ISSN: 2588-4115; Infection Epidemiology and Microbiology. 2020;6(3):165-175 10.29252/iem.6.3.165

Synergic Effect of *Curcuma longa* L. Extract with Antimicrobials against Cuban *Helicobacter pylori* Isolates

ARTICLE INFO

Article Type
Original Research

Authors
Onelkis Feliciano, *MSc*[1*]
Yandy Ybalmea, *MSc*[2]
Arianna Yglesias, *MSc*[2]
Alexis Diaz, *PhD*[2]
Rafael Llanes, *MSc*[1]
Oderay Gutierrez, *MSc*[1]

How to cite this article
Feliciano O., Ybalmea Y., Yglesias A., Diaz A., Llanes R., Gutierrez O. Synergic Effect of *Curcuma longa* L. Extract with Antimicrobials against Cuban *Helicobacter pylori* Isolates. Infection Epidemiology and Microbiology. 2020;6(3): 165-175

[1] Research, Diagnostic and Reference Center. Pedro Kouri Tropical Medicine Institute. Havana, Cuba.
[2] Department of Research, Laboratories of Biopharmaceuticals and Chemistries Productions (LABIOFAM). Havana. Cuba.

**** Correspondence***
Address: Bacteriology-mycology Department, Pedro Kourí Tropical Medicine Institute, Havana, Cuba
onelkisfs@infomed.sld.cu

Article History
Received: July 05 ,2020
Accepted: September 15 ,2020
Published: October 28 ,2020

ABSTRACT

Aims: *Helicobacter pylori* is a bacterium that colonizes the gastric mucosa and is the main cause of gastritis as well as ulcer and gastric cancer. Due to the clinical significance and international increase in *H. pylori* multidrug resistance, it is necessary to search for new strategies improving eradication rates. Natural compounds have been demonstrated to have antimicrobial effect and the ability to restore the efficacy of conventional drugs. The objective of this work was to evaluate the antimicrobial effect of the hydroalcoholic extract of *Curcuma longa* L. (Cu) against *H. pylori* isolates.

Materials & Methods: The minimum bactericidal concentration of the extract was determined by means of the MTT assay; also, the combination and dose reduction indices for levofloxacin (LVX), metronidazole (MET), and rifampicin (RIF) antimicrobial agents were determined by checkerboard format. Interaction analysis was performed using the CompuSyn program.

Findings: About 90% of *H. pylori* isolates studied (9/10) were sensitive to the hydroalcoholic extract. Synergism was observed in more than 50% of Cu-LVX, Cu-MET, and Cu-RIF combinations. Additionally, for different concentrations of the extract, reduction rates in antimicrobial agents were determined to be between 0.5 and 360 times.

Conclusions: The hydroalcoholic extract of turmeric showed a good potential to be used as an antimicrobial agent in the treatment of *H. pylori* infection, either alone or in combination with antibiotics used, suggesting the renewal of the effectiveness of conventional antimicrobials in reducing the phenomenon of antimicrobial resistance.

Keywords: *Helicobacter pylori;* Antimicrobial resistance*; Curcuma longa;* Drugs combination.

CITATION LINKS

[1] Kanna S, Maradey-Romero C, Fass R. Diagnostic tests for Helicobacter ... [2] Mahady GB, Pendland SL, Yun G, Lu ZZ. Turmerir (Curcuma longa) and ... [3] Sarkar A, De R, Mukhopadhyay A. Curcumin as a potential ... [4] Otero W, Trespalacios AA, Otero L, Vallejo M, Torres M, Pardo R, et al. Guía práctica para el ... [5] Fu L. Diagnóstico y resistencia antimicrobiana de Helicobacter pylori. Instituto de ... [6] Kwiecien S, Magierowski M, Majka J, Ptak-Belowska A, Wojcik D, Sliwowski Z et al. Curcumin: A potent ... [7] Regulación M 85-16. Requisitos para la licencia sanitaria ... [8] González RA, Vistel M. Caracterización fitoquímica de ... [9] Fernández A, Mendiola J, Acuña D, Caballero Y, Scull R, Gutiérrez Y. Actividad ... [10] Leyva M, Tacoronte JE, Marquetti MC, Scull R, Montada D, Rodriguez Y. Actividad ... [11] Llanes R, Feliciano O, Gutiérrez O, Gala A, Valdés L, Capó V, et al. Nuevos ... [12] Picazo JJ. Métodos básicos para el estudio de la sensibilidad a los ... [13] Chou TC. Theoretical Basis, Experimental Design, and Computerized ... [14] Aşkar Ş, Deveboynu Şn. Investigation of in-vitro antibacterial activity of ... [15] Cos P, Vlietinck AJ, Bander Berghe D, Maes L. Anti-infective potential of ... [16] Gibbons S. Phytochemicals for bacterial resistance – strengths, weaknesses, and opportunities. Planta Med. 2008; 74(6): 594–602 [17] Rai D, Singh JK, Roy N, Panda D. Curcuminn ... [18] Patil TR, Patil ST, Patil S, Patil A. Effect of non-antibiotic ... [19] Mahady GB, Pendland SL, Yun G, Lu ZZ. Turmerir (Curcuma longa) and ... [20] Mun SH, Joung DK, Kim YS, Kang OH, Kim SB, Seo YS, et al. Synergistic ... [21] Maliziaa T, Tejadaa M, Marchettib F, Favinib P, Pizzarellic G, Campaa M. Synergic ... [22] Cheng A, Sheng WH, Liou JM, Wang HP, Wu MS, Lin JT. Comparative in vitro antimicrobial ... [23] Ranjbar R, Mohammadi A. Synergistic effects of combined curcumin and ... [24] Sasidharan NK, Ravikumar S, Jacob J, Nambisan B. In vitro synergistic effect of ... [25] Rangel-Castañeda I, Cruz-Lozano J, Zermeño-Ruiz M, Cortes-Zarate R, Hernández-Hernández L, Tapia-Pastrana G. Drug susceptibility testing and ... [26] Judaki A, Rahmani A, Feizi J, Asadollahi K, Hafezi M. Curcumin in ... [27] Hassana S, Berchova K, Majerova M, Pokorna M, Svajdlenkaa E. In vitro synergistic effect ... [28] Lodovico S, Napoli E, Campli E, Fermo P, Gentile D, Ruberto G. Pistacia vera L. oleoresin and ... [29] Zullo V, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of ...

