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Original article

Anti-*Helicobacter pylori* activity of Egyptian medicinal plants and bacteriophages

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A R T I C L E   I N F O

A B S T R A C T

Background: *Helicobacter pylori* (*H.pylori*) is the most common cause of gastric infections worldwide. Due to antibiotic resistance and adverse effects, phytotherapy and phage therapy have been a research focus as an alternative therapy for *H. pylori* infection. **Objectives:** To assess the medicinal plant extracts and bacteriophages as a treatment of *H. pylori* infection. **Methodology:** Thirty-five gastric biopsies were cultured for *H. pylori* isolation. Screening of medicinal plants extract efficiency was done by Disc diffusion method. Minimum inhibitory concentrations of extracts were assessed. In vivo effect of Punica granatum peel extract was tested by bacterial density and histopathology in rats. Sewage water samples were screened for *H. pylori* specific bacteriophages. Single plaque isolation technique was used for phage purification. **Results:** Ten out of 35 (28.57%) patients had positive gastric biopsy for *H. pylori* by culture. Four out of 10 (40%) isolates were resistant to all antibiotics. Inhibitory effect of Rosemarinus officinalis, Syzygium aromaticum, Rhus coriaria and Ammi visagna on *H. pylori* was detected. Punica granatum extract was the most efficient in vitro. In vivo, Punica granatum peel extract caused significant reduction of bacterial density (*P<0.05*) and enhanced ulcer healing. Sewage water filtrates contained 3 types of *H. pylori* specific bacteriophages. During phage purification, phage infectivity was lost. **Conclusions:** Punica granatum peel extract revealed better in vivo activity against *H. pylori* than standard regimen antimicrobials. Other effective plants can be beneficial in *H. pylori* infection management. Loss of bacteriophage infectivity may be an obstacle to phage therapy of *H. pylori*.

Introduction

Helicobacter pylori (*H. pylori*) is a common bacterial pathogen possessing many virulence factors and proved to be associated with gastritis and peptic ulcer disease. Moreover, *H. pylori* is categorized as class I carcinogen by the International Agency for Research on Cancer (IARC) since chronic mucosal inflammation and atrophy leads to malignant transformation [1]. In addition, *H. pylori* infection is linked to several non-gastric diseases such as type 2 diabetes, ischemic cardiovascular and cerebrovascular diseases [2].

The prevalence of *H. pylori* infection shows considerable variation among countries, being lower in developed countries than developing countries. Infection prevalence reaches up to 72%, 90% of pediatric and adult population, respectively in Egypt. Such high prevalence in indigenous people is due to poor personal, food and water sanitation [3].

H. pylori is susceptible to a limited number of antimicrobials. Adding to this, the development of antimicrobial resistance is a major concern for *H. pylori* eradication. Several regimens were developed for treatment of *H. pylori* infections. Eradication failure using 1st line clarithromycin based triple regimen is increasing. Different salvage regimens comprising levofloxacin and rifabutin are being used [4]. Several countries reported high prevalence of levofloxacin resistance that doesn't permit its empiric use as salvage therapy. Additionally, failure of permanent cure caused either by resistance or reinfection is extremely common particularly in developing nations. It is obvious that the contemporary therapeutic options are inadequate, and that innovative and alternative non-antibiotic treatments or prophylactic measures are needed [5].

Phage based therapy was developed in the pre-antibiotic era by Felix d'Herelle in ex-Soviet Union, but the development of antibiotics halted the progression of this field. Bacteriophages have been a research focus as one of the possible alternative therapy for different bacteria, regardless of the existence of antibiotic resistance [6]. Using obligate lytic bacteriophage or purified phage lytic proteins for treatment of bacterial infections has been extensively and eagerly embraced as a research topic in Western countries [7]. Phage therapy possess great advantage missed in other types of biological and pharmacological therapies that in the presence of host bacteria, they are able to increase its numbers (self-amplification) by infecting the bacteria and producing virion progeny whilst minimally affecting the overall microbiota and body tissues. The advantages of phage therapy disclose the shortcoming of antibiotic. The time to substitute antibiotics by phage therapy is imminent [8].

Phytotherapy (herbal therapy or botanical therapy), entails the use of plants or plant extracts for medicinal purpose. Phytotherapy using herbs is as old as human civilization that was gradually side lined by synthetic drugs. Using plant derivatives as antimicrobials has been almost nil since the discovery of antibiotics in the 1950s till the 2000s. [9].

Owing to the increasing resistance of microorganisms to different antimicrobials more and more studies are directed for using bacteriophage and herbs as alternative therapy for infectious diseases including *H. pylori*.

This study aimed to assess the effect of isolated bacteriophages and Egyptian medicinal plants extracts on *H. pylori* that may be useful for treating *H. pylori* gastric infection.

Methodology

Study design

Thirty-five adult patients submitted to upper gastrointestinal endoscopic investigation for dyspeptic manifestation in endoscopy unit, Zagazig university hospital were enrolled. Participants who received antimicrobial therapy, H2-receptor blockers, and proton-pump inhibitors within 30 days before endoscopy were excluded. Written informed consents were obtained for agreement of participation. This study was carried out in accordance to The Code of Ethics of the World Medical Association (Declaration of Helsinki). *In vivo* study in laboratory animals had complied with the ARRIVE animal guidelines.

