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Inhibition of *Helicobacter pylori* and Its Associated Urease by Two Regional Plants of San Luis Argentina

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A B S T R A C T

The search of alternative anti-*Helicobacter pylori* agents obtained mainly of medicinal plants is a scientific area of great interest. The antimicrobial effects of *Litrahea molleoides* and *Aristolochia argentina* extracts against sensible and resistant *H. pylori* strains, were evaluated in vitro. Also, the urease inhibition activity and the effect on the *ure*A gene expression mRNA was evaluated. The *L. molleoides* and *A. argentinae* extracts showed antimicrobial activity against all strains assayed. Regardless of the extract assayed a decrease of viable count of approximately 2 log units on planktonic cell or established biofilms in *H. pylor*i strains respect to the control was observed ($p<0.05$). Also, both extracts demonstrated strong urease inhibition activity on sensible *H. pylori* ($p<0.05$). In all strains, the *ure*A gene expression was down regulation independently of extracts used. The promising results of this work suggest that both *L. molleoides and A. argentina*, two traditional medicinal plant of Cuyo region, could be used as *H. pylori* alternative treatment that could attenuate virulence of bacterium and enable the host immune system to combat infection.

Introduction

Helicobacter pylori is a Gram-negative bacterium that selectively colonizes the gastric mucosa of almost half the human population. *H. pylori* infection is considered to be a major public health issue worldwide for its implication in the etiology of gastro duodenal diseases, such as gastritis, peptic ulcers and gastric adenocarcinoma (Garza-González *et al.*, 2014). Several virulence factors, among them, the urease enzyme, contribute to the inflammation and pathologic changes observed in gastric mucosa. The microorganism converts urea into ammonia and carbon dioxide modifying the acidic gastric environment to facilitate colonization (Kusters *et al.*, 2006; Kenneth and McColl, 2010).

On the other hand, as with various bacteria studied to date, *H. pylori* can have an alternative lifestyle as a biofilm (Yonezawa *et al.*, 2010). Biofilms are important in bacterial pathogenesis due to because allows at microbes survive and spread within the host.

This natural community is characterized by cells that are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription. Also, the biofilm matrix acts as shield, protecting bacteria from host defenses and antibiotics (Carron *et al.*, 2006; Wong *et al.*, 2016).

The eradication of *H. pylori* remains a primary goal for alleviating peptic ulcer disease and preventing associated gastric malignancies (Malfertheiner, 2017). Triple therapy consisting of a proton pump inhibitor and two antibiotics, usually amoxicillin (Amx), clarithromycin (Cla) or metronidazole (Mtz) has been recommended; however the emergence of antibiotic resistance complicates the treatment. Previous studies showed that in our region the prevalence of strains resistant to Cla and Mtz is high compared to other regions of the world (Vega *et al.*, 2010).

Taking account the high *H. pylori* antibiotic resistance rates, principally in developing countries, the WHO listed *H. pylori* among 16 antibiotic-resistant bacteria that pose the greatest threat to human health and encourages the search for alternative treatments for these pathogens with high impact in Public Health (Dang and Graham, 2017).

Nowadays there is great interest in the search for alternative anti-*H. pylori* agents obtained mainly of medicinal plants used by the population for the treatment of various digestive disorders. Additionally, is needed the search for new targets of *H. pylori* inhibition mainly against those factors related to the initial step in colonization. Is well accepted that urease-negative mutant does not cause gastritis due to difficulties in colonization, therefore, specific inhibition of urease activity could be a possible strategy to eliminate the microorganism. In this sense, several authors have studied the inhibitory effect of plant extracts on urease activity (Amin *et al.*, 2013; Sahin, 2015, Sarkar *et al.*, 2016; Zhou *et al.*, 2016).

Lithraea molleoides (Vell.) Engl. (Anacardiaceae), is a tree which grows in South America and is known in Argentina as “molle blanco”. For its medicinal properties is used for the treatment of respiratory and digestive diseases among others. It is also, an ingredient of some foods such as “arrope” and “aloja” (Garro *et al.*, 2015).

Aristolochia argentina (Aristolochiaceae), popularly known as "charrúa", is used in folk medicine for gastrointestinal disorders in the region of Cuyo, Argentina.

The aim of this study was to evaluate the anti-*H. pylori* and urease inhibition activities of extracts obtained from two plants used as folk medicinal in San Luis, Argentina.

