Searching and evaluation of bacteriophagues and extracts, fractions and compounds obtained from *Passiflora tripartita* var *mollissima* and *P. tarminiana* against *Helicobacter pylori*

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Abstract:

Helicobacter pylori (*H. pylori*) is a bacterium that causes diverse gastroduodenal diseases and gastric cancer. The WHO has classified it as a type I carcinogen. Currently available treatments have lost efficacy, due to the appearance of *H. pylori* resistant strains for the antibiotics used in the therapeutic schemes. In 2017 the who publish a list of bacterias that represent a danger for human health, including *H. pylori* as a high priority bacterium, inviting to the scientific community searching for new therapeutic alternatives. Phage and phyto therapy are alternatives in study that can offer new alternatives to fight antimicrobial resistance. The aim of this study was to evaluate two alternatives based of phage therapy searching lytic phages in wastewater, fecal and gastric biopsies samples and evaluate them against reference strains of *H. pylori* and to evaluate the antimicrobial activity against *H. pylori* of extracts, fractions, and compounds from *Passiflora tripartita* var *mollissima* and *P. tarminiana*. The presence of *H. pylori* was identified in 32.2% of the fecal samples (10/31) and 64.5% of the gastric biopsy samples (60/93), it was not possible to isolate any bacteriophage with lytic activity against this bacterium. Lysis zones were identified in the *H. pylori* control strains, cultures mixed to the filtrates obtained from one sample from the Bogotá River and two from the Arzobispo river; however, the isolation of any bacteriophage was not achieved. On the other hand, a minimum inhibitory concentration (MIC) of 1000 µg/mL were found for the hydroethanolic extracts of *P. tripartita* var *mollissima* and P. *tarminiana*, in addition, a MIC of 250 µg/mL were found for the butanolic and aqueous fractions of *P. tarminiana* against the reference strains of *H. pylori*. The butanolic fraction of *P. tarminiana* was the most active against reference and clinical strains of *H. pylori*, showing an MIC between 250 and 500 µg/mL. Vitexin and rutin were found in fractions of *P. tarminiana* and rutin only in *P. tripartita* var *mollissima* fractions by High Performance Thin Layer Chromatography (HPTLC) technique. Also, electrophoretic profile for flavonoid were obtained by Capillary Electrophoresis (CE). Also, it was possible to stablish a methodology

to isolate C-glicosyl flavonoids by CPC in the butanolic fraction of *P. tarminiana* isolating five pure compounds in a short time. The results found in this work will provide knowledge for the searching and evaluation of bacteriophagues, and the evaluation of the antimicrobial activity of different extracts, fractions and compounds from *Passiflora* species studied against *H. pylori*, also showing their difficulties for both studies to compare results, showing the need for stablish standardized methods to evaluate phagues and plant extracts, fractions and or compounds in fastidious bacteria such as *H. pylori*. This study opens new opportunities for future research with the butanolic fraction of *P. tarminiana*, to evaluate the cytotoxic activity and the possible associated mechanisms of action, with projection to scale it to *in vivo* models.

Keywords: *Helicobacter pylori, bacteriophages, Passiflora tripartita, Passiflora tarminiana, flavonoids.*

1. Introduction

Helicobacter pylori (*H. pylori*) is a bacterium that causes gastroduodenal diseases and gastric cancer [1]. It is estimated that more than 50% of the world population is infected by this bacterium [2]. In addition, the World Health Organization (WHO) has classified it as a type I carcinogen [3]. Current treatment consists of the combination of one or more antibiotics such as clarithromycin, metronidazole, amoxicillin, levofloxacin, among others, and a proton pump inhibitor (PPI) in various therapeutic schemes [4]. However, currently available treatments based on triple therapies have lost effectiveness, being even lower than 60% in some countries, due to the appearance of resistant strains. A global resistance of 31.1% is estimated for clarithromycin and 80% for metronidazole [5,6]; In Colombia, resistance data to clarithromycin is 17.72%, 81.07% for metronidazole [7] and up to 27.3 for levofloxacin [8]. In 2017, the WHO made a call for the scientific community to promote the search for new therapeutic alternatives, classifying *H. pylori* as a high priority bacterium [9].

Phagotherapy consists of using viral particles capable of infecting bacteria. This strategy has already been used successfully in the treatment of infectious diseases caused by other Gram-positive and Gram-negative bacteria [10]. However, for *H. pylori*, the description of phages is still limited, although it is a growing field and currently different phages inserted into its genome have been described [11]. However, reports about specific phages capable of lysing *H. pylori* are scarce [12,13], although they have promising results that support the therapeutic potential of these viral particles in the eradication of *H. pylori*.

Another alternative studied is the use of natural products. Among the plants to be evaluated in this work are the species of the genus *Passiflora*. The *Passiflora* genus stands out for its traditional use as a sedative, as well as its anxiolytic, anti-inflammatory, gastroprotective and antimicrobial activity. Among its main compounds are the C-glycosyl flavonoids, saponins and alkaloids in a smaller proportion [14,15]. To date, there are still few reports of antimicrobial activity for species of the *Passiflora* genera against *H. pylori* [16,17]. In

accordance with the above, the objective of this work consisted in the evaluation of two therapeutic alternatives such as the search for lytic bacteriophages and the evaluation of the antimicrobial activity of extracts, fractions, and compounds of different species of *Passiflora*, of occurrence in Colombia against to *H. pylori* such as: *P. tripartita* var *mollissima* (banana passion fruit-curuba) and *P. tarminiana* (curuba de indio).

2. Materials and Methods

2.1. Searching and evaluation of bacteriophages

2.1.1. Sampling and processing of water, fecal and gastric biopsies for the bacteriophage search

All the samples were collected in the period between June 2019 and November 2020 in Bogotá D.C city under the "Permiso marco de recolección de especímenes de especies silvestres de la Diversidad Biológica con fines de investigación científica, mediante resolución 0546 del 29 de mayo de 2014 del Ministerio de Ambiente y Desarrollo Sostenible (Autoridad Nacional de Licencias Ambientales) y su actualización: Resolución No 0778 de 07 de julio de 2017. The wastewater samples were collected in 125 ml sterile bottles, in the Bogotá River: with Coordinates: (4°43'43.8"N 74°07'36.5"W), Arsovispo River: (4°37'30.1"N74°03'45.1"W), Pontevedra River (4°41'29.7"N 74° 04'51.4"W) and Boyacá Avenue with Calle 66 pipe; (4°40'52.0"N 74°05'57.7"W). As for the fecal samples and biological gastric biopsies, they were obtained from gastroenterology patients at the El Virrey Medical Center; All patients who participated in the study had to meet the inclusion and exclusion criteria to be included in the study. Among them: Not having consumed antibiotics 4 months before taking the samples. All patients were previously invited to participate and signed the informed consent. The samples were transported under refrigeration (4°C) to the Laboratorio de Bacteriología Especial of the Pontificia Universidad Javeriana. For the processing of the residual water samples, the recommendations of Didamony et al [18] were followed, performing a clarification by centrifugation at 6000g for 20min followed by the filtration of the supernatant with two types of membrane: PVDF (Polyvinylidene Fluoride) of 0.22 and 0.45 µm; and PES (Polyethersulfone) of 0.22 µm. Clear samples were directly filtered. On the other hand, for stool samples, the recommendations of Chibani-Chennoufi S.et al [19] and Born Y. et al [20] were followed; in which approximately 10 g of each sample were homogenized with 20 ml of TS solution (8.5 g NaCl, 1 g Tryptone/Liter) and Buffer SM (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 7.9 g Tris-HCl pH 7.5/Liter) in a 1:1 ratio; After homogenization, centrifugation was performed at 14,500 g/15 min/4°C and the supernatant was filtered with a 0.22 µm PES membrane. Gastric biopsies (antrum, body and incisura/patient) were incubated in 1 ml of supplemented Brucella broth at 37°C and 11% CO_2 for 48 hours, according to Abdel-Haliem et al [13]. and Cuomo et al [21]. Subsequently, each sample was macerated using activated carbon; the macerates of each sample were centrifuged at 7000 g/5 min and the supernatant obtained was filtered with a 0.22 µm PES membrane, to remove the bacterial load. The filtrates obtained from the environmental and biological samples were used in the evaluation of the presence of lytic bacteriophages against