Isolation, identification and antimicrobial susceptibility test of *H. pylori*

From each participant enrolled, three gastric biopsies were obtained, (two biopsies from the antrum and one from the fundus). Specimens were then transported to the laboratory in 200µl sterile brain heart infusion broth (BHIB) (Oxoid, UK) supplemented with 10% fetal bovine serum (Sigma-Aldrich, Germany) in sterile screw capped tubes immediately. Specimens were homogenized and inoculated on brain heart infusion agar (BHIA) (Oxoid, UK) plates supplemented with 10% fetal bovine serum and *H. pylori* selective supplement (Dent) (Oxoid, UK). The plates were incubated for 3-7 days at 37°C in an anaerobic jar (Oxoid, UK) under microaerophilic conditions using CampyGen (CN0025) (Oxoid, UK). Negative cultures were incubated for up to 14 days that may be needed for initial isolation. Identification of isolated *H. pylori* was done by Gram stained film, urease, catalase and oxidase tests [9]. Isolates were named as HZ (H is named after for *H. pylori*; Z is named after for Zagazig) followed by sample number. Stock cultures

were stored in BHIB supplemented with 10% fetal bovine serum (Sigma-Aldrich, Germany) and glycerol (20% v/v) in -20 ºC freezer.

Antimicrobial susceptibility of isolates was done by minimum inhibitory concentration (MIC) testing by E-test strips (Biomerieux, France). The E-test method is adapted to slow-growing bacteria like *H. pylori*. Briefly, 72 h old *H. pylori* culture of each isolate was suspended in brain heart infusion broth to reach a turbidity of 3 McFarland. Suspension volume of 50 µl was inoculated on Mueller- Hinton agar plates (Oxoid, UK) enriched with 10% blood. After the plates were dried, antibiotic strips were placed on plates and incubated under microaerophilic conditions for 3 days, MICs were interpreted. Clarithromycin MIC was interpreted based on Clinical and Laboratory Standards Institute (CLSI(breakpoints [10]; MICs of the other four agents (levofloxacin, tetracycline, amoxicillin and metronidazole) were interpreted based on clinical break point of European Committee on Antimicrobial Susceptibility Testing (EUCAST) [11].

Selection of *H. pylori* strains as phage isolation and purification host
Lysogenized bacterial isolates that contain temperate phages are considered undesirable isolation host. Detection of lysogeny was performed by physical phage induction for all 10 isolated *H. pylori* strains by mean of ultraviolet (UV) radiation.

Physical induction was performed by centrifugation of 5 ml of *H. pylori* culture in supplemented BHIB at 15000 rpm for 10 min at room temperature. Bacterial Pellet was re-suspended in 5 ml sterile 0.1M MgSO4. The bacteria suspension was irradiated in a sterile glass Petri dish at a distance of 16 cm from the germicidal short wave (254nm) UV lamp (15 watt) for 1 minute, 2 minutes, 3minutes, 4 minutes and 5minutes. Decimal value (D value), the time needed to reduce 90 % of colony forming unit (CFU) count, of HZ3 by the used germicidal lamb was 0.5 minute. Treatment durations were 2x, 4x, 6x, 8x and 10x estimated D value [12]. The maximum duration used was 10x D value "5 minutes", as it caused marked reduction in bacterial density that would interfere with plaque visualization.

The irradiated suspension was mixed with similar volume of molten supplemented brain heart infusion agar, mixed gently then poured and spread evenly in Petri dishes containing 15 ml of bottom solid agar. The plates were incubated under microaerophilic conditions and checked regularly for plaque formation. Bacterial suspensions without UV exposure were used as control [13].

Screening of *H. pylori* phages from sewage water and water streams
Six collected water samples (from 6 different areas of our locality) were screened for *H. pylori* specific bacteriophages by enrichment technique followed by spot test. The enrichment technique was performed according to **Adams** [14]. For this, the collected water samples were centrifuged at 15000 rpm for 10 min, and then supernatants were filtered through 0.45µm membrane-filter (Whatman microplus 21 STL).

Four *H. pylori* strains (HZ1, HZ3, HZ4, HZ28) were used for screening and isolation of *H. pylori* specific phages from water samples. Five ml of 27h broth cultures of *H. pylori* isolates were added to 25 ml water filtrates and incubated overnight in anaerobic jar (microaerophilic condition). Following incubation, the cultures were centrifuged at 15000 RPM for 10 minutes. The supernatants were filtered through a 0.45 µm membrane filter. The filtrates were screened for presence of phages by spot test. Spot test was done as described [14, 15].

Single plaque isolation procedure for *H. pylori* specific phages purification
According to **Adams** [14]lytic phages (suitable for phage therapy) were screened by checking zones of lysis (indicate phage activity). Clear plaques were regarded as an indicator of a lytic phage while turbid plaques indicate a temperate phage.

