

## In Vitro and In Vivo Activities of Chios Mastic Gum Extracts and Constituents against *Helicobacter pylori*<sup>▽</sup>

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The extracts and pure major constituents of Chios mastic gum (resin of *Pistacia lentiscus* var. *chia*) were tested for their activities against *Helicobacter pylori*. A total mastic extract without polymer (TMEWP) was prepared after removal of the contained insoluble polymer in order to ameliorate solubility and enhance in vivo activity. Administration of TMEWP to *H. pylori* SS1-infected mice over the period of 3 months with an average dose of 0.75 mg/day led to an approximately 30-fold reduction in the *H. pylori* colonization (1.5 log CFU/g of tissue). However, no attenuation in the *H. pylori*-associated chronic inflammatory infiltration and the activity of chronic gastritis was observed. To further characterize potential active mastic constituents, the TMEWP was separated into an acidic and a neutral fraction. Both were extensively characterized by nuclear magnetic resonance and mass spectroscopy to elucidate the structure of the components contained within each fraction. After chromatographic separation, the acid fraction gave the major triterpenic acids, while the neutral fraction gave several triterpenic alcohols and aldehydes. Mastic extracts and isolated pure triterpenic acids were tested for in vitro activity against a panel of 11 *H. pylori* clinical strains. The acid fraction was found to be the most active extract (minimum bactericidal concentration [MBC], 0.139 mg/ml), and the most active pure compound was isomasticadienolic acid (MBC, 0.202 mg/ml [0.443 mM]). Our results show that administration of TMEWP may be effective in reducing *H. pylori* colonization and that the major triterpenic acids in the acid extract may be responsible for such an activity.

*Pistacia lentiscus* L. is an evergreen shrub of the *Anacardiaceae* family, very common in the eastern Mediterranean area. The variety *chia* (Duham) is uniquely cultivated in southern Chios, a Greek island in the Aegean. The resin of that plant, mastic gum, is obtained as an exudate after “hurting” the trunk and branches.

Mastic gum has been used in traditional Greek medicine for various gastrointestinal disorders like gastralgia, dyspepsia, and peptic ulcer for more than 2,500 years. Ancient Greek physicians, such as Hippocrates, Dioscorides, Theophrastos, and Galenos, mentioned its properties and recommended its use. In modern times, it is used as a seasoning in Mediterranean cuisine, in the production of chewing gum, in perfumery, in dentistry, and by the local population of Chios for the relief of gastralgia and protection against peptic ulcer. Early studies involving mastic administration to rats with experimentally induced gastric and duodenal ulcers indicated a significant decrease of free acidity (2). Moreover, a double-blind clinical trial carried out on patients with symptomatic and endoscopically proven duodenal ulcer showed increased symptomatic relief in patients on mastic (1 g daily) compared to patients on

placebo, while endoscopically proven healing occurred in 70% of the patients on mastic (1).

In 1983, Barry Marshall and Robin Warren suggested that gastric inflammation and peptic ulceration were the result of an infection caused by *Helicobacter pylori* (34). In the years to follow, the presence of *H. pylori* infection was shown to be the etiologic determinant of chronic active gastritis and a major risk factor for the development of peptic ulcer disease, gastric atrophy, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (23). Antibiotic eradication schemes have proved very effective in clearing the infection; however, low patient compliance and the development of antibiotic resistance have created the need for new *H. pylori* eradication strategies. Mastic gum has been reported to possess considerable in vitro antibacterial and antifungal activities (32). A few years ago, it was specifically reported to be effective against *Helicobacter pylori* in vitro (7, 12, 21). However, in a more recent in vivo study of *H. pylori* infection, the activity of mastic gum was compared with antibiotic eradication schemes, and after a 7-day therapy no eradication of the bacterium from the stomachs of mice receiving mastic was observed (17). Finally, *H. pylori*-positive patients were treated with mastic capsules for 7 days, and they all remained *H. pylori* positive after the administration (6). The last two studies concluded that no “antibiotic-like” activity should be expected from crude mastic.

The crude resin that was used in all previous studies contained a high percentage (30%) of an insoluble and sticky polymer (poly- $\beta$ -myrcene) (33) that obviously hinders its oral administration and reduces the bioavailability of the contained active compounds. To bypass such problems, we prepared a

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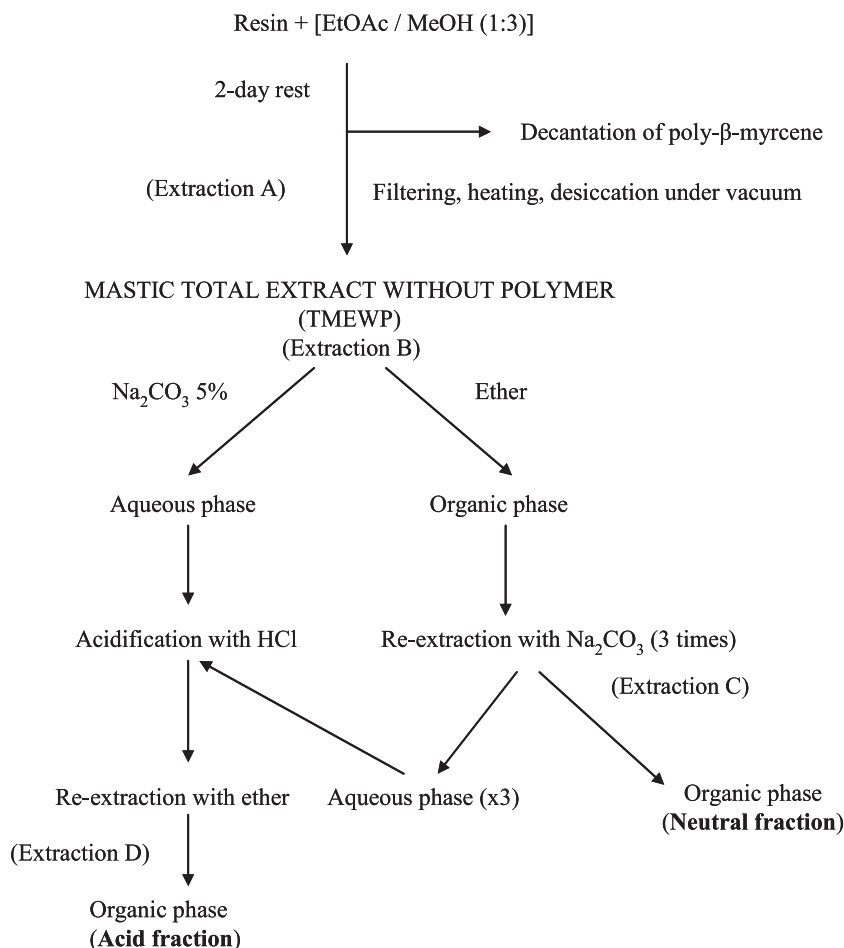


FIG. 1. Extraction of Chios mastic gum.

total mastic extract without polymer (TMEWP) and tested its activity against *H. pylori* in mice infected with the *H. pylori* SS1 strain. In addition, well-characterized mastic gum fractions as well as isolated pure compounds were tested *in vitro* against *H. pylori* in an effort to identify the most active constituents.