the bacterial strains. The filtrates were kept at -80°C with the addition of 10% glycerol (v/v). In addition to obtaining filtrates, the identification of *H. pylori* in biological samples (faeces and gastric biopsies) was conducted, to determine the presence or absence of the bacteria and thus be able to predict in a certain way the presence or absence of bacteriophages. For this, bacterial genomic DNA was extracted using the "DNeasy Blood & Tissue" Extraction Kit (Qiagen, USA), following the manufacturer's instructions. The eluates obtained were analyzed by PCR, through the amplification of a fragment of the *vacA* gene (allelic forms s1/s2).

2.1.2. Bacterial liquid culture for phage evaluation.

Liquid culture was performed for bacterial growth of *H. pylori*, following the recommendations described by Kitsos et al. [22] Duque-Jamaica et al. [23]. Douraghi. et al. [24] Joo et al [25]. and Matsuzaki et al [26]. Brucella broth was used with 10% (v/v) fetal bovine serum, 0.11% (v/v) DENT supplement (Oxoid™) and 0.52% (v/v) ISOVITALEX (Becton Dickinson); 5.6% (v/v) of inoculum was added to this medium with an OD=600nm: (Abs between 0.05 to 0.1); to obtain a final volume of 5 mL. Incubation was performed at 37°C and 11% CO2 without shaking. For each of the procedures described below, exponential growth phase liquid cultures of *H. pylori* strains NCTC 11637, NCTC 11638, 321A, 372C and PHA288C [27] were used. Additionally, *Campylobacter coli* strains CMPUJ264 and *Escherichia coli* ATCC 25922 were used as control strains, all with OD600nm: 0.2 to 0.5. Incubation without shaking was carried out for 5-7 days to obtain the *H. pylori* and C. coli strains, and 3-4 hours for *Escherichia coli*.

2.1.3. Evaluation of phage presence using plaque formation units (PFU)

For the evaluation of the presence of bacteriophages, several methods were used, which were: Single layer spot method, double layer spot method and double layer method in agar. For the first method, the bacterial suspension of each of the strains was extended in solid Brucella medium, by means of massive seeding, allowed to dry and subsequently 5 µl of each of the obtained filtrates were added dropwise, as described by Twest et al. to [28]. Incubation was conducted for 72 hours at 37°C and under microaerophilic conditions. The double-layer spot technique was performed taking into account the recommendations of Abdel-Haliem [13], Matsuzaki et al [26]. and Zhang. et al [29]: evaluating three conditions: In the first, 400 µl of each strain and 3.6 ml of soft medium with 0.5% agar were used. In the second condition evaluated, 1 ml of each strain and 3.5 ml of soft medium with 0.7% agar were used, and in the third condition, 1 ml of each strain and 3.5 ml of soft medium with 0.7% agar were used. and 5 mM calcium chloride $CaCl_2$. The mixture obtained from the three conditions evaluated was poured onto Brucella solid medium and kept at room temperature for 30 minutes until the upper layer solidified, and then 25 µl of the filtrates were added dropwise. The culture media were left at room temperature until the spots completely dried (drip) and were incubated for 72 hours at 37°C and under microaerophilic conditions. Finally, for the double-

layer agar method, two conditions based on what was described by Abdel-Haliem et al [13] were evaluated. and Matsuzaki et al (24). Briefly, in the first, 400 µl of each strain, 100 µl of each filtrate and 3.5 ml of soft medium with 0.5% agar were used, and in the second condition evaluated, 1 ml of each strain was used, 1 ml of each filtrate and 3.5 ml of medium.

2.2. Evaluation of *Passiflora* extracts, fractions, and compounds against *H. pylori*

2.2.1. *Passiflora* extraction

To obtain extracts, plant material was collected from leaves of the species *P. tripartita* var *mollissima* and *P. tarminiana* under the "Contrato de acceso a recursos genéticos y productos derivados N° 212 (RGE 0287-6) entre la Pontifica Universidad Javeriana y el Ministerio de Medio Ambiente y Desarrollo Sostenible, with deposits COL599223 (*P. tripartita*) and COL599247 (*P. tarminiana*). The plant material was dried at 35°C for 72 h and subsequently ground. Two types of extracts were obtained which consisted of: A hydroethanolic maceration at 50% ethanol/water in a 1:15 plant/solvent ratio for 48 hours and an aqueous infusion for 10 minutes in a 1:10 ratio. The extracts obtained were dried by rotary evaporation and then by lyophilization.

2.2.2. Fractionation of active extracts from active *Passiflora* extracts

From the active extracts, a liquid-liquid fractionation was performed, using solvents from least to most polarity. Briefly, 1g of each extract was resuspended in 300 mL of water and placed in a separatory funnel and, separately, ethyl acetate and butanol (100 mL of each, for three times) were passed in triplicate, stirring gently. between each pass of solvent, thus originating the fractions ethyle acetate (AcOEt), buthanolic (BuOH) and the aqueous residue (H_2O). The organic fractions obtained were dried by rotary evaporation using a rotary evaporator and the aqueous residues by lyophilization using a lyophilizer.

2.2.3. Chemical characterization of fractions of *Passiflora tripartita* var *mollissima*, and *P. tarminiana* using HPTLC and CE

For the development of the HPTLC, a CAMAG® equipment with VisionCATS® Software was used. Each of the samples were resuspended in methanol and filtered through a 0.22 µm PTFE syringe filter. Extracts and fractions were prepared at a concentration of 1000 µg/mL. All samples were applied in band. The seeding volume was 20 µL for extracts and fractions, and 2 µL for standards. A silica gel F-254 HPTLC plate with glass support (Merck) was used as stationary phase, and ethyl acetate, acetone, acetic acid and water were used as mobile phase in a ratio (6:2:1:1, v /v). For the derivatization, a natural reagent was used for the visualization of flavonoids. Visualization was performed with UV light at a wavelength of 365 nm.

On the other hand, the CE technique was used to obtain the electrophoretic profiles of *Passiflora*, using an Agilent technologies 7100® with Agilent ChemStation® software. For the analysis, the methodology of Costa et al [30] with some modifications was followed. Samples for the fractions were prepared at a concentration of 100 µg/mL in water and filtered through a 0.22 µm PTFE filter. An Agilent® capillary 56 cm long and 50 µm internal diameter with expanded detection window was used. In the first place, a pre-conditioning protocol was executed that consisted of passing 1 M NaOH for 5 min, followed by 0.5 M NaOH for 5 minutes, H_2O for 5 minutes and finally the analysis buffer (BGE) composed of sodium tetraborate 50 mM (TBS) + 20% MeOH, pH= 10 for 10 min. Each sample was injected at 50 mbar for 10 s and the analysis was performed by passing BGE for 20 min, at a voltage of 25 kV, 30 °C. The detection was performed using a diode array (DAD) at 390 nm for the visualization of flavonoids, with the verification of their corresponding spectrum. Between each sample, BGE was run for 5 min.

