

Original Article

***Helicobacter pylori* in ice cream and its control using mastic gum essential oil**

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ABSTRACT

Objective: This study aimed at assessing the prevalence of *Helicobacter* spp. in ice cream sold at Qena city (Egypt) with special reference to *H. pylori*, and to study the antimicrobial effect of mastic gum essential oil on *H. pylori*.

Materials and methods: A total of 60 small and large scale ice cream samples (30 each) were investigated for the presence of *Helicobacter* spp. Then, the essential oil of mastic gum was added to lab prepared ice cream inoculated with *H. pylori* isolate in a count of Log₆ using 2 concentrations of 0.8 and 1.6% and one group was kept as a control group. Each group was subdivided into 2 sub-groups; one was kept at -5°C and another one was kept at -20°C.

Results: *Helicobacter* spp. could be isolated from 11.7%, while, *H. pylori* could be found in 3.3% of the total examined ice cream samples. Regarding the anti-*Helicobacter pylori* effect of mastic gum essential oil added to lab prepared ice cream, in samples kept at -5°C it was found that after hardening the count fall into Log₅ in both control and 0.8% groups and log₄ in 1.6% essential mastic oil concentration. Reduction in *H. pylori* at the first week and third day of storage reached 100% where *H. pylori* couldn't be counted or isolated from both 0.8 and 1.6% concentrations, respectively. While in ice cream samples stored at -20°C, the reduction in *H. pylori* load was rapid at first, it was log₄ and log₃ in groups of 0.8% and 1.6% concentrations, respectively versus log₅ in control samples. At the third day *H. pylori* was not able to be counted or isolated at third day of storage for both concentrations.

Conclusion: All isolates recovered from small scale ice cream samples reflexing the hygienic conditions under which samples were produced. Mastic gum essential oil exhibited a powerful anti-*H. pylori* effect recommending its addition to food matrix for therapeutic purposes or as a functional food.

KEYWORDS

Essential oil; *H. pylori*; Ice cream; Mastic gum

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INTRODUCTION

Helicobacter pylori is considered as the causative agent of peptic ulcer, chronic gastritis, gastric adenocarcinoma, mucosa associated lymphoid tissue lymphoma and duodenal ulcer (Sachdeva et al., 2014), and it is the first recognized carcinogenic bacteria causing gastric cancer (Dore et al., 2001; Rahimi and Kheirabadi, 2012; Momtaz et al., 2014; Mousavi et al., 2014; Yahaghi, et al., 2014). *H. pylori* is thought to be the second dominant cause of cancer worldwide (WHO, 2010). Existence of *H. pylori* in the stomach of domestic animals, milk, meat, and gastric biopsies indicates that domestic animals and the food originated from them may act as potential sources of this bacterium (Mousavi et al., 2014). Composite foodstuffs, particularly milk, have been considered as a prospective source of human infection (Fujimura et al., 2002) due to its acidic pH, nutritional values, salt concentration and presence of high amount of activated water that assist the growth and survival of *H. pylori* for long time, and subsequently transmit to human (Fan et al., 1998; Quaglia et al., 2007). The ways by which *H. pylori* is transmitted have not been definitely assured, however, some studies indicated that oral-oral or feco-oral way of transmission may occur (Brown, 2000; Calvet et al., 2013). Food may serve as a vehicle for *H. pylori* transmission through primary contamination of food originating from animal or secondary contamination due to improper handling by human (Quaglia et al., 2007)..

For the effective treatment for *H. pylori* infections, two antibiotics and a proton pump inhibitor are traditionally used. However, the use of these antibiotics for long time may result in the development of resistance towards *H. pylori*, and also may change the normal flora of the gastrointestinal system (Murali et al., 2014), and subsequently may limit in the therapeutic options (Mégraud, 2004). Since consumers nowadays are more selective for their food items and are more demanding for natural antimicrobials, researchers are deeply involved in searching of natural antimicrobial alternatives. One of the most famous natural antimicrobials is plants' essential oil particularly essential oil of mastic gum which is Generally Recognized as Safe (GRAS) (Lucera et al., 2012).