Materials and Methods

Plant material

L. molleoides (Vell.) Engl. (Anacardiaceae) and *A. argentina* (Aristolochiaceae) were collected in San Luis, Argentina. The plants were identified by Dr. Luis A. Del Vitto and a voucher specimen has been deposited at the Herbarium of the Universidad Nacional de San Luis, voucher N°515 and Nº 9258 respectively.

Preparation of aqueous extracts

The infusion was obtained of the air-dried plant material of each species adding boiling distilled water to powder material (100 ml: 5 g) and left to stand at room temperature for ten minutes, according to Argentinean

Pharmacopoeia Argentina (2007). The residual plant material was separated by filtration and the supernatant was lyophilized in a RIFICOR® freeze dryer. The crude extracts were dissolved in double distilled water (MilliQ, Millipore), and sterilized with a 0.2-mm filter (Sartorius).

Strains and culture conditions

H. pylori NCTC 11638 (reference strain), a kind gift from Dr. Manuel López-Brea, Microbiology Service of Hospital Universitario de la Princesa, Madrid, Spain and five clinical isolates obtained from gastric antral biopsy specimens were used for this study. *H. pylori* strains were grown in Mueller-Hinton agar (MHA) supplemented with 7% horse blood (MHA-HB) and identified by microscopy, urease, catalase and oxidase tests.

Antibacterial activity of *L. molleoides* and *A. argentina*

The antibacterial activity of both plants extract against *H. pylori* strains was assayed by broth microdilution method using Mueller Hinton Broth (MHB) according to CLSI guidelines (2007). The initial inoculum corresponding at 0.5 on the Mac Farland standard ($1x10^8$ colony forming units (CFUs)/mL) was used. Serial dilutions of Amx (Sigma-AldrichCo., StLouis, MO) were used as a control in the susceptibility test. Two fold dilutions of both extracts were performed to obtain the following final concentrations: from 500 to 4 µg/mL and from 125 to 0.08 µg/mL for Cla, Amx and Mtz. Broth microdilution methods were carried out in 96-well microtitre plates as previously described (Garro *et al.*, 2015). Minimal inhibitory concentration (MIC) was measured by determining the smallest amount of extract or antibiotic needed to inhibit the visible growth of the microorganism. Resistance was defined as the Cla MIC being ≥ 1 µg/mL, Amx MIC > 0.5 µg/mL and Mtz MIC being ≥ 8 µg/mL. All tests were performed in duplicate.

Biofilm assays

Biofilms of *H. pylori* strains were obtained as previously described by Vega *et al.*, 2012. In the same assay, planktonic cell was removed at 48 h to i) viable cell counts, ii) optical microscopy and iii) RNA extraction. To quantify the biofilm, the coverslips were sampled at 48 h, rinsed three times with phosphate-buffered saline (PBS) to remove planktonic cells and biofilm debris, and vortexed for 3 min in PBS to allow cell detachment from biofilm. Viable biofilm cell counts were plated onto MHA-HB by duplicate. CFUs were counted after incubation in a microaerobic atmosphere for three days at 37ºC. Also, RNA extraction was performed. The planktonic and biofilm morphology was observed by staining with 0.1% fuchsin for 15 min and visualized with an optical microscope.

Effect of plants extract against *H. pylori* planktonic and biofilm

Subinhibitory concentrations of the *L. molleoides* and *A. argentina* extracts, corresponding to 0.5 MIC respectively were tested against planktonic and biofilm cell. The effect on established biofilm was determined as following. The biofilm obtained as previously described was washed twice with PBS. Then, was placed in Petri dish with fresh medium containing *L. molleoides* or *A. argentina* extracts at a sub-inhibitory concentration and incubated in microaerobic atmosphere at 37ºC for 26 h. Following, the coverslips was removed and washed with PBS for i) viable cell counts; ii) optical microscopy and iii) RNA extraction. Same determinations were assayed with the

plantonick cell treated with sub-inhibitory concentration of *L. molleoides* or *A. argentina* extracts.

Gene expression

Planktonic and biofilms cell coming from developed on the glass surfaces were treated with TRIzol reagent (Invitrogen) for total RNA extraction.

