Anti-inflammatory, antinociceptive, antioxidant, and antimicrobial activities of hydroalcoholic extracts of *Witheringia solanacea* L'Hér

Actividad antiinflamatoria, antinociceptiva, antioxidante y antimicrobiana de extractos hidroalcohólicos de *Witheringia solanacea* L'Hér

Running title: Pharmacological activities of *Witheringia solanacea*

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Design	X	X			
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ABBREVIATIONS: DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; DW: dry weight; E%: percentage of edema; EDTAE: ethylenediaminetetraacetic acid equivalents; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; FICA: ferrous iron chelating activity assay; FRAP: ferric reducing antioxidant power assay; GAE: gallic acid equivalents; HAT: hydrogen atom transfer; MIC: minimum inhibitory concentration; ORAC: oxygen radical absorbance capacity assay; QE: quercetin equivalents; SET: single electron transfer; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; TE: Trolox equivalents; TFC: total flavonoid content; TPC: total phenolic content.

ABSTRACT

Context: *Witheringia solanacea* has traditionally been used in Latin American medicine for its anti-inflammatory and antimicrobial properties, as well as for general pain management. However, few pharmacological studies have been conducted to validate these traditional uses.

Aims: To determine the chemical composition and evaluate selected pharmacological activities of *W. solanacea* extracts using various experimental models.

Methods: Hydroalcoholic extracts from fruit and aerial parts of *W. solanacea* were analyzed by high-performance thin layer chromatography to detect secondary metabolites. Qualitative phytochemical screening and quantification of total phenolic and flavonoid contents were performed. The antioxidant and antibacterial activities of the extracts were evaluated *in vitro*. Additionally, acute oral toxicity, analgesic, and anti-inflammatory activities of the aerial-part extract were assessed in rats.

Results: Phytochemical analysis confirmed the presence of phenolics, flavonoids, coumarins, terpenoids, triterpenes, steroids, and alkaloids. Fruit extracts exhibited higher antioxidant activity than aerial-part extracts. The extracts showed only limited antibacterial activity, with effects observed only against *E. faecalis* (MIC ≈ 5 mg/mL). The aerial-part extract was classified as non-toxic (LD_{50} > 2000 mg/kg). *In vivo*, this extract produced significant analgesic effects in the tail-flick model and significantly reduced carrageenan-induced paw edema and leukocyte infiltration, with effects comparable to those of indomethacin.

Conclusions: *W. solanacea* aerial part extract exhibits analgesic and anti-inflammatory properties that support its traditional use for pain and inflammation, although only limited antibacterial activity was observed.

Keywords: anti-inflammatory; antimicrobial; antinociceptive; antioxidant; oral toxicity; *Witheringia solanacea*.

RESUMEN

Contexto: La planta *Witheringia solanacea* ha sido utilizada tradicionalmente en la medicina latinoamericana por sus propiedades antiinflamatorias y antimicrobianas, así como para el tratamiento del dolor en general. Sin embargo, pocos estudios farmacológicos han validado estos usos tradicionales.

Objetivos: Determinar la composición química y evaluar algunas actividades farmacológicas de los extractos de *W. solanacea* mediante diversos modelos experimentales.

Métodos: Se analizaron extractos hidroalcohólicos de frutos y partes aéreas de *W. solanacea* mediante cromatografía de capa fina de alta resolución para detectar metabolitos secundarios. Además, se realizaron pruebas fitoquímicas cualitativas y determinación del contenido de compuestos fenólicos y flavonoides totales. Los extractos fueron evaluados para determinar su actividad antioxidante y antibacteriana *in vitro*. También se evaluó la toxicidad oral aguda y las actividades analgésicas y antiinflamatorias del extracto de partes aéreas en ratas.

Resultados: El análisis fitoquímico confirmó la presencia de compuestos fenólicos, flavonoides, cumarinas, terpenoides, triterpenos, esteroides y alcaloides. Los extractos de frutos mostraron una mayor actividad antioxidante que los extractos de partes aéreas. Por otro lado, los extractos presentaron una débil actividad antibacteriana, observándose efectos únicamente contra

E. faecalis (CMI ≈ 5 mg/mL). El extracto de partes aéreas se clasificó como no tóxico (DL_{50} > 2000 mg/kg). *In vivo*, este extracto produjo efectos analgésicos significativos en el modelo de retiro de la cola y redujo significativamente el edema plantar inducido por carragenina y la infiltración leucocitaria, con efectos comparables a los de la indometacina.

Conclusiones: El extracto de partes aéreas de *W. solanacea* presenta propiedades analgésicas y antiinflamatorias que respaldan su uso tradicional para el tratamiento del dolor y la inflamación; sin embargo, se observó únicamente una actividad antibacteriana limitada.

Palabras claves: antiinflamatorio; antimicrobiano; antinociceptivo; antioxidante; toxicidad oral; *Witheringia solanacea*

INTRODUCTION

Witheringia solanacea L'Hér is a small shrub (1-4 m high) that belongs to the Solanaceae family. This species is widely distributed from southern Mexico to Bolivia in South America, through Central America and the Caribbean islands. The plant is typically found between 0 and 2000 m above sea level (Pequeno et al., 2017).

W. solanacea has several medicinal ethnobotanical applications in Mesoamerica and South America. For example, in Mexico and Nicaragua, this plant is used to treat skin illnesses such as acne (Jacobo-Herrera et al., 2006; Coe, 2008). In Costa Rica, *W. solanacea* is used to treat infections; the fruit is eaten to alleviate headaches and stomachaches, and the roots are used to treat diabetes (García et al., 2006). In Panama, the whole plant and fruit are used for general body pain, skin diseases, hypertension, and as an anthelmintic (Gupta et al., 1993; Caballero-George et al., 2001). In Ecuador, the plant is widely used by several indigenous tribes; infusions and the juice from the leaves and fruit are employed to treat headaches, inflammation, skin infections, stomachaches, diarrhea, bronchitis and tuberculosis (Ballesteros et al., 2016).

Despite the extensive use of this plant in traditional Latin American medicine, few pharmacological studies have confirmed the traditional use of *W. solanacea*. Some studies have investigated the hypoglycemic activity (Herrera et al., 2011; Pequeno et al., 2021) and the activity against malaria and leishmaniasis (Chinchilla et al., 2012; Chinchilla-Carmona et al., 2014) of different extracts and parts of the plant.

Moreover, the phytochemistry of *W. solanacea* is not well known, but it has been related to *Witheringia coccoloboides*, from which seven physalins have been isolated. Physalins are steroidal lactones commonly present in the Solanaceae family and have a variety of biological activities, such as antitumor, antibacterial, and anti-inflammatory properties (Wu et al., 2021; Meira et al., 2022). Physalins B, D, and F have been isolated from the leaves of *W. solanacea.*

Physalins B and F have demonstrated anti-inflammatory effects in *in vitro* models using cell cultures (Jacobo-Herrera et al., 2006).

To date, no studies have assessed the anti-inflammatory and analgesic activities of *W. solanacea* in animal models, which could provide additional biologically relevant evidence supporting the ethnobotanical applications of this plant. Furthermore, to the best of our knowledge, no previous information has been reported regarding the evaluation of the antibacterial effect of *W. solanacea* extracts, despite its traditional use for treating infections. Given the limited pharmacological data available on *W. solanacea*, this study evaluated its antimicrobial, anti-inflammatory, and analgesic activities using hydroalcoholic extracts of fruit and aerial parts from two regions of Costa Rica. Acute oral toxicity, antioxidant capacity, and phytochemical profiles were also determined.

MATERIAL AND METHODS

Collection of plant material

Aerial parts of *W. solanacea* were collected in February 2021 in two different locations of Costa Rica: Puerto Jiménez, Puntarenas province (South Pacific Region) (8º33´14´´ N, 83º23´56´´ W) and Mora, San José province (Central Region) (9º54´ 52.9´´ N, 84º16´50.6´´ W). Taxonomic identification was performed by an expert botanist from the Nacional University of Costa Rica. A voucher specimen of the species was deposited in the "Juvenal Valerio Rodríguez" Herbarium (Nacional University of Costa Rica) under the acquisition numbers JVR5362 and JVR5363, respectively.

Preparation of extracts

Combined stems and leaves of *W. solanacea* were shade-dried and ground to a particle size of 2 mm using a blade mill (Wiley, United States). Ripe fruits were frozen at -80 °C and freeze-dried using a LABCONCO Freezone 6 system (United States). The dried fruit was milled with a food processor and sieved to a maximum particle size of 2 mm. Each plant material was macerated separately in a 1:10 ratio with 80%(v/v) ethanol for two weeks. The extract was decanted, and the plant residue was subjected to a second one-week maceration under the same conditions. The resulting extract was decanted and combined with the first extract. The pooled extract was filtered through a 0.45 µm PVDF membrane and ethanol was removed by evaporation at 40 °C (Büchi R-100, Switzerland). The remaining aqueous phase was freeze-dried to obtain the dried extracts. Four extracts were obtained, corresponding to: fruit from Mora (F1), fruit from Puerto Jiménez (F2), stems and leaves from Mora (SL1), and stems and leaves from Puerto Jiménez (SL2). Extraction yields were calculated, and the extracts were stored at -20 °C for further analyses.

