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In Vitro Activity of Olive Oil Polyphenols against *Helicobacter pylori*

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Helicobacter pylori is linked to a majority of peptic ulcers and to some types of gastric cancer, and resistance of the microorganism to antibiotic treatment is now found worldwide. Virgin olive oil is an unrefined vegetable oil that contains a significant amount of phenolic compounds. Under simulated conditions, we have demonstrated that these substances can diffuse from the oil into the gastric juice and be stable for hours in this acidic environment. In vitro, they exerted a strong bactericidal activity against eight strains of *H. pylori*, three of them resistant to some antibiotics. Among the phenolic compounds, the dialdehydic form of decarboxymethyl ligstroside aglycon showed the strongest bactericidal effect at a concentration as low as 1.3 μg/mL. Although the experimental conditions are different from other reported works, this bactericidal concentration is much lower than those found for phenolic compounds from tea, wine, and plant extracts. These results open the possibility of considering virgin olive oil a chemopreventive agent for peptic ulcer or gastric cancer, but this bioactivity should be confirmed in vivo in the future.

KEYWORDS: Olive oil; phenolic compounds; simulated digestion, *Helicobacter pylori*; antimicrobial

INTRODUCTION

The most accepted regime for the eradication of *Helicobacter pylori* infection currently includes a triple therapy, which combines the antibiotic clarithromycin and amoxicillin with a proton pump inhibitor such as omeprazole. This chemotherapy, however, sometimes produces side effects and fails to eliminate infection in 10−30% of patients (*1*). The occurrence of strains resistant to antibiotics would be expected to increase, and it is nowadays important to search for nonantibiotic substances with anti-*H. pylori* activity. Herbal extracts and essential oils have been used as traditional medicines for thousands of years all over the world, and their anti-*H. pylori* activity has been widely demonstrated in vitro (*2*−*5*). Many foodstuffs have also exhibited inhibitory activity against the growth of *H. pylori* in vitro, among others, red wine (*6*, *7*), sprouted peas (*8*), green tea (*9*), and cranberry juice (*10*). In many cases, the antibacterial activity of both herbal extracts and foodstuffs has been associated with their content in phenolic compounds, in particular, flavonoids (*11*−*13*), resveratrol (*6*), and hydrolyzable tannins (*14*). The mechanism by which phenolic compounds affect the growth of *H. pylori* is unknown, but different theories have been proposed, for example, inhibition of the urease activity (*15*), adhesion to human gastric mucus (*10*), disintegration of the outer membrane (*16*), and inhibition of VacA citotoxin activity which causes the development of inflammation and ulceration in patients (*17*, *18*).

However, in vivo studies with garlic (*19*), jalapeño peppers (*20*), cinnamon extract (*21*), broccoli (*22*), and cranberry juice (*23*) failed to eradicate *H. pylori* infection in spite of the well-reported antibacterial data obtained from in vitro experiments. With this concern in mind, researchers have recommended the consumption of these natural foods as chemopreventive agents (*6*) or in combination with antibiotics to eradicate the bacterial infection (*24*).

Virgin olive oil is one of the few edible vegetable oils that is consumed unrefined, which implies that it contains a significant amount of minor bioactive substances. Among them, phenolic compounds have received a great deal of attention over recent years because of the beneficial properties attributed to human health (*25*−*27*). Despite the myriad of papers published on olive oil and olive oil polyphenols, none has focused on the inhibition of *H. pylori* growth. We have recently discovered a very high antimicrobial activity of olive oil polyphenols against a broad spectrum of foodborne pathogens (*28*), and it was suspected that this activity could also be exerted against *H. pylori*. In addition, an early study detected that the substitution of animal fat for olive oil in the diet produced a significant reduction in the size of ulcers in patients (*29*), and another work related the consumption of olive oil with a reduction in gastric acid secretion (*30*).

There is a controversy about the changes that phenolic compounds suffer during their transit through the stomach. It

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was found that the acidic environment is capable of hydrolyzing cocoa procyanidins (*31*) and other researchers reported their stability in the gastric juice (*32*). The bound ester of chlorogenic acid is also stable at the pH of the stomach (*33*). The main phenolic compounds in olive oil are the secoiridoid aglycons of oleuropein and ligstroside, and they hydrolyze during olive storage into simple phenols such as hydroxytyrosol and tyrosol (*34*). The two latter compounds and oleuropein seemed to be stable in gastric juice (*35, 36*) but not the secoiridoid aglycons (*10*). It is of great interest to clarify this point because we found that the antimicrobial activity of olive oil polyphenols against foodborne pathogens was mainly related to certain secoiridoid aglycons (*28*).