Introduction

Helicobacter pylori infection is common worldwide, and it has been estimated that more than 60% of the population is infected by this microorganism [1]. Several studies have indicated that persistence of infection of this bacterium and the effect of virulence factors are associated with the development of different gastroduodenal diseases such as chronic gastritis, peptic ulcer, MALT-type lymphoma, and gastric carcinoma. In 1994, the Cancer Research Agency classified *H. pylori* as a type I human carcinogen [2]. For the control of gastroduodenal diseases as a consequence of *H. pylori* infection, eradication is the most appropriate solution. Standard regimens for the treatment of *H. pylori* infection include various types of antimicrobials and proton pump inhibitors [3]. However, the widespread use of antimicrobials has conditioned the high rates of antimicrobial resistance worldwide. In 2017, the World Health Organization listed 12 bacteria that threaten human health, among which high priority was given to clarithromycin-resistant *H. pylori* [4]. In a recent study in our laboratory, *H. pylori* showed 95% resistance to metronidazole and 25% resistance to clarithromycin [5]. These findings highlight the need to develop new antibacterial agents against bacterial infection with high efficacy, safety, and cost-effectiveness, which could be directed at specific cell targets.

Plant-derived compounds have been demonstrated to possess therapeutic and chemopreventive potential against different chronic diseases such as cardiovascular, neurodegenerative, and neoplastic diseases [3]. Dietary components in spices, nuts, vegetables, and fruits have been shown to suppress carcinogenesis *in vitro* [2]. Polyphenols with their effective antioxidant and anti-inflammatory properties, modulating important signaling molecules, are of great pharmacological interest. Catechins in green tea, lycopene in tomatoes, resveratrol in grapes and red wine, quercetin in apples and onions, as well as curcumin in *Curcuma longa,* to name a few, have shown considerable anticarcinogenic effects on various organs (skin, liver , chest, lung, and prostate).

Curcumin is a yellow polyphenolic pigment found in the roots of *C. longa* plant. This plant is a member of the Zingiberaceae family and grows mainly in India and Southeast Asia [6]. Some studies suggest that turmeric has various properties such as anti-inflammatory, antioxidant, anticancer, anti-proliferative, antifungal, and antimicrobial activities [2-3]. Regarding this last property, India is one of the countries that offers the most studies on this subject [6].

Internationally, in recent years, an increase in the use of herbal products for medical purposes has been observed, leading to the issuance of regulatory standards by the World Health Organization. In Cuba, the State Center for Quality Control of Medicines (CECMED) has established new policies and legal frameworks to ensure the quality, safety, and efficacy of herbal products. This study aimed to include several medicinal plants in the Cuban Pharmacopoeia of Medicinal Plants based on experimental data, among which is *C. longa* [7]. So far, studies carried out in our country on *C. longa* have addressed its phytochemical properties [8], antiparasitic activity against the causative agent of malaria [9]; and insecticidal activity against the genus Aedes [10], but its antimicrobial activity has not yet been addressed. It would be interesting to study *C. longa* in Cuba because of the pleotropic activities of the extract, the possible variation in the polyphenol content of this plant grown in different geographical areas of the world, and the risk of emergence and prevalence of multidrug-resistant (MDR) *H. pylori* isolates

in our country.

Objectives: The aim of this study was to evaluate the antimicrobial effect of *C. longa* in combination with conventional drugs against Cuban *H. pylori* strains.

Materials and Methods

Collection of plant material and preparation of the hydroalcoholic extract of *C. longa*: The rhizome of *C. longa* L. plant was collected from the province of Pinar del Rio. The selection of plant material for the subsequent preparation of the extract was done by taking into account the classification of trees in the National Botanical Garden (registration number: 9700178). The collected plants were transferred to the Chemical, Pharmaceutical, and Plastic Units belonging to LABIOFAM, where the extract was prepared in hydroalcoholic form.