Post enrichment water filtrates with positive spot test were serially diluted (1/10, 1/100, 1/1000). 100 µl of filtrates were mixed with 1 ml of susceptible host (HZ3- HZ4) cell (adjusted to 4 McFarland standard) and 3 ml of molten semi solid supplemented BHI medium (0.7% agar agar). This mixture was poured and distributed evenly onto solid BHIA plate surface. The plates were incubated at 37°C under microaerophilic conditions for 3 to 5 days and checked for presence of lytic area. The phage with clear plaques and wide lysis zone (on HZ3 plates) was chosen to be purified.

The chosen phage was propagated by successive single-plaque isolation steps to obtain homogenous plaques. Briefly, the morphologically selected plaque was picked by sterile pasture, suspended in 5 ml of 3 days broth culture of HZ3 isolate, and incubated overnight at 37 $^{\circ}$C under microaerophilic conditions. After incubation, the phage-host mixture was centrifuged at 15000 RPM for 10 min and

supernatant was filtered through membrane filter (0.45μm pore size) to remove any non-infected bacteria and bacterial debris. The filtrate was subjected to another cycle of single plaque isolation steps [16].

Phytobiotic therapy

▪ Preparation of crude plant extracts and evaluation of its effect on *H. pylori* isolates

The plants were purchased from registered herbalist (Salem Al-Awady), herbal store located in Zagazig, Sharkia, Egypt. Studied plant parts including *Mentha spicata* "leaves", *Punica granatum* "peel", *Acacia nilotica* "fruit", *Rhus coriaria* "seed", *Syzygium aromaticum* "fruit", *Rosemarinus officinalis*" leaves" and *Ammi visnaga* "seed" were ground separately and weighed. Extracts were obtained by using maceration with ethanol for 4 days and the resulting extract was subsequently filtered using Whatman filter papers and concentrated under reduced pressure at 40°C using Buchi rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland). 30 µL of different plant extracts was added aseptically to sterile discs (6 mm in diameter). Discs were placed onto HZ3 inoculated supplemented BHIA plates and incubated under microaerophilic conditions at 37°C for 72 h. Diameters of inhibition zones were exactly measured [17].

▪ Determination of minimal inhibitory concentration (MIC)

For the effective herbal extracts (*Punica granatum, Rhus coriaria, Syzygium aromaticum, Rosemarinus officinalis, Ammi visnaga*), the MIC of extracts was evaluated by mixing 700 µl of filer sterilized serially diluted extract in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) with 700 µl of selected bacterial strain (HZ3) suspension (5×10^5 CFU/ml) to have concentration range (160 to 0.156 mg/ml) in small glass tubes. Growth control and extract sterility control tubes were included. The tubes were incubated at 37°C under microaerophilic conditions and compared daily with control tubes. The lowest concentration (highest dilution) of the extract that produced no visible growth was determined as MIC [17].

▪ Inhibitory effect of *Punica granatum* peel extract fractions on *H. pylori* isolate (HZ3)

Punica granatum crude extract was fractionated using successive solvents: petroleum ether (PE), methylene chloride (MC) and ethyl acetate (EA) [18]. The process was carried out in separating funnel, and the anti *H. pylori* effect of each fraction was determined by disc diffusion method as mentioned previously [17].

▪ Inhibitory effect of each subfraction of acetyl acetate fraction on *H. pylori* isolate (HZ3)

The ethyl acetate fraction from *Punica granatum* peel was partially purified as follows : ethyl acetate extract was applied onto a silica gel 60 G column (Merck ;100g;column internal diameter 2.5cm).The column was eluted with a linear gradient manner, starting with 100 % petroleum ether (subfraction 1),petroleum ether : methylene chloride(1:1, subfraction2) and 100% methylene chloride (subfraction 3), methylene chloride : methanol by a linear gradient (2% "subfraction 4", 4% "subfraction 5", 8% "subfraction 6", 16% "subfraction 7", 32% "subfraction 8") and 100% methanol (subfraction 9). All subfractions were then concentrated using rotary evaporator at 45°C [19].

The activity of each subfraction was tested against HZ3 by disc diffusion test, previously described [17]. Diameters of inhibition zones were exactly measured.

▪ Effect of antibiotics and *Punica granatum* extract in animal model

Forty healthy male albino, 6-8 weeks old, weighing around 160-180g (Central animal house, College of Veterinary medicine, Zagazig University) were acclimatized for 1 week. Gastric ulceration and infection were done by method described by **konturek et al** [20]. Briefly, each rat had been fasted for 24 h before induction of ulcer. During the fasting period, rats were supplied with sucrose 8% (w/v) solution in NaCl 0.2% (w/v) to prevent dehydration that was withdrawn an hour before intervention.

Animals were randomly categorized into healthy control group (n=5), ulcerated non-infected (n=5), ulcerated infected non-treated group (n=10), ulcerated infected group receiving mixture of amoxicillin 50mg/kg, clarithromycin 30mg/kg, omeprazole 1 mg/kg (n=10) and ulcerated infected group receiving 600 mg/kg of *Punica granatum* extract (n=10).

The tested extract dose was adjusted according to **Patel and coworker** [21] which evaluated 600mg/kg/day as a safe dose without any recorded toxicity in rats. They didn't evaluate the toxicity of higher doses of extract in their study.