Therefore, the aim of this work was to study the anti-*H. pylory* activity of olive oil polyphenols and the stability of secoiridoid aglycons under simulated stomach conditions for the first time.

MATERIALS AND METHODS

Oil. All olive oils used were virgin olive oils of the Picual, Manzanilla, Cornicabra, Hojiblanca, and Arbequina varieties and were purchased from local department stores and were kept at room temperature between experiments.

Incubation of Olive Oil with Simulated Gastric Juice. To study the diffusion and hydrolysis of the olive oil polyphenols, we incubated 10 g of Picual virgin olive oil with 10 mL of water acidified with HCl to reach pH 2. This mixture was put in six different 50-mL centrifuge tubes which were shaken in a GFL 3005 orbital shaker for 4 h inside a 37 °C incubator. Two tubes were taken after 0.5, 1, and 4 h, were centrifuged at 9000*g* for 5 min, and the aqueous phase was collected with a Pasteur pipet. Additionally, 7400 units of pepsin from porcine stomach mucus (Sigma, MO) was added to another two tubes and was left for 4 h.

Simulation for 0.5 h without pepsin addition was repeated with Arbequina, Cornicabra, Manzanilla, and Hojiblanca oils.

To check the effect of pH on diffusion and hydrolysis of the phenolic compounds, incubation for 0.5 h at 37 °C was performed in water acidified with HCl to reach pH 2, in a sodium acetate buffer at pH 4, and in a sodium phosphate buffer at pH 7 (PBS).

Another experiment was run to study the influence of the oil:water ratio on the diffusion and hydrolysis phenomena. Five grams of Picual olive oil was incubated with 5 mL of water acidified with HCl to reach pH 2 for 0.5 h at 37 °C and a 0.1-mL sample was withdrawn. Subsequently, 5 mL of acidified water was added to the mixture and was incubated for another 0.5 h at 37 °C. A new 0.1-mL sample was withdrawn, and the sequence was repeated until the ratio oil:water was 1:4.

Immediately, after each experiment, 1.5 mL of the aqueous extract was mixed with 0.41 mL of a 119 mM sodium acetate buffer (pH 4) containing 0.2 mM of syringic acid as internal standard and was kept at −30 °C before analysis of the phenolic compounds. The oil phase was also stored at −30 °C before analysis.

Polyphenol Analysis. Phenolic extracts of olive oils were obtained following the procedure described elsewhere (*38*). Briefly, 0.6 mL of olive oil was extracted using 3 × 0.6 mL of *N,N*-dimethylformamide (DMF); the extract was then washed with hexane, and N_2 was bubbled into the DMF extract to eliminate the residual hexane. Finally, the extract was filtered through a 0.45-μm pore size nylon filter and was injected into the chromatograph.

The analysis of the polyhenols in the aqueous phase was made by directly injecting the solution into the chromatograph after filtration through a 0.45-μm pore size nylon filter.

The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters column heater module (Waters Inc., Mildford, MA). A Spherisorb ODS-2 (5 μm, 25 cm × 4.6 mm i. D., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90% water (pH adjusted to 3.0 with phosphoric acid) and 10% methanol. The concentration of the latter solvent was increased to 30% over 10 min and was maintained for 20 min. Subsequently, the methanol percentage was raised to 40% over 10 min, was maintained for 5 min, and then was increased to 50%. Finally, the methanol percentage was increased to 60, 70, and 100% in 5-min periods. Initial conditions were reached in 15 min. A flow rate of 1 mL/min and a temperature of 35 °C were used in all experiments. A Waters 996 diode array detector and a Jasco FP-920 fluorescence detector (Jasco, Tokyo, Japan) were connected in series. Quantification of the phenolic compounds was made using an internal standard (syringic acid).

Isolation of Phenolic Compounds. Polyphenols were extracted from Manzanilla virgin olive oil using a phospate buffer saline at pH 7. The analytical column, mobile phases, gradient, and equipment were the same as those used for the polyphenol analysis except the aqueous mobile phase, which was acidified with HCl to pH 4. Fractions from 80 high-performance liquid chromatography (HPLC) runs were collected peak by peak. The pooled extract for each peak (50−80 mL) was evaporated under reduced pressure close to dryness and the residue was dissolved in 1 mL of deionized water. Finally, the purity and concentration of each phenolic compound were measured by HPLC. A control run was also performed by injecting methanol and collecting all fractions of the run (75 min). The pooled fractions were evaporated close to dryness, and the residue was dissolved in 1 mL of deionized water.

