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Antimicrobial Activity of an Amazon Medicinal Plant (Chancapiedra) (*Phyllanthus niruri* L.) against *Helicobacter pylori* and Lactic Acid Bacteria

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The potential of water extracts of the Amazon medicinal plant Chancapiedra (*Phyllanthus niruri* L.) from Ecuador and Peru for antimicrobial activity against *Helicobacter pylori* and different strains of lactic acid bacteria such as *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum* was investigated. *H. pylori* was inhibited by both water extracts in a dose dependent manner, whereas lactic acid bacterial growth was not affected. Both extracts contained ellagic acid and hydroxycinnamic acid derivatives and exhibited high free radical scavenging linked-antioxidant activities (89%). However, gallic acid was detected only in the Ecuadorian extract. Preliminary studies on the mode of action of Chancapiedra against *H. pylori* revealed that inhibition may not involve proline dehydrogenase-based oxidative phosphorylation inhibition associated with simple mono-phenolics and could involve ellagitannins or other non-phenolic compounds through a yet unknown mechanism. This study provides evidence about the potential of Chancapiedra for *H. pylori* inhibition without affecting beneficial lactic acid bacteria. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: Phyllanthus niruri L.; *Helicobacter pylori*; *Lactobacillus*; antimicrobials; phenolic antioxidants.

INTRODUCTION

Helicobacter pylori is a gram-negative, microaerophilic and spiral-shaped bacterium that has been implicated in gastritis, peptic ulcer disease and over long exposure potentially to gastric carcinoma (Malfertheiner *et al*., 2010). *H. pylori* colonize the stomach of approximately half of the world's population and unless specifically treated, infection with this gastric pathogen is lifelong (Voravuthikunchai and Mitchell, 2008). *H. pylori* induces a strong inflammatory response within the host, thereby releasing a high level of host-derived toxic oxygen species, but *H. pylori* can survive and colonize persistently in the harsh conditions of the gastric mucosa. To combat such forms of oxidative stress, *H. pylori* expresses several key biochemical responses that include the oxidative stress resistance enzymes superoxide dismutase and catalase, a family of peroxiredoxins and newly described antioxidant proteins such as the NADPH quinone reductase (Wang *et al*., 2006).

Treatment of *H. pylori* infection consists of multiple therapies that combine a wide range of antibiotics such as tetracycline, amoxicillin, imidazoles and a few selected macrolides (clarithromycin). However, the increase in antibiotic resistance and host-associated side effects reduces the efficacy of such treatments (Kusters *et al*., 2006). In view of the incomplete cure achieved with antibiotic-based therapy due to resistant strains, investigations on naturally occurring antimicrobial compounds found in dietary, medicinal plants and herb extracts as alternative and/or as bioactive agents supporting antibiotics for treating *H. pylori* infection have excellent potential.

Phyllanthus niruri L. (Euphorbiaceae) is a small erect annual herb growing up to 30–40 cm in height and is indigenous to the Amazon rainforest and other tropical areas including South East Asia, Southern India and China (Girach *et al*., 1994). This plant has a long history in herbal medicine systems and has been used widely in different regions of the world for the treatment of various diseases. *In vitro* studies have shown that water extracts of *P. niruri* L. inhibit the calcium oxalate crystal growth and aggregation in human urine suggesting that it may interfere with the early stages of stone formation and may represent an alternative treatment of urolithiasis (Sighinolfi *et al*., 2007). Further studies have revealed the antihypertensive (Iikuza *et al*., 2006), the antioxidant and hepatoprotective potential of this medicinal plant (Harish and Shivanandappa, 2006). These therapeutic properties have been linked to the presence of active phytochemicals such as flavonoids, alkaloids, terpenoids, lignans, tannins, coumarins and saponins from various parts of *P. niruri* L. (Bagalkotkar *et al*., 2006). However, the potential of this medicinal plant against human pathogens such as *H. pylori* still remains unknown.

The possibility of a combined therapy including phenolic phytochemical-linked medicinal plants and lactic acid bacteria with probiotic potential on *H. pylori* inhibition may constitute novel and low cost strategies

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towards the solution to *H. pylori*-associated peptic ulcers and active chronic gastritis as well as their long term impacts.

Probiotic bacteria are defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host. Probiotic activity has been associated with Lactobacilli, Bifidobacteria, *Streptococcus*, *Enterococcus*, non pathogenic *E. coli* and *Saccharomyces boulardii* (FAO/OMS, 2002). It has been shown that probiotic bacteria prevent infection of pathogenic bacteria both through activation of the host's immune system and through direct competition of the probiotic bacteria with the pathogen. There is good evidence that *H. pylori* is killed by lactobacilli both *in vitro* and to a limited extent *in vivo* (Zou *et al*., 2009).

Based on the above rationale, the objective of this research was to evaluate the potential of water extracts of Chancapiedra (*Phyllanthus niruri* L.) from Peru and Ecuador for antimicrobial activity against *Helicobacter pylori* and different strains of lactic acid bacteria with probiotic potential such as *Lactobacillus acidophilus*, *Lactobacillus casei* and *Lactobacillus plantarum*. Further, preliminary investigations into the mode of action of high phenolic–water extracts of *P. niruri* L. on *H. pylori* inhibition were evaluated based on the rationale that certain simple phenolics could be proline analog mimics and may regulate antimicrobial activity through inhibition of proline oxidation via proline dehydrogenase (Shetty and Lin, 2005).

MATERIALS AND METHODS

Materials. The commercial product from Ecuador which included dried leaves of *Phyllanthus niruri* L. (Euphorbiaceae) (brand 'La Cholita') was purchased from an Ecuadorian Store in Hadley, MA (USA). Commercially available fresh leaves of *Phyllanthus niruri* L. were purchased in a local market in Lamas-San Martin (Peru) (High Amazon) in January 2008, and then dehydrated at 65–70 °C in a hot air oven until constant weight. Specimens were authenticated based on comparisons to similar specimens confirmed previously by Rutter (1990) and Mejia and Renjifo (2000).