#### MATERIALS AND METHODS

**General procedures.** Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker DRX 400 and Bruker AC 200 spectrometers ( $^1\text{H}$  [400 and 200 MHz] and  $^{13}\text{C}$  [50 MHz]); chemical shifts are expressed in ppm downfield from tetramethyl silane. The  $^1\text{H}$ - $^1\text{H}$  and the  $^1\text{H}$ - $^{13}\text{C}$  NMR experiments (distortionless enhancement by polarization transfer, correlated spectroscopy [COSY], COSY for long-range couplings, heteronuclear multiple quantum coherence, heteronuclear multiple-bond correlation, and nuclear Overhauser effect spectroscopy [NOESY]) were performed using standard Bruker microprograms. Gas chromatography-mass spectrometry (MS) analysis was performed on a Finnigan GCQ Plus mass spectrometer. Electron impact and chemical ionization-MS spectra were determined on a Finnigan GCQ Plus mass spectrometer using  $\text{CH}_4$  as the chemical ionization reagent and high-resolution mass spectrometry on an AEI MS-90 spectrometer. Medium-pressure liquid chromatography (MPLC) was performed with a Büchi model 688 apparatus on columns containing Si gel (type 60; 20 to 40  $\mu\text{m}$ ; Merck).

**Extraction process.** Commercial mastic gum was supplied by the Chios Mastic Growers Association, which is the exclusive worldwide producer of the resin. A quantity of mastic gum (500 g) was diluted in ethyl acetate (500 ml), and then methanol (1,500 ml) was added (Fig. 1). The mixture was let stand, and after a period of 2 days, a layer of poly- $\beta$ -myrcene (150 g) was decanted. The clear

supernatant solution was obtained by filtration, and the solvent mixture was evaporated in a rotary evaporator at  $45^\circ\text{C}$  with an 80-kPa vacuum (extraction A). The resulting semisolid residue was dried in a desiccator at  $70^\circ\text{C}$  and 1,000-mbar vacuum and gave a white powder (350 g). The TMEWP was freely soluble in ethanol, which is feasible for the crude resin only under protracted heating. TMEWP was partitioned between aqueous 5%  $\text{Na}_2\text{CO}_3$  (1 liter) and ether (3.5 liter) as described by Barton (5) (extraction B). The organic phase was reextracted three times with 5%  $\text{Na}_2\text{CO}_3$  (1 liter each time) (extraction C) and afforded the neutral fraction of mastic (135 g) as the organic phase. The aqueous phase was added to that of extraction B and acidified with 1 N HCl (3 liters). The acidic solution was reextracted with ether (6 liters) (extraction D), and the organic phase afforded the acid fraction of mastic (190 g).

**Comparison of crude mastic gum and TMEWP chemical profile.** Crude mastic gum and the TMEWP were submitted to thin-layer chromatography over silica gel using a mixture of  $\text{CH}_2\text{Cl}_2$  and methanol (MeOH) (98/2) as the solvent system. The chromatographic zones were detected after spraying with a solution of vanillin and sulfuric acid in methanol. The chromatograms of both substances were identical except for poly- $\beta$ -myrcene, which was present only in the crude resin (data not shown).

**Isolation of pure triterpenic acids and neutral compounds.** A part of the acidic fraction (20 g) was submitted to MPLC over normal-phase silica gel first with a cyclohexane- $\text{CH}_2\text{Cl}_2$  gradient (from cyclohexane- $\text{CH}_2\text{Cl}_2$  at 50/50 to  $\text{CH}_2\text{Cl}_2$  at 100%) and then with a  $\text{CH}_2\text{Cl}_2$ -MeOH gradient (from  $\text{CH}_2\text{Cl}_2$  at 100% to  $\text{CH}_2\text{Cl}_2$ -MeOH at 80/20). The total solvent volume used was 17.3 liters. A total of 24 fractions were obtained, the separation of which was decided on the basis of thin-layer chromatography (with several systems used as solvents), and the chromatographic zones were detected as mentioned above (the same separation procedure was followed in all chromatographic separations mentioned below). Fraction 11 (1.252 g) was separated by MPLC over normal-phase silica gel with a  $\text{CH}_2\text{Cl}_2$ -MeOH gradient (from  $\text{CH}_2\text{Cl}_2$  at 100% to  $\text{CH}_2\text{Cl}_2$ -MeOH at 80/20;