RNA quantification was performed by spectrophotometric measurement using a NanoDrop ND- 1000 (NanoDrop Technologies) and each RNA sample was adjusted to give a final concentration of 2 ng/ µl. The *ure*A, and housekeeping 16S rRNA genes were analyzed. cDNA was performed with random hexamer and 200 U Moloney murine leukaemia virus reverse transcriptase (Invitrogen). The identification of amplified fragments of 411, and 390 bp for *ure*A, and 16S rRNA genes respectively, was performed with 1.8% agarose gel electrophoresis. The gels were stained with GelRed Nucleic Acid Gel Stain (Biotium Inc), visualized under UV light and photographed. The DNA fragment size was determined by comparison with molecular weight markers with a range of 50 to 1000 bp. Semi-quantification of the bands was performed with an image analyzer (ImageJ WCIF) against the constitutive gene 16S rRNA. The ratio for *ure*A gene respect to the constitutive gene and the ratio of the expression of *ure*A gene for an extract respect to the control were established. Values less than 1 correspond to a decrease in expression and values greater than 1 correspond to an increase in expression.

Inhibitory effect on urease activity

The inhibitory effect of the *L. molleoides* and *A. argentinae* extracts on urease activity was measured using a modification of the method described by Malekzadeh *et al.,* (2001). In brief, 2.850 µl of urea broth, 50 µl of either extract, and 100 µl of *H. pylori* culture medium were added to a glass tube. The initial *H. pylori* number in the urea broth was about 1×10^8 CFU/ml, and the final concentrations of the both extracts were 4, 8, 16, 32 and 64 µg/ml. Double distilled water (50 µl) was added instead of either extract, as the control. The mixture was incubated in a microaerobic atmosphere at 37°C for 3 h, and subsequently the absorbance of the culture at 560 nm was measured using a spectrophotometer. The inhibitory effect on urease activity was calculated using Eq.: Inhibitory effect of urease activity (%) = (1 – Absorbance of sample) / (Absorbance of control) × 100.

Statistical analysis. Statistical analysis was performed using Graph Pad Prism version 5.00 for Windows and Graph Pad in Stat version 3.00 for Windows (Graph Pad Software, San Diego, California, USA, www. graphpad.com). All data are expressed as the mean ± S.E.M. (Standard Error of Mean). A probability of $p<0.05$ was considered significant.

Results and Discussion

The treatment of *H pylori* infection is very important to prevent the development of severe diseases such as gastric cancer and lymphoma, as well as to the resolution of peptic ulcer disease, and dyspepsia symptoms (Kavitt and Cifu, 2017). However, antimicrobial treatment does not achieve the much required success in the eradication because the resistance developed by the microorganism. A potential source for therapeutic use against *H. pylori* strains from natural sources is encouraging given the worldwide sensitivity studies performed using medicinal plant extracts (Sarkar *et al.,* 2016).

The results for antibacterial activity (MICs values µg/ mL) of plant extracts, Amx, Cla and Mtz, against the reference *H. pylori* and

five clinical strains are showed in table 1. The *L. molleoides* and *A. argentinae* extracts showed inhibitory activity against strains assayed. There was no difference in antibacterial effect between *L. molleoides* and *A. argentina* extracts. For all strains, except NCTC 11638 and HP796, the MIC values for *A. argentinae* extract were lower than *L. molleoides* extract with MIC ranging from 64 to 8 µg/mL and 125 to 8 µg/mL respectively. The Cla and Mtz resistant strains showed higher MIC values for any *L. molleoides* or *A. argentinae* extract. However, taking into account the activity for either of the two extract on sensible and resistant *H. pylori* strains, the results obtained are significant and could be considered for its use as natural alternative treatment. All strains were sensible to Amx.

Table 2 shows the results of the antimicrobial effect on *H. pylori* strains in both planktonic and biofilm state treated with sub-inhibitory concentration of *L. molleoides* or *A. argentina* using the viable count method. Regardless of the extract used we observed a decrease of approximately 2 log units on planktonic cell or established biofilms in resistant *H. pylor*i strains with respect to the control ($p<0.05$). The HP105 and HP109 sensible *H. pylori* strains in planktonic state treated with either extracts showed a decrease of viable cell of 3 log units ($p<0.05$) and in biofilm state the decrease of viable cell was of 2 log units ($p<0.05$). The resistant *H. pylori* strains, HP796 and HP173, showed a decrease of viable count 33 fold and 32.3 fold respectively at sub-inhibitory concentration of *A. argentina* ($p<0.05$). When used a sub-inhibitory concentration of *L. molleoides* a similar decrease of viable count, 28 and 29 folds for same strains, was observed.