2.2.4. Purification of the butanolic fraction of *P. tarminiana* using centrifugal partition chromatography (CPC)

From the butanolic fraction of *P. tarminiana*, which was the most active fraction against reference and clinical strains of *H. pylori*, the purification of flavonoids was carried out by centrifugal partition chromatography (CPC) in a PLC- Gilson® CPC with a 250 mL rotor capacity. We followed the methodology of Costa et al [31], with some modifications. Briefly, a gradient solvent system of AcOEt, BuOH and H_2O (1:X:1) was used, changing the proportion of BuOH [0.05 (System A), 0.1 (System B), 0.5 (System C) and 1.0 (System D)]. The lower aqueous phase of system A was used as the stationary phase while the upper organic phase of each solvent system was used as the mobile phase. The column was filled with the stationary phase at a speed of 500 rpm and a flow of 5 mL/min and then, the mobile phase was added in an ascending mode and the retention of the stationary phase of this system was verified, which was 42.9% (Vs-98 mL). After the column was equilibrated, 500 mg of sample were injected in ascending mode, and each solvent system was passed for 25 min at a flow rate of 5 mL/min and at a speed of 1500 rpm. A volume of 10 mL was collected for each subfraction until a number of 50 subfractions were obtained. Finally, each subfraction was verified by CCD using silica gel as stationary phase and mobile phase of ethyl acetate, acetone, acetic acid and water in a ratio (6:2:1:1, v/v). For the derivatization, a natural reagent was used for the visualization of flavonoids. Visualization was performed with UV light at a wavelength of 365 nm.

2.8. Evaluation of antimicrobial activity of extracts, fractions, and compounds of *Passiflora tripartita* var *mollissima* and *P. tarminiana* against *H. pylori.*

For the evaluation of antimicrobial activity, agar dilution technique was used following the the Clinical and Laboratory for Standards Institute (CLSI) recommendations, considered this technique, the gold standard for the evaluation of antimicrobial activity in slow-growing

microorganisms [32]. Each of the extracts and fractions was initially dissolved in 20% v/v DMSO solution and then filtered on a 0.22 µm sterile syringe filter. Subsequently, a sterility test was performed on each of the fractions in BHI agar.

For the antimicrobial activity tests, each extract or fraction to be evaluated was mixed with Mueller Hinton II agar supplemented with horse blood at 7% v/v and Isovitalex at 0.4% v/v, thus not exceeding a concentration of 1% DMSO in the culture medium. For each extract, the concentrations of 1000, 500 and 250 µg/mL for the extracts, and 500, 250 and 125 µg/mL for the fractions were evaluated. The antimicrobial activity of the extracts and fractions were evaluated against reference strains of *H. pylori*: NCTC 11637 and NCTC 11638, which are in the collection of microorganisms of the Pontificia Universidad Javeriana with CMPUJ122 and CMPUJ123 codes, respectively. For the seeding of each of the *H. pylori* strains in the media with extract or fraction, an inoculum of each strain used was prepared, transferring colonies in the exponential growth phase of the microorganisms (96 hours of incubation) in tubes containing sterile physiological saline solution 0.45% w/v and adjusting the concentration of the inoculum by turbidity to the 2.0 McFarland standard (1 x 10^8 cells/mL) (94). Finally, the plates were incubated at 37 °C under microaerophilic conditions (11% CO2). for 72h. The fractions with the best antimicrobial activity were subsequently evaluated against clinical strains of *H. pylori* (Ex1C, Ex2C, Ex3A, Ex4A) with different susceptibility profiles to clarithromycin, levofloxacin, metronidazole, and amoxicillin, following the same methodology described above. The strains were taken from previous studies of the research group whose names were changed for ethical reasons [27]. The resistance of these strains against the four antimicrobials evaluated was determined, using a simplified agar dilution method, preparing only the plates corresponding to the resistance cut-off concentration. The resistance breakpoints were considered for the interpretation of susceptibility for clarithromycin, levofloxacin, and amoxicillin (≥1 µg/mL) and metronidazole (≥ 8µg/mL) (94–96). The resistance profiles are shown in Table 1 (see table 1)

Table 1: Resistant profiles of clinical strains of *H. pylori* used for antimicrobial evaluation.

Strain	**MIC(µg/mL)**			
	Claritromycin (CLA)	**Levofloxacin (LEV)**	**Metronidazole (MTZ)**	**Amoxicillin (AMX)**
Ex1C	>1 (R)	≤1(S)	≤8(S)	≤1(S)
Ex2C	≤1 (S)	>1(R)	>8(R)	≤1(S)
Ex3A	>1 (R)	>1(R)	>8(R)	≤1(S)
Ex4A	≤1(S)	≤1(S)	>8(R)	≤1(S)

Additionally, purchased standards of vitexin, quercetin, caffeine, and catechin were evaluated at concentrations of 20, 10, and 5 µg/mL each, both for the reference strains and for the clinical strains. As controls for this assay, we used: A viability growth control of *H. pylori* in medium without treatment, either, at the beginning and at the end of the procedure; an *H. pylori* vehicle control in 1% DMSO and an *H. pylori* inhibition control with amoxicillin

at 0.125 µg/mL. Once the incubation was finished, the minimum inhibitory concentration (MIC) of each fraction was determined, defined as the minimum concentration where visible growth was observed.

3. Results

3.1. Wastewater, fecal and gastric sampling obtaining

A total of 36 wastewater samples were collected corresponding to the Bogotá river (n=30), the Arsovispo river (n=2), the Pontevedra channel (n=1) and the Boyacá Avenue with Calle 66 channel (n=3). Additionally, fecal samples were collected from 31 patients and gastric biopsies from 31 patients with samples from the corpus, antrum and incisure of each one. During the processing of the first environmental samples, the evaluation of two filtration membranes was carried out: PVDF (Polyvinylidene Fluoride) of 0.22, 0.45 µm and PES (Polyethersulfone) of 0.22 µm, finding a greater filtration capacity in the PES membrane. At the end of the processing of the samples, 36 residual water filtrates, 31 stool filtrates and 93 gastric biopsy filtrates were obtained, using 0.22 µm PES membrane filters, which guaranteed the sterility of the filtrates to be evaluated.

3.2. Searching of phagues using spot, simple layer and double agar layer

The evaluation of the presence of bacteriophages in samples of residual water, feces and gastric biopsies, only allowed obtaining growth inhibition zones (lysis plates) in one sample from the Bogotá River and two from the Arzobispo River (Figure 1). The inhibition zones were extracted and evaluated by liquid enrichment culture, from this culture the D.O was evaluated to obtain a bacterial growth curve. The analysis of these curves allowed to show growth restriction only in the strain NCTC 11638 compared with the lysis plate obtained by the simple layer method and the initial filtering. The cultures in which lytic activity was found were filtered and confronted again with all the selected *H. pylori* strains, using the direct method in double layer with serial dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. However, in these assays, neither growth inhibition nor lytic activity was observed. Reason for which, in this study, bacteriophages were not isolated.