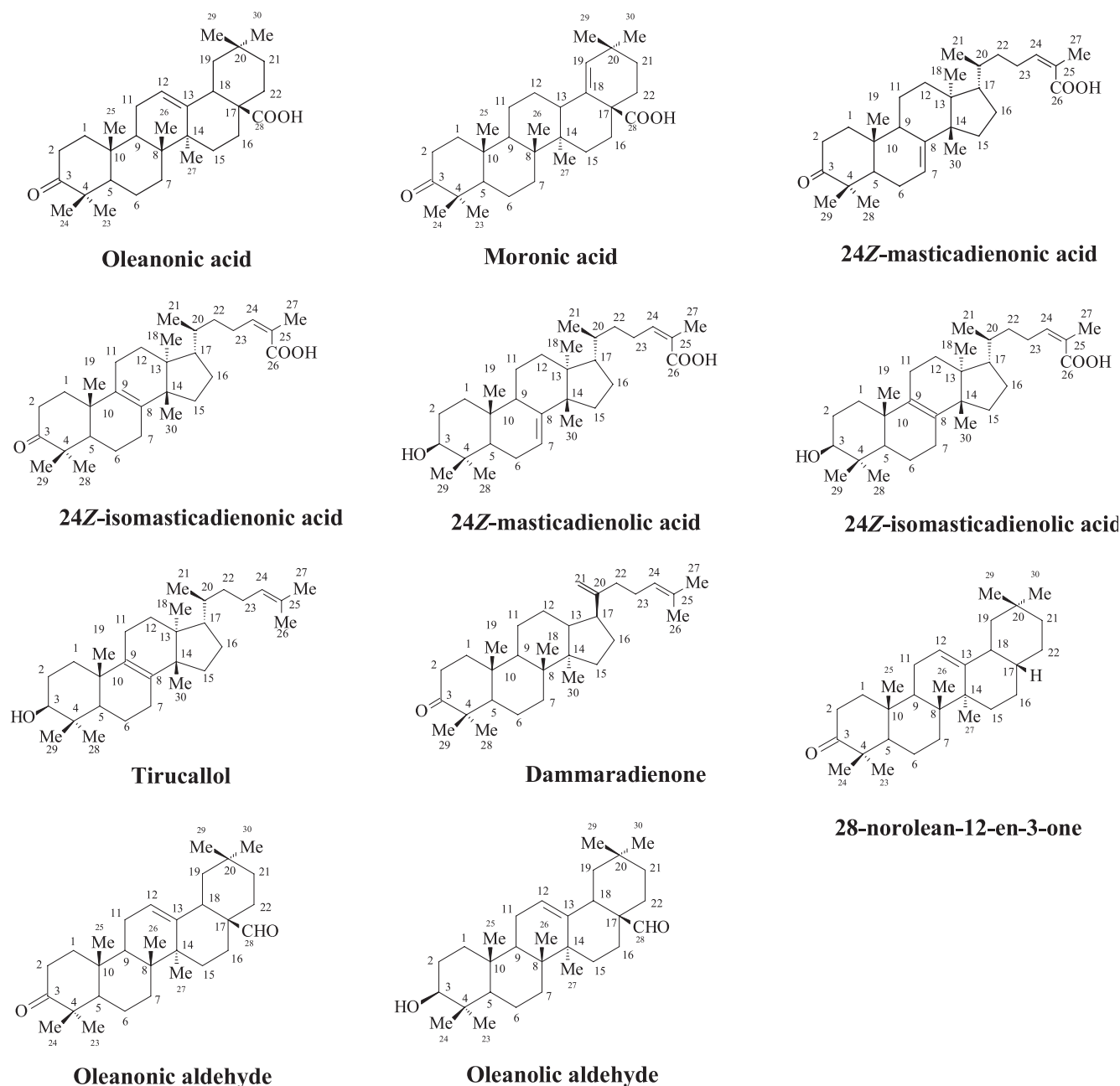


FIG. 2. Triterpenic compounds isolated from mastic acidic and neutral fractions.

total solvent volume used, 2.1 liters) and afforded oleanonic acid (515 mg) (29, 30) and moronic acid (338 mg) (13). Fraction 12 (2.431 g) was separated by MPLC over normal-phase silica gel eluted with a  $\text{CH}_2\text{Cl}_2$ -MeOH gradient (from  $\text{CH}_2\text{Cl}_2$  at 100% to  $\text{CH}_2\text{Cl}_2$ -MeOH at 80/20; total solvent volume used, 3.3 liters) and afforded 24Z-masticadienonic acid (1.1 g) (5, 8, 22) and 24Z-isomasticadienonic acid (1.0 g) (26). Fraction 17 (198 mg) was separated by column chromatography over silica gel eluted with a  $\text{CH}_2\text{Cl}_2$ -MeOH gradient (from  $\text{CH}_2\text{Cl}_2$ -MeOH from 99/1 to 80/20; total solvent volume used, 1.1 liter) and afforded 24Z-masticadienolic acid (85 mg) (9, 22) and 24Z-isomasticadienolic acid (92 mg) (24). The molecular structures are displayed in Fig. 2. All the above constituents were identified by one-dimensional (1D) and 2D NMR and MS and by comparison with data in the literature. Full NMR data for 24Z-isomasticadienonic acid, which have never been reported, are presented below.

**24Z-Isomasticadienonic acid.** White "powder," m.p. 166 to 167°C;  $[\alpha]_D^{25}$ : +34° (c 1.10, MeOH), EI-MS (*m/z*): 454 (34), 439 (100), 421 (62), 393 (13), 271 (11),

257 (19).  $^1\text{H-NMR}$  ( $\text{CDCl}_3$ , 400 MHz):  $\delta$  = 0.70 (3H, s, H-18), 0.82 (3H, s, H-30), 0.86 (3H, d,  $J$  = 5.8 Hz, H-21), 0.98 (3H, s, H-28), 1.02 (3H, s, H-19), 1.04 (3H, s, H-29), 1.08 (1H, H-22 $\alpha$ ), 1.19 (1H, H-15 $\alpha$ ), 1.38 (1H, H-20), 1.45 (1H, H-17), 1.46 (1H, H-22 $\beta$ ), 1.48 (1H, H-15 $\beta$ ), 1.57 (1H, H-1 $\alpha$ ), 1.64 (1H, H-5), 1.67 (2H, H-12), 1.80 (2H, H-6), 1.84 (3H, s, H-27), 1.88 (1H, H-16 $\alpha$ ), 1.90 (2H, H-11), 1.99 (1H, H-16 $\beta$ ), 1.93 (1H, H-1 $\beta$ ), 2.06 (2H, dd,  $J$  = 7 Hz, 14 Hz, H-7), 2.39 (1H, H-23 $\alpha$ ), 2.42 (1H, H-2 $\alpha$ ), 2.49 (1H, H-2 $\beta$ ), 2.51 (1H, H-23 $\beta$ ), 6.01 (1H, t,  $J$  = 7 Hz, H-24).  $^{13}\text{C-NMR}$  ( $\text{CDCl}_3$ , 100 MHz):  $\delta$  = 15.51 (C-18), 18.53 (C-21), 19.74 (C-27), 20.22 (C-6), 20.51 (C-19), 21.06 (C-28), 21.32 (C-16), 24.15 (C-30), 26.65 (C-29), 26.83 (C-23), 27.42 (C-7), 28.01 (C-11), 29.74 (C-15), 30.65 (C-12), 34.55 (C-2), 35.51 (C-1), 35.80 (C-22), 36.39 (C-20), 37.09 (C-10), 49.99 (C-13), 47.23 (C-4), 49.99 (C-14), 49.99 (C-17), 51.42 (C-5), 125.81 (C-25), 132.61 (C-9), 134.63 (C-8), 147.35 (C-24), 173.40 (C-26), 218.32 (C-3).