Gastric ulcer was induced by intragastric administration of 100 µL acetic acid. Daily, 0.1mL of freshly cultivated *H. pylori* (HZ3) suspension was delivered via a sterile gastric cannula for intragastric administration for 7 days after ulcer induction [20].

One day after the last intragastric administration of *H. pylori*, one healthy control, one ulcerated non-infected and one ulcerated infected rats were euthanized under ether anesthesia. Under aseptic conditions, the stomach was divided into two identical halves. One half was sent to microbiology laboratory to confirm that control and ulcerated non-infected group, were pathogen free and to confirm that infection was established and assess the mean of *H. pylori* density in different infected groups. While the other half of stomach was sent to histopathological laboratory for preparation of paraffin section for histopathological analysis using Hematoxylin and Eosin (H&E) for confirmation of ulcer progression. Giemsa stain was used for detection of *H. pylori* in gastric sections.

After 168 h post infection (hpi) (a week), surviving rats from different groups were euthanized under ether anesthesia. Following death or euthanizing rats, the stomach was divided into two identical halves [22]. One half was weighed and homogenized in 10 mL of BHI broth then serially diluted. Aliquots 10 μL of different dilutions were inoculated onto supplemented BHIA plates incubated at 37℃ under microaerophilic conditions for 5 days. The colonies were counted, and bacterial density was assessed (quantitative assessment). The other half was used for histopathological analysis for follow up of infection progress and response to therapy.

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Statistical significance was evaluated using analysis of variance test (ANOVA), followed by the post hock test (Bonferroni test). Chi-square was used to compare categorical variables by using computerized software (SPSS version 23, Chicago, IL, USA)

Results

Only 10 out of 35 patients (28.57%) had gastric biopsies positive for *H. pylori*. Four out of 10 (40%) isolates were resistant to all tested antibiotics and the highest bacterial resistance was against clarithromycin and amoxicillin (without statistical significance *P>0.05*). The least resistance is reported against levofloxacin 60% (without statistical significance *P>0.05*). *Helicobacter pylori* isolate HZ3 had levofloxacin MIC = 64 μg/ml **(Table 1)**.

None of H. *pylori* isolates exposed to UV irradiation for different durations produced lytic area. *H. pylori* isolates (HZ1, HZ3, HZ4, HZ28) were used as isolation host for bacteriophage because they are resistant to all tested antibiotics. The filtrates of two water sources gave lytic areas only on *H. pylori* isolates HZ3, HZ4. Meanwhile, the other tested water filtrates did not give lytic area with any of tested *H. pylori* isolates.

Three phenotypes (size and appearance) of plaques were detected including, two large plaques (one of them had turbid appearance while the other appeared clear) and the third phenotype was clear pinpoint **(Table 2)**. During phage purification process, the selected phage lost its infectivity of HZ3.

We found that *Punica granatum* peel crude extract showed the widest inhibition zone = 23 mm by disc diffusion and the lowest recorded MIC against HZ3 (MIC=10 mg/ml). Accordingly, this extract was used for further study **(Table 3)**. Interestingly, ethyl acetate fraction of *Punica granatum* extract on *H. pylori* (HZ3) is the only fraction having apparent *H. pylori* inhibitory effect **(Table 4)**.

Nine subfractions of *Punica granatum* extract ethyl acetate fraction, were obtained by column chromatography. Subfraction obtained with 32% MCl: Mth had the largest inhibition zone = 18 mm **(Table 5)**

A statistically significant reduction (*P<0.05*) of bacterial density following crude extract administration. There was also significant difference between bacterial density following use of standard regimen of *H. pylori* treatment and peel extract for one week *(P<0.05)*. Both regimens didn't eradicate the bacteria **(Table 6)**.

The histopathological study of gastric sections of different animal groups is shown in **figure (1)**

- Non-treated group (A III)**:** deep ulcer with gross detachment of surface mucous epithelium. (Green arrow) and marked infiltration of inflammatory cells (Yellow arrow) at the ulcer sites and mucous glands, (B III)**:** *H. pylori* bacteria (blue arrow) at the stomach lumen.
- Antibiotic treated group (A IV)**:** marked degeneration of surface mucous epithelium with increased depth of peptic ulcer with gross detachment of surface epithelium (Green arrow), the mucous gland showed degenerative changes and marked inflammatory infiltrate (Yellow arrow). (B IV): The lumen of stomach and ulcer site showed large numbers of *H. pylori* cells (blue

arrow) free and some were attached to the surface epithelium

- *Punica granatum* peel extract treated group: (A V): showed moderate ulcerative changes and mild detachment of surface mucous

epithelium (Green arrow), mild infiltration of inflammatory (B V): There was moderate amount of clumped coccoid *H. pylori* (blue arrow) attached to the damaged detached epithelium.

Table 1. Susceptibility of isolated *H. pylori* to different antibiotics by E-test.