Unless noted, all chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

Strains used. *Helicobacter pylori* (strain ATCC 43579, which originated from human gastric samples) was obtained from the American Type Culture Collection (Rockville, MD).

The lactic acid bacteria strains used in this study were the following: *Lactobacillus bulgaricus* was isolated in a previous study (Apostolidis *et al*., 2007). *Lactobacillus acidophilus* was supplied by Rosell Institute Inc. Montreal, Canada (Lot#: XA 0145, Seq#:00014160), *Lactobacillus plantarum* (1) (ATCC 9019), *Lactobacillus plantarum* (2) (NRRL B4496), *Lactobacillus casei* (1) (ATCC 343, PLP-XYL) and *Lactobacillus casei* (2) (PLP-3537).

Extract preparation. A total of 10 g of powdered dried sample was added to 100 mL of distilled water and refluxed at 95 °C for 30 min and cooled. The extracts were then filtered through filter paper (Whatman No. 2) and made up to 100 mL with distilled water (100 mg/mL of sample concentration). The pH of the aqueous extracts were corrected to 6–8 and centrifuged at 9300 × **g** for 30 min. An aliquot of the supernatant was re-centrifuged at 3000 rpm for 10 min before each assay.

Total phenolics assay. The total phenolics were determined by the standard Folin-Ciocalteu method as modified by Shetty *et al*. (1995). Briefly, 1 mL of diluted extract (50 mg/mL) was transferred into a test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample 0.5 mL of 50% (vol/vol) Folin-Ciocalteu reagent was added and mixed. After 5 min, 1 mL of 5% Na_2CO_3 was added to the reaction mixture and allowed to stand for 60 min. The absorbance was read at 725 nm. The standard curve was established using various concentrations of gallic acid in 95% ethanol, and the results were expressed as mg of gallic acid equivalent per gram of sample dry weight (dw).

Antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) inhibition assay. The standard DPPH scavenging activity was determined by an assay modified by Kwon *et al*. (2006). To 1.25 mL of 60 μM DPPH in 95% ethanol, 250 μL of diluted extract (50 mg/mL) was added, and the decrease in absorbance was monitored after 1 min at 517 nm ($Abs517_{extract}$). The absorbance of a control (distilled water instead of sample extract) was also recorded after 1 min at the same wavelength ($Abs517_{control}$). Therefore, the percentage of inhibition was calculated by:

$$\% \text{ Inhibition} = \frac{(Abs517_{control}) - (Abs517_{extract})}{(Abs517_{control})} \times 100$$

High performance liquid chromatography (HPLC) analysis of phenolic profiles. The extracts (2 mL) were filtered through a 0.2 μm filter. A volume of 5 μL of extract was injected using an Agilent ALS 1100 autosampler into an Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a DAD 1100 diode array detector. The solvents used for gradient elution were (A) 10 mM phosphoric acid (pH 2.5) and (B) 100% methanol. The methanol concentration was increased to 60% for the first 8 min and to 100% over the next 7 min, then decreased to 0% for the next 3 min and was maintained for the next 7 min (total run time, 25 min). The analytical column used was Agilent Zorbax SB-C_{18}, 250 × 4.6 mm i.d., with packing material of 5 μm particle size at a flow rate of 1 mL/min at room temperature. During each run the absorbance was recorded at 306 nm and 333 nm and the chromatogram was integrated using an Agilent Chemstation enhanced integrator. Pure standards of chlorogenic acid, gallic acid and ellagic acid in 100% methanol were used to calibrate the standard curves and retention times.

Preparation of starter culture of *Helicobacter pylori*. *Helicobacter pylori* was cultured according to Stevenson *et al*. (2000). Standard plating medium (*H. pylori* agar plates) were prepared by using 10 g of special peptone (Oxoid Ltd, Basingstoke, England) per liter, 15 g of granulated agar (Difco Laboratories, Becton, Dickinson and Co., Sparks, MD) per liter, 5 g of sodium chloride

(EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water.

Broth media were prepared by 10 g of special peptone (Oxoid Ltd, Basingstoke, England) per liter, 5 g of sodium chloride (EM Science, Gibbstown, NJ) per liter, 5 g of yeast extract (Difco) per liter, 5 g of beef extract (Difco) per liter of water. A volume of 1 mL of stock *H. pylori* was added into test tubes containing 10 mL of sterile broth media. They were incubated at 37 °C for 48 h before being used for inoculating by the spread plate technique. The active culture was then spread on *H. pylori* agar plates to make bacterial lawn for the agar-diffusion assay.

Agar-diffusion assay. The antimicrobial activity of sample extracts was analysed by the agar-diffusion method. The assay was done aseptically using sterile 12.7 mm diameter paper disks (Schleicher & Schuell, Inc., Keene, NH) to which 100 μL of test extracts were added. Saturated disks were placed onto the surface of seeded agar plates. Controls consisted of disks with distilled water only. Treated plates were incubated at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak Campy container system sachets (Becton, Dickinson and Co., Sparks, MD). The diameter of the clear zone (no growth) surrounding each disk was measured and the zone of inhibition was determined and expressed in mm. For the dose dependency studies, the sample extract (100 mg/mL) was diluted to 75, 50 and 25 mg/mL with distilled water. The antimicrobial activity of *Phyllanthus niruri* L.-associated pure phenolic compounds such as chlorogenic acid, ellagic acid and gallic acid was also evaluated at two concentrations: 0.1 mg/disk and 1 mg/disk. Each experiment was repeated two times and consisted of three replicates each time (three disks per sample or treatment).