A part of the neutral fraction (17.2 g) was submitted to column liquid chromatography over normal-phase silica gel with a cyclohexane- $\text{CH}_2\text{Cl}_2$  gradient

(from cyclohexane at 100% to  $\text{CH}_2\text{Cl}_2$  at 100%) to afford 22 fractions. The total solvent volume used was 21 liters. Fraction 5 (839 mg) was separated by MPLC over normal-phase silica gel with a cyclohexane- $\text{CH}_2\text{Cl}_2$  gradient (from 90/10 to 100%  $\text{CH}_2\text{Cl}_2$ ; total volume used, 2 liters) and afforded tirucallol (110 mg) (23) and dammaradienone (128 mg) (20). Fraction 8 (533 mg) was separated by MPLC over normal-phase silica gel eluted with a cyclohexane- $\text{CH}_2\text{Cl}_2$  gradient (from 80/20 to 100%  $\text{CH}_2\text{Cl}_2$ ; total volume used, 1.5 liters) and afforded 28-norolean-12-en-3-one (206 mg) (20). Fraction 10 (396 mg) was separated by MPLC over normal-phase silica gel eluted with a cyclohexane- $\text{CH}_2\text{Cl}_2$  gradient (from 80/20 to 100%  $\text{CH}_2\text{Cl}_2$ ; total volume used, 1.4 liters) and afforded oleanonic aldehyde (152 mg) (25) and oleanolic aldehyde (98 g) (3). The molecular structures are displayed in Fig. 2. All the above constituents were identified by 1D and 2D NMR and MS and by comparison with data in the published literature.

The neutral fraction was also submitted to analytical separation by gas chromatography-MS. Comparison of mass spectra with MS data library Wiley 275.1 and data in the literature (4) resulted in the identification of the five compounds mentioned above as the major neutral ones, while several minor diterpenes or triterpenes with aldehyde, ketone, or hydroxyl groups were detected on the basis of molecular weight but not thoroughly identified.

**In vitro test of mastic total extract, fractions, and pure compounds.** Minimum bactericidal concentrations (MBC) were evaluated utilizing *H. pylori* reference strain CCUG 38771 and another 10 clinical strains belonging to the Hellenic Pasteur Institute collection, isolated from gastric antrum biopsies from patients suffering from gastritis or duodenal or gastric ulcer (LAVHP-1 to LAVHP-10). *H. pylori* isolates were stored in brain heart infusion broth (BHIB) supplemented with 20% glycerol at  $-80^\circ\text{C}$ . All strains prior to use were cultured twice under microaerophilic conditions (CampyPak Plus; Becton-Dickinson, Cockeysville, MD) for 24 h at  $37^\circ\text{C}$ , in Chalgren's-Wilkins (CHW) agar plates supplemented with 7% (vol/vol) horse blood and 1% (vol/vol) Vitox (Oxoid, Basingstoke, United Kingdom). Liquid cultures of *H. pylori* bacteria to a density of  $10^9$  CFU/ml were prepared by suspension in BHIB (Oxoid) supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) and 0.25% yeast extract (Oxoid). Successive twofold dilutions of each mastic extract or pure compound in BHIB medium were placed in sterilized 96-well flat-bottom microplates (Sarstedt, Numbrecht, Germany) within a total volume of 100  $\mu\text{l}$ . All extracts were tested at a final concentration range of 0.049 to 1.560 mg/ml, with the exception of the acidic fraction, for which successive twofold dilutions ranged from 0.060 to 1.920 mg/ml. To each well containing the mastic dilutions, approximately  $10^7$  CFU of *H. pylori* bacteria were added within a 100- $\mu\text{l}$  volume, and the microplates were incubated at  $37^\circ\text{C}$  for 24 h with continuous agitation under microaerophilic conditions. Thereafter, viability of *H. pylori* was evaluated by determination of viable CFU in CHW agar plates following incubation at  $37^\circ\text{C}$  for 48 h under microaerophilic conditions. The MBC was defined as the lowest concentration of mastic extract or pure compound that killed at least 99.9% of the CFU contained in the original inoculum. The mean MBC for each mastic preparation was determined as the average of three independent experiments.

**Infection of mice with *H. pylori* strain SS1.** Specific-pathogen-free 6- to 8-week-old female C57BL/6 mice were obtained from the Central Animal Facility of the Hellenic Pasteur Institute. They were housed according to relevant Greek national legislation, fed a commercial diet, and given water ad libitum, except as otherwise stated. *H. pylori* infections by the SS1 strain were carried out as described before (27, 28). Briefly, freshly prepared aliquots (100  $\mu\text{l}$ ;  $10^8$  CFU) of *H. pylori* strain SS1 in BHIB (Oxoid) were administered to mice via orogastric inoculation, three times within a week. Accordingly, all noninfected control mice were inoculated with the same volume of plain BHIB.

**Administration of TMEWP in vivo.** TMEWP was diluted into ethanol at a concentration of 180 mg/ml and then dissolved into a final aqueous solution of 180  $\mu\text{g}/\text{ml}$ . The extract was administered through the animals' drinking water, starting 1 month following initial *H. pylori* infection and for 3 more months. The following groups of animals were included in the study: *H. pylori*-infected mice administered TMEWP (SMH;  $n = 10$ ); uninfected mice administered TMEWP (SM;  $n = 10$ ); and *H. pylori*-infected mice left untreated (SH;  $n = 10$ ) as a control group. Animal weight was monitored throughout the whole observation period as a measure of health of the animals. Daily water consumption was measured, and a mean daily TMEWP consumption per animal was calculated to be 0.75 mg throughout the whole administration period. In addition to the above therapeutic in vivo protocol, we conducted a preliminary prophylactic study where C57BL/6 mice ( $n = 5$ ) were administered TMEWP for a week prior to *H. pylori* infection, and we assessed *H. pylori* colonization 2 weeks after the initial infection.

**Assessment of *H. pylori* colonization levels.** At end of the 3-month observation period, blood samples were collected via the tail vein and animals were sacrificed by cervical dislocation. Excised stomachs were dissected along the lesser curva-

ture, and *H. pylori* detection in the gastric tissue was accomplished by *H. pylori* quantitative culture and PCR. For *H. pylori* SS1 quantitative culturing, pre-weighed half-stomach samples were homogenized in thioglycolate medium (Oxoid), serially diluted in phosphate-buffered saline, and plated on CHW agar plates with antibiotics (vancomycin, 10  $\mu\text{g}/\text{ml}$ ; trimethoprim, 10  $\mu\text{g}/\text{ml}$ ; polymyxin B,  $10^4$  IU/liter; amphotericin B, 2  $\mu\text{g}/\text{ml}$ ; nalidixic acid, 10  $\mu\text{g}/\text{ml}$ ; bacitracin, 30  $\mu\text{g}/\text{ml}$ ; fluorocytosine, 5  $\mu\text{g}/\text{ml}$ ; all from Sigma, St. Louis, Mo.). The cultures were incubated under microaerophilic conditions at  $37^\circ\text{C}$  for up to 6 days. *H. pylori* colonies were visualized on the basis of urease activity, and results were expressed as log CFU per gram of gastric tissue. The minimum bacterial density detected by this method was 100 CFU per gram. Qualitative *H. pylori* detection in the gastric samples was performed by *H. pylori*-specific PCR utilizing primers for the *ureC* (*glmM*) gene as described before (18). Genomic DNA for the detection of *H. pylori* by PCR in tissue samples or bacterial colonies was isolated by using a DNeasy tissue kit (QIAGEN).