Strains and Growth Conditions. Eight isolates of *H. pylori* were used in this study. The type strain LMG 19449 and strains LMG 18041 and LMG 8775 were obtained from the Belgian Coordinated Collections of Micro-organisms (BCCM/ LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, B-9000 Gent, Belgium), and five clinical strains were obtained from human gastric biopsy specimens. Isolation of strains was performed on Columbia blood agar base (CM331 Oxoid, Basingstoke, United Kingdom) with 5% blood and *H. pylori* Selective Supplement (Dent, SR147, Oxoid). Identification of isolates was based on Gram staining, oxidase$^+$, catalase$^+$, and urease$^+$ (*1*). All strains were routinely grown in a solid medium consisting of BBL Brucella Broth (Becton, Dickinson and Co., Sparks, MD 21152) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, A-4061 Pasching, Austria), and 1.5% agar (medium BB−FBS). Plates were incubated under water-saturated conditions at 37 °C in jars (GENbox, bioMérieux, 69280 Marcy l'Etoile, France). Microaerophilic conditions were generated with GENbox microaer (bioMérieux) generators. Columbia agar + 5% horse blood (bioMérieux) was also used in some instances (medium COH). Strains were stored at −80 °C in BHI (Oxoid) plus 20% glycerol.

Antibiotic Susceptibility Testing. The strains were investigated for antibiotic resistance using the E-test (AB Biodisk, Sweden) on both BB−FBS and COH plates under microaerophilic incubation conditions and following the manufacturer's instructions. Amoxicillin, clarithromycin, metronidazole, and tetracycline were tested as recommended (*39*).

Effect of Olive Oil Extract on *H. pylori*. Ten grams of virgin olive oil (Manzanilla cultivar) was mixed with 10 mL of PBS (pH 7) at room temperature for 5 min with occasional vortexing. After centrifugation at 9000*g*, the aqueous phase was collected and used for the experiments. Bacterial suspensions in PBS of each strain were mixed with olive oil extract at 5%, 10%, and 20% concentrations. Cell density was calculated to obtain 5 Log CFU/mL as the initial population. After 5 min of contact at room temperature, surviving colony forming units (CFU) were counted on BB−FBS following incubation at 37 °C for 3−6 days under microaerophilic conditions. Each experiment was carried out twice, and duplicates and controls with no olive oil extract were always included. Another experiment was conducted with type strain LMG 19449 and olive oil extract at 0%, 1%, and 5%, studying survival along time from 0 to 60 min. The LMG 19449 strain was chosen for this experiment because it is the type strain of *H. pylori*.

Antimicrobial Effect of Isolated Phenolic Compounds on *H. pylori*. Strain LMG 19449 in PBS × 2 was mixed 1:1 with compounds obtained by HPLC as described above. Each compound was tested at 5% of its concentration reported in **Table 2**. After 1 h at room temperature, surviving CFU was counted as explained above.

Table 1. Phenolic Compounds (Micromoles per Kilogram) in the Olive Oils Studied

	olive oil variety				
polyphenol	Manzanilla	Picual	Arbequina	Hojiblanca	Cornicabra
hydroxytyrosol	479.6	176.8	179.6	159.2	164.1
hydroxytyrosol glycol	8.8	26.9	14.3	16.4	10.8
tyrosol	159.4	52.3	59.9	121.6	54.1
Hy-AC[a]	130.8	500.4	202.6	51.9	1.5
Hy-EDA	740.5	923.4	559.4	296.2	1332.7
Ty-EDA	283.5	202.6	179.8	160.2	427.1
1-acetoxypinoresinol	72.2	18.7	133.9	54.4	9.2
pinoresinol	52.7	154.2	107.6	86.0	104.4
Hy-EA	535.8	622.7	85.0	454.2	778.6
Ty-EA	249.6	253.6	17.5	240.8	497.2

[a] Hy-AC, hydroxytyrosol acetate; Hy-EDA, dialdehydic form of decarboxymethyl oleuropein algycon; Ty-EDA, dialdehydic form of decarboxymethyl ligstroside aglycon; Hy-EA, oleuropein aglycon; Ty-EA, ligstroside aglycon.