Proline growth response assay. A model for the mode of action of phenolic phytochemicals was developed based on the rationale that simple mono-phenolics in phytochemical profiles could behave as proline analogs or proline analog mimics and are likely to inhibit proline oxidation via proline dehydrogenase (Shetty and Wahlqvist, 2004). Further, the likely inhibitory effects of phenolic phytochemicals should be overcome by proline if the site of action is proline dehydrogenase.

Bacterial lawns of *H. pylori* were prepared as described previously. Plating media were prepared by using standard plating medium as described in the agar-diffusion assay with some modifications. Proline (Sigma, Louis, MO) was added into the medium to a final concentration of 0.5, 1, 3 and 5 mM). Different concentrations of sample extracts (25, 50, 75 and 100 mg/mL) were added to paper disks (100 μL) using a micropipette. Saturated disks were placed onto surface of seeded agar plates. Plates were incubated at 37 °C for 48 h in BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak EZ Campy container system sachets (Becton, Dickinson and Co., Sparks, MD). The diameter of the inhibition zone surrounding each disk was measured (mm) and the zone of inhibition was determined.

Lactic acid bacteria proliferation assay. Initially 100 μL of frozen stock from the lactic acid bacterial strains was inoculated into 10 mL MRS broth (Difco) and incubated for 24 h at 37 °C. Then, 100 μL of the 24 h grown strain was re-inoculated into 10 mL MRS broth for 24 h at 37 °C. Sample extracts (50 mg/mL of concentration) were filter sterilized using sterile filters Millex GP 0.22 μm (Millipore Corporation, Bedford, MA). Filter sterilized sample extracts (1 mL) and 100 μL of the 48 h grown strain (diluted 10 times with sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37 °C for 24 h. A control with 1 mL of sterile distilled water instead of sample extract was also included. An initial evaluation of the lactic acid bacteria proliferation was carried out only in MRS broth (Difco) by measuring the optical density at 600 nm of the re-suspended cells in 1 mL of distilled water (500 μL aliquot previously centrifuged at 10000 × **g** for 10 min) after 3, 6, 9, 12 and 24 h of growth. Based on the results from the previous evaluation, the experiment was repeated using the plate counting technique. Filter sterilized sample extracts (dose of 5 mg/mL in final medium reflecting plate assay exposure of *Helicobacter pylori*) and 100 μL of the 48 h grown strain (diluted 100 times with sterile distilled water) were added into 9 mL of MRS broth tubes and incubated at 37 °C for 12 h. A control with 1 mL of sterile distilled water instead of the sample extract was also included. A volume of 100 μL from the appropriate dilution was plated on MRS agar (Difco) and incubated in anaerobic BBL GasPak jars (Becton, Dickinson and Co., Sparks, MD) with BD GasPak EZ anaerobe container system sachets (Becton, Dickinson and Co., Sparks, MD) at 37 °C for 48 h to determine the CFU/mL. The plate counting was done at time 0 and after 6 and 12 h of bacteria growth.

Statistical analysis. All experiments were performed by duplicate and the analysis at every time point from each experiment was carried out in triplicate ($n = 6$). In the case of HPLC analysis, the experiments were performed at least in triplicates. Results were expressed as mean standard deviation. Data were subjected to paired Student's *t*-test and 1- or 2-way analysis of variance (ANOVA) with the Tukey's test ($p < 0.05$) for mean comparisons using the Statgraphics Centurion XV software package (StatPoint Inc., Rockville, MD).

RESULTS AND DISCUSSION

Total phenolics, antioxidant activity by DPPH and HPLC analysis

Table 1 shows the total phenolic contents of water extracts of Chancapiedra (*Phyllanthus niruri* L.) from different origins (Ecuador and Peru) correlated to their free radical scavenging linked-antioxidant activity measured by the DPPH assay.

The total phenolic contents were higher in the Peruvian extract than in the Ecuadorian extract (73 ± 2 and 65 ± 2 mg/g dw, respectively; $p < 0.05$). However, the antioxidant activity was quite similar in both extracts (89%). Specific phenolic compounds were detected and quantified by HPLC-DAD (Table 2) and the chromatograms of both Peruvian and Ecuadorian water extracts

Table 1. Total phenolics and DPPH scavenging activity of water extracts of Chancapiedra (*Phyllanthus niruri* L.) from Ecuador and Peru[a]

Origin	Total phenolics (mg gallic acid/g sample dw)	DPPH scavenging activity (%)
Ecuador	65 ± 2^{b}	88.9 ± 0.1^{a}
Peru	73 ± 2^{a}	88.93 ± 0.08^{a}

[a]Values are mean ± SD; columns with different letters indicate statistically significant differences ($p < 0.05$, $n = 6$).

Table 2. Phenolic composition of water extracts of Chancapiedra (*Phyllanthus niruri* L.) measured by high performance liquid chromatography-diode array detector[a]

	Phenolic compound (mg/g dw)		
Origin	Hydroxycinnamic acid[b]	Gallic acid	Ellagic acid
Ecuador	2.98 ± 0.02^{b}	1.29 ± 0.01	1.8 ± 0.1^{a}
Peru	8.63 ± 0.01^{a}	n.d.	1.5 ± 0.1^{a}

[a]Values are mean ± SD;

[b]expressed as chlorogenic acid; n.d., not detected. Columns with different letters indicate statistically significant differences ($p < 0.05$, $n = 3$).

are shown in Fig. 1. Hydroxycinnamic acid derivatives and likely caffeoyl derivatives due to the similarity of their UV spectra to that showed by chlorogenic acid were detected in both extracts, although the content in the Peruvian extract was significantly higher than in the Ecuadorian extract (8.63 ± 0.01 and 2.98 ± 0.02 mg/g dw expressed as chlorogenic acid, respectively). In addition, Peruvian and Ecuadorian extracts of *P. niruri* L. had similar contents of ellagic acid (Table 2), but gallic acid was detected only in the Ecuadorian water extract (1.29 ± 0.01 mg/g dw). These differences indicate the important effect of the sample origin on phenolic profiles of *P. niruri* L water extracts.