The characteristic helical shape of bacterium is a prerequisite for to successfully carry out the invasion and subsequent colonization in the gastric mucosa (Wang *et al.,* 2010). In this study, optical microscopy showed morphological changes in the presence of either *L. molleoides* or *A. argentina* extracts. Untreated cells had a helical shape, whereas the extract-treated cells had coccoid shape. The figure 1 shows the results obtained with NCTC 11638 untreated and treated with *L. molleoides* or *A. argentina* in both cell states. The morphology changes at coccoid shapes of *H. pylori* strains have been associated with to loss of culturability and reduction in the total amounts and integrity of RNA (Worku *et al.,* 1999; Lee *et al.,* 2016). The results obtained is according to the observed for reduction in viable counts. The ability of *H. pylori* to form biofilm it facilitates the colonization, also gives resistance to mucus turnover and gastric peristalsis. Interestingly, in this work, we demonstrate that both extracts act on the established biofilm of sensitive and resistant *H. pylori* strains.

Table.1 MICs of *Lithraea molleoides* and *Aristolochia argentina* extracts and antibiotic for six Strains of *Helicobacter pylori*

H. pylori	MIC (µg/ml) plants extracts and Antibiotic				
Strains	Cla	Mtz	Amx	*L. molleoides*	*A. argentina*
NCTC 11638	0.5	0.25	0.25	16	16
HP796	4	16	0.5	125	125
HP173	4	16	0.5	125	64
HP105	0.25	1	0.125	16	8
HP109	0.5	2	0.25	16	8
HP857	0.25	1	0.25	64	32

[a]Values are average derived from two determinations

Table.2 Viable counts of *Helicobacter pylori* planktonic and biofilm cell treated with sub-inhibitory concentration of *Lithraea molleoides* or *Aristolochia argentina*.

H. pylori	State cell		*Litrahea molleoides*		*Aristolochia argentina*	
strains	Planktonic	Biofilm	Planktonic	Biofilm	Planktonic	Biofilm
NCTC 11638	$2.5x10^{8}$±1.0	$2.3x10^{4}$±0.3	$6.5x10^{6}$±0.1 [a]	$1.5x10^{2}$±0.3 [b]	$6.3x10^{6}$±0.3 [c]	$1.3x10^{2}$±0.6 [d]
HP796	$2.5x10^{9}$±0.1	$4.5x10^{4}$±0.1	$8.9x10^{7}$±0.1 [a]	$3.4x10^{2}$±0.1 [b]	$7.5x10^{7}$±0.1 [c]	$3.6x10^{2}$±0.3 [d]
HP173	$2.1x10^{9}$±0.3	$3.5x10^{4}$±0.3	$7.3x10^{7}$±0.1 [a]	$2.9x10^{2}$±0.3 [b]	$6.5x10^{7}$±0.1 [c]	$3.0x10^{2}$±0.3 [d]
HP105	$1.4x10^{9}$±0.2	$1.3x10^{4}$±0.2	$4.1x10^{6}$±0.3 [a]	$1.7x10^{2}$±0.6 [b]	$3.3x10^{6}$±0.3 [c]	$1.5x10^{2}$±0.6 [d]
HP109	$1.5x10^{9}$±0.3	$1.7x10^{4}$±0.3	$5.1x10^{6}$±0.2 [a]	$1.3x10^{2}$±0.3 [b]	$4.9x10^{6}$±0.5 [c]	$1.1x10^{2}$±0.8 [d]
HP857	$3.2x10^{9}$±0.3	$5.3x10^{4}$±0.3	$4.3x10^{7}$±0.3 [a]	$2.8x10^{2}$±0.2 [b]	$3.7x10^{7}$±0.1 [c]	$2.7x10^{2}$±0.3 [d]

Data are represented as means log10 ± *S.D.* of total cells counts of two independent experiments
[(a-d)] mean values with different superscripts for planktonic or biofilm state treated with *L. molleoides* or *A. argentina* are significantly different

Table.3 Inhibitory effects of extracts on the urease activity in *Helicobacter pylori* strains

