanin content in this study was significantly lower than that of freeze-dried samples extracted with acetone. However, the values obtained were similar to those from studies using fresh fruit and alcoholic solvents (methanol and ethanol), and the differential pH method.

The differential pH method, while commonly used, is not very sensitive and requires corroboration with more precise methods. To address this shortcoming, anthocyanin quantification was also performed using high-performance liquid chromatography (HPLC). The total anthocyanin content (TAC) determined using cyanidin chloride (CyCl) as an internal standard was 1.47 ± 0.01 mg/g FW for the hydrolyzed extract (Ea) and 1.30 ± 0.00 mg/g FW for the pure extract (Ep).

Antioxidant activity

Flavylium cation structure enables radical electron delocalization within sp^2 orbitals, leading to radical stabilization. The antioxidant efficacy of anthocyanins is influenced by their degree of hydroxylation, acylation, and glycosylation (**Esquivel-Alvarado *et al.***, 2020; **Liu *et al.,*** 2018; **Zhang *et al.***, 2013). Hydroxylation and methoxy groups on the C ring enhance antioxidant activity, with hydroxyl groups (-OH) contributing more significantly than methoxy groups ($-OCH_3$). Consequently, the antioxidant capacity of anthocyanidins increases in the following order: pelargonidin < peonidin < cyanidin < malvidin < petunidin < delphinidin. Glycosylation, on the other hand, reduces the antioxidant potential of anthocyanins because sugar residues diminish electron delocalization, proton donation, and chelating ability. As a result, anthocyanidins, which lack glycosylation, demonstrate greater activity than anthocyanins, while acylation contributes to the glycosylation process (**Esquivel-Alvarado *et al.***, 2020; **Liu *et al.***, 2018; **Jiang *et al.***, 2012).

ABTS

The antioxidant activity was assessed by determining the IC_{50} values for AA and Ep. The respective linear equations obtained were y = 10.424x + 0.5909 for AA and y = 0.0481x + 9.9099 for Ep (**Figure 3**), which both demonstrated a good linear fit, with coefficients

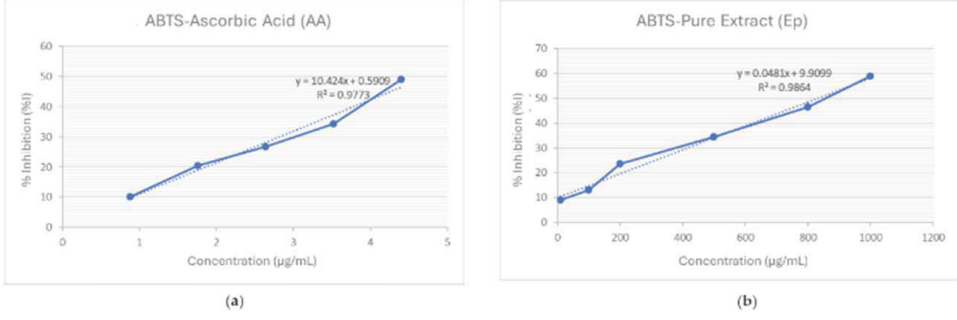

Figure 3. Inhibition percentage (%I) vs. concentration (ug/mL) curve of **(a)** ascorbic acid (AA) and **(b)** Vaccinium floribundum (mortiño) in the ABTS assay

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. 2026
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of determination close to 1, specifically 0.9773 for AA and 0.9864 for Ep. The IC_{50} values calculated for AA and Ep were 4.74 ± 0.12 µg/mL and 833.47 ± 29.65 µg/mL, respectively. These results indicate that the antioxidant activity of the extract (Ep) is approximately 175 times lower than that of ascorbic acid (AA).

DPPH

As in the previous essay, the IC_{50} values for ascorbic acid (AA) and the extract (Ep) were determined to assess antioxidant activity. The respective equations derived were y = 35.007ln (x) - 24.073 for AA and y = 18.626ln (x) - 85.169 for Ep (**Figure 4**). A strong linear fit was observed, as indicated by the coefficients of determination for AA and Ep (0.9348 and 0.9548, respectively). The IC50 values calculated were 8.30 ± 0.22 µg / mL for AA and 1418.01 ± 188.59 µg / mL for Ep. These results show that the antioxidant activity of the extract is approximately 170 times lower than that of ascorbic acid.