Chemical characterization of the extracts

Extracts solutions (10 mg/mL) were prepared in a 1:1 methanol-water mixture, and 25 µL were applied onto HPTLC-grade silica gel 60 F254 plates (20 x 10 cm; Millipore, Germany). Samples were sprayed as 8 mm x 1 mm bands using the ATS4 autosampler (CAMAG, Switzerland) at 150 nL/s, with nitrogen as the propellant gas. The first band was positioned 20 mm from the left edge of the plate, and the solvent front was set at 80 mm from the bottom. After application, the plates were dried for 30 seconds using the ADC2 automatic developing chamber (CAMAG, Switzerland). The chamber was then saturated with the mobile phase. Following saturation, the plates were developed and dried for 5 minutes. Chromatographic development was conducted at room temperature (24 ± 3 °C) and relative humidity (51 ± 3 %). Chromatograms were visualized under visible light or UV light (254 or 366 nm) using a TLC visualizer (CAMAG, Switzerland).

Conditions for the detection of phenolic compounds by HPTLC

For the detection of highly polar phenolic compounds (e.g. glycosidic phenolics), a single development system was employed using a mobile phase composed of butan-2-ol: *n*-butanol, ethyl acetate, and formic acid (60:40:15:10). The development chamber was saturated for 20 minutes prior to use. For low-polarity phenolic compounds (e.g. non-glycosidic phenolics), a two-step development system was applied. Plates were first developed with a mobile phase of toluene, ethyl acetate, and formic acid (60:45:3), followed by a second elution using toluene, ethyl acetate, *n*-hexane, and formic acid (60:30:10:3). The chamber was saturated for 10 minutes before each elution step, and plates were dried in the ADC2 development chamber prior to the second development. After development, plates were derivatized using potassium ferricyanide and ferric chloride reagents (detection of phenolics), aluminum chloride reagent (detection of flavonoids) or potassium hydroxide solution (detection of coumarins, flavonoids, and quinones). All reagents were prepared and applied according to Lock de Ugaz (1994).

Conditions for the detection of terpenoids by HPTLC

Terpenoids were detected using a mobile phase of chloroform, methanol, and water (100:40:5) after 20 minutes of chamber saturation. Following development, plates were derivatized with either anisaldehyde/sulfuric acid or Liebermann-Burchard reagent, both prepared and applied according to Lock de Ugaz (1994).

Conditions for the detection of alkaloids by HPTLC

Alkaloids were detected using a mobile phase of chloroform, methanol, and ammonium hydroxide (47.5:47.5:5) after 20 minutes of chamber saturation. Following development, plates were derivatized with Dragendorff's reagent, as described by Lock de Ugaz (1994).

Additional qualitative tests

Additionally, the extracts underwent preliminary phytochemical screening to identify secondary metabolite groups, including carbohydrates, reducing sugars, amino acids and peptides, saponins, tannins, and cardiac glycosides, following standard protocols (Lock de Ugaz, 1994; Tiwari et al., 2011).

Determination of Total Phenolic Content (TPC)

TPC of the extracts were determined in triplicate using the 96-well Folin-Ciocalteu method described by Bobo-García et al. (2015). Results were expressed as the mean concentration of gallic acid equivalents (GAE) (mg GAE/g dry weight (DW)) ± standard error of the mean (SEM).

Determination of Total Flavonoid Content (TFC)

TFC of extracts was measured in triplicate using a modified aluminum chloride method based on Magalhães et al. (2012). Quercetin was used as the standard at a concentration range of 20–60 µg/mL. TFC values were expressed as mean quercetin equivalents (QE) (mg QE/g DW) ± SEM.

Evaluation of antioxidant activity

Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC values were determined following the method of Kenny et al. (2015), with minor modifications. Fluorescence decay curves of standard and samples were recorded over 1 hour. A Trolox calibration curve was constructed within a linearity range of 20-60 µM. Each sample was analyzed in triplicate, and antioxidant capacity was expressed as mean Trolox equivalents (TE) (µmol TE/g DW) ± SEM.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP values were measured using a microplate-adapted version of the method described by Işıl et al. (2010). Trolox standards (20-100 µM) were prepared in methanol. Extract concentrations were adjusted to fall within the linear range of the calibration curve. Aliquots of 20 µL of Trolox or extract solutions were dispensed into microplate wells, followed by the addition of 130 µL of 30 mM HCl, 25 µL of 1.2% (w/v) potassium ferricyanide, and 25 µL of

0.1% (w/v) iron (III) chloride hexahydrate. Methanol was used as the blank for the standard instead of Trolox, while distilled water replaced potassium ferricyanide in the extract blanks to correct for intrinsic absorbance. Plates were incubated at room temperature, and the absorbance was measured at 700 nm using a Synergy HT microplate reader (BioTek Instruments, USA). Each assay was performed in triplicate, and results were reported as mean TE (µmol TE/g DW) ± SEM.

2,2- Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay

The concentration of extract required to reduce 50% of DPPH radicals (IC_{50}) was determined using a microplate assay method adapted from Kenny et al. (2015). The IC_{50} values were obtained by interpolation from dose-response curves fitted with the Hill equation. Each determination was conducted in triplicate, and antioxidant activity was reported as mean IC_{50} (µg/mL) ± SEM.

Ferrous Iron Chelating Activity (FICA) assay

FICA was assessed following the procedure described by Santos et al. (2017), with minor modifications. A 0.3 mM iron (II) chloride solution was used as iron (II) source, and the EDTA standard curve ranged from 25-100 µM. Each assay was performed in triplicate, and results were expressed as mean EDTA equivalents (EDTAE) (µmol EDTAE/g DW) ± SEM.

Evaluation of antibacterial activity

Staphylococcus aureus (ATCC 6538), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 15442), *Escherichia coli* (ATCC BAA-2452), *Klebsiella pneumoniae* (ATCC 10031), and *Salmonella enterica* subsp. *enterica* serovar Typhimurium (known as *S. typhimurium*) (ATCC 14028) were employed as test microorganisms. The minimum inhibitory concentration (MIC) of the extracts was determined using the broth microdilution method, following the guidelines established by the European

Committee for Antimicrobial Susceptibility Testing (EUCAST, 2003) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). Extracts were tested at a maximum concentration of 10 mg/mL. Ciprofloxacin hydrochloride was used as the positive control for *E. coli* and *E. faecalis*, while ceftriaxone disodium salt was used for the remaining strains. Dimethyl sulfoxide (DMSO) was employed as the solvent for the extracts, at a final concentration not exceeding 1% (v/v) in the culture broth. For antibiotic controls, the culture broth was used without any added solvent.

Experimental animals

Sprague–Dawley rats (11-12 weeks old) were obtained from the Biological Testing Laboratory (LEBi), University of Costa Rica. All experimental protocols were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA 038-2020) and complied with the International Guiding Principles for Biomedical Research Involving Animals (CIOMS). Animals were maintained under standard conditions (22 ± 2 ◦C, light/dark cycles of 12 h) and *ad libitum* access to food and water.

Due to limited fruit availability, only the stem and leaf extract (SL2) was used for *in vivo* tests. SL2 was selected over SL1 because of its higher total phenolic content and antioxidant activity, suggesting a greater presence of bioactive compounds.

Acute oral toxicity test

The acute oral toxicity of SL2 was evaluated according to OECD guideline 423. A single oral dose of 2000 mg/kg (dissolved in distilled water) was administered to six female Sprague-Dawley rats (11 weeks old, 260 ± 15 g) fasted overnight. A control group received distilled water. Animals were observed at 30, 60, 120, 180, and 240 minutes after treatment and daily for 14 days. Clinical signs (e.g. tremors, convulsions, dehydration, salivation, diarrhea, lethargy), as well as respiratory, circulatory, autonomic, and central nervous system function, were monitored. Body weight was recorded every two days. At the end of the observation period, animals were euthanized (150 mg/kg i.p. pentobarbital overdose), and necropsies were performed to assess macroscopic changes in target organs.