For the preparation of the turmeric extract, the plant material was dried at room temperature and pulverized, then the extraction process was carried out by maceration for 7 days in 80% ethanol according to the Public Health Branch Standard 311 [7]. The liquid obtained was extracted by decantation, filtered on filter paper, and packed in glass containers. The solvent was removed by evaporation under reduced pressure for 24 hours. At the time of use, the dry extract was weighed and diluted in 70% ethanol to obtain a hydroalcoholic solution at a concentration of 10 mg/mL. This solution was transferred to the Helicobacter National Reference Laboratory (HNRL) of IPK and stored at 4°C until further evaluation.

***H. pylori* isolates and culture conditions**: For the evaluation of the extract antimicrobial effect, 10 isolates were selected from the strains which were isolated from patients with gastroduodenal symptoms and conserved at -80ºC in tryptone soy broth plus 20% glycerol in HNRL. The strains selection was based on the resistance phenotype. Of the 10 isolates included, 7 were resistant to some of the antimicrobials used in the first-, second-, and third-line therapy (metronidazole, clarithromycin, levofloxacin, and rifampicin), and 3 were resistant to at least three antimicrobial families (MDR isolates). The susceptibility values were determined in a previous study [5]. The reference strain *H. pylori* ATCC 43504 was used in different studies.

The isolates were seeded on Columbia agar medium (Biolife, Italy) supplemented with 10% ram blood, 1% fetal bovine serum (Biochrom, Germany), and VCNT as an inhibitory supplement (vancomycin, colistin, nystatin, and trimethropim) (Biolife, Italy). The plates were then incubated under microaerophilia conditions (10% CO_2, 5% O_2, and 85% N_2) (Campy Packs, Oxoid sachets) in a 2.5 L jar (Oxoid, England) for 72 hours at 37 °C. For the re-identification of *H. pylori* colonies, urease tests and Gram staining were performed [11]. Bacterial suspensions were prepared with a turbidity similar to McFarland Scale 3 ($1x10^8$ CFU/mL) in Brain-Heart broth (Biolife, Italy) with enrichment and inhibitory supplements of Vitox (Biolife, Italy) and VCNT (Biolife, Italy), respectively.

Determination of *C. longa* extract inhibitory concentration against *H. pylori* isolates: The evaluation of the extract antimicrobial effect against the standardized Brucella broth culture, prepared as described above, was performed by means of the 96-well microtiter-plate method (Greiner BioOne, Germany). To do so, 50 µL of *H. pylori* bacterial suspension dissolved in Brucella broth culture medium was added to a flat-bottom 96-well cell culture plate in the presence of double serial dilutions of turmeric with different concentrations (3.12-200 µg/mL) in a final volume of 100 µL. Dimethyl sulfoxide (DMSO) as a solvent was evaluated under conditions similar to the extract. Bacteria dissolved in culture medium with no treatment represented growth control. The

antimicrobial metronidazole in a concentration range of 2-256 µg/mL was used as the reference drug. The plates were incubated for 24 hours at 37 °C, then 3- (4,5-dimethylthiazol-2-yl) -2,5 diphenyl tetrazolium (MTT) (0.5 mg/ mL) was added to all wells, and the plates were incubated for an additional 3 hours. After the incubation period, 80 µL of DMSO was added, and the plates were incubated for an additional 15 minutes.

Absorbance was measured by an ELISA plate reader (Revelation Dynex Technologies, Spain) at 570 nm. The absorbance at a wavelength of 630 nm was used as reference. With the determined absorbance values, the percentage of growth inhibition was calculated using the following equation: % inhibition = 100- (A570 (BT) / A570 (NBT)) x100; where BT is the bacteria treated with the extract, and NBT is the non-treated bacteria with the extract or control. The evaluation was carried out using three replicates in each case, and three tests were performed. Using the cell viability values, the IC_{50} (concentration of the extract that reduces bacterial growth by half) was determined for each treatment. The IC_{50} values of the extract were determined from the concentration-effect curves (DO600nm/ MTT) by means of sigmoidal regression analysis (GraphPad Sofware 5.01 San Diego California, USA). The absorbance values determined in the cell viability assay were used to calculate the viability percentage using the following expression: % = (abs treated / abs control) x 100.

In vitro* evaluation of the combined antimicrobial activity of *C. longa* extract and conventional drugs against *H. pylori: Evaluation of the antimicrobial activity of turmeric extract in combination with antimicrobials used in the first-, second-, and third-line therapy (metronidazole, levofloxacin and rifampin) was carried out using the checkerboard method [12]. For this purpose, two metronidazole resistant isolates (9A and 31C), two levofloxacin resistant isolates (35C and 48A), and two rifampicin resistant isolates (12A and 20A) were selected. Double serial dilutions (from 0 to 2 times the IC_{50} value) of the extract and the minimum inhibitory concentration (MIC) of the antimicrobial were inoculated in a microplate under conditions similar to those described in the previous section. The affected fraction (Fa) was calculated for each treatment using the following expression: 100-percentage of viable cells/100. Fa values were entered into CompuSyn software (Program for the quantification of synergism and antagonism in drug combination, Version 1.0, ComboSyn, USA) to obtain the combination index (CI) and dose reduction index (DRI). A CI <1 is defined as synergism, CI = 1 or very close to 1 is defined as additivity, and CI > 1 is defined as antagonism. While DRI > 1 and DRI <1 indicate favorable and unfavorable dose reduction, respectively, DRI= 1 indicates that there is no dose reduction [13]. Additionally, the results were also plotted in the form of an isobologram constructed using the drug combination indices according to CompuSyn program. In the isobologram, the Y axis represents the dose of *C. longa,* while the X axis shows the antimicrobial dose. The interpretation of this graph was carried out as follows: the area under the diagonal line that intercepts the two ends of both axes represents the synergism area, points on this line represent the additivity area, and the area above this line represents the antagonism area.