Flavonoids such as quercetin, rutin, quercitrin, astragalin and catechin have been identified in extracts from *P. niruri* L. (Bagalkotkar *et al*., 2006). Methyl brevifolin carboxylate, a coumarin derivative, was also isolated from leaves of *P. niruri* L. and showed a vasorelaxant effect on rat aortic rings (Iikuza *et al*., 2006). Among the polymeric molecules, ellagitannins such as geraniin, repandusinic acid and corilagin have been also found in extracts of *P. niruri* L. (Bagalkotkar *et al*., 2006). According to results from this study, neither flavonoids nor ellagitannins were identified in aqueous extracts of *P. niruri* L. under the conditions of the conducted experiments. However, the presence of hydroxycinnamic acids likely caffeoyl derivatives was recently reported by our laboratory in a sample from Ecuador (Ranilla *et al*., 2010).

Ellagic acid itself is not thought to be naturally present in plants. Instead, polymers of gallic acid and hexahydroxydiphenoyl (HHDP) are linked to glucose centres to form a class of compounds known as ellagitannins (hydrolysable tannins) (Rice-Evans *et al*., 1996). When two gallic acid groups become linked side-by-side within a tannin molecule, the HHDP group is formed. After hydrolysis, the HHDP acid is dehydrated followed by spontaneous lactonization forming ellagic acid (Aguilera-Carbo *et al*., 2008). Furthermore, gallotannins, the other group of hydrolysable tannins, yield glucose and gallic acid upon hydrolysis (Puupponen-Pimiä *et al*., 2005). This rationale would

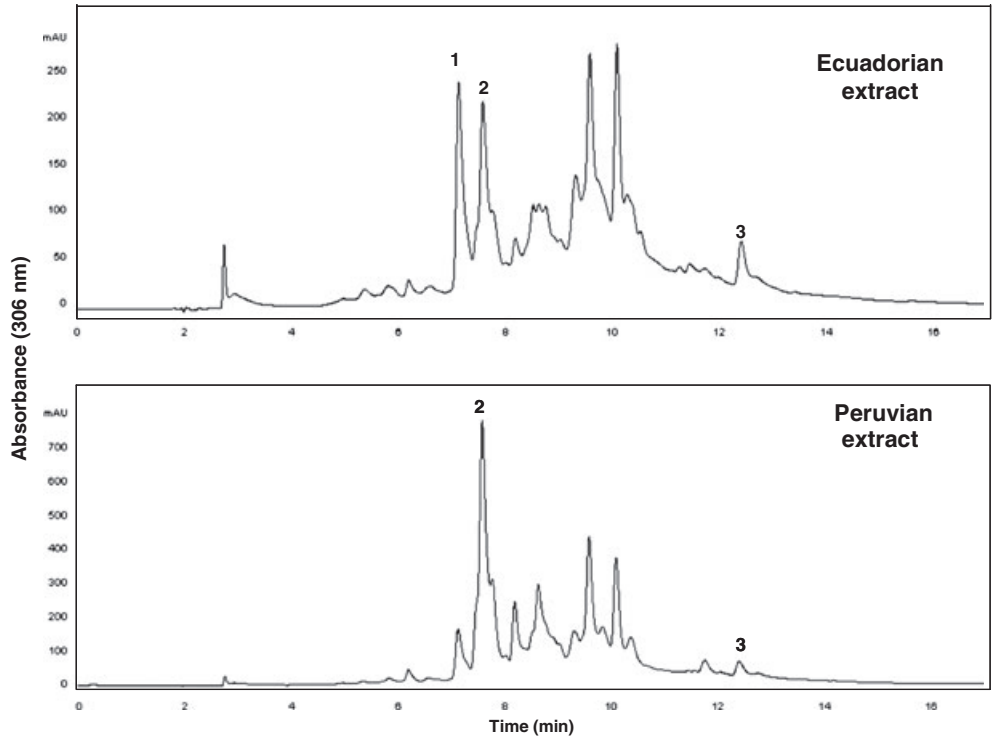

Figure 1. Chromatograms of water extracts of Chancapiedra (*Phyllanthus niruri* L.) 1, gallic acid; 2, hydroxycinnamic acid; 3, ellagic acid. Peak areas were integrated at 306 nm for quantification of detected phenolic compounds.

explain the presence of ellagic acid and gallic acid in water extracts of Chancapiedra likely derived from ellagitannins or gallotannins partially hydrolysed after the hot water extraction undertaken in this study.

Antibacterial activity against *Helicobacter pylori* and proline growth response

Both water extracts of Chancapiedra (*Phyllanthus niruri* L.) from Ecuador and Peru showed similar anti-*Helicobacter pylori* activities and a dose-dependency trend was observed when four different concentrations of sample were tested (25, 50, 75 and 100 mg of dried sample/mL) (Figs 2 and 3).

In order to determine if Chancapiedra linked-specific phenolics detected by HPLC-DAD are involved on its anti-*H. pylori* activity, the antimicrobial effect of pure phenolic compounds such as ellagic acid, gallic acid and chlorogenic acid was also evaluated by the agar-diffusion assay at two concentrations (0.1 and 1 mg/disk). However, no inhibition was observed (data not shown) in all cases.