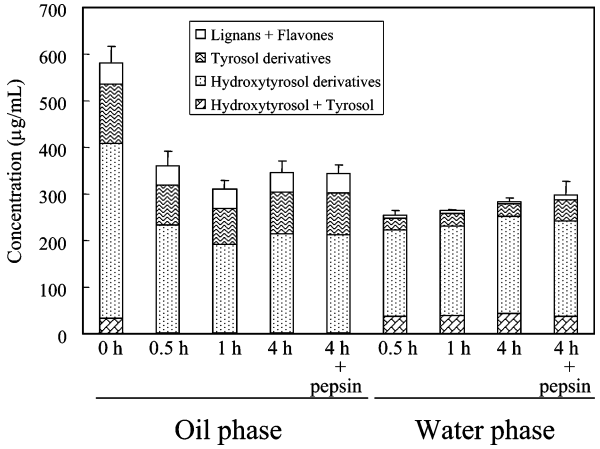

Figure 1. Distribution of olive oil polyphenols between the oily and the aqueous phases after simulation of the stomach conditions with a Picual virgin olive oil. Bars mean the standard deviation of two replicates. Lignans: 1-acetoxypinoresinol and pinoresinol; flavones: apigenin and rutin; tyrosol derivatives: dialdehydic form of decarboxymethyl ligstroside aglycon and ligstroside aglycon; hydroxytyrosol derivatives: hydroxytyrosol glycol, hydroxytyrosol acetate, dialdehydic form of decarboxymethyl oleuropein aglycon and oleuropein aglycon.

RESULTS

Diffusion and Stability of Olive Oil Polyphenols under Simulated Gastric Juice. Table 1 shows the phenolic composition of the different olive oils used for the experiments. As could be expected, there were great differences among olive varieties (*40*). The aglycons of oleuropein and ligstroside were the main polyphenols in oils, followed by the simple phenols hydroxytyrosol, tyrosol, hydroxytyrosol acetate, and lignans. Incubation at 37 °C of the Picual virgin olive oil in water acidified with HCl to reach a pH 2 gave rise to diffusion from the oil to the aqueous phase of approximately half of the phenolic compounds (**Figure 1**). This physical phenomenon was not time-dependent because the difference in phenolic compounds between the aqueous extracts obtained at 0.5 and 4 h of gastric simulation was minimal. Indeed, we observed that the majority of polyphenols diffused from oil into the aqueous phase during the first 5 min of contact (data not shown). It can also be observed in **Figure 1** that the addition of pepsin to the acidified water did not influence the diffusion yield of phenolic compounds. Additionally, its use did not contribute significantly to alter the phenolic composition in the aqueous solution. Another interesting finding reflected in **Figure 1** is the fact that the secoiridoid aglycons (dialdehydic form of decarboxymethyl oleuropein aglycon, dialdehydic form of decarboxymethyl ligstroside aglycon, oleuropein aglycon, and ligstroside aglycon) did not change significantly in the acidified water up to 4 h. Likewise, the concentration of tyrosol and hydroxytyrosol slightly increased during the 4 h of gastric simulation. The concentration of both lignans (1-acetoxypinoresinol and pinoresinol) and flavones (apigenin and rutin) in the acidic environment did not present significant changes during the 4-h study.

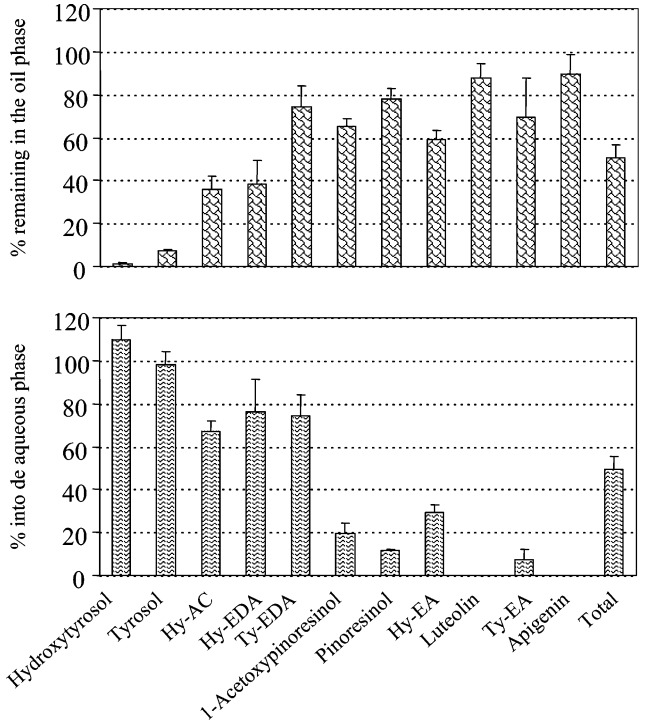

Figure 2. Distribution of olive oil polyphenols between the oily and the aqueous phases after stomach simulation for 0.5 h. Data are mean ± standard deviation of five monovarietal olive oils (Manzanilla, Picual, Hojiblanca, Arbequina, and Cornicabra). See **Table 1** for abbreviation identification.