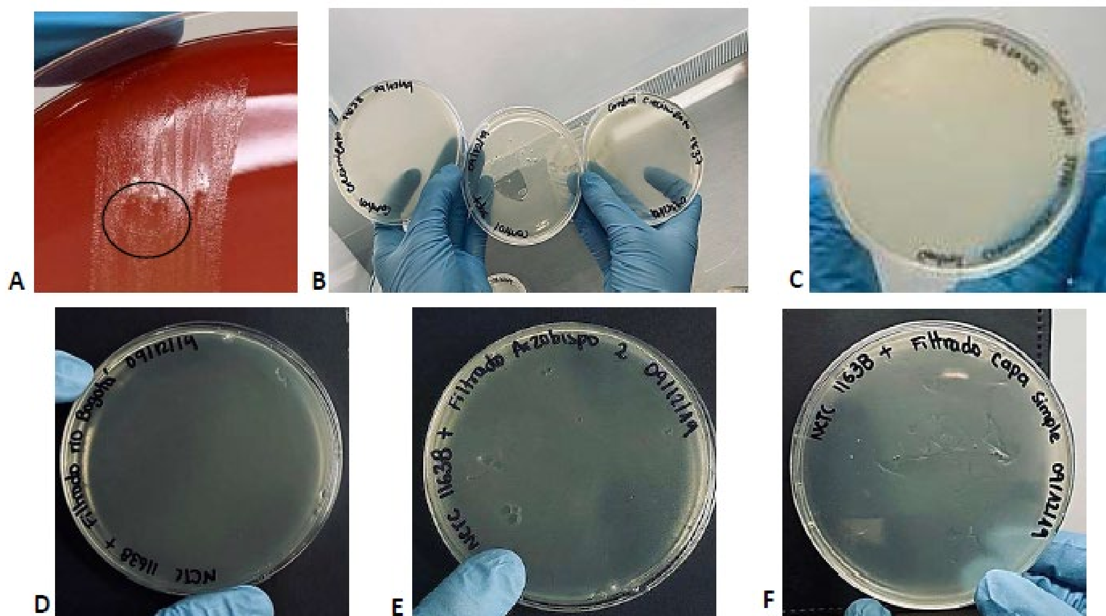


Figure 1. Inhibition zones of *H. pylori* growth. **A.** Bogotá river using simple spot method **B**. Growing and sterility controls. **C.** Growth control *H. pylori* NCTC 11638 **D.** Bogotá River using direct double layer **E.** Arzobispo river using direct double **F**. Plaque evaluation of Bogotá River using double plate.

3.3. Chemical characterization of extracts and fractions of *Passiflora tripartita* var *mollissima* and *Passsiflora tarminiana*.

3.3.1. Chromatographic profiles:

Chromatographic profiles were obtained for the visualization of flavonoids and saponins from the extracts of, *P. tripartita* var *mollissima* and *P. tarminiana* (Figures 1 and 2). For the profiles obtained from the *Passiflora* extracts, the presence of flavonoids can be observed in all the *Passiflora* extracts, with different profiles between species. On the other hand, for the visualization of the saponin profiles, the presence of saponins cannot see clearly for *P. tripartita* and *P. tarminiana*, and the presence of this type of metabolite cannot be confirmed.

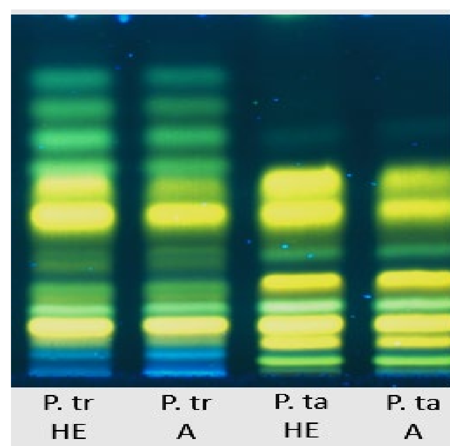


Figure 2. High Performance Thin Layer Chromatography for visualization of specific chromatographic profiles for flavonoids: **P.tri HE**: *P. tripartita* var *mollissima* Hydroethanolic, **P.tri A**: *P. tripartita* var *mollissima* Aqueous, **P.tar HE**: *P. tarminiana* Hydroethanolic and **P.tar A**: *P. tarminiana* Aqueous. Fe. Silica gel. Fm: ethyl acetate-acetone-acetic acid-water (6:2:1:1). Rv: Natural reagent / 366nm.

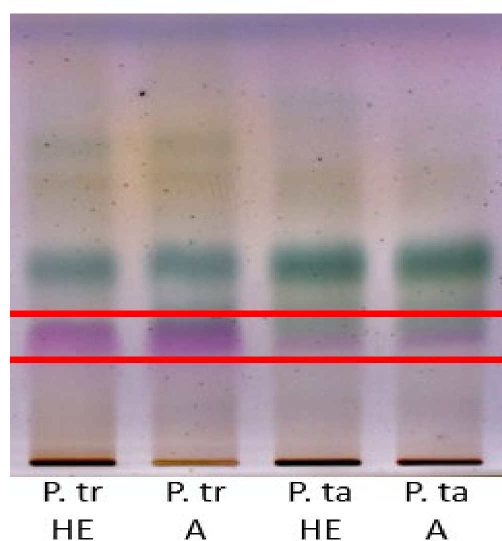


Figure 3. High Perfomance Thin Layer Chromatography (HPTLC) for visualization of specific chromatographic profiles for violet saponins, **P.tri HE:** *P. tripartita* var *mollissima* Hydroethanolic, **P.tri A:** *P. tripartita var mollissima* Aqueous, **P.tar HE:** *P tarminiana* Hydroethanolic and **P.tar A:** *P. tarminiana Aqueous*.

For the profiles obtained for the fractions, as can be seen in Figure 3, the presence of yellow-orange, yellow-green and orange bands was observed, which are associated with the presence of flavonoid-type compounds, taking into account the developer and the wavelength used [33]. Additionally, qualitatively, an increase in the concentration of flavonoids for each of

the butanolic fractions obtained with respect to the crude extract. Additionally, the presence of vitexin can be evidenced with a Retention Factor (Rf) = 0.78 in both *P. tripartita* and *P. tarminiana* and rutin with an Rf = 0.16 in *P. tarminiana*, under the chromatographic conditions used.

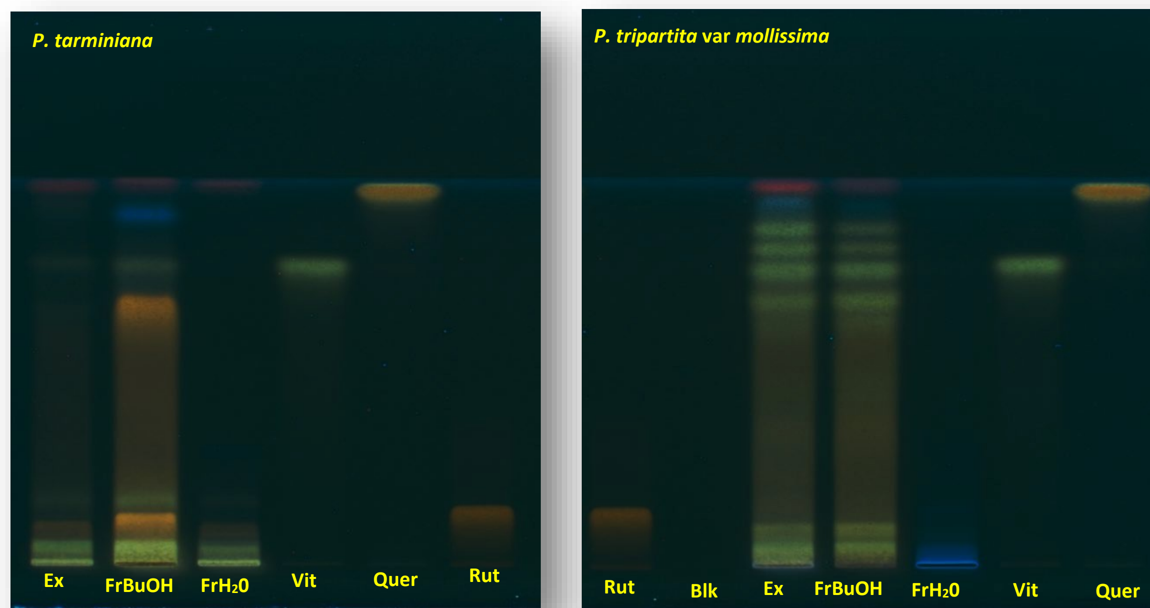


Figure 4. Chromatographic profiles of fractions of *P. tarminiana* and *P. tripartita* var *mollissima* by HPTLC. Stationary phase: Silica Gel F254, Mobile phase: Ethyl acetate: Acetone: Acetic acid: H_2O 6:2:1:1. Developer: Natural Reagent UV 366 nm. **Ex:** Crude Extract; **FrBuOH:** Butanolic fraction; **FrH$_2$O:** Aqueous Fraction; **Vit:** Vitexin; **Quer:** Quercetin; **Rut:** Rutin. **Blk:** Blank