Previous studies have provided clues in animal cell and bacteria model systems that phenolics could modulate cellular redox response through the proline-associated pentose phosphate pathway (Shetty and Wahlqvist, 2004; Lin *et al*., 2005). Therefore, the rationale for the proline growth response assay was to evaluate if simple mono-phenolics in extracts of Chancapiedra behave as proline analogs or proline mimics and if so, could they inhibit proline oxidation via proline dehydrogenase (PDH) at the plasma membrane level in prokaryotic cell disrupting the oxidative phosphorylation linked-proton motive force (PMF) resulting in inhibition of the bacterium (Kwon *et al*., 2007; Lin *et al*., 2005). If this is the case then the addition of proline could overcome the inhibition of proline analog type phenolics with aromatic ring structure (Shetty and Lin, 2005).

The results obtained in the present study indicate that the anti-*H. pylori* activity of water extracts of Chancapiedra is not associated with the proline dehydrogenase-based oxidative phosphorylation inhibition. The addition of proline into the *H. pylori* agar media could not reverse the strong inhibitory activity of the extracts even when higher doses of proline were tested (Fig. 4). Therefore, mechanisms involving membrane related breakdown or alterations linked to biphenolics and polymeric phenolics not detected by HPLC may be responsible for the anti-*H. pylori* activity of *P. niruri* L. extracts by using different mechanisms of action.

Ellagitannin-rich plant extracts have been shown to possess inhibitory activity against multiple antibiotic-resistant *H. pylori* strains. Voravuthikunchai and Mitchell (2008) using *in vitro* assays reported that both aqueous and ethanol extracts from *Punica granatum* exhibited high activity against different strains of *H. pylori*. Previous studies have reported that *Punica granatum* fruits are rich in ellagitannins such as punicalagin which also had potent inhibitory effect on methicillin-resistant *Staphylococcus aureus in vitro* (Machado *et al*., 2002).

Hydrolysable tannins have higher water solubility than other compounds and this property may be important for their promising antibacterial activities against *H. pylori in vitro*. Monomeric hydrolysable tannins including ellagitannins such as geraniin and corilagin revealed especially stronger anti-*H. pylori* activity than oligomeric hydrolysable tannins (Funatogawa *et al*., 2004). During the bacterial killing process, monomeric ellagitannins rapidly aggregate *H. pylori* cells into clusters and fuse them, which suggests that these compounds act on the membrane or membrane-linked surface structures of *H. pylori* (Funatogawa *et al*., 2004). Further, monomeric ellagitannins exhibited a dose-dependent membrane damaging activity and possibly this property contribute to their anti-*H. pylori* action (Funatogawa *et al*., 2004).

Another possible mechanism of the antibacterial action of phenolics is associated to the bacterial antiadhesion activity. Adhesion of bacteria is a vital prerequisite for successful microbial colonization and infection. High molecular-weight constituents of cranberry juice have been shown to inhibit adhesion of *H. pylori* to immobilized human mucus, erythrocytes and cultured gastric epithelial cells (Burger *et al*., 2002). It was suggested that ellagitannins rich cranberry juice may also inhibit the adhesion of bacteria to the stomach *in vivo*, and may prove useful for the prevention of stomach ulcer that is caused by *H. pylori* (Burger *et al*., 2002) or for the eradication of *H. pylori* in combination with proton pump inhibitors in patients receiving treatment with a conventional triple therapy (Shmuely *et al*., 2007).

Based on the above rationale, the strong anti-*H. pylori* activity found in water extracts of Chancapiedra may be attributed to the presence of ellagitannins such as geraniin or corilagin, compounds that have been previously identified in the leaves of *Phyllanthus niruri* L. (Bagalkotkar *et al*., 2006). Most likely, these ellagitannins were partially hydrolysed after the hot water extraction resulting in the formation of ellagic acid, gallic acid and other ellagitannin derivatives. However, further studies are needed to confirm this premise.

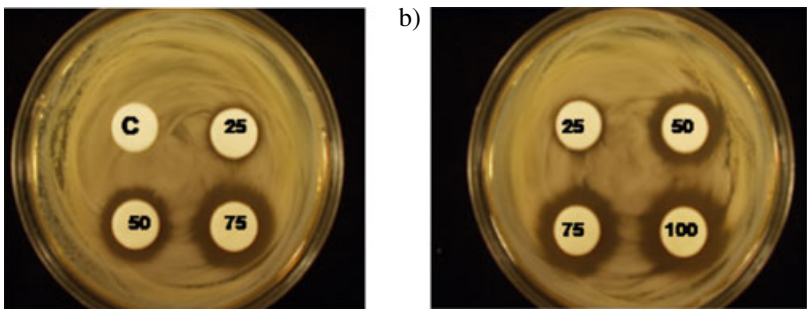

Figure 2. *Helicobacter pylori* inhibition of the same Chancapiedra (*Phyllanthus niruri* L.) water extract at different doses of sample concentration: (a) 25, 50 and 75 mg/mL; (b) 25, 50, 75 and 100 mg/mL. C, distilled water as a control. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

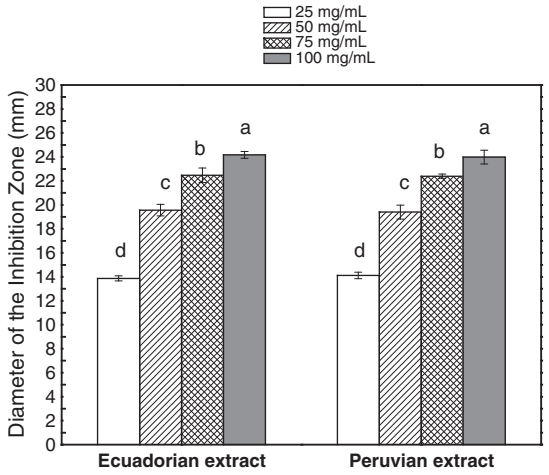

Figure 3. Anti-*Helicobacter pylori* activity of two water extracts of Chancapiedra (*Phyllanthus niruri* L.) from Ecuador and Peru at four different doses of sample concentration. Bars with the same letters at the same extract concentration indicate no significant differences ($p > 0.05$). Bars with different letters at each sample origin data (Ecuadorian or Peruvian) indicate statistically significant differences ($p < 0.05$, $n = 6$).