The concentration of each polyphenolic compound in both phases of Manzanilla gastric simulation is presented in **Figure 2**. As could be expected from a previous work (*27*), the more polar the compound the more complete the diffusion reached from the oil into the acidified water (**Figure 2**). Hydroxytyrosol and tyrosol totally diffused into the acid water whereas the diffusion yield of lignans, flavones, and oleuropein and ligstroside aglycons was very low. Overall, half of the total polyphenols initially present in the oil diffused into the aqueous solution.

Because pH in gastric juice increases during food digestion, we studied the diffusion of phenolic compounds at a pH higher than 2 (**Figure 3**). The diffusion yield was higher at pH 4 and 7 than at pH 2 for both Arbequina and Picual virgin olive oils studied.

During the digestion, the ratio 1:1 between aqueous and oily phases is not always exact, usually, the aqueous phase increases with digestion time with respect to the oil phase. Therefore, to achieve realistic results, the amount of phenolic compounds diffused from the oil to the acidified water was also evaluated by increasing the volume of the acidified water with time (**Figure 4**). The extraction of total polyphenols increased when the ratio water:oil was raised from 1 to 2 but remained unchanged at a higher ratio (2 to 4). This behavior was also similar for the complex secoiridoid aglycons.

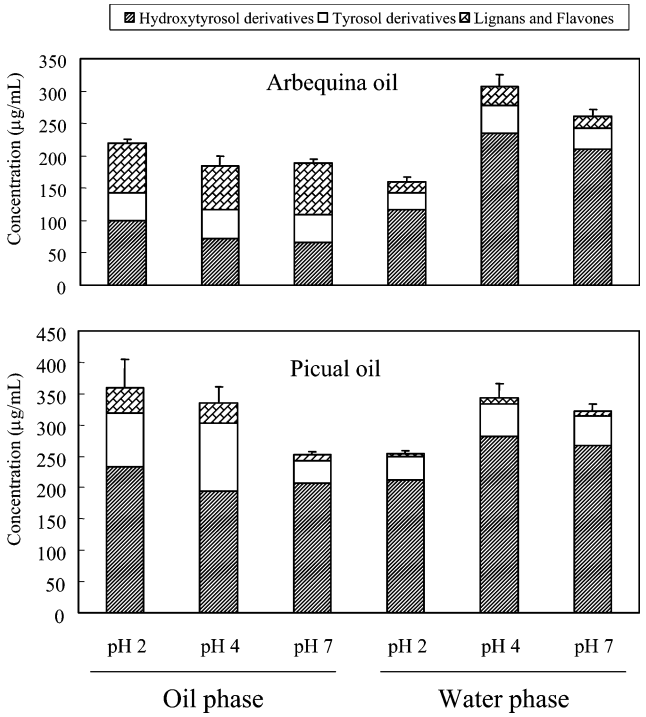

Figure 3. Effect of the pH on the distribution of olive oil polyphenols between the oily and aqueous phases after simulation of stomach conditions for 0.5 h. Bars mean the standard deviation of two replicates. Hydroxytyrosol derivatives: hydroxytyrosol, hydroxytyrosol glycol, hydroxytyrosol acetate, dialdehydic form of decarboxymethyl oleuropein aglycon and oleuropein aglycon; tyrosol derivatives: tyrosol, dialdehydic form of decarboxymethyl ligstroside aglycon and ligstroside aglycon; lignans: 1-acetoxypinoresinol and pinoresinol; flavones: apigenin and rutin.