3.3.2. Electrophoretic profiles for fractions of *Passiflora*

The capillary electrophoresis technique is an attractive analytical technique, since it allows the use of small amounts of sample and reagents. Thanks to this, the electrophoretic profiles were obtained for the visualization of flavonoids from the fractions of *P. tripartita* and *P. tarminiana* (figures 4 and 5). The identification of flavonoids was made from the comparison of their UV spectra. For the *P. tarminiana* fractions, a complex profile of flavonoids was visualized in relation to the number of peaks; In addition, in accordance with the HPTLC analysis, it was possible to visualize a higher concentration of flavonoids in the butanolic fraction. The profiles obtained for *P. tarminiana* have not been reported until the time of the study. In *P. tripartita*, a more complex profile of flavonoids is observed compared to *P. tarminiana* when observing a greater number of peaks.

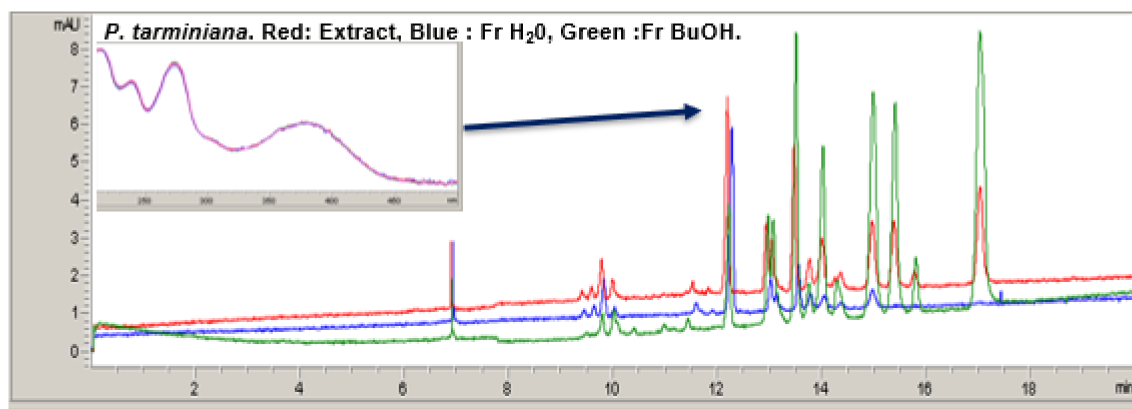


Figure 5. Electrophoretic profile of fractions obtained from *P. tarminiana*: (Red: Extract, Blue = Fr H_2O, Green = Fr BuOH. Electrophoretic conditions: Capillary 56 cm, 50 µm internal diameter, expanded detection window. Run = 5 min Buffer TBS 50 mM+ MeOH 20% pH= 10, 20 min 50 mM TBS Buffer, Injection 50mbar 10s, Voltage 25 kV, 30°C, DAD Detection 390 nm.

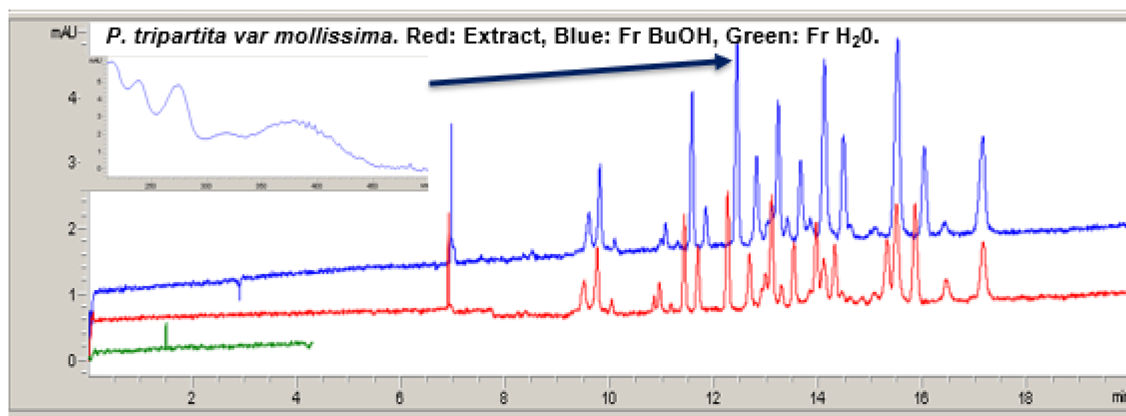
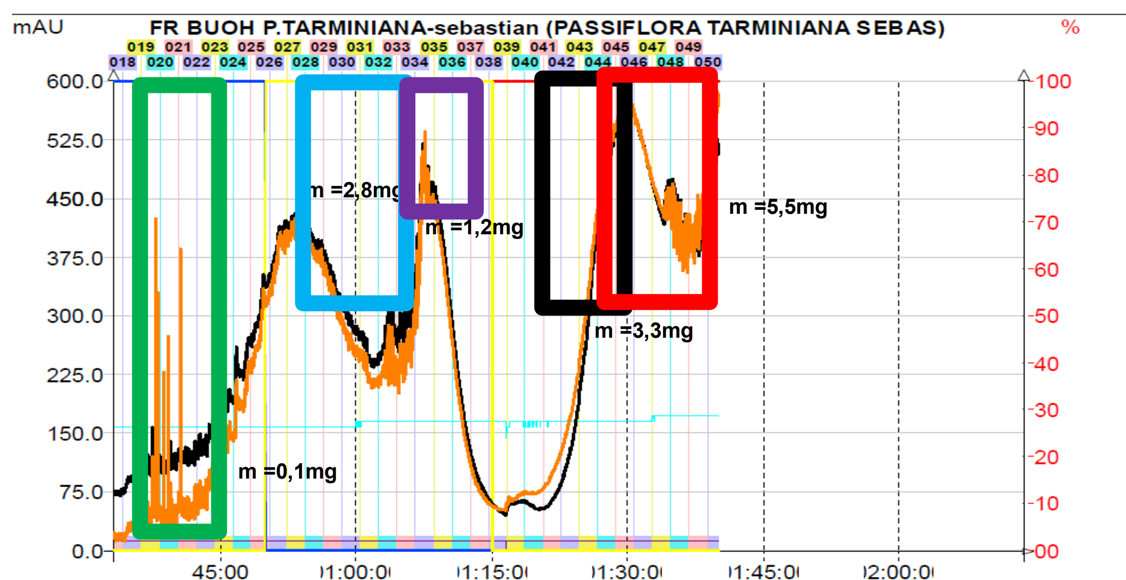


Figura 6. Electrophoretic profile of fractions obtained from *P. triparita* var *mollissima* A) (Red = Extract, Blue: Fr BuOH, Green: Fr H_2O). Electrophoretic conditions: 56 cm capillary, 50 µm internal diameter, expanded detection window. Run= 5 min Buffer TBS 50 mM+ MeOH 20% pH= 10, 20 min 50 mM TBS buffer, Injection 50 mbar 10 s, Voltage 25 kV, 30 °C, DAD Detection 390 nm.