Findings

***In vitro* antimicrobial activity of *C. longa* extract against *H. pylori* isolates:** As shown in Table 1, the extract of *C. longa* showed antimicrobial activity against 9 of the 10 *H. pylori* clinical isolates evaluated (90%). The mean inhibitory concentration values of the

Table 1) Mean inhibitory concentration (IC_{50}) values of *C. longa* extract against *H. pylori* isolates.

Isolates‡	IC_{50} ± SD
9A	0.35 ± 0.13
12A	0.29 ± 0.02
14A¥	26 ± 4.08
20A¥	106 ± 8.20
20C¥	120 ± 2.83
31C	3.9 ± 0.01
35A	1.03 ± 0.16
39C	70 ± 9.80
48A	1.06 ± 0.05
58A	26 ± 3.18
ATCC 43504	40 ± 4.55

Legend: ‡ laboratory code, IC_{50}: medium inhibitory concentration, SD: standard deviation, ¥ multidrug resistant isolates. Results are presented as the mean ± SD of three independent experiments

extract against resistant and MDR isolates ranged from 0.29-120 µg/mL. Isolate 20C was found to be resistant to the extract with a value greater than 100 µg/mL.

Combined antimicrobial effect of *C. longa* extract and antimicrobials on *H. pylori* isolates: When evaluating the antimicrobial effect of the combination of *C. longa* extract with antimicrobials, 36 values of combination index and dose reduction index were obtained for each isolate. The combination of *C. longa* with metronidazole and rifampicin was turned out to have antagonistic effect against isolates 9A and 12A, interactions in 9A were classified as strong antagonism. In the same strain, four points showing synergism coincided with the MIC value of metronidazole (256 µg/mL) combined with a concentration range of 0.175 to 0.021 µg/mL of *C. longa* extract (Figure 1). The behavior of isolates 48A and 35A was interesting since synergism with MIC was only observed for levofloxacin (32µg/mL) and low concentrations of the extract.

Numerous synergistic interactions were observed in isolates 20A and 31C due to the combination of the extract with rifampicin as well as with metronidazole, respectively. In these interactions, both the isolates also showed the highest values of dose reduction index for both products, reaching the order of 4.15 x 1014 times the MIC value of rifampicin.

As shown in the isobologram internal points, all the isolates studied affected by the synergistic effect, independently of the antimicrobial used. For example, for rifampicin and metronidazole, great synergistic and antagonistic effects were observed with values lower than the MIC of the antimicrobial. It is important to highlight the positive behavior of the combination of rifampicin with *C. longa* on the MDR 20A isolate, since in patient carrying this isolate, this antimicrobial is the only treatment option. Homogeneous behavior was only observed for levofloxacin in combination with low concentrations of *C. longa* extract, which is encouraging for this antimicrobial used in the second-line treatment.

Table 2 shows the best combination and dose reduction values for *C. longa* extract and the antimicrobials used against the 6 *H. pylori* isolates evaluated. Synergism was observed, which was even strong in some, with dose reduction values of both compounds, ranging from 1.83-28 to 287.30 times the value of the product concentration. The isolate 9A showed the best DRI values of 4.57 and 28, 287.30 times the concentration of *C. longa* extract and metronidazole, respectively. It is worth noting that isolate 20A, also resistant to the extract, showed the lowest combination rate for rifampicin, with high DRI values of 8.49 and 6,664.31 times for both drugs.