**Histopathologic analysis of gastric tissue samples.** Excised stomachs were opened along the lesser curvature, and the longitudinal half was fixed in 10% neutral buffered formalin solution, embedded in paraffin, and processed for histopathologic analysis. Antral, body, and cardioesophageal mucosa samples were examined in the same section. Eleven serial longitudinal 4- $\mu\text{m}$  sections were cut from each specimen; 9 of them were stained with hematoxylin-eosin for evaluation of gastric inflammation, and 2 were stained by the May-Grünwald Giemsa stain method for the assessment of *H. pylori* colonization. The bacterial density and the pathological characteristics of the gastric mucosa were assessed according to the updated Sydney system (11). Histopathologic evaluation was performed with no prior knowledge of the identity of the samples by the histopathologist.

**Determination of serum anti-*H. pylori* immunoglobulin G levels.** Immunoglobulin G (IgG) levels of anti-*H. pylori* antibodies were detected in the serum samples collected by an in-house enzyme-linked immunosorbent assay method. Briefly, 15  $\mu\text{g}$  of *H. pylori* SS1 antigen produced by sonication and subsequent dialysis (SpectraPor; cutoff pore size, 8 kDa) was used to coat 96-well plates. Collected mouse serum samples (diluted 1/50) were primarily incubated in the plates for 24 h at  $4^\circ\text{C}$ , and then rabbit anti-mouse IgG (whole molecule)-peroxidase conjugate (Sigma) was used for the secondary incubation (2 h at  $37^\circ\text{C}$ ). Color was developed by addition of *o*-phenylenediamine (Sigma), and optical density at 492 nm was measured in a Sunrise microtiter plate reader (Tekan).

**Statistical analysis.** Analysis of the results from the in vivo experiments was performed with respect to *H. pylori* colonization by two-tailed unpaired *t* test with Welch correction and with respect to the associated gastritis by the Wilcoxon rank sum test due to the ordinal nature of the data. A *P* value of  $<0.05$  was considered significant in both tests.

## RESULTS

**Isolation of TMEWP, acid and neutral fractions, and triterpenic compounds.** The TMEWP was obtained from crude mastic gum in a 70% proportion using a modification of the method described by Barton (5). Ether was replaced by ethyl acetate, a more convenient and nonexplosive solvent, in order to minimize the risk when manipulating large quantities of resin.

The TMEWP was further divided into two fractions, an acidic and a neutral one. The acidic fraction of TMEWP after several chromatographic separations afforded the major triterpenic acids (Fig. 2) oleanonic acid (515 mg), moronic acid (338 mg), 24Z-masticadienonic acid (1.1 g), 24Z-isomasticadienonic acid (1.0 g), 24Z-masticadienolic acid (95 mg), and 24Z-isomasticadienolic acid (102 mg). The neutral fraction, after similar treatment, afforded five neutral triterpenic compounds: tirucallol (110 mg), dammaradienone (128 mg), 28-norolean-12-en-3-one (206 mg), oleanonic aldehyde (152 mg), and oleanolic aldehyde (98 mg).

All the above constituents were identified by NMR ( $^1\text{H}$ ,  $^{13}\text{C}$ , distortionless enhancement by polarization transfer, COSY, heteronuclear multiple quantum coherence, heteronuclear multiple-bond correlation, and NOESY) and MS and by com-



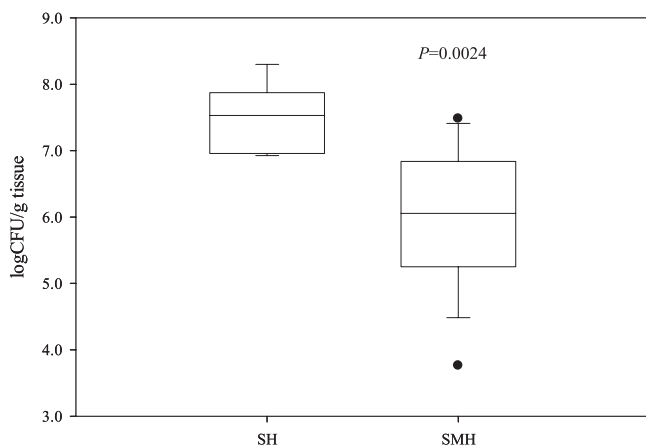


FIG. 3. *H. pylori* colonization in *H. pylori*-infected mice following continuous administration of total mastic extract without the polymer (SMH;  $n = 10$  animals) or left untreated (SH;  $n = 9$  animals). Viable *H. pylori* counts are expressed in log CFU/g gastric tissue. A moderate 1.5-log reduction in *H. pylori* colonization was observed ( $P = 0.0024$ ) in the SMH study group compared to the SH control group.

parison with data in the literature. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for 24Z-isomasticdienonic acid have never been reported, and the stereochemistry of the double bond at position 24 of masticdienonic acid, isomasticdienonic acid, masticdienonic acid, and isomasticdienonic acid has never been studied. In all cases, the NOESY correlation of Me-27 with the double bond proton H-24 confirmed the stereochemistry as Z. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of 24Z-isomasticdienonic acid are reported herein for the first time.