3.3.3. Purification of buthanolic fraction of *Passiflora tarminiana* using CPC

Considering the good antimicrobial activity obtained for the butanolic fraction of *P. tarminiana* against reference strains and clinical strains of *H. pylori* detailed below, it was decided to purify the flavonoids in this fraction by means of CPC. As can be seen in figure 11, by using this technique it was possible to obtain fifty subfractions which were analyzed by TLC. The analysis allowed the separation of 5 compounds in subfractions 20-22, (green box, m=0.1 mg); 29-32, (yellow box, m= 1.2mg); 34-36, (purple square m= 2.8 mg); 42-44, (black box, m= 3.3mg) and 45-49: (red box, m= 5.5mg). By revelation using natural reagent, these compounds were found to be of the flavonoid type.



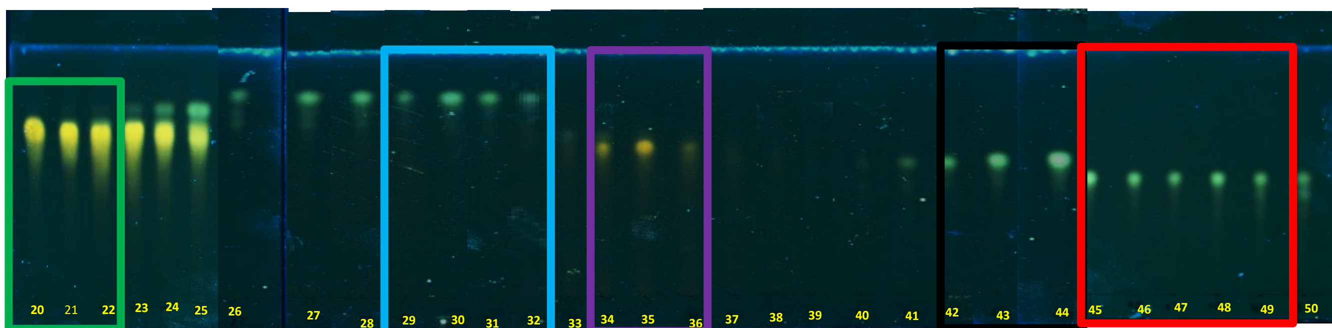


Figure 7. Centrifugal partition chromatogram of the butanolic fraction of *P. tarminiana*: A) Final system: AcOEt gradient: BuOH: $H_2$0 A (1: 0.05:1) B (1: 0.1: 1) C (1: 0.5: 1) D (1: 1: 1) 25 min, 1500 rpm. Fe: Aqueous phase solution A Fm: Organic phases of each solvent system A, B, C and D. Sample: 500 mg, injection in ascending mode, flow: 5 mL/min. Detection: Bottom: Thin layer chromatography for the visualization of flavonoids from the subfractions of the butanolic fraction of *P. tarminiana*. Fe. Silica gel. Fm: Ethyl acetate-acetone-acetic acid-water (6:2:1:1). Rv: Natural reagent / 366nm.

3.4. Evaluation of antimicrobial activity of extracts, fractions, and compounds on reference strains of *H. pylori*

In this study, hydroethanolic and aqueous extracts of *Passiflora* species occurring in Colombia were evaluated against reference strains of *H. pylori*. Using the agar dilution technique, it was possible to evaluate extracts obtained from leaves of *P. tripartita* var *mollissima* and *P. tarminiana* against *H. pylori* by measuring the minimum inhibitory concentration (MIC). In table 2, the hydroethanolic extracts of *P. tripartita* var *mollissima* and *P. tarminiana* showed antimicrobial activity with a MIC = 1000µg/mL.

Table 2: Antimicrobial activity of crude extracts obtained from *Passiflora tripartita* var *mollissima* and *P. tarminiana* against reference strains of *H. pylori*.

Extract	MIC NCTC 11637 (µg/mL)	MIC NCTC 11638 (µg/mL)
P. tripartita EtOH/$H_2$0	1000	1000
P. tripartita I 1:10	>1000	>1000
P. tarminiana EtOH/$H_2$0	1000	1000
P. tarminiana I 1:10	>1000	>1000

Subsequently, the active extracts were fractionated by liquid-liquid fractionation, thus obtaining the Ethyl Acetate fractions, BuOH and the aqueous fraction. Acetate fractions were excluded from this study due to their low yield and difficulty to be scaled up (data not shown).

As shown in Table 3, the antimicrobial activity of the *P. tripartita* var *mollissima* and *P. tarminiana* fractions improved compared to the initial crude extracts whose MIC was 1000µg/mL. The fractions with the highest activity were the butanolic fraction and the aqueous fraction of *P. tarminiana* whose MIC obtained was 250 µg/mL. The butanolic fraction of P. *tripartita* var *mollissima* showed a MIC = 500 µg/mL, while its aqueous fraction did not present antimicrobial activity.

Table 3: Antimicrobial activity of fractions obtained from *P. tripartita* var *mollissima* y *P. tarminiana*, against reference strains of *H. pylori.*

Extract	Fraction	MIC NCTC 11637 (µg/mL)	MIC NCTC 11638 (µg/mL)
P. tarminiana EtOH/H_20	*Passiflora tarminiana* BuOH	250	250
P. tarminiana EtOH/H_20	*Passiflora tarminiana* H2O	250	250
P. tripartita EtOH/H_20	*Passiflora tripartita var mollissima* BuOH	500	500
P. tripartita EtOH/H_20	*Passiflora tripartita var mollissima* H2O	>500	>500
Amoxicillin		MIC < 0125µg/mL	

The BuOH and H_20 fraction of *P. tarminiana* were evaluated against clinical strains of *H. pylori* using the agar dilution technique. To determine the resistance profile of the clinical strains, the CLSI breakpoints for Clarithromycin and Metronidazole [2], Wang et al [34] and Wuepperhorst et al [35] for levofloxacin and amoxicillin were used. Interestingly, as seen in Table 4, The Ex1C strain and the Ex4A strain which are resistant to clarithromycin and metronidazole, respectively. For the Ex2C and Ex3A strains, a higher concentration of the fraction is necessary to inhibit these strains (MIC = 500 µg/ml).

Table 4. Antimicrobial activity of extracts and fractions of *P. tarminiana* against clinical strains of *H. pylori.*

Extract	Fraction	MIC (µg/mL)					
		NCTC 11637	**NCTC 11638**	**Ex1C**	**Ex2C**	**Ex3A**	**Ex4A**
P. tarminiana EtOH/H_20	*P. tarminiana* Fr BuOH	250	250	250	500	500	250
P. tarminiana EtOH/H_20	*P. tarminiana* Fr H20	250	250	500	>500	500	500
Clarithromycin (CLA)		< 1	< 1	**>1 (R)**	≤ 1	**>1(R)**	≤ 1
Metronidazole (MTZ)		< 8	< 8	≤ 8	**>8(R)**	**>8(R)**	**>8(R)**
Levofloxacin (LEV)		< 1	< 1	≤ 1	**>1 (R)**	**>1(R)**	≤ 1
Amoxicillin (AMX)		< 1	< 1	≤ 1	≤ 1	≤ 1	≤ 1

For the evaluation of standards, the compounds vitexin, quercetin and catechin were evaluated at concentrations of 20 µg/mL, 10 µg/mL and 5 µg/mL against reference strains and clinical strains of H. pylori. From the results obtained in Table 5, none of the standards evaluated showed activity against H. pylori at a concentration of 20 µg/mL or less.