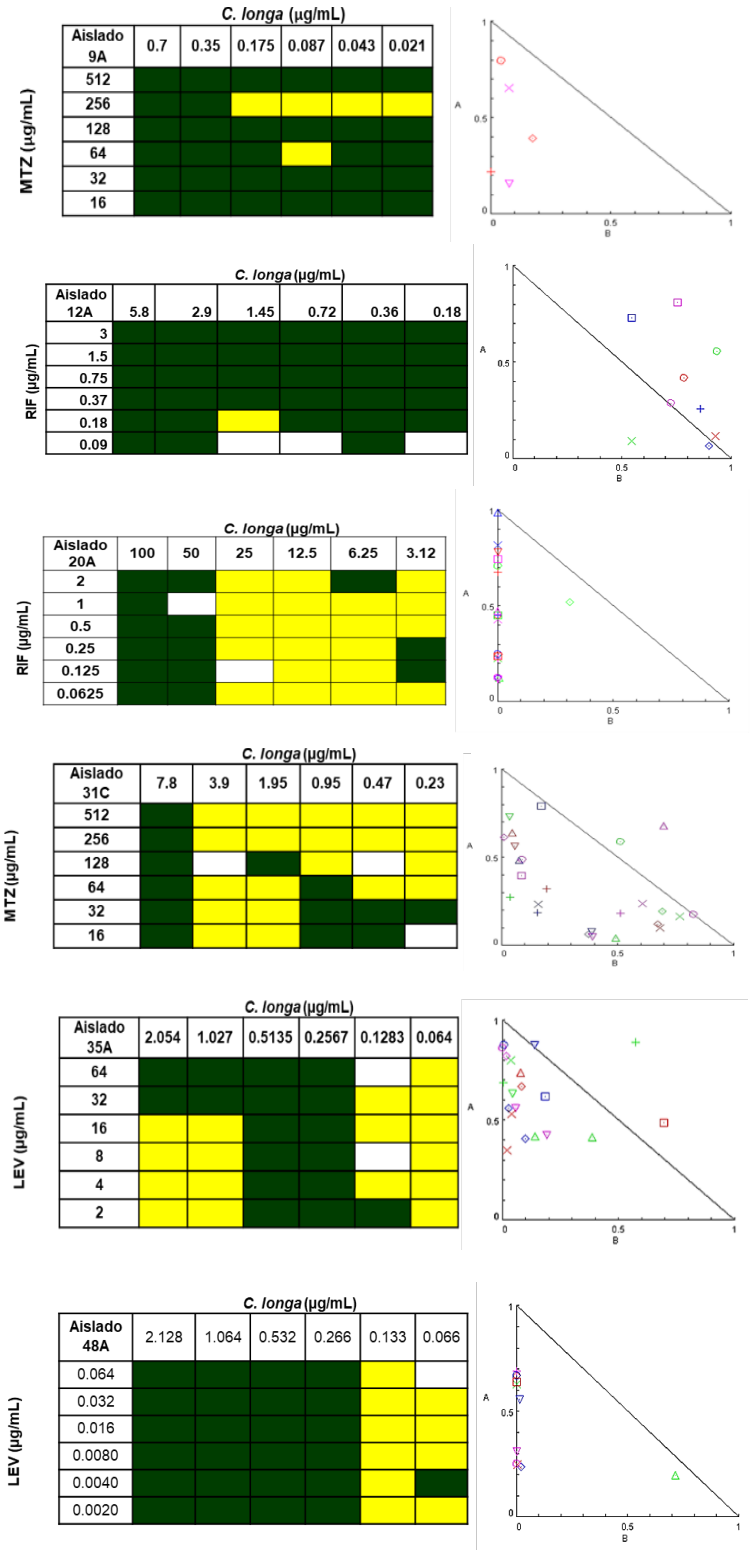

Figure 1) A schematic representation of the checkerboard test and the isobologram obtained with CompuSyn program Version 1.0 for the evaluation of the combined effect of *C. longa* and antimicrobials against *H. pylori* isolates 9A, 12A, 20A, 31C, 35A, and 48A. The green zone represents an antagonistic effect, the white zone represents an additive effect, and the yellow zone represents a synergistic effect. On the right side of the diagram, a isobologram is plotted, the Y axis of which represents the dose of *C. longa* while the X axis represents the dose of antimicrobial. The area under the diagonal line that intercepts the two ends of both axes represents the area of synergism, points on this line represent the additivity area, and the area above the line represents the antagonism area.

Table 2) Dose reduction indices analysis of the best combinations between *C. longa* and antimicrobials against the 6 *H. pylori* isolates.

Isolates‡	AMB	Best Combination of C. longa + AMB (µg/mL)	CI	Type and Degree of harmacological interaction	DRI C. longa	DRI AMB
9A	MTZ	0.175 + 256	0.219	Strong synergism	4.57	28 287.30
12A	RIF	0.145 + 0,187	0.638	Synergism	1.83	10.97
20A	RIF	3.12 + 1	0.118	Strong synergism	8.49	6 664.31
31C	MTZ	0.39 + 512	0.311	Synergism	3.65	6.44
35A	LVX	0.064 + 8	0.368	Synergism	2.87	49.45
48A	LVX	0.066 + 2	0.254	Strong synergism	3.95	800

Legend: ‡ laboratory code; AMB: antimicrobial; MTZ: metronidazole; RIF: rifampicin; LVX: levofloxacin; CI: combination index; IRD: Dose Reduction Index, IRD> 1 is considered favorable, and <1 is considered unfavorable. IC and IRD values were generated by Compusyn 1.0 program. The type and degree of pharmacological interaction was estimated according to Chou, 2006.

Discussion

Antimicrobial resistance is currently of great international interest [4]. The global resistance rates of *H. pylori* to metronidazole and clarithromycin range between 26.7-17.2%, which is higher in developing countries compared to developed countries. In a recent study carried out in Cuba, *H. pylori* isolates showed 94.7, 56.3, 25, and 6.2% resistance against metronidazole, levofloxacin, clarithromycin, and rifampin, respectively [5]. In addition, MDR strains resistant to the second- and third-line treatment were identified. Currently, a large number of investigations have been focused on the addition of medicinal plants to conventional treatments, which could increase eradication rates [14].