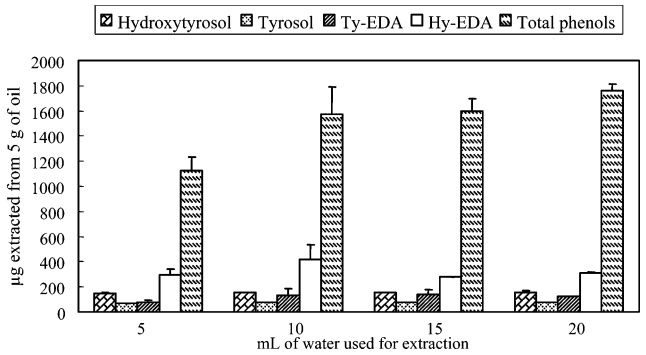

Figure 4. Effect of the oil:acidified water ratio on the extraction yield of polyphenols from a Picual virgin olive oil after stomach simulation for 0.5 h. Bars mean the standard deviation of two replicates. See **Table 1** for abbreviation identification.

Anti-*H. pylori* Effect of Olive Oil Polyphenols. Among the *H. pylori* strains tested, LMG 19449 and LMG 18041 were resistant to metronidazole, and strain V7 was resistant to metronidazole and clarithromycin (MICs > 256 μg/mL). All strains were susceptible to amoxicillin and tetracycline. Previous studies of isolates from the Valme Hospital (Seville) revealed resistance to metronidazole in 29% of *H. pylori* strains and to clarithromycin in 10% (*41*). An aqueous olive oil extract of the Manzanilla variety was used to run in vitro experiments on *H. pylori* survival. **Table 2** shows the polyphenol content of this extract, which was rich in the simple phenols hydroxytyrosol and tyrosol and in the dialdehydic form of decarboxymethyl oleuropein aglycon.

Table 2. Content in Olive Oil Polyphenols of the Aqueous Extract Obtained after 0.5 Min of Contact between 10 g of Manzanilla Virgin Olive Oil and 10 mL of PBS Solution

polyphenol	concentration (μM) (standard deviation
hydroxytyrosol	485.7 ± 20.8
hydroxytyrosol glycol	10.0 ± 0.6
tyrosol	203.6 ± 0.1
Hy-ACa	95.4 ± 0.5
Hy-EDA	491.6 ± 35.0
Ty-EDA	84.8 ± 6.3
1-acetoxypinoresinol	21.4 ± 4.0
pinoresinol	4.7 ± 1.4
Hy-EA	129.6 ± 5.0
Ty-EA	18.8 ± 0.6

[a] Hy-AC, hydroxytyrosol acetate; Hy-EDA, dialdehydic form of decarboxymethyl oleuropein aglycon; Ty-EDA, dialdehydic form of decarboxymethyl ligstroside aglycon; Hy-EA, oleuropein aglycon; Ty-EA, ligstroside aglycon.

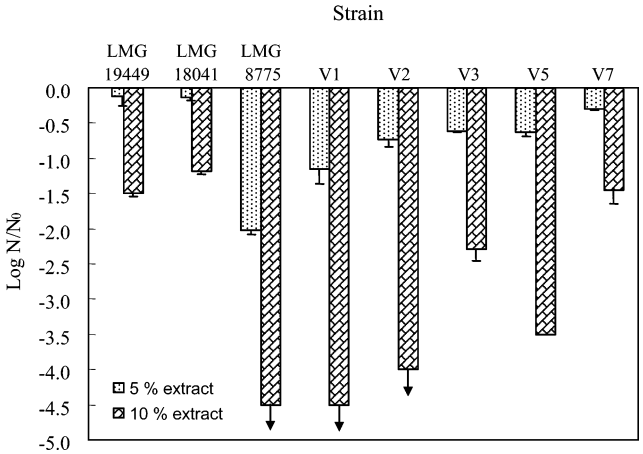

Figure 5. Bactericidal activity (Log N/N_0) of diluted aqueous extract of Manzanilla virgin olive oil against *H. pylori* strains. $N_0 = 5.2 \pm 0.3$ CFU/mL inoculated. N = CFU/mL after 5 min of contact. Arrows mean that number of viable cells was under the detection limit. Bars mean standard deviation of two replicates.

Preliminary experiments disclosed that undiluted olive oil extract killed all strains after 5 min of contact. Thus, diluted extract was used to compare the bactericidal effect against the eight strains studied. Aqueous extract at 20% concentration in PBS killed all bacteria ($N_o = 5.2 \pm 0.3$ Log CFU/mL). *H. pylori* survived after 5 min of contact in a concentration lower than 10% (**Figure 5**). However, three (LMG 8775, V1, and V2) of the eight strains tested were very sensitive to the olive oil extract and did not survive after 5 min of contact with 10% extract. The most resistant strains were LMG 19449 and LMG 18041. As expected, a concentration effect was found for all strains tested; a 10% extract killed more cells than a 5% extract. A 5% concentration of the Manzanilla oil extract means about 19 μg/mL of total polyphenols in this solution.