Mastic gum is the resin of *Pistacia lentiscus*, and has been used to treat abdominal pain, dyspepsia and gastric and duodenal ulcer (Al Said et al., 1986). The essential oil of mastic gum is a valuable product named mastic oil comprising of a mixture of a several compounds, and most of them have antimicrobial properties (Tassou and Nychas, 1995; Magiatis et al., 1999). It is excessively used in confectionery products as food additive, in the perfume manufacture and also as a component in

cosmetics and health products. Mastic oil displays a variety of pharmacological properties, such as antimicrobial (Koutsoudaki et al., 2005; Paraschos et al., 2011), anti-inflammatory (Heo et al., 2006), and antileukaemic (Loutrari et al., 2006).

The anti-*H. pylori* activity *in vitro* of mastic oil has been documented by several researchers (Koutsoudaki et al., 2005; Paraschos et al., 2011; Miyamoto et al., 2014). Since food has complex media and the interactions between antimicrobials and food macromolecules, particularly proteins and fat, may influence the performance of the antimicrobial activity (Shelef, 1983), the antimicrobial effect of mastic oil on *H. pylori* inoculated in ice cream was investigated in this study. To our knowledge, this is the first report concerning the anti-*H. pylori* effect of mastic essential oil in dairy food matrix. The present work aimed at isolating and confirmation of *H. pylori* from ice cream samples sold at Qena (Egypt) markets, and to investigate the anti-*H. pylori* activity of mastic gum essential oil on *H. pylori* inoculated in lab prepared ice cream.

MATERIALS AND METHODS

Samples: A total of 60 small and large scaled ice cream samples (30 each) were examined for the presence of *H. pylori*. The samples were collected from different localities at Qena city, Egypt, and were transferred in ice box to the lab as soon as possible for further studies.

Isolation of *H. pylori* from ice cream: Isolation of *H. pylori* was done according to the technique described by Stevenson et al. (2000). Melted ice cream samples were enriched using *H. pylori* Special Peptone Broth (HPSPB) supplemented with 5% sterile horse serum and *H. pylori* selective supplement (Oxoid, SR0147E) (5 mg/L vancomycin, 2.5 mg/L trimethoprim, 2.5 mg/L cefsulodine and 2.5 mg/L amphotericine B). Incubation was done at 37°C for 48 h in an atmosphere of 6% O₂, 10% CO₂ and 84% N₂, which supplied by gas generating kits (Oxoid BR 56) in an anaerobic jar. Then, HPSPA supplemented with 5% sterile horse serum and SR0147E was streaked using a loop full of the enrichment broth of each sample. The plates were incubated at 37°C for 3-7 days under the same microaerobic condition as mentioned before. All the cultured plates were inspected after 3, 5 and 7 days. Suspected colonies grew slowly and recognized by its small shape not exceeding 2 mm in diameter, circular, convex and translucent as described by Naclmmkin and Skirrow (1998). The obtained isolates were identified morphologically and biochemically as clarified by Zenner (1999). The bacterial culture showing urease positive reaction and negative reaction of hippurate hydrolysis and nitrate reduction was identified as *H. pylori*.