Evaluation of antinociceptive activity by the tail flick test

Antinociceptive effects were assessed using the tail flick test described by D'Amour and Smith (1941), with a Tail Flick Unit-Thermal Stimulation device (Ugo Basile, model 7360). Male Sprague–Dawley rats (12 weeks old, 350 ± 20 g) were randomized into four groups (n = 8) and treated i.p. with SL2 extract dissolved in saline solution (500 or 1000 mg/kg), morphine (5 mg/kg) as positive control, or saline solution (1 mL) as negative control. Tail withdrawal latency was measured 10 minutes before (pre-treatment latency), and at 30, 60, 90, and 120 minutes after administration of treatments (post-treatment latency). All measurements were performed at the midpoint of the distal third of the tail. A 20 s cut-off time was applied to prevent tissue damage. Results were expressed as percentage of antinociception (A%) according to the following formula: *A% = (post-treatment latency – pre-treatment latency) / (cut-off time – pre-treatment latency) x 100.*

Evaluation of anti-inflammatory activity by the carrageenan-induced paw edema model

The anti-inflammatory effect was assessed according to the method described by Winter et al. (1962), with minor modifications. Male Sprague–Dawley rats (11 weeks old, 300 ± 20 g) were randomized into four groups (n = 8) and treated i.p. with SL2 extract dissolved in saline solution (250 or 500 mg/kg), indomethacin (50 mg/kg) as positive control, or saline (1 mL) as negative control. After one hour, 0.1 mL 1% (w/v) of carrageenan was injected subplantarly into the right hind paw, and 0.1 mL of saline into the left paw. Paw thickness was measured in duplicate at 1, 2, 4, 6, and 24 hours using a digital caliper (Kroeplin, model C330). Results were expressed as percentage of edema (E%) according to the following formula: *E% = (right paw size – left paw size) / (left paw size) x 100.*

Histology of paw tissue

At the end of the anti-inflammatory evaluation, animals were euthanized by decapitation. Subplantar skin samples were fixed in 10% formalin-PBS for 48 h, processed, embedded in paraffin, sectioned (5 μm), and stained with hematoxylin–eosin. Histopathological evaluation focused on tissue congestion and leukocyte infiltration.

Statistical analysis

Results were expressed as means ± SEM. Data from antioxidant activity assays were analyzed by one-way ANOVA followed by Tukey's *post hoc* tests and Pearson´s correlation. *In vivo* results were analyzed using two-way repeated measures ANOVA followed by Dunnett´s multiple comparisons test. A p value < 0.05 was considered statistically significant. Analyses were performed using the GraphPad Prism® version 8.

RESULTS

Chemical characterization of the extracts

Data from the detection of different groups of metabolites in the ethanolic extracts obtained from *W. solanacea* are summarized in Table 1. HPTLC chromatograms are shown in Figs. 1 to 4. Fruit extracts contained a more diverse array of phenolic compounds compared to extracts from stems and leaves (Fig. 1A). Moreover, most of the phenolics present in the extracts were highly polar. The flavonoids present in F1 and F2 consisted of a mixture of polar and non-polar compounds, with F1 containing the greatest number of such compounds (Fig. 1B and Fig. 2B). Coumarins were only detected in SL1 and SL2 under low-polarity HPTLC development conditions; however, under high-polarity elution conditions, coumarins were also found in F2 but not in F1 (Fig. 1C and Fig. 2C).

288 **Table 1.** Results of qualitative tests for the detection of metabolite groups in *W. solanacea* extracts.

Detected metabolites	**Extract**			
	F1	**F2**	**SL1**	**SL2**
Metabolites detected by HPTLC				
Phenolics	+	+	+	+
Flavonoids	+	+	+	+
Quinones	-	-	-	-
Coumarins	-	+	+	+
Terpenoids	+	+	+	+
Triterpenes and Steroids	+	+	+	+
Alkaloids	+	+	-	-
Metabolites detected by qualitative phytochemical screening				
Carbohydrates (Molisch test)	+	+	+	+
Carbohydrates (Benedict test)	+	+	+	+
Amino acids and peptides (Ninhydrin test)	+	+	+	+
Amino acids and peptides (Biuret test)	+	+	+	+
Saponins (Foam test)	-	-	-	-
Tannins (Protein precipitation test)	-	-	-	-

Cardiac glycosides (Kedde test)	-	-	-	-
Cardiac glycosides (Keller-Killiani test)	-	-	-	-

+: Positive result; -: Negative result. F1: Fruit extract from Mora; F2: Fruit extract from Puerto Jiménez; SL1: Stem and leaf extract from Mora; SL2: Stem and leaf extract from Puerto Jiménez.

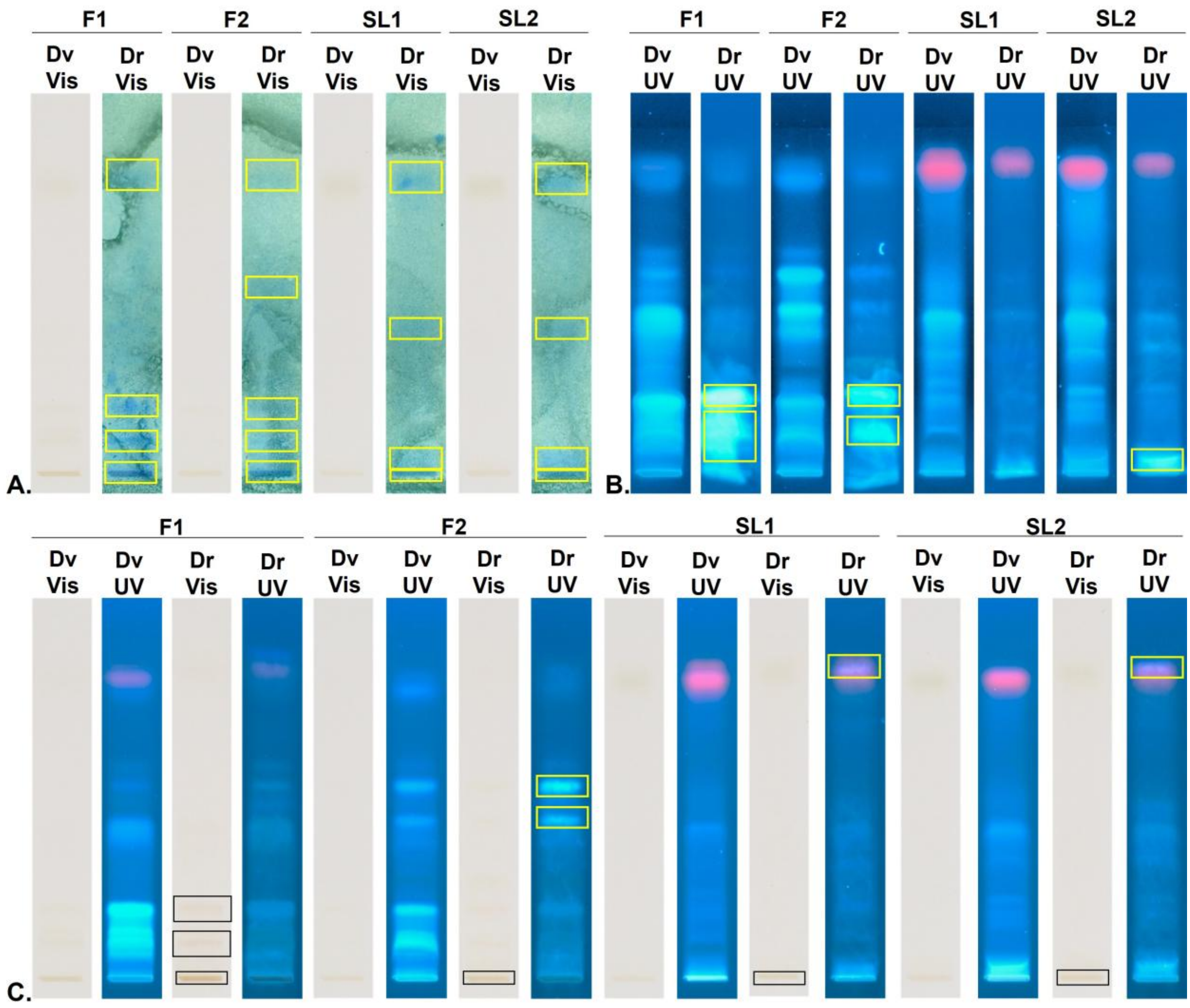


Figure 1. Chromatograms showing the detection of polar phenolic compounds in *W. solanacea* extracts. **A)** General phenolics (derivatization with potassium ferricyanide and ferric chloride reagent). **B)** Flavonoids (derivatization with aluminum chloride reagent). **C)** Coumarins, flavonoids, and quinones (derivatization with potassium hydroxide reagent).

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; UV: chromatogram under UV light (366 nm); Vis: chromatogram observed under visible light. Major positive changes are highlighted with yellow or black boxes.