	Inhibitory effect on urease activity (%) *L.molleoides*					Inhibitory effect on urease activity (%) *A. argentina*				
H. pylori	Extract concentration (µg/ml)					Extract concentration (µg/ml)				
strains	4	8	16	32	64	4	8	16	32	64
NCTC 11638	11± 0.5	27± 0.7	29± 1.1	31± 0.6	33± 0.8 *	9± 1.3	29±0.9	31±0.7	33±1.0	35±0.7 *
HP796	9± 0.9	14± 1.3	16± 1.5	18± 1.1	25± 1.3	11± 0.6	19±1.3	23±0.9	25±1.3	27±1.1
HP173	10± 1.3	13± 0.3	15± 2.3	17± 1.1	23± 2.1	13± 1.8	17±1.5	21±2.1	23±1.5	25±1.3
HP105	18± 0.6	31± 0.5	33± 1.7	36± 0.7	39± 1.5 *	21± 1.2	35±1.0	38±1.5	40±0.5	43±0.9*[a]
HP109	21± 0.9	33± 1.1	35± 0.7	38± 1.3	44± 0.9 *	23± 1.0	39±1.3	40±1.1	42±1.1	46±0.5*
HP857	19± 2.3	30± 0.5	32± 1.0	36± 1.5	41± 1.3 *	21± 1.7	37±1.1	39±0.7	43±1.5	45±1.0 *

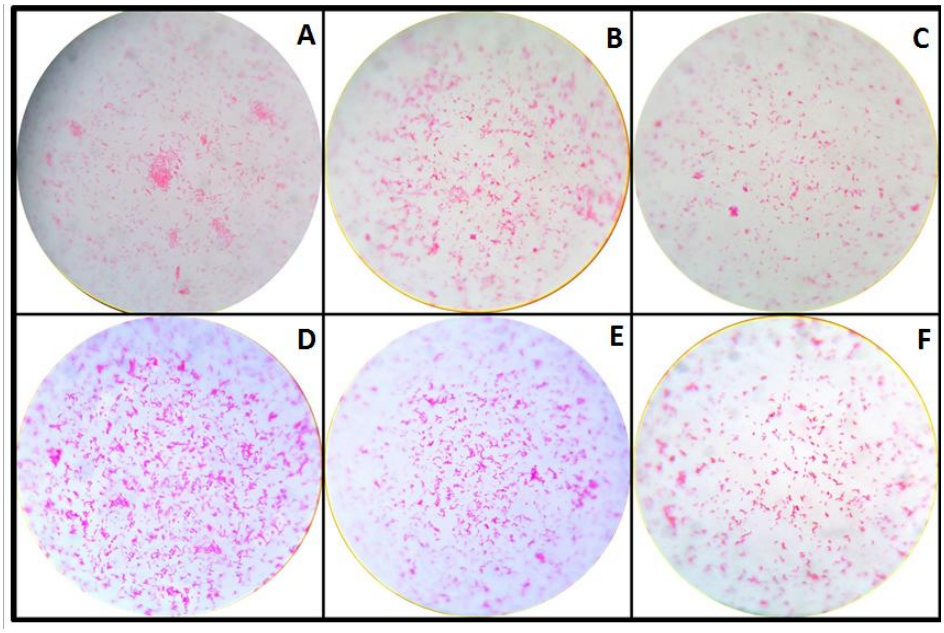
*: $P<0.05$

Table.4 Effect of extracts on the urease gene expression in *Helicobacter pylori* strains

H. pylori strains	Effect of *L. molleoides* /fold change		Effect of *A. argentina* / fold change	
	Planktonic	Biofilm	Planktonic	Biofilm
NCTC 11638	-2.9	-1.9	-2.7	-2.1
HP796	-2.1	-1.7	-1.9	-1.5
HP173	-1.9	-1.3	-1.7	-1.7
HP105	-3.5	-2.1	-3.9	-2.3
HP109	-3.7	-2.3	-3.5	-2.5
HP857	-3. 9	-2. 5	-3.7	-2.7

Data are represented as means of two independent experiments

Fig.1 Morphological changes of NCTC 11638 *Helicobacter pylori* strain in the presence of *Lithraea. molleoides* or *Aristolochia argentina* extracts. A: planktonic and D: biofilm control; B: planktonic and E: biofilm treated with a sub-inhibitory concentration of *L. molleoides* extract; C: planktonic and F: biofilm treated with a sub-inhibitory concentration of *A. argentina* extract. The optical micrographs stained with 0.1% fuchsin showed conversion of the helical to coccoid form (arrow) after treated with either extracts. Results are representative of two independent experiments.