Membrane stabilization (MS) assay

Anthocyanins' anti-inflammatory activity has been corroborated by their ability to inhibit several substances and pro-inflammatory enzymes, such as tumor necrosis factor alpha (TNF-α), cyclooxygenase-2 (COX-2), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), nitric oxide (NO), C-reactive protein (CRP), inducible nitric oxide synthases (iNOS), and lipoxygenase (LOX) (**Kozłowska & Dzierżanowski**, 2021). It also has an attenuating power on proinflammatory genes (tumor necrosis factor (TNF), interleukins (ILs), cyclooxygenase (COX), prostaglandin-endoperoxidase synthase, tenascin) and the ability to suppress chemokine genes (e.g., chemokine ligands and interferon alpha-inducible proteins) (**Chen *et al.***, 2008). Besides, anthocyanins have been shown to increase the amount of the enzymes glutamate-cysteine ligase (GCL) and glutamate-cysteine ligase-modifying subunit (GCLM), which influence the reduction of glutathione. It has even been reported that they have been able to inhibit nuclear factor kappa B (NF-κB), an important factor within the NF-κB pathway of inflammation that is responsible for cytokine production and transcriptional control (**Kozłowska & Dzierżanowski**, 2021). Some anthocyanins with an anti-inflammatory effect on these markers are cyanidin-3-glucoside, cyanidin-3-rutinoside, delphinidin-3-rutinoside, malvidin-3-glucoside, cyanidin-3-O-sophoroside, and cyanidin-3-O-sambubioside (**Chen *et al.***, 2008; **Sharma & Lee**, 2022; **Kozłowska & Dzierżanowski**, 2021). The model we used in our study was the membrane stabilization assay, since it enables the comparison of the erythrocyte membrane with the lysosomal membrane of macrophages, responsible for the inflammatory process. This means that by stabilizing its membrane, the release of protease is avoided and bactericidal enzymes are controlled (inflammatory response), while the hemolysis inhibition or stabilization shows mortiño extracts' potential to reduce the inflammatory response (**Qamar *et al.***, 2021).

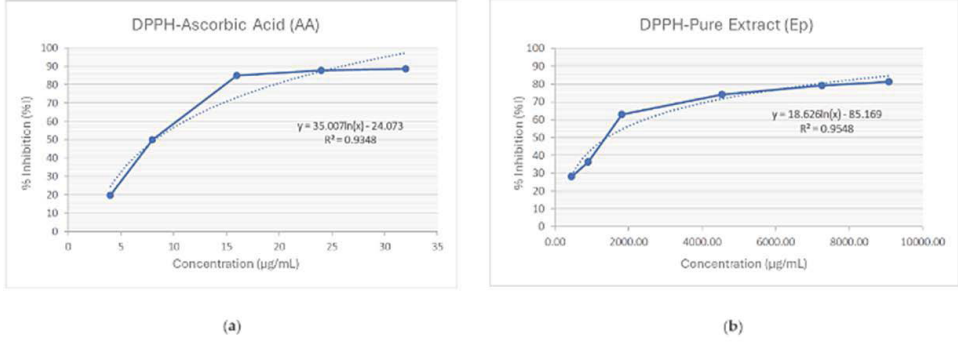

Figure 4. Inhibition percentage (%I) vs. concentration (ug/mL) curve of **(a)** ascorbic acid (AA) and **(b)** Vaccinium floribundum (mortiño) in the the DPPH assay

We evaluated, therefore, several standards for comparison sake: anthocyanidins (Cy and Df), the flavonol quercetin (Qc), a natural compound with known anti-inflammatory properties, and diclofenac sodium (Ds), a commercial anti-inflammatory drug. Anthocyanidins and Qc demonstrated comparable anti-inflammatory activity, with Qc exhibiting the highest percentage of inhibition among the natural compounds. The maximum inhibition percentages achieved at 1 µg/mL were 32.31, 34.26, and 37.68 % for Cy, Df, and Qc, respectively.