Isolates	Clarithromycin[a] S ≤0.25 µg/ml I >0.25, <1 µg/ml R ≥1 µg/ml	Levofloxacin[b] S<1 µg/ml R ≥1 µg/ml	Tetracycline[b] S<1 µg/ml R≥1 µg/ml	Amoxicillin[b] S ≤ 0.12 µg/ml R > 0.12 µg/ml	Metronidazole[b] S<8 µg/ml R ≥8 µg/ml	Total antibiotic resistance/total antibiotics tested	P value
HZ1	3 (R)	2 (R)	4 (R)	8 (R)	64 (R)	5/5 (R)	
HZ3	8 (R)	64 (R)	8 (R)	2 (R)	24 (R)	5/5 (R)	
HZ4	4 (R)	8 (R)	4 (R)	8 (R)	16 (R)	5/5 (R)	
HZ8	4 (R)	0.25 (S)	0.5 (S)	8 (R)	12 (R)	3/5 (R)	
HZ25	0.125 (S)	2 (R)	4 (R)	0.094 (S)	2 (S)	2/5 (R)	
HZ28	1 (R)	64 (R)	8 (R)	16 (R)	12 (R)	5/5 (R)	
HZ31	2 (R)	0.5 (S)	0.25 (S)	2 (R)	16 (R)	3/5 (R)	
HZ33	6 (R)	0.5 (S)	0.125 (S)	8 (R)	4 (S)	2/5 (R)	
HZ34	0.094 (S)	0.5 (S)	4 (R)	1 (R)	32 (R)	3/5 (R)	
HZ35	1 (R)	4 (R)	8 (R)	0.094 (S)	4 (S)	3/5 (R)	
Resistant isolates/ total isolates	8/10 (80%)	6/10 (60%)	7/10 (70%)	8/10 (80%)	7/10 (70%)		0.846

a) CLSI breakpoint and interpretive criteria only for clarithromycin, b) Clinical break point of EUCAST 2015 for other antibiotics.
(R) Resistant / (S) sensitive. Isolates were given the letters HZ for H. pylori (H) isolated from Zagazig (Z) followed by the serial number of patient enrolment in the study.

Table 2. Morphology of plaques isolated from water samples.

H. pylori isolates number	water source	Plaques morphology	
		Plaques Size	Plaques appearance
HZ3	A	Pinpoint	Clear
		Large	Turbid
	B	Large	Clear
HZ4	A	Pinpoint	Clear
	B	Large	Turbid

A: Sewage tanks of Zagazig water station, B: Moese canal
HZ3: H. pylori isolated from 3rd gastric biopsy, HZ4: H. pylori isolated from 4th gastric biopsy

Table 3. Inhibition zone diameter of *H. pylori* isolate (HZ3) by different extracts and its corresponding MIC.

Plant extract (mg/disk)	Used part of plant	Extraction Yield% and form	Family name	Common name	Inhibition zone diameter by mm on HZ_3 (MIC mg/ml) *
Mentha spicata (15)	leaves	4 fluid	Lamiaceae	Garden mint	-
Acacia nilotica (66)	fruit	7.62 fluid	Fabaceae	Egyptian thorn	-
Punica granatum peel extract (30)	peel	20 fluid	Lythraceae	Pomegranate	23 [10] *
Rosemarinus officinalis (43)	leaves	17.2 fluid	Lamiaceae	Rosemary	18 [40] *
Syzygium aromaticum (55)	fruit	22 fluid	Myrtaceae	Clove	14 [80] *
Rhus coriaria (22)	seed	14.8 fluid	Anacardiaceae	Sumac	12 [80] *
Ammi visnaga (171)	seed	22.8 fluid	Apiaceae	Khella	9 [160] *

*Numbers between superscripted parentheses indicates the MIC (mg/mL)

Table 4. Inhibition zone diameter of *H. pylori* isolate (HZ3) by various fractions of *Punica granatum* peel extract.

Extract fraction	Inhibition zone(mm)
Petroleum ether fraction	-
Methylene chloride fraction	-
Ethyl acetate fraction	18

Table 5. Column chromatographic fractionation of ethyl acetate fraction of *Punica granatum* peel extract and subfractions inhibition zone diameter on HZ3.

| Eluent	PE subfraction 1	PE: MCl 50% subfraction 2	MCl 100% subfraction 3	MCl: Mth					Mth100% subfraction 9
				2% subfraction 4	4% subfraction 5	8% subfraction 6	16% subfraction 7	32% subfraction 8	
Inhibition Zone (mm) of HZ_3	-	-	-	-	12	10	14	18	-

PE: petroleum ether, **MCl:** methylene chloride, **Mth:** methanol.

Table 6. *In vivo* effect of *Punica granatum* crude extract and antibiotics on *H. pylori* density after 7 days of treatment.

Rat group	Mean of *H. pylori* density in infected rat groups before intervention (CFU/mL)	Density of *H. pylori* in stomach after 7 days of treatment or no treatment (CFU/mL)	*P* value
III	$30.98 \pm 4.931 \times 10^5$	$343.92 \pm 2.7 \times 10^{5\,a}$	0.000
IV	$30.98 \pm 4.931 \times 10^5$	$40.89 \pm 2.91 \times 10^{4\,b}$	0.000
V	$30.98 \pm 4.931 \times 10^5$	$15.6 \pm 1.4 \times 10^{3\,c}$	0.000
P value		0.000	

III –*H. pylori* infected without treatment; **IV**- *H. pylori* infected and treated with standard triple therapy; **V** - *H. pylori* infected and treated with 600 mg/kg body weight of *Punica granatum* extract. Calculated mean is for triplicate measurements $\pm$ SD; [a-b-c] means with different superscripts in the same column are considered statistically significant.