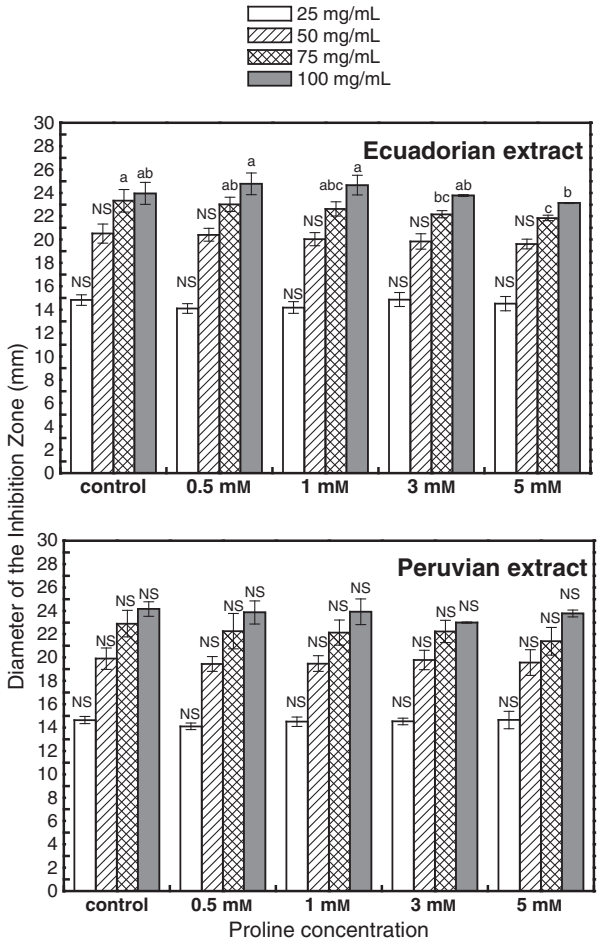

Figure 4. Effect of different concentrations of Chancapiedra extracts (*Phyllanthus niruri* L.) (mg/mL) with proline (0.5, 1, 3 and 5 mM) on anti-*Helicobacter pylori* activity. Control, without proline. Bars with different letters at the same extract concentration indicate statistically significant differences ($p < 0.05$, $n = 6$). NS, no significant differences at the same extract concentration ($p > 0.05$).

As mentioned above, the total phenolic contents and the HPLC phenolic profiles of *P niruri* L. extracts varied according to the origin. However, extracts from Ecuador and Peru did not have significant differences in their free radical scavenging-linked antioxidant activities (Table 1) and probably this characteristic could be also correlated to their similar anti-*H. pylori* activities. Soluble phenolics have been suggested to exert their antimicrobial effect by causing hyperacidification via proton donation at the plasma membrane interface of the microorganism and intracellular cytosolic acidification, excess of which can disrupt H^+-ATPase required for ATP synthesis (Kwon *et al.*, 2007). The inhibition of *H. pylori* by *P. niruri* L. water extracts could be due to the proton donating effect of soluble phenolic antioxidant compounds which may inhibit cytosolic dehydrogenases when acidification is high. Further, phenolics may have the ability to quench free electrons from the electron transport chain along the bacterial membrane or inhibit dehydrogenases linked proton efflux. This could disrupt the flow of the electrons at the level of cytochromes and inhibit the growth of bacteria by disrupting the protons required for oxidative phosphorylation (Shetty and Wahlqvist, 2004). Ellagitannins have been shown to possess strong scavenging properties against free radicals indicating their potential ability to donate protons (Reddy *et al.*, 2007). This provides clues that they could modulate interfacial and cytosolic acidity which could be inhibitory if not countered by dehydrogenases.

P. niruri L. has been reported to contain alkaloids, terpenes, lignans, saponins, besides phenolic compounds such as ellagitannins and flavonoids (Bagalkotkar *et al.*, 2006). The anti- *H. pylori* activity of lignans has been reported previously (Toyoda *et al.*, 2007). Thus, inhibition of *H. pylori* by water extracts of Chancapiedra may be likely associated to the synergistic action of such compounds in combination with ellagitannins and other soluble phenolic derivatives. *P. niruri* L. has demonstrated antibacterial actions against *Staphylococcus*, *Micrococcus* and *Pasteurella* bacteria (Veeramuthu *et al.*, 2006). However, the potential of this plant for inhibiting the gastric cancer linked-*H. pylori* is reported for the first time in this study.

Effect on probiotic lactic acid bacteria proliferation

An initial evaluation of the effect of Chancapiedra (*Phyllanthus niruri* L.) water extracts on the proliferation of lactic acid bacteria with probiotic potential was carried out by measuring the bacterial cell concentration at 600 nm after 3, 6, 9, 12 and 24 h of growth in MRS broth (Fig. 5). A non-probiotic microorganism (*Lactobacillus bulgaricus*) was also included in the screening step. Overall, the addition of water extracts of *P. niruri* L. (5 mg/mL of sample concentration into the MRS broth, reflecting plate assay exposure of *Helicobacter pylori*), independent of the sample origin, did not affect the growth of *Lactobacillus acidophilus*, *Lactobacillus casei* 1 and 2, and *Lactobacillus plantarum* 1 and 2 when compared with the control (distilled water). Statistically, some differences were observed at 12 h of growth ($p < 0.05$). Therefore, the experiment was repeated by using the more accurate plate counting technique at 6 and after 12 h of growth in liquid media