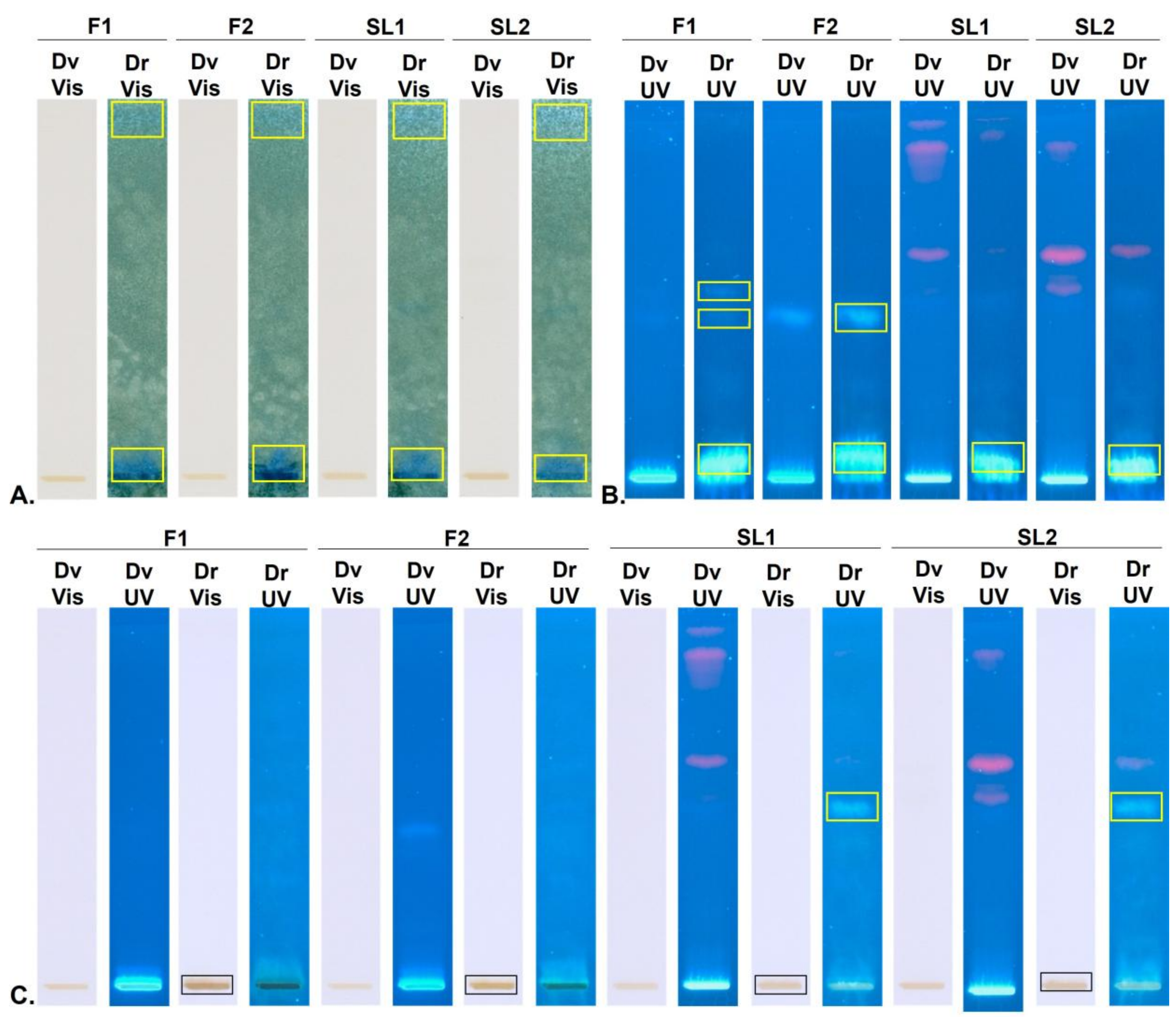


Figure 2. Chromatograms showing the detection of non-polar phenolic compounds in *W. solanacea* extracts. **A)** General phenolics (derivatization with potassium ferricyanide and ferric chloride reagent). **B)** Flavonoids (derivatization with aluminum chloride reagent). **C)** Coumarins, flavonoids, and quinones (derivatization with potassium hydroxide reagent).

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; UV: chromatogram under UV light (366 nm); Vis: chromatogram observed under visible light. Major positive changes are highlighted with yellow or black boxes.

Extracts were also rich in terpenoids (Fig. 3). Differences were observed between extracts of the same plant material collected from different regions; moreover, F1 and SL1 showed a more diverse terpenoid profile compared to F2 and SL2, respectively. The extracts were particularly abundant in triterpenoid and steroidal compounds (Fig. 3B). The fruit extracts also showed the presence of alkaloids, with F1 and F2 exhibiting a similar profile for this group of metabolites (Fig. 4). However, SL1 and SL2 were negative for the presence of alkaloids.

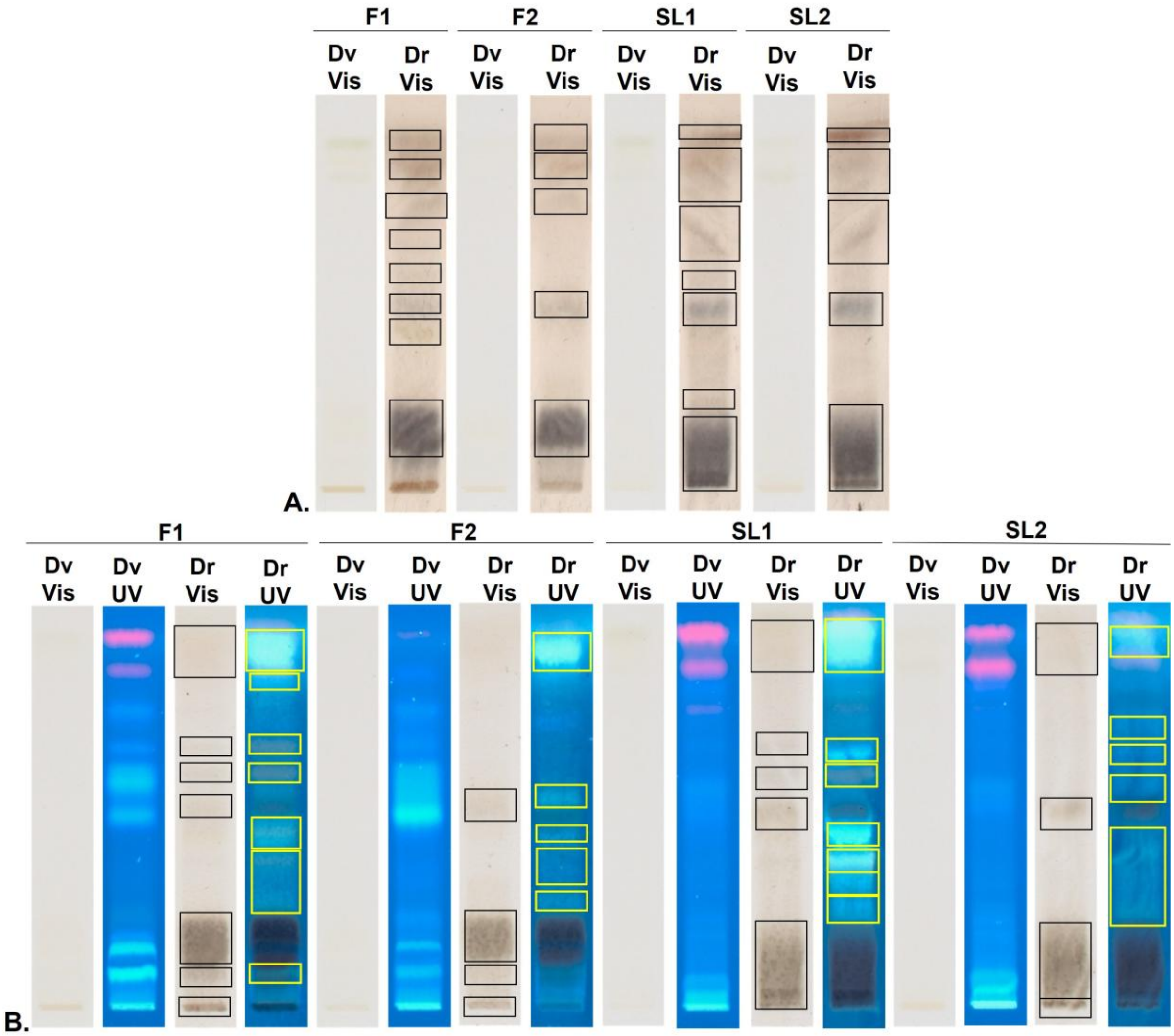


Figure 3. Chromatograms showing the detection of terpenoids in *W. solanacea* extracts. **A)** General terpenoids (derivatization with anisaldehyde/sulfuric acid reagent). **B)** Steroids and triterpenoids (derivatization Liebermann-Burchard reagent).

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; UV: chromatogram under UV light (366 nm); Vis: chromatogram observed under visible light. Major positive changes are highlighted with yellow or black boxes.