Figure 1. Photomicrograph of histopathologic section stained by H&E (A) and Giemsa stain (B) Non-treated group (III), antibiotic treated group (IV) and extract treated group (V).
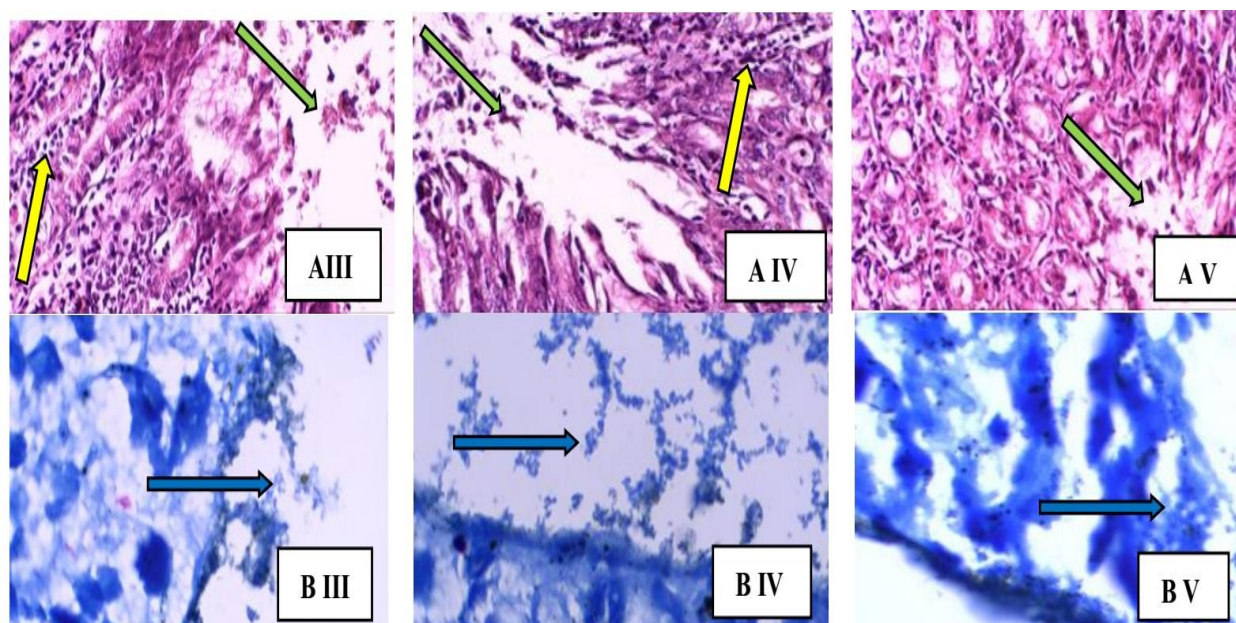
Discussion

Helicobacter pylori infection is an important health problem worldwide. The World Gastroenterology Organization (WGO) reported that the *H. pylori* prevalence in Egypt was 90% in adults. Average prevalence of *H. pylori* infection in world's population is 50%. *Helicobacter pylori* resistance is an ever-increasing problem that is growing around the world [3].

Microbiological culture of gastric mucosal biopsy revealed positive growth of *H. pylori* in 10 out of 35 (28.57%) specimens. In contrast **Taj and coworkers** [23] in their study reported positive culture from gastric biopsy to be 9%. Meanwhile, these results were less than that obtained in two studies [24, 25] which reported *H. pylori* recovery from gastric biopsy in 44% and 34.8 % of cases respectively.

Helicobacter pylori positive culture ascertains *H. pylori* infection, but negative culture does not exclude it. The factors contributing to variability in results of *H. pylori* detection among studies may be the nature of gastric disorder and used culture media. The site, size and bacterial density of biopsy specimen as well as patchy distribution of *H. pylori* on target mucosa may contribute to variability between studies.

Seven out of 10 *H. pylori* isolates (70%) were resistant to metronidazole. In contrast to an Egyptian study [23] that reported 100% resistance to metronidazole by using disc diffusion method. Less resistance percentages were reported in an Italian study [26] using agar dilution test where only 34.69% of *H. pylori* isolates were metronidazole resistant. An Egyptian study using molecular method for detection of metronidazole resistance reported only 25% of isolates to be metronidazole resistant [27]. This great variation in metronidazole resistance prevalence may be due to difference in methodology of susceptibility testing as well as difference of studied population.

Resistance to clarithromycin was (80%) which agrees with **Di Giulio et al** [26] who reported that the percentage of resistance to clarithromycin was 72.44% as well as in another Egyptian study clarithromycin resistance was reported to be 66.19 % by genetic based assessment of resistance [28].