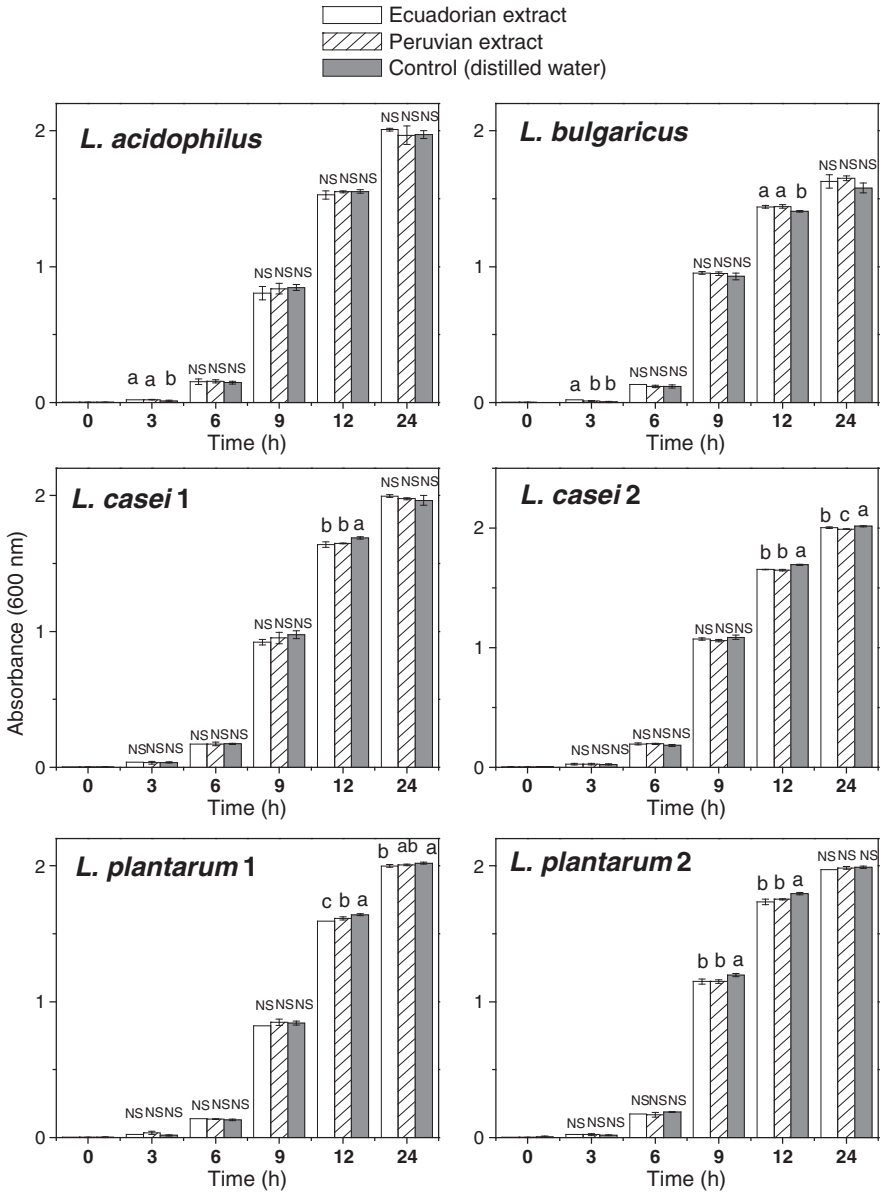

Figure 5. Evaluation of the effect of Chancapiedra (*Phyllanthus niruri* L.) water extracts on lactic acid bacteria proliferation (absorbance at 600 nm). Bars with different letters at the same time of incubation indicate statistically significant differences ($p < 0.05$, $n = 6$); NS, no significant differences ($p > 0.05$).

(MRS broth). As shown in Fig. 6, the growth of evaluated *Lactobacillus* strains was not inhibited by any of the *P. niruri* L. extracts (5 mg/mL of sample concentration into the MRS broth) ($p > 0.05$).

Similar to the above studies, Puupponen-Pimiä *et al.* (2001) using the liquid culture technique, observed that the ellagitannin rich-raspberry extract did not inhibit the growth of *Lactobacillus paracasei, Lactobacillus rhamnosus* and *Bifidobacterium lactis* at low concentrations (1 mg/mL). However, when a five times higher concentration of raspberry extract was used, the growth of tested *Lactobacillus* strains was clearly inhibited. The same authors also reported that growth of selected Gram-negative bacteria such as *Salmonella* and some *E. coli* strains were generally inhibited by high phenolic-extracts from common Finnish berries, whereas no inhibition was observed against Gram-positive lactic acid bacteria. They hypothesized that observed variations may reflect differences in cell surface structures between Gram-negative and Gram-positive bacteria. In particular, the outer membrane of Gram-negative bacteria functions as a preventive barrier against hydrophobic compounds.

Funatogawa *et al.* (2004) using *in vitro* assays, indicated that monomeric ellagitannins did not have antibacterial activity against non-pathogenic *E. coli* (a normal inhabitant of the human intestinal tract), suggesting that hydrolysable tannins may be able to inhibit *H. pylori* without affecting potentially beneficial and well adapted intestinal bacterial flora. Further, the same authors highlighted the anti-*H pylori* activity of hydrolysable tannins without affecting the viability of gastric epithelial cells. In addition, similar to the current results, a recent study from our laboratory also showed the potential of polyphenol rich-tea extracts against *H. pylori* and the lack of inhibition of selected beneficial lactic acid bacteria. Besides the possible effect due to differences in cellular wall configuration, differences in metabolism linked to