In the present study, the antimicrobial properties of *C. longa* extract were evaluated against clinical isolates of *H. pylori*. International literature recommends that in order for a crude extract to be considered promising, it must have an inhibitory concentration value below 100 µg/mL [15-16]. The extract showed a good antimicrobial effect on 9 out of 10 resistant and MDR isolates evaluated with mean inhibitory concentration values between 0.29-100 µg/mL. The sensitivity shown by the strains suggests that curcumin, the active ingredient in the extract, acts by different mechanisms from antimicrobials to inhibit bacterial growth. Among these mechanisms are: 1) inhibition of dynamic assembly of FtsZ (bacterial protofilament) which polymerizes Z-shaped cell rings to promote cell division [17] and 2) inhibition of the enzyme shikimate dehydrogenase (SDH), which participates in the synthesis pathway of aromatic amino acids and folates.

The Shikimate route, as it is called, does not exist in humans or animals; thus, SDH is considered as an alternative target for treatment. Curcumin is a non-selective competitor to SDH, and in *H. pylori*, this enzyme is encoded by *aroE* gene [17]. The variation in theinhibitory concentrations observed between the isolates are due to differences in the assimilation of turmeric by the bacteria

and not due to genetic polymorphisms in *aroE* gene [18].

On the other hand, one of the MDR isolates (20C) was resistant to *C. longa* extract. This could be due to differences in the intrinsic resistance to the active component of the extract or due to low concentrations of the compound with antimicrobial activity, because the extracts are a mixture of active and inactive chemical components.

There are no previous studies in Cuba, evaluating the antibacterial activity of *C. longa*. A previous investigation evaluating the turmeric extract, isolated from leaves, against *Plasmodium falciparum* parasite obtained mean inhibitory concentrations greater than 100 mg/mL [9]. In another study, evaluation of 65 *H. pylori* isolates in India showed a range of inhibitory concentrations between 5-50 µg/mL [7]. For their part, Mahady et al. (2002) [19] obtained the grow inhibition of 19 isolates of *H. pylori* using both curcumin and hydroalcoholic extract of turmeric. Although the extract has shown antibacterial activity in various studies, differences in the inhibition concentration values of the product persist. These differences are due to variations in the chemical content of the extract, which depend on the extraction method used, the method of determining antimicrobial activity, the doses evaluated, the geographical origin of the plant, as well as the stage of development and extraction of the product [18].

Turmeric extract has also been evaluated against nosocomial pathogens such as extended-spectrum beta-lactamase-producing *Echerichia coli*, *Pseudomona aeruginosa*, resistant *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococus aureus* (MRSA) with higher concentration values of 4.87-78 mg/mL [20].

The use of multiple drugs makes it possible to address diverse targets, different subpopulations, or several diseases at the same time. The use of various drugs with different modes of action may also direct the effect against a certain target and make the treatment more effective. The possible favorable benefits of developing synergism includes; 1) increasing therapeutic efficacy, 2) decreasing the dose while maintaining the same efficacy and preventing toxicity, 3) minimizing the development of antimicrobial resistance, and 4) providing selective synergism against the therapeutic target (efficacy synergism) and not against the host (toxic antagonism). For these therapeutic benefits, the drug combination has been widely used and has become a main alternative in the treatment of the most deadly diseases such as cancer and infectious diseases, including AIDS [15].

The first interactional study performed on the eradication of *H. pylori* infection was focused on a combination of macrolides and proton pump inhibitors (PPIs) [21]. In this study, researchers reported the potentiation of the macrolides effect in the presence of PPIs, lanzoprazole, and omeprazole against 38 *H. pylori* isolates. In a subsequent study by Chen et al. (2015) [22], by combining antimicrobials used in the treatment of bacterial infection, they found synergism with clarithromycin only in susceptible *H. pylori* isolates. These findings were decisive in demonstrating *in vitro* that monotherapy was not effective not only due to the antimicrobial resistance but also due to the location of bacteria in the gastric mucus, making it more difficult for a simple drug to exert its action.

The combination of *C. longa* extract with metronidazole, levofloxacin, and rifampin in the present study showed a synergistic effect on all the isolates, although in two isolates, it was only due to the antibiotic MIC value evaluated. There are not many reports in the international literature about the effect of turmeric in combination with antimicrobials against *H. pylori*. The most representative is a recent *in vivo* study on Balb/c mice inoculated with *H. pylori* [23]. No significant difference was observed between the groups of mice treated with curcumin and antimicrobials, separately,

but synergism was observed when combining the drugs, achieving a reduction in the levels of gastrin, IFN-γ, and some enzymes involved in lipid peroxidation in animals.