Regarding resistance to amoxicillin, it was reported to be 80%. This agrees with resistance prevalence in other Egyptian studies [24, 25], which detected resistance in 90% and 68.8% respectively. On the other hand, very low resistance rate was reported in Italy, where amoxicillin resistance was only 1.02% [26].

This high resistance rate may be a secondary resistance as a result of previous use of these antibiotics either as a part of *H. pylori* eradication therapy (1st line triple therapy used in *H. pylori* infection treatment in Egypt) or other infectious disease with very common use of amoxicillin combined with clavulanic acids in Egypt.

For selection of bacterial host for phage enrichment and isolation, we tried to identify and use non-lysogenized bacterial strains. Non-lysogenized bacterial strains are usually used in phage isolation because many prophages alter the bacterial host, making it resistant to phage super infection [29]. In our study, no plaques were detected from any of *H. pylori* isolates upon phage induction by the physical method. This may be due to actual absence of prophage from these isolates, inadequacy of induction protocol in current study or the need of certain additives (calcium, magnesium or even tryptophan) to help prophage to adsorb onto host cell and form visible plaques on a lawn. Occasionally induced prophages produce micro plaques impossible to be seen [30]. Another study conducted by **Lehours and coworkers** detected phage DNA by genome sequencing in *H. pylori* isolates but failed to reveal lysis plaques upon phage induction although the

induced phage particles were visualized by electron microscopic imaging [12].

A rule of thumb for bacteriophages detection and isolation from nature is to use the sources where the host bacteria are abundant. Phages for gastrointestinal bacteria are readily isolated from fecal material and sewage. In our study sewage water and freshwater streams were a potential source of *H. pylori* specific phages as 2 of 6 water sources contained *H. pylori* specific bacteriophages. The existence of *H. pylori* specific bacteriophage in environment suggests persistence of *H. pylori* in contaminated water and its ability to withstand harsh conditions. **Morton and Bardhan** also isolated *H. pylori* specific bacteriophages from sewage water [15].

Three types of plaques (large turbid, large clear and pen point) were isolated after enrichment of water by *H. pylori* isolates HZ1, HZ3, HZ4 and HZ28 separately. During phage purification by five transfer steps using HZ3 isolate, the selected phage lost its infectivity.

There are many reasons for infectivity loss and interruption of phage development during every stage of phage replicative cycle. The main mechanism disturbing bacteria and phage co-evolution is spontaneous mutation. *Helicobacter pylori* has a great genomic plasticity, presenting high rates of mutation [31].

Infectivity loss may be through phage adsorption inhibition due to bacterial surface protein mutations. *Helicobacter* pylori exhibits exceptionally high rates of DNA point mutations due to lack in DNA repair system. Presence of surface mutants of bacterial host during phage infection, causes them to rapidly become the dominant phenotype and subsequently become resistant to phage infection [32]**.**

In addition, CRISPR (clustered regularly interspaced short palindromic repeats) systems are rapidly evolving defense mechanisms against foreign genetic elements derived from bacteriophages and other exogenous genes. They are loci containing multiple, short direct repeats separated by spacers (Short segments of DNA homologous to phage DNA). Spacers' sequences help recognition and destruction of homologues extrinsic gene. On the flanks of these spacer repeats regions, several CRISPR-associated (*cas*) genes, that encode for enzymes that attack invading phage genome with subsequent phage genome break down and phage replication failure. A major hindrance of phage

therapy is the spontaneous development of bacterial resistance to phage infection [31].

Helicobacter pylori isolate (HZ3) which is resistant to all tested antibiotics, lost its susceptibility to phage during steps of phage purification. In antimicrobial susceptibility, the studied strain (HZ3) exhibited high level resistance to Levofloxacin. According to a Japanese study of levofloxacin resistance, double mutation of gyrase enzyme existed when MIC of levofloxacin ≥ 32 μg/ml, while low level resistance was associated with single mutation that support high mutability of HZ3 strain. Such high mutability can confer resistance to both phage and antibiotics [33].

On the other hand, a phenomenon of self-targeting by CRISPR/Cas system (resembling autoimmunity) can modulate bacterial genome and may delete resistance genes in bacteria leading to increase bacterial susceptibility to antibiotic. So CRISPR/Cas system confers bacterial resistance to phage but may induce susceptibility to antibiotics [34]. Many antibiotics regimens lost their effectiveness in *H. pylori* eradication due to emergence of resistance. In addition, antibiotics are associated with different adverse effects. Therefore, several medicinal plants have been increasingly used owing to their antibacterial properties as complementary or alternative to antibiotics. Natural products of plant origin are potential sources for the discovery and development of new effective agents against infections.

Among 7 tested crude extracts; only 5 extracts gave antibacterial activity against the selected *H. pylori* isolate. The antimicrobial activity, detected by inhibition zone diameter, was: *Punica granatum* (23mm) > *Rosemarinus officinalis* (18mm) > *Syzygium aromaticum* (14mm) > *Rhus coriaria* (12mm) > *Ammi visnaga* (9mm).

Menthe specata had no antibacterial effect on *H. pylori* and no inhibition zone was reported. On the other hand, another study reported weak anti-*Helicobacter pylori* activity of *Mentha specata* [35].