The pathogenesis of *H. pylori* is attributed to its ability to colonize the gastric epithelium and its virulence factors (Ansari and Yamahoka, 2017). The human immune system cannot eliminate because of the bacterial ability of immune evasion. Thus, neutralizing or suppressing the expression of virulence factors that allow attenuates the pathogenicity of the bacterium and facilitate the elimination for the host innate immune system is desired to overcome infection (Fernebro, 2011). In this sense, urease inhibitors had been isolated from some plants and herbs (Amin *et al.,* 2013; Hřibová *et al.,* 2014; Modolo *et al.,* 2015). We studied two regional plants that have not been screened yet for its urease inhibition activities in *H. pylori* strains in

order to determine its effect on the main factor of colonization and virulence of the bacterium. Table 3 show the inhibitory effects of *L. molleoides* and *A. argentina* extracts on the urease activity in *H. pylori* strains. The *A. argentina* extract showed higher urease inhibitory activity than *L. molleoides* for all concentrations tested in all strains; however, no statistically significant differences were observed. Respect to sensible *H. pylori* strains, both extracts demonstrated to be strongest that on resistant *H. pylori* strains. The urease activity of sensible *H. pylori* strains decreased by more than 20% at 64 µg/ml concentration respect to 4 µg/ml of either extracts ($p<0.05$), whereas that the resistant strains decreased by about 12%. Our results confirm the antibacterial activity of *L. molleoides* and *A. argentina* extracts against *H. pylori* strains for inhibition of urease activity. Lee *et al.,* 2016 using the same method demonstrated the inhibition of urease of methanol and ethanol *Inula britannica* extracts at 10 mg/mL concentration against three reference strains Mtz sensible. On the other hand, on a total of 42 aqueous plants extracts using the phenol–hypochlorite method showed antiurease activity with a range of 17.8-80.0% inhibition at a concentration 0.2 mg/mL (Hřibová *et al.,* 2014). Amin *et al.,* 2013 in the *H. pylori* urease inhibitory assay, using methanol and acetone extracts of *Acacia nilotica* and *Calotropis procera* demonstrated significant inhibition depending to the concentration and type of the extract (Amin *et al.,* 2013).

The presence of sublethal levels of antibiotics can alter the expression of genes related to the bacterial stress and virulence on a transcriptional level (Andersson and Hughes, 2014). In order to determine the molecular mechanism of action of both extract, the expression of the *ure*A gene induced by sub-inhibitory concentration of either extracts, was investigated by RT-PCR and semicuantified using ImageJ. Table 4 shows the effect of both extracts on expression of *ure*A gene mRNA. In all strains, the *ure*A gene expression was down regulation independently of extracts used. The sensible *H. pylori* strains showed an expression markedly reduced of *ure*A gene mRNA ranging 3.5 to 3.9 fold, when the strains were treated with either extracts at sub-inhibitory concentration respect to the control; however no difference statistical was observed. The *ure*A gene expression from cell of *H. pylori* in a biofilm established showed slightly lower down regulation compared to planktonic cells with both extracts. This suggests that *L. molleoides* and *A. argentina* extracts decrease *ure*A gene expression and agree according to the results obtained in the assay of inhibition of urease activity.

Bioactive compounds of medicinal plants possess recognized antimicrobial properties. The results obtained in this work can be linked to alkaloids, aristolochic acid and terpenes present in *A. argentina* among other active principles. Gutkind *et al.,* demonstrated that *A. argentina* extract have antibacterial and antifungal activity (Gutkind *et al.,* 1981). On the other hand, *L. molleoides* possess phenolic acids, mannitol, flavonoids as bioactive compounds for which we demonstrated activity against sensible and resistant *H. pylori* strains (Garro *et al.,* 2015). Additionally, we demonstrated that *L. molleoides* extract confers gastroprotective properties against gastric ulcers induced by different necrotizing agents in Wistar rats (Garro *et al.,* 2015).

The decrease of urease activity of all the *H. pylori* strains obtained in this work is of therapeutic significance because the plants extract assayed would be potent anti-*H. pylori* agents used alone or in combination with antibiotics. The virulence genes are important by assesses druggable targets in the search for new tools in the antibacterial arsenal. Also, could be used as a potential combinatorial approach of anti-virulence therapeutics (Schroeder *et al.,* 2017). In this sense, the proton-pump inhibitors such as omeprazole and lansoprazole currently used in the treatment of *H. pylori* infections combined with antibiotics are potent urease inhibitors.

The results obtained with two extract of traditional medicinal plant used in the Cuyo region, Argentina are promising because the extracts demonstrated to be very effective against *H. pylori* strains sensible and resistant and in planktonic or biofilm state. Additionally, we demonstrated the inhibitory effect on the urease activity of two extracts and first time determined a molecular mechanism by decrease of *ure*A gene expression mRNA. This could attenuate bacterium and enable the host immune system to combat infection.

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