The combined antibacterial activity of turmeric extract has been explored in other resistant bacteria. Sasidharan et al. (2014) [24] used the checkerboard method and obtained a significant synergistic effect by combining turmeric with third-generation cephalosporins against bacteria associated with diarrheal diseases. Other authors obtained a reduction in the MIC of oxacillin, ampicillin, ciprofloxacin, and norfloxacin when evaluating these antimicrobials in combination with turmeric against MRSA isolates [20]. A recent study conducted on enterotoxigenic *E. coli* strains obtained a synergistic effect by evaluating three concentrations of turmeric in combination with 12 antimicrobials using the disc diffusion method [25].

Currently, there are varied studies that have shown the synergistic effects of plant extracts components in combination with different antimicrobials against *H. pylori*, with a possible improvement in eradication rates [26]. Evaluation of the aqueous extract of *Hibiscus sabdariffa* L. was shown to have a good synergistic effect in combination with clarithromycin and metronidazole against *H. pylori* isolates with a 40 and 46-fold dose reduction, respectively [27]. On the other hand, oleoresin from *Pistacia vera* L. plant in combination with levofloxacin with values lower than the MIC of natural product was shown to produce a synergistic effect by eliminating the biofilm formation of *H. pylori* MDR isolates [28].

The aforementioned reports share some commonalities in that they have been conducted on isolates resistant to antimicrobials used in conventional treatments. However, evaluation of *H.* pylori susceptible isolates could be interesting by taking into account the possible reduction in antimicrobial doses used in these cases. The authors recognize the absence of clarithromycin powder for drug combination studies as a limitation of the study. Nevertheless, it is important to denote that the mechanisms of pharmacological synergism are related to the action on multiple targets of bacterial cell wall and creating pharmacokinetic and physicochemical effects, such as improving solubility and bioavailability of compound, as well as the action on resistance mechanisms [29]. According to the best of our knowledge, this is the first work in Cuba, showing the effect of *C. longa* extract in combination with conventional drugs against *H. pylori* strains. Future studies would be required to better investigate the antimicrobial efficacy of each of the active compounds of *C. longa* against Cuban *H. pylori* strains.

Conclusion

The extract of *C. longa* showed a good potential as an antimicrobial agent against most of the resistant and multidrug-resistant *H. pylori* clinical isolates, making it a possible therapeutic alternative against the emergence of bacterial resistance. The combination of drugs with the plant extract *in vitro* caused a strong synergism with minimal inhibitory concentrations of antimicrobial as well as a decrease in the dose of both products, which make it possible to use it as a complement in the treatment of *H. pylori* infection.

Acknowldgements: The authors would like to thank Dr. Lianet Monzote (PhD) for his opportune advice.

Ethical Permissions: The protocol was approved by the Ethical Review Committee of the IPK (CEI-PK 72-18).

Conflict of Interest: The authors declare that there is no conflict of interest.

Authors' Contributions: Conceptualization: Onelkis Feliciano, Rafael Llanes and Alexis

Diaz; Data curation and formal analysis: Onelkis Feliciano, Yandy Ybalmea, Arianna Yglesias and Oderay Gutierrez; Investigation: Onelkis Feliciano, Yandy Ybalmea; Project a dministration: Arianna Yglesias; Supervision: Rafael Llanes. Writing of original draft: Onelkis Feliciano All authors read and approved the final manuscript.

Fundings: Biopharmaceutical laboratories (LABIOFAM) supported this research.

Consent to participate: Not applicable.

References

1. Kanna S, Maradey-Romero C, Fass R. Diagnostic tests for Helicobacter pylori. Gastroenterol Endosc News. 2013; 11(Special edition): 51-8.
2. Mahady GB, Pendland SL, Yun G, Lu ZZ. Turmerir (Curcuma longa) and curcumin inhibit growth of Helicobacter pylori, a group 1 carcinogen. Anticancer Res. 2002; 22(6C): 4179-81.
3. Sarkar A, De R, Mukhopadhyay A. Curcumin as a potential therapeutic candidate for Helicobacter pylori associated diseases. World J Gastroenterol. 2016; 22(9): 2736-48.
4. Otero W, Trespalacios AA, Otero L, Vallejo M, Torres M, Pardo R, et al. Guía práctica para el diagnóstico y tratamiento de la infección por Helicobacter pylori en adultos. Rev Col Gastroenterol. 2015; 30(1): 17-33.
5. Fu L. Diagnóstico y resistencia antimicrobiana de Helicobacter pylori. Instituto de Gastroenterología, abril-septiembre 2018 [tesis de maestría]. Instituto de Medicina Tropical Pedro Kouri; 2018.
6. Kwiecien S, Magierowski M, Majka J, Ptak-Belowska A, Wojcik D, Sliwowski Z et al. Curcumin: A potent protectant against esophageal and gastric disorders. Int J Mol Sci. 2019; 20(6): 1477.
7. Regulación M 85-16. Requisitos para la licencia sanitaria de fabricación y comercialización de los preparados vegetales y medicamentos naturales en los centros de producción local. Cuba. MINSAP-CECMED. Abril 2016; páginas 1-14.
8. González RA, Vistel M. Caracterización fitoquímica de la Curcuma longa L. Rev Cubana Quím. 2015; 27(1): 9-18.
9. Fernández A, Mendiola J, Acuña D, Caballero Y, Scull R, Gutiérrez Y. Actividad antimalárica y citotoxicidad de extractos hidroalcohólicos de seis especies de plantas usadas en la medicina tradicional cubana. Rev Cub Med Trop. 2011; 63(1): 52-7.
10. Leyva M, Tacoronte JE, Marquetti MC, Scull R, Montada D, Rodriguez Y. Actividad insecticida de aceites esenciales de plantas en larvas de Aedes aegypti (díptera: Culicidae). Rev cub Med Trop 2008; 60(1): 78-82.
11. Llanes R, Feliciano O, Gutiérrez O, Gala A, Valdés L, Capó V, et al. Nuevos conocimientos sobre el diagnóstico y la resistencia antimicrobiana de Helicobacter pylori en Cuba. Rev Anales Acad Ci Cuba. 2014; 2(4): 1-9.
12. Picazo JJ. Métodos básicos para el estudio de la sensibilidad a los antimicrobianos. Procedimientos de microbiología clínica. 2000:1-54. Libro
13. Chou TC. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. Pharmacol Rev. 2006; 58(3): 621–81.
14. Aşkar Ş, Deveboynu Şn. Investigation of in-vitro antibacterial activity of Curcuma longa commercial extract. Eurasian JHS. 2018; 1(1):1-6.
15. Cos P, Vlietinck AJ, Bander Berghe D, Maes L. Anti-infective potential of natural products: How to develop a stronger in vitro proof-of-concept. J Ethnopharmacol. 2006; 106:290–302.