We also observed that the bactericidal activity of the oil extract was time-dependent (**Figure 6**). A 5% oil extract did not show a significant effect on the culturable cells of *H. pylori* LMG 19449 after 5 min of contact but reduced the number of cells by more than 4 Logs after 30 min of contact. In fact, only 1% extract showed a significant bactericidal activity after 60 min of contact.

In view of these results, each single phenolic compound detected in the aqueous extract was isolated by HPLC and was tested at 5% of its concentration in this extract (**Table 2**). *H. pylori* LMG 19449 was incubated with the isolated compounds

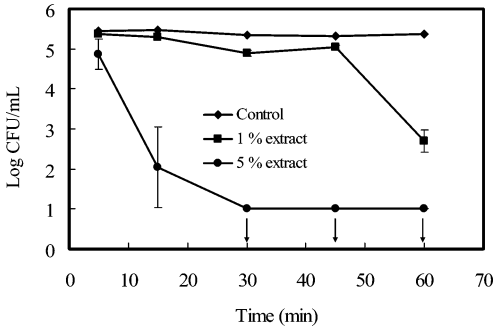

Figure 6. Survival of *H. pylori* LMG 19449 along time of contact with diluted aqueous extracts from Manzanilla virgin olive oil. Arrows mean that number of viable cells was below the detection limit. Bars mean standard deviation of two replicates. Where error bars are not visible, determinations are within the size of the symbols on the graph.

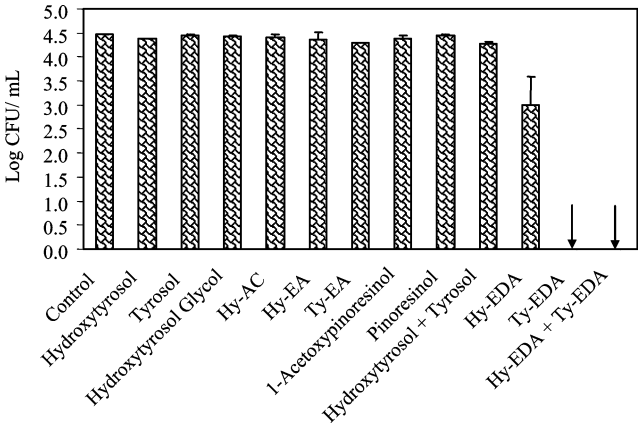

Figure 7. Survival of *H. pylori* LMG 19449 after 60 min contact with isolated phenolic compounds. Each compound was tested at 5% of its content in the aqueous extract reflected in **Table 2**. Arrows mean that the number of viable cells was below the detection limit. Bars mean the standard deviation of two replicates. See **Table 1** for abbreviation identification.

for 60 min. Results are presented in **Figure 7**. None of the phenolic compounds studied except the dialdehydic form of decarboxymethyl oleuropein aglycon (Hy-EDA) and, in particular, the dialdehydic form of decarboxymethyl ligstroside (Ty-EDA) showed significant bactericidal effect. Also, three elenolic acid derivatives were tested, and they did not exert any significant bactericidal effect. In consequence, Ty-EDA was the compound responsible for the major bactericidal activity of the olive oil extract, followed by Hy-EDA. Incubation with Ty-EDA decreased the number of culturable cells by more than 4 Logs at a concentration as low as 26 μM, which means only 1.3 μg/mL (**Figure 7**). In spite of the fact that other phenolic compounds were present in the aqueous extract at higher concentrations than Ty-EDA, this substance alone exerted a noticeable bactericidal activity against *H. pylori*.