**In vivo activity of mastic TMEWP against *H. pylori* infection in vivo.** During the course of the experiment, none of the participating mice died and no statistical difference was observed between the three groups with regard to animal weight gain (data not shown). Mean water consumption was approximately the same for each animal group and ranged from 4.2 to 4.5 ( $\pm 0.1$  [standard error of the mean]) ml, regardless of the presence of TMEWP. Average extract consumption was the same for both SM and SMH groups, calculated at 0.75 mg per mouse and taking into account mean water consumption volumes. In the control uninfected group, SM, absence of *H. pylori* infection was confirmed by PCR, serology, or *H. pylori* culture of the gastric samples. In the untreated *H. pylori*-infected SH group, the presence of *H. pylori* was confirmed by PCR and quantitative culturing in 9 out of 10 animals. In the *H. pylori*-negative animal, further analysis of serum anti-*H. pylori* IgG antibodies confirmed the absence of infection and, therefore, it was excluded from the study. The rest of the animals were positive for the presence of *H. pylori* by PCR, and the mean *H. pylori* colonization was calculated at  $7.5 \pm 0.18$  log CFU/g of tissue. In the TMEWP-treated *H. pylori*-infected SMH group, the presence of *H. pylori* was confirmed by PCR in all the animals, and the mean *H. pylori* colonization was calculated at  $6.0 \pm 0.35$  log CFU/g of gastric tissue. A statistically significant reduction in *H. pylori* viable counts was calculated between the two animal groups ( $P = 0.0024$ ) (Fig. 3). These results were also consistent with the histopathologic observations, showing reduced colonization of the bacterium in the gastric antrum

TABLE 1. *H. pylori* colonization in *H. pylori*-infected mice treated with TMEWP

Location	Group	No. of mice of grade <sup>a</sup> :				$P^b$
		0	1	2	3	
Antrum	SH	1	3	3	2	0.031
	SMH	3	6	1	0	
Corpus	SH	5	4	1	0	0.038
	SMH	10	0	0	0	

<sup>a</sup> Colonization grades are according to the updated Sydney system (11), as follows: normal, 0; mild, 1; moderate, 2; marked, 3.

<sup>b</sup> Statistical analysis with reference to the SH control group, done by Wilcoxon rank sum test. Both correlations shown were significant.

and corpus in the TMEWP-treated SMH group ( $P = 0.031$  for antrum,  $P = 0.037$  for corpus) (Table 1).

*H. pylori*-specific IgG antibodies are the dominant antibody class present in the sera of chronically *H. pylori*-infected mice and may serve as an indicator of successful *H. pylori* infection. A significant difference in anti-*H. pylori* IgG antibody titers was observed between *H. pylori*-infected (groups SH and SMH) and noninfected control mice (SM). However, no statistical difference was detected between the TMEWP-treated SMH group and the untreated *H. pylori*-infected SH group with regard to serum anti-*H. pylori* IgG levels (Fig. 4).

Histopathologic evaluation of the gastric mucosa revealed a mild induction of chronic gastritis in the antrum and the corpus fundus in the animals of the untreated *H. pylori*-infected SH group (Tables 2 and 3). Gastric samples were characterized by mild infiltration of the lamina propria with scattered lymphocytes and neutrophils. Chronic inflammatory infiltration in the antrum was mild in eight animals and moderate in one animal. In the corpus, only six animals developed mild chronic inflammatory infiltration (Table 2). The activity of chronic gastritis that developed was mild to moderate (mild in six animals, moderate in two animals) (Table 3) in the antrum and milder in the corpus (normal in seven animals, mild in two animals)

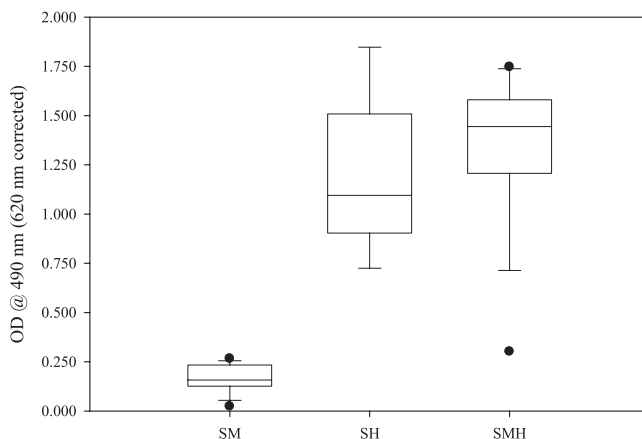


FIG. 4. Serum anti-*H. pylori* IgG antibody response in *H. pylori*-infected animals treated with total mastic extract without the polymer (SMH) or left untreated (SH). Uninfected control mice that received total mastic extract without the polymer are also depicted (SM). Mouse sera were diluted to 1:50. No difference in anti-*H. pylori* titers was observed at 12 weeks postinfection between the SMH and SH groups.

TABLE 2. Chronic inflammatory infiltration<sup>a</sup> in *H. pylori*-infected mice treated with TMEWP

Location	Group	No. of mice of grade <sup>b</sup> :			P <sup>c</sup>
		0	1	2 <sup>a</sup>	
Antrum	SH	0	8	1	0.713
	SMH	0	8	2	
Corpus	SH	3	6	0	0.569
	SMH	7	3	0	

<sup>a</sup> Lymphocyte infiltration.<sup>b</sup> Histopathology grades are according to the updated Sydney system (11) as follows: normal, 0; mild, 1; moderate, 2.<sup>c</sup> Statistical analysis with reference to the SH control group, done by Wilcoxon rank sum test.

(Table 3). No development of glandular atrophy or intestinal metaplasia was observed, as the time interval from the onset of infection was too short. In the extract-treated *H. pylori*-infected SMH group, the development of chronic gastritis was similar to the SH group (Tables 2 and 3), with marginally reduced numbers of neutrophils infiltrating the lamina propria in some cases. However, statistical analysis with regards to chronic inflammatory infiltration or the development of chronic active gastritis revealed no significant differences between the two animal groups. The collected data from the animal studies suggested that continuous administration of 0.75 mg TMEWP to *H. pylori*-infected mice may moderately reduce *H. pylori* colonizing numbers without a profound effect on the associated gastritis. However, prophylactic administration of TMEWP did not prevent *H. pylori* colonization (data not shown).

**In vitro activity of mastic total extract, fractions, and compounds against *H. pylori* strain SS1.** Having observed a moderate antimicrobial effect in vivo against *H. pylori*, we proceeded to investigate the potential in vitro anti-*H. pylori* activity of TMEWP and its acidic and neutral fractions against a panel of 10 clinical isolates of *H. pylori* and the CCUG 38771 reference strain (Table 4). Figure 5 depicts characteristic kill curves for strains LAVHP-6 (one of the least susceptible strains) and LAVHP-7 (the most susceptible strain). Mastic extracts exhibited concentration- and strain-dependent bactericidal activities. More specifically, in all strains tested, without exception the acidic fraction exhibited the highest activity, with a mean MBC of 0.136 mg/ml, followed by the TMEWP (MBC, 0.256 mg/ml). Reduced activity was observed for the neutral fraction of the TMEWP (0.638 mg/ml). Up to twofold differences were observed in the MBC between individual strains tested, and

TABLE 3. Activity of chronic gastritis<sup>a</sup> in *H. pylori*-infected mice treated with TMEWP