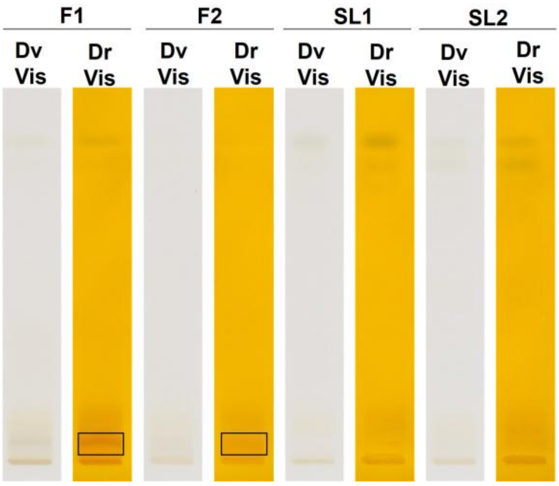


Figure 4. Chromatograms showing the detection of alkaloids in *W. solanacea* extracts by derivatization with Dragendorff reagent.

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; Vis: chromatogram observed under visible light. Major positive changes are highlighted with black boxes.

Determination of TPC, TFC and antioxidant activity

The results of the TPC and TFC assays are presented in Fig. 5A and Fig. 5B. Fruit extracts showed higher levels of TPC and TFC compared to stem and leaf extracts. F1 had the highest TPC value (59.1 ± 0.9 mg GAE/g), whereas F2 showed the highest TFC level (127.7 ± 5 mg QE/g).

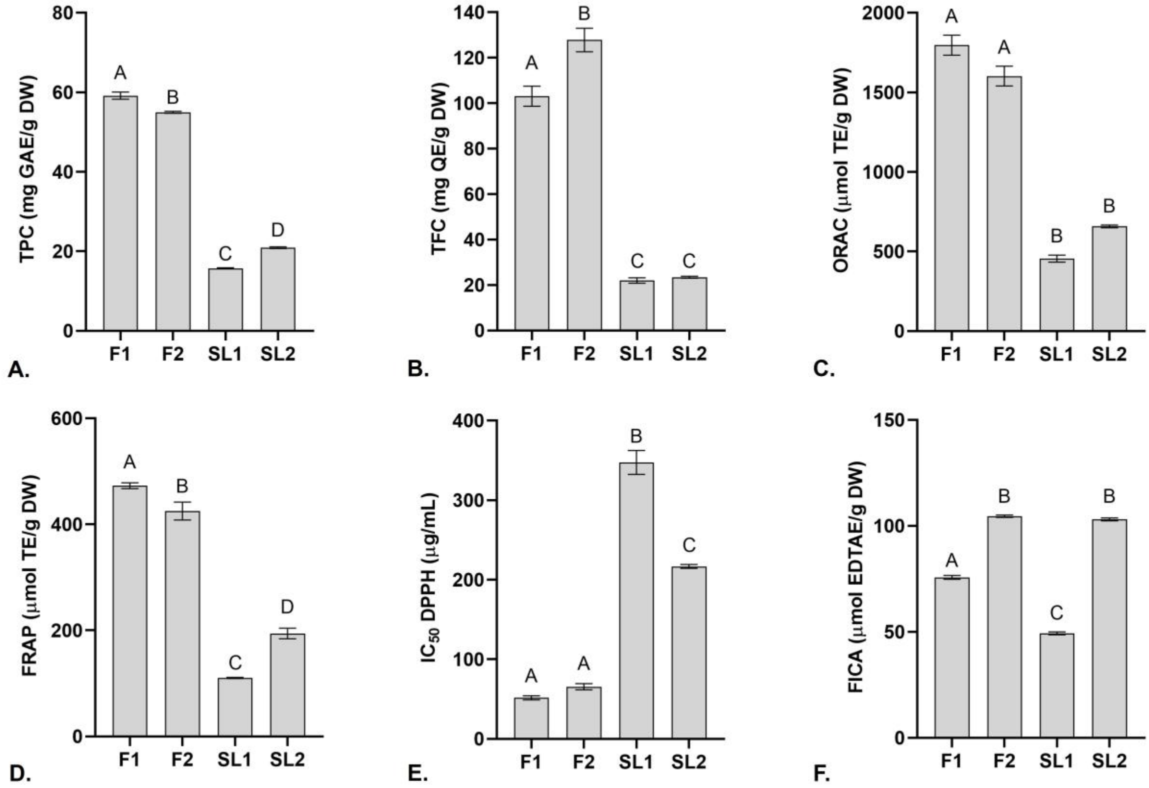
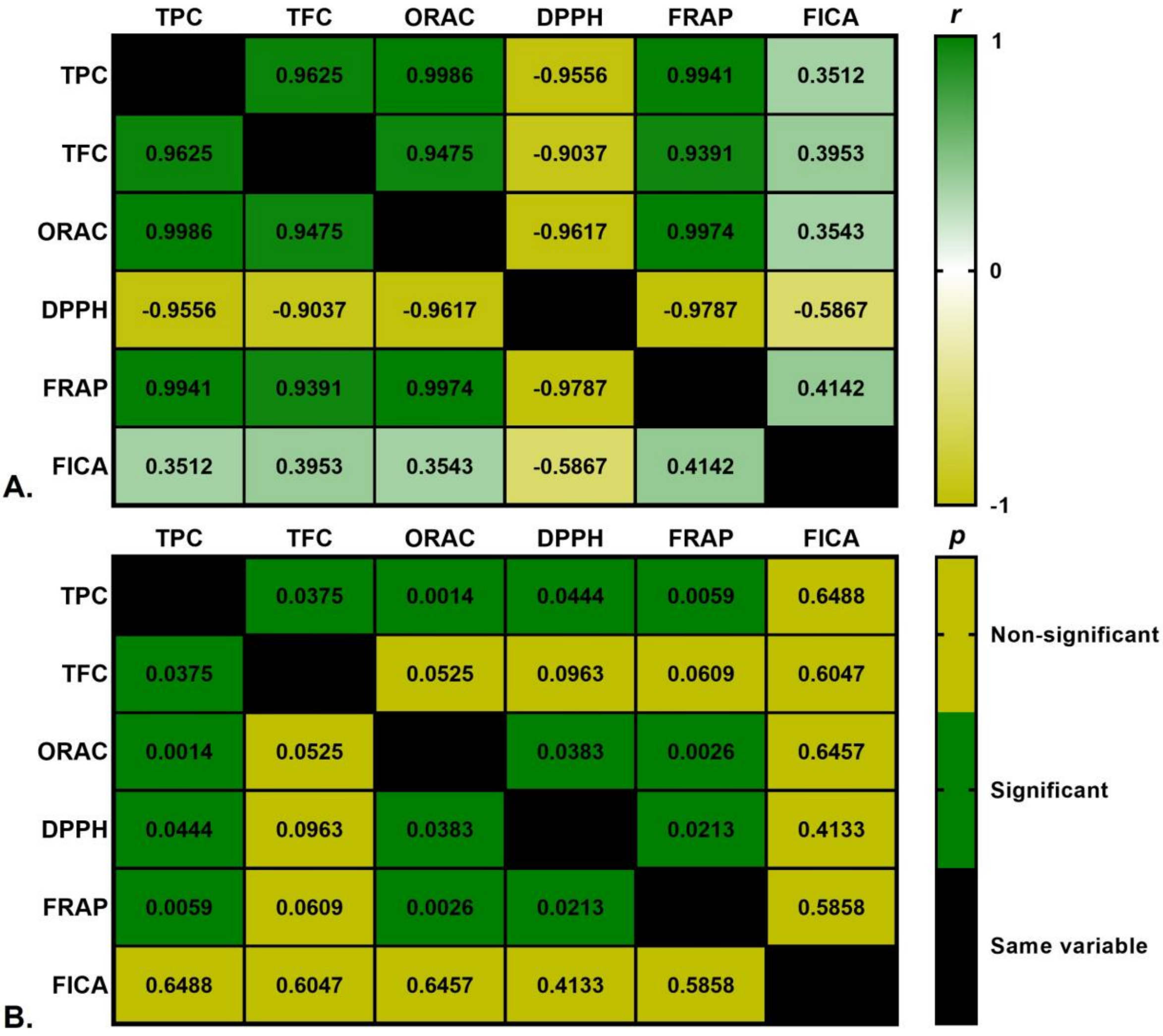


Figure 5. Determination of TPC, TFC, and antioxidant activity of *W. solanacea* extracts. **A)** TPC values. **B)** TFC values. **C)** ORAC values. **D)** FRAP values. **E)** IC_{50} DPPH values. **F)** FICA values.

Different capital letters above the bars indicate significant differences ($p < 0.05$). Results are expressed as mean ± SEM (n = 3). DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; DW: dry weight; EDTAE: ethylenediaminetetraacetic acid equivalents; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; FICA: ferrous iron chelating activity assay; FRAP: ferric reducing antioxidant power assay; GAE: gallic acid equivalents; ORAC: oxygen radical absorbance capacity assay; QE: quercetin equivalents; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; TE: Trolox equivalents; TFC: total flavonoid content; TPC: total phenolic content.