Our results were consistent with the anti-inflammatory activity reported by **Curinambe *et al.,*** 2024, who used ethanolic extracts and performed the membrane stability (MS) test by hypotonicity in erythrocytes, demonstrating an *in vitro* anti-inflammatory effect similar to indomethacin, with an inhibition percentage of 20 to 35%. On the other hand, they found that the mixture of anthocyanins had a greater effect, i.e., they had a summative effect and allowed greater inflammatory power (**Qamar *et al.,*** 2021). This is consistent with our results, where anthocyanidins had a similar anti-inflammatory effect, and the extract demonstrated greater anti-inflammatory activity, possibly due to the mixture of anthocyanins present in the mortiño extract (**Table 1**).

Two-way and one-way ANOVA tests were conducted to determine significant differences and assess the anti-inflammatory activity of various treatments and the blueberry extract, respectively. Our results indicated that the values of concentration, treatment, and the interaction between these two were statistically significant ($p < 0.05$) (**Table 2**). This means that both variables influenced the anti-inflammatory activity of the mortiño extract.

Finally, we noticed that the total anti-inflammatory activity of the extract was influenced by the additive effect of anthocyanins, with flavonoids and phenolic acids contributing to this effect. These compounds effectively reduce the production of pro-inflammatory mediators, as they reduce inflammation by inhibiting pro-inflammatory enzymes (COX-2, 5-LOX), suppressing inflammatory signaling pathways (NF-kB, MAPK), and lowering pro-inflammatory cytokines (IL-1β, TNF-α, IL-6). They also act as potent antioxidants, mitigating oxidative stress and modulating immune cell activity to decrease inflammatory reactions (**Al-Khayri *et al*.**, 2022; **Kiriyama *et al.***, 2024). As can be seen in quercetin (a type of flavonoid), there is an important effect that could explain the biological activity of the total extract, given that flavonoids and phenolic acids are also responsible for this biological effect.

Antimicrobial activity

Kirby-Bauer method. Here, we assessed antimicrobial activity by measuring the diameter of inhibition halos. According to the classification criteria, halos <6 mm in diameter indicate resistance, those >6 mm are considered intermediate, and those 9 to 14 mm in diameter are classified as sensitive (**Dangles & Fenger**, 2018; **Esquivel-Alvarado *et al.***, 2020). The test evaluated the antimicrobial effectiveness of the 50% (E50) and 100% extracts (E100), as well as the corresponding antibiotics against *P. aeruginosa, S. saprophyticus, E. coli, and C. tropicali*s (**Table 3**). This means that the use of 100% mortiño extract against *P. aeruginosa* resulted in a 44.67% inhibition, with an inhibition halo of 8.68 mm. Our findings are comparable to those reported by **Masaquiza** (2018), who observed larger inhibition zones of 25.33 mm and 18.00 mm using encapsulated mortiño extract. In contrast, *S. saprophyticus* was classified as sensitive, as the mortiño extract at 50% concentration achieved a 57.67% inhibition, while at 100% concentration, inhibition reached 52.33%, which is consistent with **Chonlon & Espinoza's** (2022) and **Quispe & Soncco's** (2022) findings.

Likewise, a 24.50% inhibition against *E. coli* was obtained with the 50% extract, which increased to 39.33% with the 100% extract. These values are similar to those reported by **Guamán** (2022), who found a 28.8% inhibition. Finally, *C. tropicalis* showed the most favorable response to the treatment. The 100% extract produced a 73.33% inhibition, compared to 52.83% with the 50% extract, which supports **Escobar's** (2021) conclusion that higher extract concentrations lead to greater antimicrobial inhibition.

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. 2026
doi: https://doi.org/10.18257/raccefyn.4045

Table 1. Anti-inflammatory activity of standards and mortiño extract (Vaccinium floribundum) evaluated using the membrane stabilization assay under thermal stress conditions. Data expressed as mean ± standard deviation (SD) (n = 3). Different lowercase letters indicate statistically significant differences among treatments according to Tukey's post hoc test ($p < 0.05$). Cy: cyanidin; Df: delphinidin; Qc: quercetin; Ds: diclofenac sodium; Ep: mortiño extract