Location	Group	No. of mice of grade <sup>b</sup> :			P <sup>c</sup>
		0	1	2 <sup>a</sup>	
Antrum	SH	1	6	2	0.369
	SMH	5	2	3	
Corpus	SH	7	2	0	0.967
	SMH	8	1	1	

<sup>a</sup> Neutrophil infiltration.<sup>b</sup> Histopathology grades are according to the updated Sydney system (11) as follows: normal, 0; mild, 1; moderate, 2.<sup>c</sup> Statistical analysis with reference to the SH control group, done by Wilcoxon rank sum test.TABLE 4. Minimum bactericidal concentrations of mastic extracts<sup>a</sup> on *H. pylori* strains

Strain	MBC (mg/ml)		
	Total extract	Acidic fraction	Neutral fraction
CCUG 38771	0.390	0.240	1.560
LAVHP-1	0.195	0.120	0.390
LAVHP-2	0.195	0.120	0.780
LAVHP-3	0.195	0.120	0.780
LAVHP-4	0.390	0.120	0.780
LAVHP-5	0.195	0.120	0.390
LAVHP-6	0.390	0.120	0.780
LAVHP-7	0.090	0.060	0.390
LAVHP-8	0.390	0.120	0.390
LAVHP-9	0.195	0.240	0.390
LAVHP-10	0.195	0.120	0.390

<sup>a</sup> With the exception of the acidic fraction, for which successive twofold dilutions ranged from 0.060 to 1.920 mg/ml, all other extracts were tested at a final concentration range of 0.049 to 1.560 mg/ml.

only in the case of LAVHP-7 strain was a higher susceptibility against the TMEWP and its acidic fraction observed.

Having obtained the highest activity with the acidic fraction of the TMEWP, we proceeded to test the isolated pure acidic compounds for anti-*Helicobacter* activity. Highest overall activity was obtained consistently and for all 11 of the *H. pylori* strains tested with isomasticdienolic acid, with a mean MBC of 0.202 mg/ml (0.443 mM), followed by masticdienolic (0.220 mg/ml [0.482 mM]), oleanonic (0.292 mg/ml [0.643 mM]), and moronic acid (0.310 mg/ml [0.683 mM]) (Table 5). Interestingly, the 3-oxo derivatives, isomasticdienonic and masticdienonic acids, showed reduced activity compared to the corresponding 3-hydroxyl derivatives.

## DISCUSSION

All previous in vivo studies evaluating activity of mastic gum against *H. pylori* have used a crude mastic preparation which contained a high percentage (30%) of an insoluble and sticky polymer. We speculated that the presence of the polymer hindered potential in vivo activity of mastic during oral administration, and for this reason we prepared TMEWP. We have verified that the chemical consistency of TMEWP was virtually identical to that of crude mastic gum, except for the absence of the polymer, and it also presented better solubility properties and increased concentration of active constituents. Previous animal studies for the determination of the potential anti-*Helicobacter* activity of mastic gum were organized on a short-term administration schedule. However, we have extended our administration and hence tested the TMEWP activity over a period of 3 months.

In the present study we utilized an established *H. pylori* infection model to evaluate the potential therapeutic effect of continuous TMEWP administration on *H. pylori* colonization and development of associated gastritis. This model involves the mouse-adapted *H. pylori* Sydney strain 1 (SS1), which colonizes the C57BL/6 mouse heavily and leads to the development of appreciable levels of gastritis closely mimicking human *H. pylori* infection (16, 19, 31). The particular infection model