Regarding the antioxidant activity assays, fruit extracts exhibited the strongest antioxidant activity in ORAC, FRAP, and DPPH assays (Fig. 5C, Fig. 5D, and Fig. 5E). F1 was the most potent extract in these assays, with ORAC, FRAP, and DPPH IC_{50} values of 1796 ± 63 µmol TE/g, 472.7 ± 5.4 µmol TE/g, and 51.6 ± 2.5 µg/mL, respectively. In the FICA assay (Fig. 5F), F2 and SL2 were the most active, with FICA values of 104.6 ± 0.5 µmol EDTAE/g and 103.1 ± 0.7 µmol EDTAE/g, respectively.

On the other hand, Fig. 6 indicates a significant correlation between TPC and TFC values and the antioxidant activities evaluated by ORAC, DPPH and FRAP assays. However, the results from the FICA assay did not show a correlation with either TPC or TFC.



	TPC	TFC	ORAC	DPPH	FRAP	FICA
TPC		0.9625	0.9986	-0.9556	0.9941	0.3512
TFC	0.9625		0.9475	-0.9037	0.9391	0.3953
ORAC	0.9986	0.9475		-0.9617	0.9974	0.3543
DPPH	-0.9556	-0.9037	-0.9617		-0.9787	-0.5867
FRAP	0.9941	0.9391	0.9974	-0.9787		0.4142
FICA	0.3512	0.3953	0.3543	-0.5867	0.4142	

	TPC	TFC	ORAC	DPPH	FRAP	FICA
TPC		0.0375	0.0014	0.0444	0.0059	0.6488
TFC	0.0375		0.0525	0.0963	0.0609	0.6047
ORAC	0.0014	0.0525		0.0383	0.0026	0.6457
DPPH	0.0444	0.0963	0.0383		0.0213	0.4133
FRAP	0.0059	0.0609	0.0026	0.0213		0.5858
FICA	0.6488	0.6047	0.6457	0.4133	0.5858	

Figure 6. Pearson's correlation matrix. **A)** Correlation coefficients (*r*). **B)** *p*-values matrix.

DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; FICA: ferrous iron chelating activity assay; FRAP: ferric reducing antioxidant power assay; ORAC: oxygen radical absorbance capacity assay; TFC: total flavonoid content; TPC: total phenolic content.

Determination of antibacterial activity

Table 2 summarizes the results from the broth microdilution assay. SL2 was the only active extract, with a MIC value of 5 mg/mL against *E. faecalis*, which is classified as exhibiting mild antibacterial activity based on the criteria reported by Bussmann et al. (2010).

Table 2. Antibacterial activity of *W. solanacea* extracts against selected bacterial strains.

Bacteria	**Minimum Inhibitory Concentration**				
	F1 (mg/mL)	**F2 (mg/mL)**	**SL1 (mg/mL)**	**SL2 (mg/mL)**	**Control* (µg/mL)**
S. aureus (ATCC 6538)	>5	>5	>5	>5	4
S. epidermidis (ATCC 12228)	>5	>5	>5	>5	2
E. faecalis (ATCC 29212)	>5	>5	>5	**5**	0.5
P. aeruginosa (ATCC 15442)	>5	>5	>5	>5	32
E. coli (ATCC BAA-2452)	>5	>5	>5	>5	0.03
K. pneumoniae (ATCC 10031)	>5	>5	>5	>5	0.03
S. typhimurium (ATCC 14028)	>5	>5	>5	>5	0.06

*Control: ciprofloxacin chloride for *E. coli* and *E. faecalis*, or ceftriaxone disodium salt for other bacteria. F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez.

Acute toxicity test

The acute toxicity test revealed that oral administration of SL2 extract at a single dose of 2000 mg/kg did not cause mortality in the rats during the 14 days of the assay. Since the median lethal dose (LD_{50}) is greater than 2000 mg/kg, this extract is classified as non-toxic according to OECD guideline 423. Moreover, the extract did not cause significant changes in behavior, general appearance, or any signs of toxicity during 14 days following administration. Body weight gain in both control and treated groups was similar. Necropsy conducted 14 days post-administration revealed no significant macroscopic alterations in the target organs.

Antinociceptive activity

The results of the antinociceptive activity of the SL2 extract are presented in Fig. 7. The tail-flick test showed a significant ($p = 0.0334$) increase in baseline latency time (i.e., the time required to withdraw the tail) 30 min after the treatment with 1000 mg/kg of SL2 extract compared to the control group. No significant effect was observed at later time points or with the 500 mg/kg dose. As expected, morphine (5 mg/kg) caused a significant increase in latency time at 30, 60, and 90 minutes post-treatment compared to the control ($p = 0.0005$, $p = 0.0009$, and $p = 0.0119$, respectively). Administration of the vehicle did not significantly affect the nociceptive threshold.

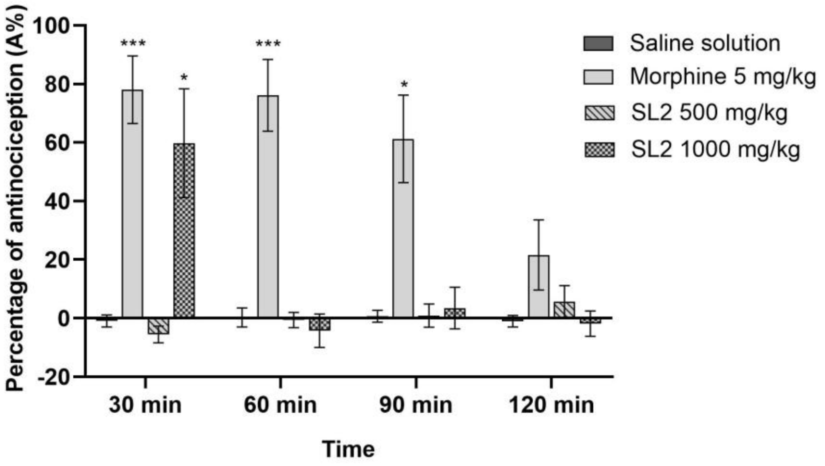


Figure 7. Antinociceptive effect of the stem and leaf extract from Puerto Jiménez (SL2) in the tail flick test in rats.

Results are presented as the mean ± SEM of the percentage of antinociception (n = 8). Statistical significance was determined by two-way repeated measures ANOVA followed by Dunnett´s multiple comparisons test. **p* < 0.05, ****p* < 0.001 compared to control (saline solution).

Anti-inflammatory activity

The results of the anti-inflammatory activity of the SL2 extract are shown in Fig. 8. Injection of carrageenan into the hind paw tissue of rats pretreated with vehicle (control group) caused a progressive increase in paw edema that persisted for 24 hours, with a maximum effect at 4 and 6 hours. Pretreatment with 500 mg/kg of SL2 extract significantly reduced the edema induced by carrageenan at 2 and 4 hours post-injection compared to the control group (p = 0.0209 and p= 0.0043, respectively). No significant reduction was observed at 1, 6 or 24 hours, although a trend toward decreased edema was noted across all time points, similar in magnitude to the indomethacin group. Pretreatment with 250 mg/kg of SL2 extract significantly (p = 0.0222) reduced edema only at 4 hours post-injection. No significant reduction in inflammation was observed at 1, 2, 6, or 24 hours. Indomethacin (50 mg/kg) produced a significant reduction in paw edema at 2 and 4 hours post-injection as compared to the control group (p = 0.0229 and p = 0.0143, respectively).

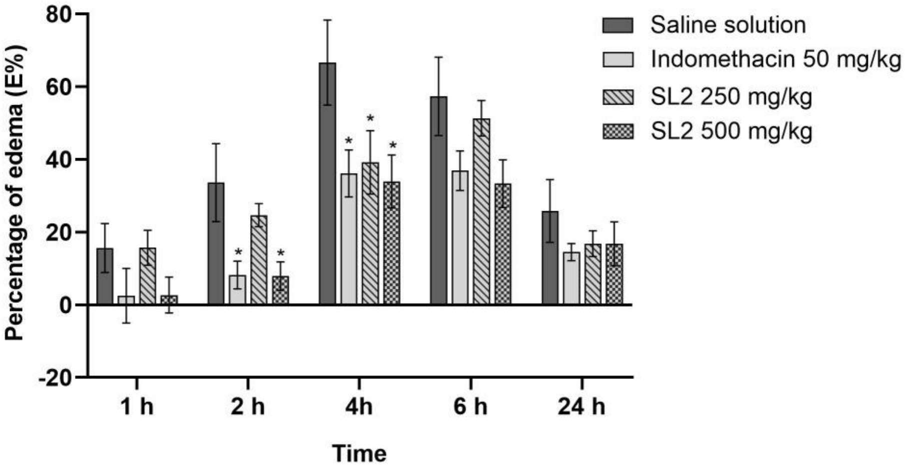


Figure 8. Anti-inflammatory effect of the stem and leaf extract from Puerto Jiménez (SL2) extract in carrageenan-induced paw edema in rats.