Table 5. Antimicrobial activity of standars against reference and clinical strains of *H. pylori.*

Standard (Compound)	MIC (µg/mL)					
	NCTC11637	**NCTC11638**	**Ex1C**	**Ex2C**	**Ex3A**	**Ex4A**
Vitexine	>20	>20	>20	>20	>20	>20
Quercetine	>20	>20	>20	>20	>20	>20
Catechin	>20	>20	>20	>20	>20	>20

4. Discussion:

H. pylori is a bacterium capable of infecting and colonizing gastric epithelial tissue; it is estimated that 60% of the world population is infected by this microorganism. In 100% of those infected, it produces chronic gastritis and 20% of these will progress to pathologies such as gastric ulcers, gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma [36–38]. Evidence has shown that *H. pylori* is related to about 90% of gastric adenocarcinomas, which is why the WHO has ratified it as a type 1 carcinogen. Taking this relationship into account, it is known that the eradication of *H. pylori* is the best strategy in the prevention of gastric cancer; however, therapeutic success rates reported around the world are between 60-70%, an incredibly low figure considering that

pharmacological therapy, empirical, should have figures above 90% [3,36,37,39,40]. This phenomenon can be explained by the resistance of *H. pylori* that it expresses against most of the antibiotics included in the treatment schemes. In 2017, the WHO published a list of 16 antibiotic-resistant bacteria that impact human health, including *H. pylori* as a high-priority bacterium. [9,36,37,40]. The increase in antibiotic resistance has been related to lack of adherence to treatment, which has led to the lack of an ideal scheme to eradicate the infection. Reasons that make evident the need to seek alternative treatments such as those investigated in this work, which are based on phagotherapy and phytotherapy [41,42]. In this work we explore new therapeutic alternatives that have been successful for the treatment of infections caused by resistant microorganism to antibiotics. Among the available alternatives, the search option for lytic bacteriophages that could be proposed as a phage therapy and the evaluation of plant extracts derived from species of the genus *Passiflora* were selected for the investigation.

4.1. Searching and evaluation of bacteriophagues in environmental and clinical samples.

Various studies have proposed a large number of factors that can intervene in a productive viral infection and that result in a successful isolation of bacteriophages; within these, the culture conditions stand out, based on the pH and bioavailability of calcium and magnesium ions, necessary for viral adsorption, by favoring electrostatic interactions that are important in the binding of the phage to the bacterial receptor [43,44]. According to the controls used with the WG5 strain of *E. coli* and the coliphage isolated from this strain, it was possible to establish that the presence of $CaCl_2$ did not influence the recovery of phages in *H. pylori*, in agreement with the methodology for the isolation of bacteriophages reported for this bacterium. Several studies have tested different concentrations of salts, being successful in the recovery of bacteriophages such as those reported by Zhang et al [29], where they found adsorption percentages of P1 phage in *E. coli* close to 100, in the concentration of 5 mM of CaCl2. Another study by Moldovan et al. found a higher percentage of adsorption of phage λ in *E. coli* at concentrations of 10-2 M of magnesium sulfate MgSO4 [44]; or in the case of Chen et al, who found that the lysis of *Lactobacillus plantarum* by phage P1 occurred without the presence of divalent cations [45]. However, they found that lysis with clear plaque formation was faster in the presence of Ca2+; Contrary to the presence of Mg2+ or absence of cations, where the plates were diffused or not cleared [45], for which the concentration of salts present in the medium can be decisive for the isolation of bacteriophages.

The concentration of bacteriophages in the sample is another relevant factor to consider, since a higher concentration could favor a possible phage-host interaction, given the presence of both the phage and its host in the same niche [43]. Trying to guarantee a good concentration of bacteriophages in the analyzed samples, in this work, the molecular identification of *H. pylori* was carried out in 31 stool samples and 93 gastric biopsy samples; using the PCR technique with the identification of the *vacA* gene; which, allowed to confirm its presence in these samples. PCR analyzes for the *vacA* gene were positive in 32.2% of stool samples (10/31) and 64.5% of gastric biopsy samples (60/93); antrum 58% (18/31), notch 67.7% (21/31) and body 67.7% (21/31). Although, these results suggest that the bacterium was present in most of the samples, the tests carried out for the isolation of bacteriophages did

not achieve their objective. This may be related to the presence and concentration of phages in the analyzed samples, for which a greater number of samples will be analyzed in the future, as well as methods of bacteriophage concentration, such as those reported by Sanchez et al () or Vale et al (14).

Additionally, other factors such as pH, nutritional conditions, inoculum concentration, membrane pore size, among others, can influence the obtaining of bacteriophages, however, according to studies by other authors, conditions like those used in this study. The strains used in the study may also influence a greater or lesser susceptibility to bacteriophages. Studies carried out in our research group evaluated the presence of prophages in Colombian strains of *H. pylori*, through the molecular identification of the integrase and holin genes, reporting that 12.6% of the Colombian strains evaluated were positive for integrase and only 4.2% were positive. For both genes [46], these results suggest a lower prevalence of prophages in American isolates of *H. pylori* compared to that reported in isolates from other geographical locations. This can be extrapolated to the lytic phages that can infect this bacterium, which could indicate that these strains may have a lower susceptibility to infection by bacteriophages.

To reach a real application of phage therapy in *H. pylori* infection, more studies are required to demonstrate its effectiveness and safety *in vitro* and *in vivo*. There are some approaches such as the one carried out by Cuomo et al [21] who have analyzed the affectation of the Hpϕ phage in combination with lactoferrin and hydroxyapatite nanoparticles for the treatment of infected gastric cells, obtaining a bacterial reduction from an initial concentration of 104 approximately to 101 CFU and anti-inflammatory activity with decreased relative expression of TNF-α and IFN-β genes, however, much more information needs to be generated. Similarly, there is no information on the resistance mechanisms that *H. pylori* may have against infection by bacteriophages and the specific receptors.

4.2. Searching and evaluation of plant extracs, fractions and compounds against reference and clinical strains of *H. pylori*.

On the other hand, we studied the antimicrobial activity of some extracts, fractions, and compounds of *Passiflora* genus that are native in Colombia: *P. tripartita* var *mollissima* and *P. tarminiana* against *H. pylori*. Chemical identification of saponins and C- glicosil flavonoids of extracts and fractions of *P. tripartita* var *mollissima* and *P. tarminiana* were made by HPTLC and CE. No saponines were observed in both type of extracts: Hydroethanolic and aqueous. On the opposite, flavonoids were seen by HPTLC technique for both: extracts and fractions of both *Passiflora* species. The above results of extracts and fractions identified the presence of rutin and vitexin in *P. tarminiana* and vitexin in *P. tripartita* var *mollissima*. This results coincide with those reported by Zucolotto et al [47], Simigortis et al [48], Sepúlveda et al [49] and Castellanos et al [50] who visualized vitexin in *P. tripartita* var *mollissima* by HPLC -DAD and HPLC-MS; HPLC-DAD-ESI-MS/MS, UHPLC-DAD and UHPLC-TOF-MS and 1H NMR respectively, on the other hand Sepúlveda et al (13), also detected the presence of vitexin in *P. tarminiana* by means of

UHPLC-DAD. For rutin compound, it is the first time that this compound has been observed in *P. tarminiana* by HPTLC.