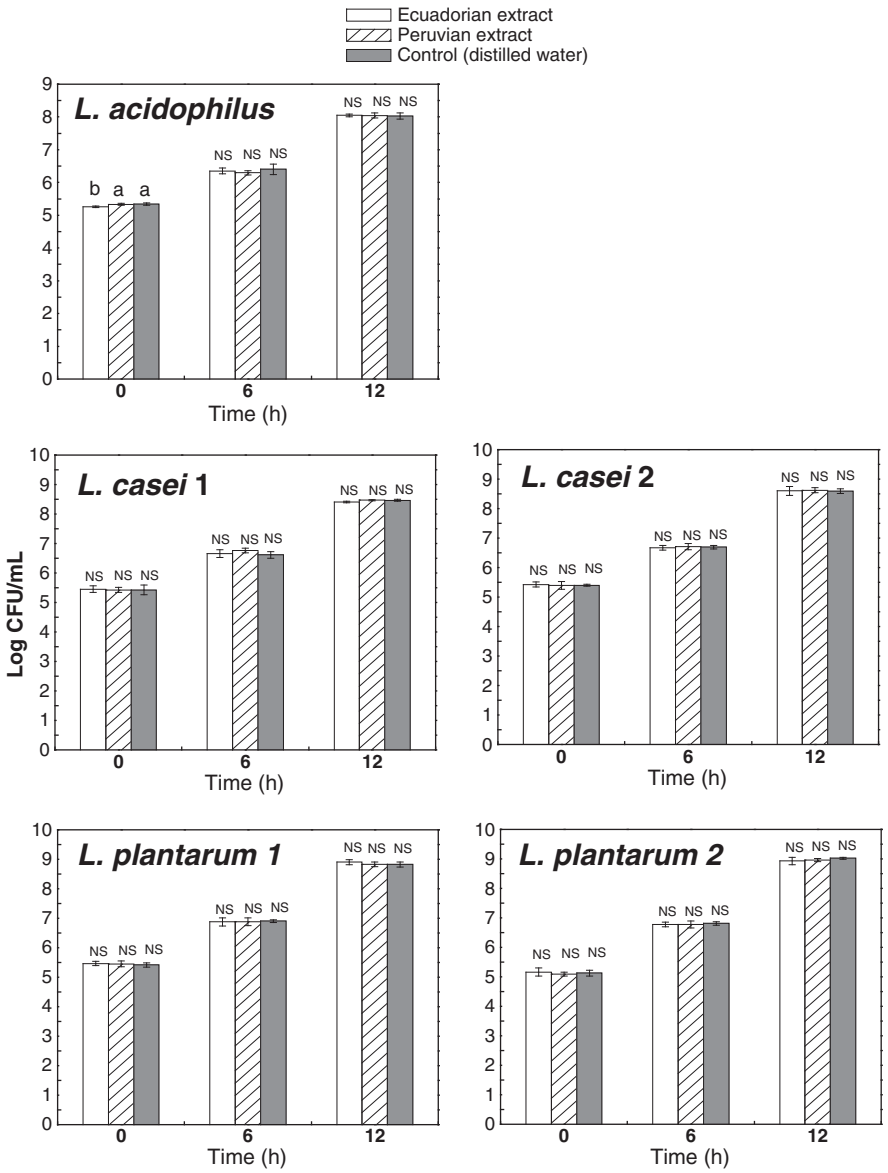

Figure 6. Effect of Chancapiedra (*Phyllanthus niruri* L.) water extracts on lactic acid bacteria proliferation (CFU/mL). Bars with different letters at the same time of incubation indicate statistically significant differences among samples ($p < 0.05$, $n = 6$); NS, no significant differences ($p > 0.05$).

energy generation (partial oxidative phosphorylation in microaerophilic bacteria such as *H. pylori* in contrast to substrate level phosphorylation in lactic acid bacteria) may also be involved (Ankolekar *et al.*, 2011).

New therapies applied for the treatment of *H. pylori* infection suppress not only *H. pylori* but also intestinal bacterial flora, which causes side effects such as abdominal pain and diarrhea (Wermeille *et al.*, 2002). In this study, the water extracts of *P. niruri* L. inhibited *H. pylori* but at the same dose the growth of evaluated lactic acid bacterial strains with probiotic potential was not inhibited. This shows the promise of Chancapiedra extracts for potential treatment or prevention of *H. pylori* infections without likely causing side effects linked to inhibition of beneficial intestinal probiotic bacteria.

The challenge of overcoming *Helicobacter pylori* antibiotic resistance has focused research on the isolation and characterization of new antimicrobial compounds from a variety of natural sources including medicinal plants. In this study, water extracts of the medicinal plant Chancapiedra (*Phyllanthus niruri* L.) from Ecuador and Peru inhibited the human pathogen *H. pylori* in a dose dependent manner. The total phenolic contents (73 ± 2 and 65 ± 2 mg/g dw, for Peruvian and Ecuadorian extract, respectively) and the HPLC phenolic profiles of each extract varied according to the sample origin. However, the free radical scavenging linked-antioxidant activity assessed by the DPPH method was high and similar in both extracts (89%). The major phenolic compounds were ellagic acid and hydroxycinnamic acid derivatives in both extracts, but gallic acid was detected only in the Ecuadorian water extract. Insights into the mode of action of Chancapiedra-linked phenolics on *H. pylori* inhibition indicated that the anti-*H. pylori* activity of this plant may not be via proline dehydrogenase-based oxidative phosphorylation inhibition. Therefore, soluble polymeric phenolics such as ellagitannins or other non-phenolic compounds are probably involved and may act in a synergistic manner, potentially affecting membrane coupled energy function. Independent of the sample origin, lactic acid bacteria with probiotic

potential such as *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Lactobacillus casei* were not inhibited by Chancapiedra water extracts at a dose of 5 mg/mL, which inhibited *H. pylori*. These results suggest that Chancapiedra could be used potentially for the prevention or treatment of *H. pylori* infections with the additional advantage of not affecting beneficial intestinal probiotic lactic acid bacteria. However, research on the elucidation of the exact mechanism for the anti-*H. pylori* activity of Chancapiedra and further *in vivo* studies on the beneficial effects are needed.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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