Muestra	Conc. (µg/mL)	A ± SD			%I ± SD			
Ct		0.7497	±	0.0245???				
Anti-inflammatory activity with various treatments - Membrane stability: Temperature								
Df	0.05	0.5703	±	0.0017	23.92	±	2.26	fg
	0.1	0.5538	±	0.0032	26.13	±	2.83	efg
	0.2	0.5438	±	0.0021	27.47	±	2.26	defg
	0.5	0.5343	±	0.0012	28.73	±	2.49	defg
	0.8	0.5172	±	0.0039	31.01	±	2.08	cdef
	1	0.4928	±	0.0186	34.26	±	1.59	cd
Cy	0.05	0.5815	±	0.0345	22.44	±	2.68	g
	0.1	0.5524	±	0.0038	26.31	±	2.85	efg
	0.2	0.5401	±	0.0031	27.95	±	2.58	defg
	0.5	0.5351	±	0.0014	28.63	±	2.30	defg
	0.8	0.5248	±	0.0028	30.00	±	2.63	def
	1	0.5075	±	0.0021	32.31	±	1.94	cde
Qc	0.05	0.5647	±	0.0004	24.67	±	2.41	fg
	0.1	0.5511	±	0.0029	26.49	±	2.18	efg
	0.2	0.5452	±	0.0009	27.28	±	2.26	defg
	0.5	0.5345	±	0.0032	28.70	±	2.73	defg
	0.8	0.5225	±	0.0018	30.30	±	2.48	def
	1	0.4672	±	0.0008	37.68	±	2.08	c
Ds	0.05	0.1540	±	0.0268	79.46	±	4.23	b
	0.1	0.0870	±	0.0056	88.39	±	1.12	a
	0.2	0.0702	±	0.0019	90.63	±	0.45	a
	0.5	0.0533	±	0.0019	92.89	±	0.47	a
	0.8	0.0443	±	0.0002	94.09	±	0.20	a
	1	0.0359	±	0.0038	95.21	±	0.44	a
Anti-inflammatory activity with mortiño extract - Membrane stability: Temperature								
Ep	0.05	0.1675	±	0.0244	77.66	±	2.94	a
	0.1	0.1132	±	0.0019	84.90	±	0.35	b
	0.2	0.1040	±	0.0009	86.13	±	0.36	b
	0.5	0.0751	±	0.0047	89.99	±	0.70	c
	0.8	0.0684	±	0.0005	90.87	±	0.36	c
	1	0.0558	±	0.0012	92.56	±	0.15	c

A= absorbance y SD= standard deviation

Minimun inhibitory concentration (MIC)

We found that higher concentrations led to greater percentages of inhibition. In the case of *P. aeruginosa*, cyanidin and delphinidin inhibited bacterial growth at 2.5 µg/mL, with 82.99% and 89.88% inhibition, respectively. In contrast, the 50% extract showed

Table 2. Two-way ANOVA (treatment × concentration) for standard compounds and one-way ANOVA for mortiño extract in the membrane stabilization assay under thermal stress conditions. Statistical significance: $p < 0.05$

	Df	Suma square	Mean square	F Value	P value
Two-way ANOVA - Anti-inflammatory activity with various treatments					
Concentration (Conc)	5	1038	208	40.641	$3.95x10^{-16}$
Treatment	3	51176	17059	3339.16	$< 2x10^{-16}$
Conc:Treatment	15	152	10	1.989	0.0366
Residuals	48	245	5		
One-way ANOVA - Anti-inflammatory activity with mortiño extract					
Concentration	5	440.3	88.06	55.39	$7.13x10^{-8}$
Residuals	12	19.1	1.59		

Table 3. Inhibition halos (mm) of the fluid extract of *Vaccinium floribundum* Kunth in antibiogram (Kirby-Bauer method) against pathogenic microorganisms in urinary tract infections

	P. aeruginosa	***S. saprophyticus***	***E. coli***	***C. tropicalis***
E50	No inhibition	9.46 ***	7.47 **	9.17***
E100	8.68**	9.14***	8.36 **	10.39***
Cefepime	25.11***	-	-	-
Nitrofurantoin	-	25.64***	-	-
Streptomycin	-	-	25.14***	-
Fluconazole	-	-	-	No inhibition

(*): Resistance; (**): Intermediate; (***): Sensivity; (-): Not applicable

a minimum inhibitory concentration (MIC) of 12,500 µg/mL with a 71.45% inhibition, while the 100% extract's MIC was 25,000 µg/mL, with a 66.37% inhibition. These values are comparable to those reported by **Sanhueza *et al*.** (2023), who observed inhibition rates between 60% and 80%.