Results are presented as the mean ± SEM of the percentage of edema (right hind paw) relative to non-inflamed control (left hind paw) (n = 8). Statistical significance was determined by two-way repeated measures ANOVA followed by Dunnett´s multiple comparison test. *$p < 0.05$ compared to control (saline solution).

The histological biopsies of the rat paw 24 hours after carrageenan injection are presented in Fig. 9. Paw tissue from the control group pretreated with vehicle (Fig. 9A) showed typical signs of acute inflammation, including leukocyte infiltration and congestion, compared to normal paw tissue (Fig. 9B). Pretreatment with indomethacin decreased paw swelling and leukocyte infiltration (Fig. 9E). Pretreatment with 500 mg/kg of SL2 extract similarly reduced swelling and leukocyte infiltration to a comparable extent (Fig. 9C). Paw tissue from animals pretreated with 250 mg/kg of SL2 extract showed more leukocyte infiltration and congestion than the group treated with the higher dose (Fig. 9D).

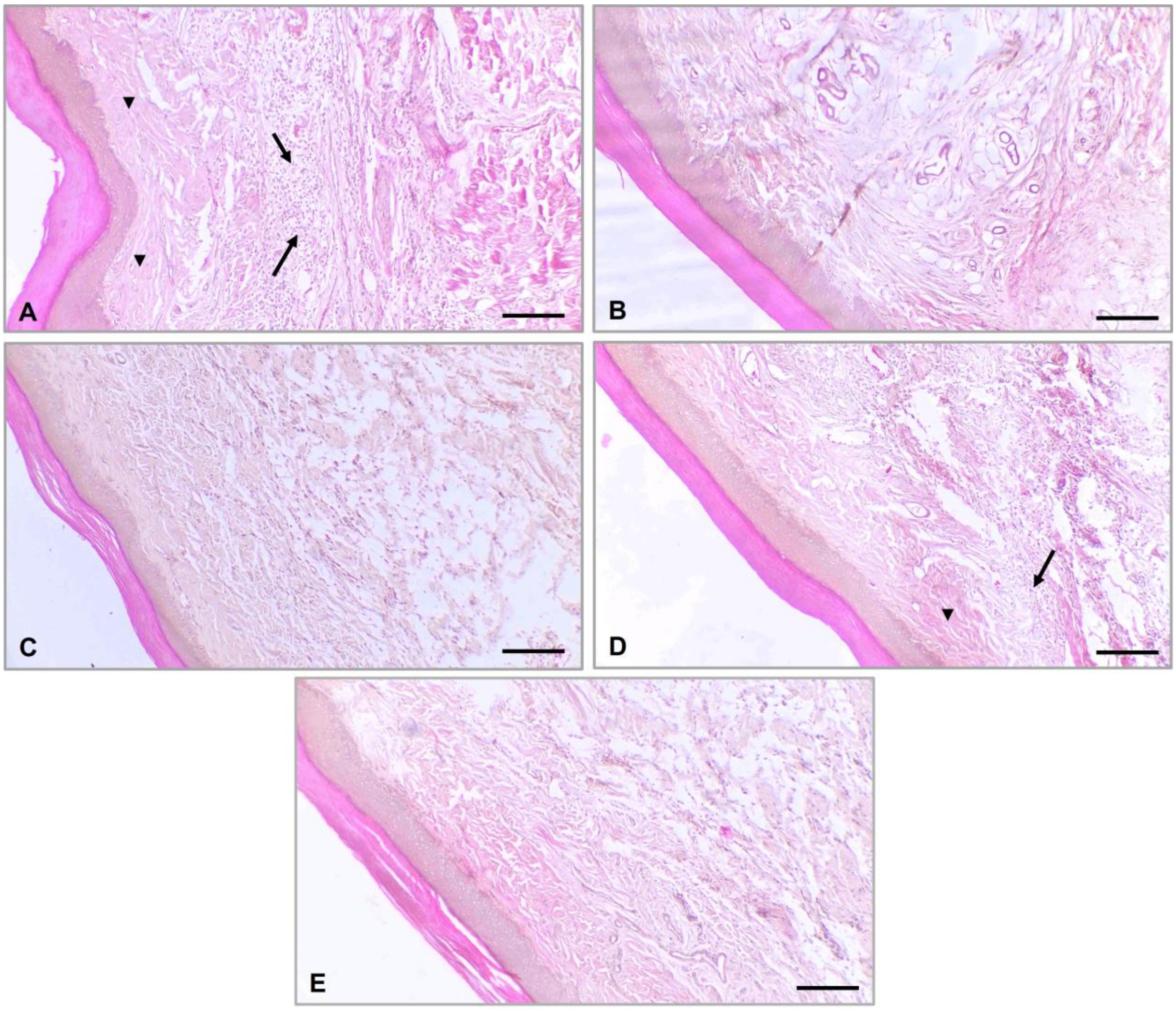


Figure 9. Paw tissue dissected 24 hours after injection of **A)** carrageenan or **B)** saline solution in control groups; **C)** 500 mg/kg, **D)** 250 mg/kg of stem and leaf extract from Puerto Jiménez (SL2); and **E)** 50 mg/kg of indomethacin. Arrows indicate leukocyte infiltration. Arrowheads indicate congestion and swelling in the tissue. Hematoxylin–eosin staining. Scale bar = 50 µm.

DISCUSSION

In Latin American countries, *W. solanacea* has traditionally been used for various purposes, including as an anti-inflammatory and antimicrobial agent, and for general pain relief. Ethnobotanical reports describe the use of different parts of the plant – such as fruits, stems, and leaves - prepared using diverse methods (Gupta et al., 1993; Caballero-George et al., 2001; García et al., 2006; Jacobo-Herrera et al., 2006; Coe, 2008; Ballesteros et al., 2016). Given the diversity in traditional preparations, ethanolic extracts of each plant material were prepared for chemical characterization and biological evaluation.

The primary finding from chemical characterization was the variation among extracts from different plant parts and collection sites. Phytochemical screening and HPTLC analysis revealed the presence of phenolics, flavonoids, coumarins, terpenoids, alkaloids, triterpenes, and steroids. Variation in phytochemical profiles between plant parts is typical in medicinal plants (Ak et al., 2020). Furthermore, chemical differences related to collection sites have been reported in other species (Camara et al., 2021). F1 and SL1 were collected in Costa Rica´s Central Valley (cooler, moderate rainfall), while F2 and SL2 were from the South Pacific Region (hotter, more humid). Environmental factors such as altitude, temperature, solar radiation, and precipitation are known to influence the metabolite profiles (Liu et al., 2016). Genotypic variation may also contribute to the observed differences (Camara et al., 2021). These aspects warrant further in-depth investigation.

The extracts were rich in triterpenoid and steroidal compounds, consistent with previous reports of these metabolite groups in species of the *Witheringia* genus, including *W. solanacea* (Pequeno et al., 2017). Previous studies have isolated physalins —steroidal lactones— from this species (Jacobo-Herrera et al., 2006).

As expected for Solanaceae species, fruit extracts were positive for alkaloids. Several alkaloids from this family exhibit anti-inflammatory activity (Xia et al., 2021). Thus, identifying the alkaloids in *W. solanacea* fruit and evaluating their anti-inflammatory potential is a promising research direction. Unfortunately, this analysis could not be conducted due to limited fruit availability.

Consistent with qualitative assays, *in vitro* analyses revealed differences in TPC and TFC values, and antioxidant activity among extracts from different regions, likely due to environmental variation. Fruit extracts showed higher TPC and TFC values, and stronger antioxidant activities compared to stem and leaf extracts. Similar differences in TPC, TFC, and antioxidant activity among plant parts have been reported in related species, such as *Withania somnifera* (Alam et al., 2012; Sahoo et al., 2024) and *Physalis peruviana* (Ertürk et al., 2017), although their extracts exhibited lower values than those of *W. solanacea* in the present study.

The antioxidant capacity was evaluated using assays based on different chemical mechanisms. Phenolic compounds and flavonoids are known to stabilize free radicals and to chelate transition metals involved in Fenton and Haber-Weiss reactions (Perron and Brumaghim, 2009). A positive correlation was observed between TPC/TFC and antioxidant activities in the ORAC, DPPH, and FRAP assays. In contrast, no such correlation was found with FICA results, possibly due to differences in compounds responsible for metal chelation. This hypothesis is supported by the observed variations in phenolic composition among the extracts.