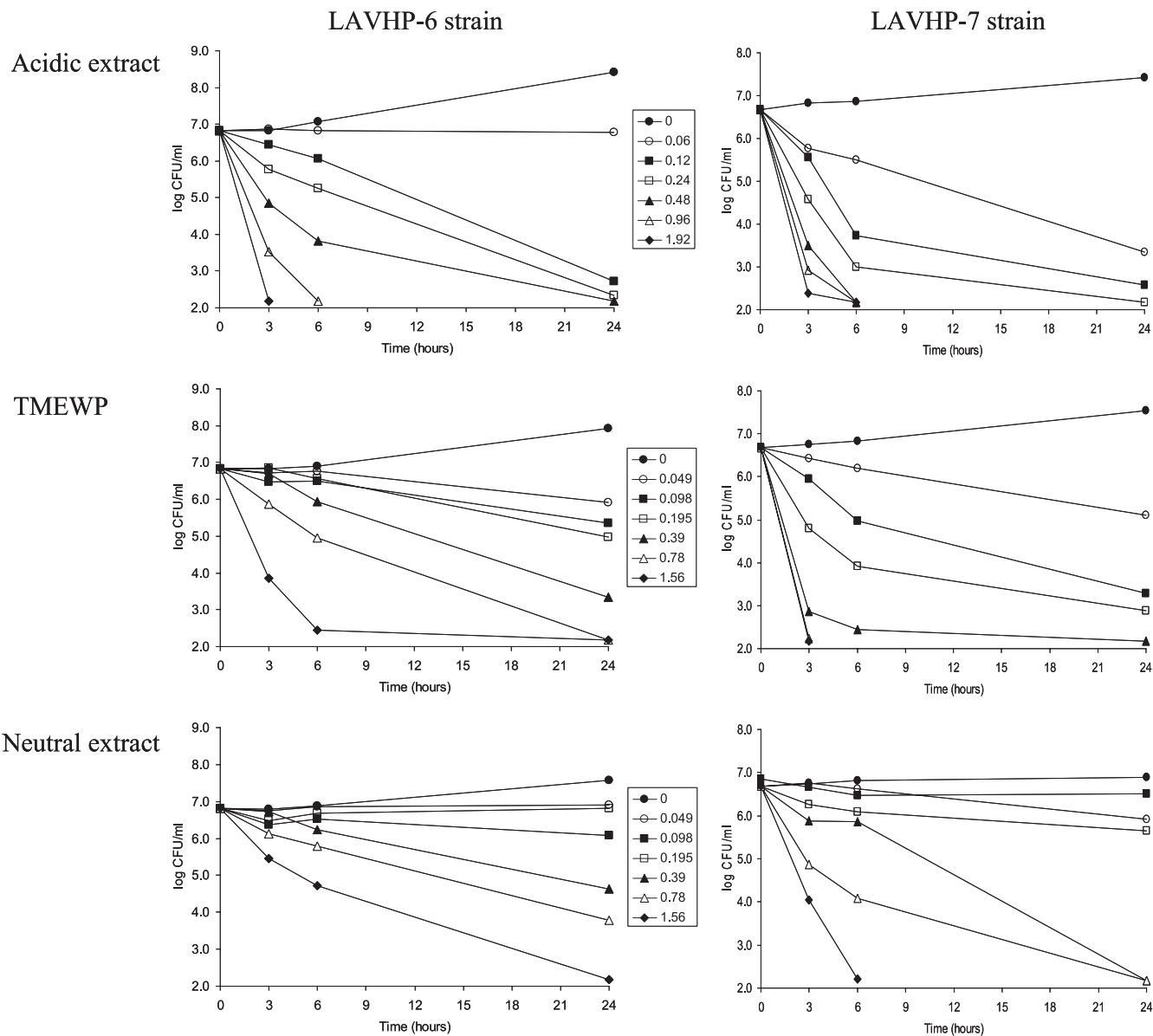


FIG. 5. Bactericidal activity of mastic gum extracts against *H. pylori* in a liquid medium. *H. pylori* strains LAVHP-6 (more resistant strain) and LAVHP-7 (most susceptible strain) were cultured under microaerophilic conditions in BHI as described in Materials and Methods and exposed to acidic, TMEWP, and neutral fractions at the concentrations depicted in the legend. After further incubation, viability was determined at each time point.

TABLE 5. Mean MBC of mastic triterpenic acid compounds within the mastic acidic fraction against *H. pylori*<sup>a</sup>

Substance	Mean MBC in mg/ml (mM)
24Z-Isomasticadienonic acid.....	0.333 (0.733)
24Z-Masticadienonic acid .....	0.350 (0.770)
24Z-Isomasticadienolic acid.....	0.202 (0.443)
24Z-Masticadienolic acid.....	0.220 (0.482)
Oleanonic acid.....	0.292 (0.643)
Moronic acid.....	0.310 (0.683)

<sup>a</sup> *H. pylori* strains are described in Table 4.

has been successfully utilized in the past to evaluate potential anti-*H. pylori* activity of a number of agents, such as antibiotics (14, 15) or lactic acid-producing bacteria (27, 28).

Our experiments showed that the mastic total extract could moderately reduce *H. pylori* colonization in the antrum and corpus of the stomach. The reduction in colonization levels calculated was approximately 30-fold, in the range of 1.5 log CFU/g of tissue. These results were in concurrence with the visible reduction in *H. pylori* colonization observed in the histopathology evaluations. However, such a moderate fall in *H. pylori* colonizing numbers could not support any attenuation of the *H. pylori*-associated chronic active gastritis. We have documented no such decline in either neutrophilic or lymphocytic

infiltration within the lamina propria. Equally, no reduction was observed with reference to serum anti-*H. pylori* IgG antibodies between the mastic-treated and nontreated animal groups. Collectively, these results are in line with earlier observations involving the same infection model, in which 7-day monotherapy with crude mastic tear diluted in 100% ethanol failed to eradicate *H. pylori* (17). Nevertheless, we were able to document a moderate drop in colonization levels possibly by increasing the bioavailability of the active mastic constituents, following polymer removal. Another main advantage of our study was the application of a longer period of administration and observation. In this way we were able to administer 0.75 mg of TMEWP (the approximate equivalent of 1 mg total mastic gum) continuously through the animal water supply, for a period of 3 months, as opposed to only 1-week therapy regimens reported in other studies. Our mode of administration mimicked more closely real life conditions, where active mastic constituents can be released in a sustained release mode following daily consumption of mastic gum. Although 3 months proved too short a period for the mastic total extract to eradicate *H. pylori*, results suggest that in real life, habitual daily consumption of mastic gum over a much longer period may well create conditions favoring a decrease in *H. pylori* colonization levels. However, we observed no prophylactic activity of TMEWP on *H. pylori* infection, although our observations depend on a small number of animals. Equally, to date no epidemiological data exist to support such a prophylactic effect among the mastic gum-consuming population of Chios Island.

A detailed in vitro investigation was performed in order to identify the potentially most active fractions in mastic extracts. The TMEWP was further separated into an acidic and a neutral fraction, and the antimicrobial activities of these fractions, as well as that of mastic total extract, were determined. We observed a moderate activity for the total mastic extract against a panel of 11 *H. pylori* strains at a mean MBC of 0.256 mg/ml, which is higher than that previously reported against the *H. pylori* SS1 strain for mastic gum preparations diluted in ethanol (17). This could well be attributed to differences in methodology and the differential susceptibility of diverse *H. pylori* isolates, as our in vitro data clearly demonstrated. The acidic fraction of the TMEWP was found to be the most active, with an MBC as low as 0.136 mg/ml, consistently in all strains tested. In order to identify the most active constituent among the contained triterpenic acids, the acid fraction was further submitted to several chromatographic separations that afforded the six major constituents. They were all tested against the same *H. pylori* strains as mastic extracts, and the most active one was found to be isomasticdienolic acid. None of the pure compounds was more active than the whole acid fraction, suggesting that, although the anti-*H. pylori* activity of mastic could be located particularly in the acidic fraction, probably the final activity is a result of synergy between all the acid constituents. Although it is the first case in which specific mastic acid triterpenic compounds have been shown to exhibit anti-*H. pylori* activity, other tetracyclic acids similar to those contained in mastic have been reported to be active against *Escherichia coli* (35). In our study, however, due to limitations in the isolation and purification steps, we were not able to test the potential

antimicrobial in vivo activity of individual triterpenic compounds.

Emerging antibiotic resistance and reduced patient compliance are the main reasons for failed *H. pylori* eradication therapy. In addition, the considerable expense of the antibiotic regimen coadministered with a proton pump inhibitor creates the need for alternative antibiotics for combination therapy. The present study demonstrated that a mastic gum extract without the polymer constituent poly- $\beta$ -myrcene was effective in reducing *H. pylori* colonization levels by 30-fold in infected mice over an administration period of 3 months and that the activity could be attributed to triterpenic acids within the acid fraction of mastic extracts. These results suggest that removal of the constituent poly- $\beta$ -myrcene polymer can produce an enhanced therapeutic moiety which may exhibit anti-*H. pylori* activity, detectable over a shorter time period, bearing all the advantages of a nutritional product. We plan to evaluate further the true therapeutic potential of the acidic and neutral fractions of mastic gum in extensive animal studies following administration of the respective triterpenic constituents, alone and in combination, to assess synergic effects. The role of such mastic constituents will also be assessed with reference to potential synergy with currently used antibiotic eradication schemes.

Finally, our results also suggest that habitual long-term mastic consumption may be effective in moderating *H. pylori* colonization, although to date no epidemiological data exist to support the hypothesis of a reduced prevalence of *H. pylori* infection among the mastic gum-consuming population of Chios Island.

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