Difference between results may be due to difference of extract purity, difference in extract composition or difference in bacterial strains. Variation in antimicrobial effect of the extract of the same plant species in different studies may be due to difference in geographic areas of plant growth also agro-climatic difference within the same geographic area, even the developmental stage of the plant [36].

Acacia nilotica fruit extract showed no effect on *H. pylori* isolates. **Amin and coworkers** in their study reported significant inhibitory effect of ethanol and acetone extract of *Acacia nilotica* leaves and flowers on 2 *H. pylori* isolates. The estimated MIC ranged from 8-64 μg/ml that was strong moderate activity [37]. This marked difference in results was due to difference in plant part studied. Although *Acacia nilotica* fruit extract exhibits significant antibacterial effect against multiple Gram positive and Gram-negative bacteria in different studies but none of these studies included *H. pylori* bacteria [38].

Mahernia et al in their study reported inhibitory effect of *Rhus coriaria* extract on urease enzyme but didn't study the antimicrobial effect on *H. pylori*. The acidic environment of stomach plays an important role in gastric colonization by *H. pylori*. Urease enzyme of *H. pylori* allows its survival by neutralizing the acidic environment. When Mahernia and coworkers examined the mechanism by which the treatments exert activity against *H. pylori*, urease activity was inhibited in vitro. Considering this, it was tempting to speculate that the *in vivo* inhibitory activity against *H. pylori* infection could also result from the inhibition of the urease activity [39].

Regarding the inhibition zone diameter of *H. pylori* by *Syzygium aromaticum* extract (14 mm) was in the range of inhibition zone diameters reported by **El-Shouny and coworkers** (11-24 mm) in their study on pandrug resistant *H. pylori* isolates [40].

Punica granatum is an ancient fruit widely consumed all over the world. In current study MIC of *Punica granatum* crude extract against *H. pylori* (10 mg/ml) was within the range reported by **Voravuthikunchai and Mitchel** [41].

Effective fractions and subfractions of ethyl acetate extract yielded inhibition zones that were less compared with the *Punica granatum* crude extract. This may be due to a synergistic effect of different fractions and subfractions of crude extract.

Neither use of antibiotics nor *Punica granatum* peel extract administration *in vivo* for 1 week caused complete eradication of *H. pylori* infection. Obviously, colony count after 1 week of intervention showed significant reduction after use of Peel extract ($p<0.05$). On the other hand, antibiotic use was associated with increase bacterial count when compared with CFU before treatment, although it significantly controlled multiplication of *H. pylori* in comparison with non-treated group.

Although, *H. pylori* isolate (HZ3) was susceptible to peel extract *in vitro*, but less susceptible *in vivo*. This discrepancy can be due to the highly protected habitat of the organism in stomach and may be short gastric-transit time. The nature of *H. pylori* growth *in vivo*, bacterial survival in an organized biofilm may provide some sort of protection [42]. In the histopathologic section stained by the Giemsa stain, transformation of *H. pylori* into coccoid forms could explain this survival, although this point remains controversial. Indeed, *H. pylori* have a helical bacillary appearance in favorable conditions which undergoes transformation into coccoid forms under unfavorable conditions. Several authors have proposed that coccoid forms are a mechanism by which *H. pylori* can survive harsh environmental conditions or is able to convert to resistant forms under therapeutic stress [43]. The reduced *in vivo* effect of peel extract on *H. pylori* may be due to unadjusted extract dose or treatment duration. The acidic environment of stomach may play degradative effect on plant extract.

Concerning histological and microbiological staining of gastric sections, antibiotic treated group showed more inflammatory infiltrates, marked mucosal ulceration and abundant bacterial clumps attached to epithelial cells. On the other hand, peel crude extract controlled mucosal ulceration, inflammatory cellular infiltrates as well as bacterial growth and adherence to epithelial cells. That significant anti-inflammatory as well as healing induction effect of *Punica granatum* peel extract was noticed in other studies. Pomegranate peel extracts, significantly decreases mucosal injury and histochemical study showed increase in distribution of polysaccharides secretion among glandular tissue [44]. **Chauhan and coworkers** stated that peel extract of *Punica granatum* have gastric cytoprotective effects through enhancement of defensive mucin secretion, glycoproteins and decrease oxidative stress mainly through promoting antioxidant status [45].

Conclusion

Punica granatum peel extract revealed better *in vivo* activity against drug resistant *H. pylori* than standard regimen antimicrobials. *Punica granatum* peel extract also induced anti-inflammatory and healing promotion effect. Other Egyptian medicinal plants have anti-*Helicobacter pylori* effect including *Rosemarinus officinalis, Syzygium aromaticum, Rhus coriaria* and *Ammi visnaga* can be beneficial in *H. pylori* infection management. Loss of

bacteriophage infectivity may be an obstacle to phage therapy of *H. pylori*.

Recommendation

Clinical trials of *Punica granatum* peel extract as supplementary treatment of *H. pylori* gastric infections and ulcers are recommended. Further investigation of active antimicrobial components of *Punica granatum* peel extract. Further investigation of *H. pylori* specific bacteriophages for identification of best therapeutic phage type or phage combination.

Conflict of interest: none

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