Antibacterial activity was assessed due to traditional use of *W. solanacea* for treating infections (García et al., 2006; Jacobo-Herrera et al., 2006). Only the stem and leaf extract of *W. solanacea* from Puerto Jiménez showed mild activity against *E. faecalis*, a pathogen involved in external and internal infections. Although limited, these findings are noteworthy due to the lack of prior antibacterial studies, despite its traditional use.

In vivo studies were conducted only with the stem and leaf extract due to limited fruit availability. SL2 was selected based on higher TPC and antioxidant activity than SL1, suggesting greater concentration of active metabolites.

Toxicological evaluation is essential for medicinal plant research. Thus, the acute oral toxicity of SL2 was assessed prior to its pharmacological evaluation. Results indicated no acute toxicity at doses up to 2000 mg/kg. This is a relevant finding, given the lack of previous toxicological studies for *W. solanacea* or related species. These results are consistent with previous studies conducted with extracts from plants of the Solanaceae family, in which many species have been shown to exhibit low acute oral toxicity in animal models at doses of up to 5000 mg/kg (Parra et al., 2001; Epoh et al., 2019; Moussaoui et al., 2020).

Pain is a common symptom associated with numerous medical conditions. In Latin America, *W. solanacea* is traditionally used to treat headaches and general pain (Gupta et al., 1993; Caballero-George et al., 2001; García et al., 2006); although no pharmacological studies had been reported prior to this work. The tail flick test is a well-established method for assessing analgesic properties, measuring the latency of the tail flick reflex following thermal stimulation, which activates pain responses at spinal and/or supraspinal levels (Barrot, 2012). Although the SL2 extract of *W. solanacea* produced an analgesic effect only during the first 30 minutes of the test, this finding is noteworthy considering its traditional use for pain relief and the absence of previous pharmacological evidence.

The phenolic-rich profile of *W. solanacea* may explain its analgesic potential, as such compounds possess antinociceptive activity (Sun and Shahrajabian, 2023). Additionally, some physalins—compounds identified in *W. solanacea*—have been reported to possess analgesic properties (Wu et al., 2021; Jacobo-Herrera et al., 2006). These components may contribute to the observed analgesic effects of the plant. However, further studies should be conducted using additional nociception and pain models to confirm this activity and to identify the specific active metabolites involved.

The carrageenan-induced paw edema model, which mimics acute inflammation, was used to assess anti-inflammatory activity. This model involves two phases: an early phase involving the release of mediators such as histamine, serotonin, and bradykinin, and a later phase characterized by increased cyclooxygenase (COX) and nitric oxide synthase activity, leading to edema and leukocyte infiltration (McKim et al., 2016). The SL2 extract reduced edema at 2 and 4 hours and decreased leukocyte infiltration at 24 hours, similar to indomethacin, suggesting inhibition of pro-inflammatory enzymes. These findings indicate a modulatory effect on acute inflammation; however, further mechanistic studies are needed.

Previous research demonstrated that physalins B and F (but not D) from *W. solanacea* modulate NF-κB in cell models (Jacobo-Herrera et al., 2006). Other studies have confirmed the anti-inflammatory effects of physalins through suppression of cytokines (Meira et al., 2022). However, *in vivo* evidence of anti-inflammatory activity for *W. solanacea* was previously lacking. The present findings provide novel *in vivo* confirmation that supports its traditional medicinal use.

Finally, antioxidant polyphenols may contribute to the observed anti-inflammatory effects (Roy et al., 2022). Phenolic acids and flavonoids have been shown to mitigate oxidative stress in inflammation models, including carrageenan-induced paw edema, in which oxidative stress is a key component (Albarakati, 2022). Thus, in addition to physalins, polyphenols are likely contributors to the anti-inflammatory activity of *W. solanacea*. Future studies could include fractionation of the active extract to isolate specific anti-inflammatory or analgesic compounds, and assessment of their effects on inflammatory mediators (e.g. COX, cytokines) to elucidate mechanisms.

CONCLUSION

Extracts from the aerial parts of *W. solanacea* demonstrated anti-inflammatory and antinociceptive effects with no observed acute toxicity. These results support the traditional use of the plant for treating inflammation and pain. In contrast, antibacterial activity was weak. The observed anti-inflammatory and analgesic effects may be attributed to antioxidant phenolics, flavonoids, and known physalins present in the extracts. *W. solanacea* therefore represents a promising source of compounds for managing inflammation and pain.

CONFLICT OF INTEREST

The authors declare that they have no competing financial interests that could influence the results reported in this paper.

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DECLARATION OF GENERATIVE AI IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT in order to check grammar, spelling, and clarity. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the published article.

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Legends of figures

Figure 1. Chromatograms showing the detection of polar phenolic compounds in *W. solanacea* extracts. **A)** General phenolics (derivatization with potassium ferricyanide and ferric chloride reagent). **B)** Flavonoids (derivatization with aluminum chloride reagent). **C)** Coumarins, flavonoids, and quinones (derivatization with potassium hydroxide reagent).

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; UV: chromatogram under UV light (366 nm); Vis: chromatogram observed under visible light. Major positive changes are highlighted with yellow or black boxes.

Figure 2. Chromatograms showing the detection of non-polar phenolic compounds in *W. solanacea* extracts. **A)** General phenolics (derivatization with potassium ferricyanide and ferric chloride reagent). **B)** Flavonoids (derivatization with aluminum chloride reagent). **C)** Coumarins, flavonoids, and quinones (derivatization with potassium hydroxide reagent).

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; UV: chromatogram under UV light (366 nm); Vis: chromatogram observed under visible light. Major positive changes are highlighted with yellow or black boxes.

Figure 3. Chromatograms showing the detection of terpenoids in *W. solanacea* extracts. **A)** General terpenoids (derivatization with anisaldehyde/sulfuric acid reagent). **B)** Steroids and triterpenoids (derivatization Liebermann-Burchard reagent).

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; UV: chromatogram under UV light (366 nm); Vis: chromatogram observed under visible light. Major positive changes are highlighted with yellow or black boxes.

Figure 4. Chromatograms showing the detection of alkaloids in *W. solanacea* extracts by derivatization with Dragendorff reagent.

Dr: observation after derivatization; Dv: observation after development; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; Vis: chromatogram observed under visible light. Major positive changes are highlighted with black boxes.

Figure 5. Determination of TPC, TFC, and antioxidant activity of *W. solanacea* extracts. **A)** TPC values. **B)** TFC values. **C)** ORAC values. **D)** FRAP values. **E)** IC_{50} DPPH values. **F)** FICA values.

Different capital letters above the bars indicate significant differences ($p < 0.05$). Results are expressed as mean ± SEM (n = 3). DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; DW: dry weight; EDTAE: ethylenediaminetetraacetic acid equivalents; F1: fruit extract from Mora; F2: fruit extract from Puerto Jiménez; FICA: ferrous iron chelating activity assay; FRAP: ferric reducing antioxidant power assay; GAE: gallic acid equivalents; ORAC: oxygen radical absorbance capacity assay; QE: quercetin equivalents; SL1: stem and leaf extract from Mora; SL2: stem and leaf extract from Puerto Jiménez; TE: Trolox equivalents; TFC: total flavonoid content; TPC: total phenolic content.

Figure 6. Pearson's correlation matrix. **A)** Correlation coefficients (*r*). **B)** *p*-values matrix.

DPPH: 2,2-diphenyl-1-picrylhydrazyl assay; FICA: ferrous iron chelating activity assay; FRAP: ferric reducing antioxidant power assay; ORAC: oxygen radical absorbance capacity assay; TFC: total flavonoid content; TPC: total phenolic content.

Figure 7. Antinociceptive effect of the stem and leaf extract from Puerto Jiménez (SL2) in the tail flick test in rats.

Results are presented as the mean ± SEM of the percentage of antinociception (n = 8). Statistical significance was determined by two-way repeated measures ANOVA followed by Dunnett´s multiple comparisons test. $^{*}p < 0.05$, $^{***}p < 0.001$ compared to control (saline solution).

Figure 8. Anti-inflammatory effect of the stem and leaf extract from Puerto Jiménez (SL2) extract in carrageenan-induced paw edema in rats.

Results are presented as the mean ± SEM of the percentage of edema (right hind paw) relative to non-inflamed control (left hind paw) (n = 8). Statistical significance was determined by two-way repeated measures ANOVA followed by Dunnett´s multiple comparison test. $^{*}p < 0.05$ compared to control (saline solution).

Figure 9. Paw tissue dissected 24 hours after injection of **A)** carrageenan or **B)** saline solution in control groups; **C)** 500 mg/kg, **D)** 250 mg/kg of stem and leaf extract from Puerto Jiménez (SL2); and **E)** 50 mg/kg of indomethacin.

Arrows indicate leukocyte infiltration. Arrowheads indicate congestion and swelling in the tissue. Hematoxylin–eosin staining. Scale bar = 50 µm.