*A*t a 2.5 µg/mL MIC, cyanidin exhibited a higher inhibition (93.05%) compared to delphinidin (64.78%). As for the extracts, the 50% concentration showed a 3,125 µg/mL MIC, with 52.29% inhibition, while the 100% extract had a 6,250 µg/mL MIC, with a slightly higher inhibition of 52.81%, confirming its effectiveness. Similarly, **Lamija *et al.*** (2021) reported favorable inhibition values.

Lower inhibition percentages were observed for *E. coli* at 2.5 µg/mL: cyanidin and delphinidin showed inhibition values of 40.96% and 42.99%, respectively. For the 50% extract, the MIC was 12,500 µg/mL, with a 35.04% inhibition, while the 100% extract showed a 2,500 µg/mL MIC, with a 18.55% inhibition. These findings are consistent with those by **Tempera *et al.*** (2010), who reported an inhibition percentage of 50.9%.

Finally, in *C. tropicalis,* cyanidin and delphinidin showed a 0.63 µg/mL MIC, with inhibition percentages of 12.08% and 13.97%, respectively. The 50% extract resulted in a 3,125 µg/mL MIC, with 8.87% inhibition, while the 100% extract showed a 6,250 µg/mL MIC, with a 12.88% inhibition. These results are comparable to those reported by **Musuña & Valera** 2020, who found a 79% inhibition with a 264 µg/mL MIC, which supports its effectiveness as a potential treatment for urinary tract infections (**Table 4**).

Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales. 2026
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Table 4. MIC and inhibition percentage (%I) of the fluid extract of *Vaccinium floribundum* (mortiño) and anthocyanidins Cy and Df against pathogenic microorganisms in urinary tract infections

		P. aeruginosa	*S. saprophyticus*	*E. coli*	*C. tropicalis*
E50	MIC (µg/mL)	12500	3125	12500	3125
	%I	71.45 ± 1.22	55.29 ± 1.44	35.04 ± 2.87	8.87 ± 5.54
E100	MIC (µg/mL)	25000	6250	25000	6250
	%I	66.37 ± 2.44	52.81 ± 0.26	18.55 ± 9.33	12.88 ± 2.08
Cy	MIC (µg/mL)	2.5	2.5	2.5	0.63
	%I	82.99 ± 0.93	93.05 ± 3.09	40.96 ± 13.01	12.08 ± 5.68
Df	MIC (µg/mL)	2.5	2.5	2.5	0.63
	%I	89.88 ± 1.36	64.78 ± 6.11	42.99 ± 4.27	13.97 ± 3.27

Conclusions

We found that cyanidin, identified by TLC and HPLC based on maximum retention times and absorbance, was the predominant anthocyanidin in *Vaccinium floribundum* (mortiño) extract. Total anthocyanin content varied depending on the method used, with values of 0.416 ± 0.03 mg C3G Eq/g FW (pH differential method) and 1.47 ± 0.01 and 1.30 ± 0.00 mg/g FW (HPLC analysis of acidified and pure extracts), respectively.

The antioxidant activity was relatively low under the conditions evaluated, which may be related to the extraction solvent and its selectivity toward anthocyanins. Regarding the anti-inflammatory activity, the extract exhibited a maximum inhibition of 92.56% and 12.10% at 1000 µg/mL in membrane stabilization assays induced by temperature and hypotonicity, respectively. In the temperature-induced assay, the activity was comparable to that of diclofenac, suggesting relevant bioactive potential.

Additionally, the extract showed antimicrobial activity against all tested microorganisms, with the highest effects observed against *S. saprophyticus* and *C. tropicalis* at 50% and 100% concentrations. Overall, these results indicate that anthocyanin-rich extracts from *Vaccinium floribundum* possess anti-inflammatory and antimicrobial activities, supporting their potential as a source of bioactive compounds. However, further studies are required to evaluate their efficacy and safety *in vivo*.

Acknowledgments

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Author contributions

J.M.: Mortiño extract obtention, botanical classification of the species in QUPS herbarium, quantification methodology, manuscript review, and drafting. **A.E.:** Evaluation of antioxidant and anti-inflammatory activity. **D.V.:** ATCC strain reactivation and antimicrobial activity evaluation. **M.M.:** Data analysis and contribution of materials, ATCC strains, and reactants.

Conflicts of interest

The authors declare no conflicts of interest.

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