DISCUSSION

In contrast to other refined edible vegetable oils, virgin olive oil possesses a considerable amount of phenolic compounds with many beneficial properties attributed to human health (*14*, *26*, *27*). Furthermore, a strong bactericidal activity against certain foodborne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Shigella sonnei*, *Salmonella enterica*, and others has recently been found (*28*). In the present study, we have discovered a strong anti-*H. pylori* activity exerted by olive oil extracts rich in phenolic compounds for the first time. This activity was even effective against some antibiotic resistant strains and, which is more important, a very low concentration of the olive oil extract was necessary. Consumption of olive oil may decrease acid secretion in the gastrointestinal tract (*30*), and it has also been associated with a reduction in the size of peptic ulcers (*29*), but it had never been related to a bactericidal action against *H. pylori*. Recently, it was reported a bactericidal effect of fatty acids and monoglycerides on *H. pylori* (*42*) although oleic acid, the main fatty acid in olive oil, did not show any effect. In fact, in our study we have demonstrated that the phenolic compounds that diffused from the oil into the simulated gastric juice were the substances responsible for the strong anti-*H. pylori* activity. In particular, Ty-EDA accounted for most of the anti-*H. pylori* activity detected in these extracts. It is important to note the low concentration (<1.5 μg/mL) of this compound needed to kill the *H. pylori* cells in vitro. The concentration of the antibiotics clarithromycin and amoxicillin required to kill the bacteria is lower (*43*), but olive oil is a food and not a medicine, and, therefore, its anti-*H. pylori* activity should be considered preventive.

Therefore, consumption of virgin olive oil may be advantageous in comparison to other edible vegetable oils which do not contain phenolic compounds. It should also be stressed that the concentration needed to kill *H. pylori* by phenolic compounds from other food sources is much higher than that found for Ty-EDA. Thus, the bacteria were sensitive to more than 100 μg/mL of tea catechins (*24*), 12−25 μg/mL of resveratrol (*6*), 12 μg/mL of flavonoids from medicinal plants (*11*), and 20−100 μg/mL of essential oils (*2*).

Ty-EDA is a complex phenolic compound present in most virgin olive oils in concentrations up to 240 μg/mL (*40*) which has recently been attributed to ibuprofen-like activity (*44*). It is not a very high lypophilic substance and can be hydrolyzed during olive oil storage (*34*). Therefore, a question arose from these points: Can this compound, as well as the other secoiridoid aglycons, diffuse from the oil into the gastric juice and remain stable during digestion? Vissers et al. (*36*) incubated hydroxytyrosol, tyrosol, and oleuropein in gastric juice for 2 h and they did not detect any changes or hydrolysis reaction on these compounds. Additionally, these results were confirmed with oleuropein in simulated gastric juice (*35*). By contrast, it has been reported (*37*) that the secoiridoid aglycons of the olive oil suffered hydrolysis during incubation with simulating gastric juice for 4 h, and an increase in the simple phenols hydroxytyrosol and tyrosol was also observed. It was necessary to clarify this subject because we have demonstrated in this study that the secoiridoid aglycons, in particular, Ty-EDA and Hy-EDA, are the strongest anti-*H. pylori* compounds. Our results revealed that half of the total amount of polyphenols diffused from the oil into the simulated gastric juice, and the secoiridoid aglycons remained stable for up to 4 h of incubation at 37 °C. In our opinion, the controversy occurs because of the differences in the storage method of the aqueous samples before analysis. We detected that the secoiridoid aglycons hydrolyzed during storage of the olive oil extracts at pH 2 in the refrigerator and, in consequence, we buffered all the aqueous extracts up to pH 4 immediately after taking the samples. Doing that, we did not find any significant hydrolysis of the secoiridoid aglycons for up to 4 h of the simulated digestion.

Therefore, Ty-EDA and Hy-EDA must diffuse into the gastric juice in vivo, and they are stable for hours in the acidic environment and are available for their anti-*H. pylori* activity. The antibacterial treatment of *H. pylori* is difficult because of

the habitat occupied by the organism below the layer of mucus adherent to gastric mucosa (*45*), and it could be a good reason to explain the failure of the in vivo experiments carried out with different foodstuffs (*19*−*21*, *33*). However, in view of the low concentration required to exert bactericidal action against *H. pylori* by the dialdehydic form of decarboxymethyl ligstroside aglycon, it is promising to carry out studies in vivo with virgin olive oil to prevent and control peptic ulcers and gastric cancer caused by this bacteria.

In conclusion, the presented results have shown that olive oil polyphenols can diffuse from the oil into the gastric juice, the more polar the compound the more complete the diffusion reached. Overall, half of the total polyphenols initially present in the oil diffused into the simulated gastric juice. Furthermore, the results indicate that the secoiridoid aglycons are not hydrolyzed in the acidic environment of the gastric juice. It has been just demonstrated that these secoiridoid aglycons, in particular, the dialdehydic form of decarboxymethyl ligstroside aglycon, are the most powerful anti-*H. pylori* compounds of the olive oil. Thus, these results open the possibility of considering virgin olive oil a chemopreventive agent for peptic ulcer or gastric cancer, but this bioactivity should be confirmed in vivo in future.

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