16. Gibbons S. Phytochemicals for bacterial resistance – strengths, weaknesses, and opportunities. Planta Med. 2008; 74(6): 594–602
17. Rai D, Singh JK, Roy N, Panda D. Curcumin inhibits FtsZ assembly: An attractive mechanism for its antibacterial activity. Biochem J. 2008; 410(1): 147-55.
18. Patil TR, Patil ST, Patil S, Patil A. Effect of non-antibiotic antimicrobial Curcuma Longa on Helicobacter Pylori Infection. Int J Pharmacogn Chinese Med. 2018; 2(5): 000150.
19. Mahady GB, Pendland SL, Yun G, Lu ZZ. Turmerir (Curcuma longa) and curcumin inhibit growth of Helicobacter pylori, a group 1 carcinogen. Anticancer Res. 2002; 22(6C):4179-81
20. Mun SH, Joung DK, Kim YS, Kang OH, Kim SB, Seo YS, et al. Synergistic antibacterial effect of curcumin against methicillin-resistant Staphylococcus aureus. Phytomedicine 2013; 20(8-9): 714-8.
21. Maliziaa T, Tejadaa M, Marchettib F, Favinib P, Pizzarellic G, Campaa M. Synergic interactions of macrolides and proton-pump inhibitors against Helicobacter pylori: A comparative in-vitro study. J Antimicrob Chemother. 1998; 41(Suppl 2): 29-35.
22. Cheng A, Sheng WH, Liou JM, Wang HP, Wu MS, Lin JT. Comparative in vitro antimicrobial susceptibility and synergistic activity of antimicrobial combination against Helicobacter pylori isolates in Taiwan. J Microbiol Inmunol Infect. 2015; 48(1):72-9.
23. Ranjbar R, Mohammadi A. Synergistic effects of combined curcumin and antibiotic in ameliorating an animal model of Helicobacter pylori infection. Biomed Res. 2018; 29(8): 1702-7.
24. Sasidharan NK, Ravikumar S, Jacob J, Nambisan B. In vitro synergistic effect of Curcumin in combination with third generation cephalosporins against bacteria associated with infectious diarrhea. Biomed Res Int. 2014; 2014: 561456.
25. Rangel-Castañeda I, Cruz-Lozano J, Zermeño-Ruiz M, Cortes-Zarate R, Hernández-Hernández L, Tapia-Pastrana G. Drug susceptibility testing and synergistic antibacterial activity of Curcumin with antibiotics against enterotoxigenic Escherichia coli. Antibiotics. 2019; 8(2): 43.
26. Judaki A, Rahmani A, Feizi J, Asadollahi K, Hafezi M. Curcumin in combination with triple therapy regimes ameliorates oxidative stress and histopathologic changes in chronic gastritis–associated Helicobacter pylori infection. Arq Gastroenterol. 2017; 54(3):177-82.
27. Hassana S, Berchova K, Majerova M, Pokorna M, Svajdlenkaa E. In vitro synergistic effect of Hibiscus sabdariffa aqueous extract in combination with standard antibiotics against Helicobacter pylori clinical isolates. Pharm Biol. 2016; 54(9):1736–40.
28. Lodovico S, Napoli E, Campli E, Fermo P, Gentile D, Ruberto G. Pistacia vera L. oleoresin and levofloxacin is a synergistic combination against resistant Helicobacter pylori strains. Sci Rep. 2019; 9(1): 4646.
29. Zullo V, Hassan C, Giorgio F, Rosania R, Ierardi E. Mechanisms of Helicobacter pylori antibiotic resistance: An updated appraisal. World J Gastrointest Pathophysiol. 2011; 2(3): 35-41.