4.2.1. Chemical characterization of extracts and fractions of *Passiflora tripartita var mollissima* and *P. tarminiana.*

By CE, the electropherogram obtained for *P. tripartita* var *mollissima* were similar compared to Costa et al 2016 [30], with only a little variations of migration time for the three group of peaks. These variations may be due to differences in pH, capillary length, and some variations in voltage, which can change the electrosmotic flow, making the separation of the peaks more separated and delayed, but increasing their resolution and peak separation. Additionally for our knowledge, no electropherogram analysis has been obtained for *P. tarminiana,* which is the first report for electrophoretic profile for this specie. For both, *P. tripartita* and *P. tarminiana*, a reading of 390 nm was used for the detection of flavonoids, due to the bathochromic effect formed by the borate-flavonoid complex, making the wavelength usually used (330-350 nm) increase [30]For both *P. tripartita* and *P. tarminiana*, a reading of 390 nm was used for the detection of flavonoids, due to the bathochromic effect formed by the borate-flavonoid complex, making the wavelength usually used (330-350 nm) increase (91)

Centrifugal Partition Chromatography (CPC) is a technique that uses two liquid phases that are immiscible and are retained by centrifugal force, which can allow a large amount of a molecule to be recovered with high purity in the shortest possible time, without the use of solid supports [51]. Using this technique, it was possible to obtain the separation of 5 flavonoid-type compounds, with an interesting degree of purity observed by HPTLC, from a single injection of 500 mg of the butanolic fraction of *P. tarminiana* in a gradient solvent system. Solvent system selection is the most crucial step in achieving an efficient separation. This test can be carried out in a test tube where, by means of agitation, it is verified that the 2 phases form in the shortest possible time (ideally less than 30 seconds) [51]. Then, by means of a chromatographic or CE technique, the affinity that the compounds have for the light or heavy phase of the compounds present in the fraction is checked. From the results obtained, it was possible to establish that the use of a gradient system can be a good strategy for the separation of flavonoids, since it was observed that, as the concentration of BuOH increases, the compounds tend to pass to the organic phase. Although, despite having subfractions with pure compounds that need to be verified by purity test, but with low mass due to the small amount of sample used, the high reproducibility of the method would allow the technique to be replicated again with a greater amount of initial sample and starting from a greater amount of fraction, it would be possible to obtain a greater mass of the purified compounds.

4.2.1. Antimicrobial evaluation of extracts and fractions of *Passiflora tripartita var mollissima* and *P. tarminiana* against reference and clinical strains of *H. pylori.*

Various reports support the importance of finding new alternatives to eradicate *H. pylori* infection, due to the increase in resistant strains [42,52,53]. Hydroethanolic extracts of *P. tripartita* var *mollissima* and *P. tarminiana* showed a MIC =1000µg/mL and aqueous extracts did not shown activity (MIC >1000 µg/mL). Then, a liquid-liquid fractionation was performed, thanks for the fractionation, antimicrobial activity of *P. tripartita* var *mollissima* and *P. tarminiana* showed better results against reference strains of *H. pylori* with a MIC=250µg/mL. Some authors have established criteria for classified the activity of and extract according to the MIC obtained. In the criteria given by Holetz et al [54], an extract as: Inactive (MIC >1000µg/mL); Weak (500<MIC<1000µg/mL); Moderate (100<MIC<500µg/mL) and Good (MIC<100µg/mL). Our best fractions, the butanolic fraction and the aqueous fraction of *P. tarminiana* obtained a MIC= 250 µg/mL, being fractions with moderate activity. Other criteria established by Wang et al [55] also classified the extract with moderate-weak activity (MIC 100-1000 µg/mL). More criteria must be defined for the continuation of the *in vivo* assays, these results must be complemented with studies that evaluate the *in vitro* cytotoxic activity in a cell model suitable for the pathogenesis of the microorganism, that is, in the case of *H. pylori*, in human gastric adenocarcinoma (HSA) cells and together with the results obtained, analyse and consider scaling up to an *in vivo* animal model

Subsequently, the buthanolic and the aqueous fraction of *P. tarminiana* were evaluated against clinical strains of *H. pylori* with different resistant profiles to clarithromycin, levofloxacin, metronidazole, and amoxicillin (see Table 1). *H pylori* can develop resistance to these antibiotics, by making changes to its genetic material, through point mutations. In the case of the BuOH fraction, its MIC is maintained for strains with resistance to clarithromycin (Ex1C) and metronidazole (Ex4A) (MIC=250 µg/mL), and for strains with multi-resistance (Ex2C and Ex3A), a higher concentration of the fraction is necessary to inhibit them (MIC= 500 µg/mL). Considering that the fraction maintained its MIC against *H. pylori* resistant strains to these antibiotics, it can be inferred that this fraction has a different mechanism of action than the antimicrobials to which *H. pylori* is resistant. However, this is a preliminary phase of the study where it is necessary to confirm by means of molecular tests to establish the mutations present in the resistant strains and correlate them with the results obtained, as well as to determine the possible mechanisms of action that may be involved. associated with the fractions respectively, which would open the possibility of considering treatments based either only on the fraction or in conjunction with the antimicrobials already used in the treatment according to the mutations found.

According to the literature, it has been observed that flavonoids can have different mechanisms of action in *H. pylori*. For example, some flavonoids such as apigenin, present in *Passiflora* species, have *in silico* affinity for HsrA, an OmP-like protein whose function is to function as a homeostatic regulator, synchronizing metabolic functions and virulence in the availability of nutrients and cell division, also, it regulates the response to oxidative stress

[56]. Another study carried out by Krol et al [57] using the broth microdilution technique, evaluated the activity against *H. pylori* of vitexin and isovitexin from *Foenugraeci semen* seeds, with a (MIC=30µg/mL) against *H. pylori*, orientin presented an activity of (MIC =125µg/mL) while other compounds such as isovitexin, vicenin-2, vicenin 3 did not present antimicrobial activity (MIC>125µg/mL). Although the results obtained did not find antimicrobial activity with respect to vitexin, comparison with the results found by Krol et al 2021 is not possible, because different methodologies were used in that study, such as broth microdilution. Other compounds such as isoorientin, present in *P. tripartita* var *mollissima*, have been shown to have *in vivo* activity against microorganisms of the intestinal microbiota in BALB/c mice, reducing lipopolysaccharide (LPS) levels; however, activity against *H. pylori* and its mechanism of action have yet to be determined [58].

When comparing the results obtained with those reported in the literature, several reports use inadequate methods for the analysis of metabolites with antimicrobial activity against *H. pylori* to those recommended by the CLSI, such as the disk diffusion method, broth microdilution, among others, so the data obtained cannot be compared with those of other studies that use these techniques. As mentioned by Li et al [59], it is especially important to emphasize that dilution in agar is considered the gold standard for evaluating the antimicrobial activity of compounds against *H. pylori*. Additionally, according to Li et al, methods such as disk diffusion and broth microdilution are not appropriate for evaluating slow-growing microorganisms such as *H. pylori* and their use is not recommended since the former does not generate MIC values and the latter requires further optimization of liquid medium for growth of H. pylori [58]. Therefore, it is recommended to use solid media to determine antimicrobial activity against *H. pylori*. What has been said above allows us to conclude that it is also necessary to call for the scientific community to standardization of the evaluation of the antimicrobial activity of natural products against microorganisms, with particular emphasis on the use of dilution in agar according to what was done in this work with *H. pylori*. Working with interdisciplinary research groups is essential for the study of plant extracts where chemistry and microbiology allow the generation of more robust and comparable results.

5. Conclusions

We evaluated two alternatives to find anti-*H. pylori* activity based of phage and phytotherapy. Different techniques and procedures were tested for the isolation of bacteriophages in environmental samples (wastewater and soil) and clinical samples (faeces and biopsies). However, no lytic phages have been found so far. On the other hand, hydroethanolic extracts of *P. tripartita* var *mollissima* and *P. tarminiana* showed antimicrobial activity against reference strains of *H. pylori* with a (MIC =1000 µg/mL), the presence of flavonoids and absence of saponins were identified by HPTLC. Additionally, the butanolic fraction of *P. tarminiana* was the best fraction against reference and clinical strains of *H. pylori* with metronidazole and clarithromycin resistance with a (MIC =250µg/mL), and this fraction requires more concentration against multi-resistant *H. pylori* clinical strains (MIC

=250µg/mL). Antimicrobial activity can be related to C-glicosil flavonoids present in the fractions, but more studies are required to compare that.

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7. Conflict of interest

The authors have no conflicts of interest to declare.

8. References

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