Confirmation of *H. pylori* isolates by PCR: QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was used to perform DNA extraction from *H. pylori* according to the manufacturer's instructions with slight modification. In brief, 200 μ L of the sample suspension was incubated with 10 μ L of proteinase K and 200 μ L of lysis buffer at 56°C for 10 min. Next, 200 μ L of 100% ethanol was added to the lysate and samples were washed and then, centrifugation was done. Finally, nucleic acid was eluted with 100 μ L of elution buffer. PCR was done to confirm the *H. pylori* isolates using the primer sequences (EHC-U; 5'-CCCTCAGCCATCAGTCCCAAAA-3' and EHC-L; 5'-AAGAAGTCAAAAACGCCCAAAAAC-3'), as described by [Van Zwet et al. \(1993\)](#). The primers were synthesized from Metabion (Germany), and were used for the amplification a 417-bp fragment of the flanking region of *H. pylori ureC* gene. The PCR mixture reaction (25 μ L) consisted of 12.5 μ L of Emerald Amp Max PCR Master Mix (Takara, Japan), 2 μ L of each primer of 20 pmol, 6.5 μ L of water, and 2 μ L of DNA template. Amplification of DNA was accomplished with 30 cycles of the following: primary denaturation was at 94°C for 10 min, annealing at 62°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1xTBE buffer at room temperature and gradients of 5V/cm were used. Gel analysis was accomplished by loading 30 μ L of the products in each gel slot and a 100-bp DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. Finally, photographing of the gel was carried out by a gel documentation system (Alpha Innotech, Biometra).

Extraction of mastic essential oil: The essential oil was extracted from the raw samples by steam distillation of mastic gums and water mixture in ratio of 1:10 for 5 h at 1.5 pressure and 105-110°C ([Miyamoto et al., 2014](#)). Part of the mastic gum used in this study was obtained as a gift and the other part was bought from different herbal markets.

Detection of anti-*H. pylori* effect of mastic gum essential oil: Fresh culture of *H. pylori* was adjusted with sterile distilled water to obtain turbidity equivalent to 0.5 McFarland turbidity standards (1.5×10^8 /mL). One millilitre of *H. pylori* culture suspension was used to inoculate Muller Hinton agar supplemented with 7% horse blood without antibiotics obtaining a count of 1.5×10^7 cells/mL. Then blank paper disks were placed on the surface of the inoculated agar plates and saturated with 20 μ L of mastic oil and incubation at 37°C for 48 h in an atmosphere of 6% O₂, 10% CO₂ and 84% N₂, using an anaerobic jar and gas generating kits (Oxoid BR 56).

The antibacterial activities were estimated by measuring a diameter of the inhibition ring ([Miyamoto et al., 2014](#))

Detection of MIC and MBC of mastic gum essential oil: The inhibitory effect of mastic gum essential oil on *H. pylori* was examined using Broth Dilution Method, as described by [Gkogka et al. \(2013\)](#). In brief, sloppy agar was made by preparing a Muller Hinton broth supplemented with 7% horse blood without antibiotics and with the addition of 0.2 % w/v bacteriological agar. Serial two-fold dilutions of mastic essential oil (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8% v/v) were prepared by vortexing the different concentrations of essential oil with the sloppy agar. Then, 1 mL of the bacterial suspension was added in each sloppy agar tube (containing 1 mL sloppy agar) to obtain a final concentration of log₇/mL, and the used concentrations of mastic oil mentioned above were used in duplicate for achieving final concentrations in the range of 0.05 to 6.4% v/v. 2 negative controls were prepared (one is containing mastic oil without bacterial inoculation and another one is inoculated with *H. pylori* without addition of mastic gum oil). After incubation under the condition described above, the Minimal Inhibitory Concentration (MIC) that is the lowest concentration of mastic oil that results in no growth of the inoculum and the Minimum Bactericidal (MBC) is the minimum concentration that kill bacteria was determined by plating the clear tube and the first concentration that showed turbidity.

Manufacturing of ice cream in the laboratory: Ice cream was prepared using ice cream powder and cool sterilized milk according to label instruction of the producer. Then the prepared ice cream was divided into 3 groups, each group consisted of 2 portions (100 mL each). Inoculum of *H. pylori* was added to each portion of the 3 groups to obtain count of log₆ CFU/mL after through mixing. Then, a group was treated with 0.8% v/v of mastic oil, while another group was treated with 1.6% v/v. The last group was kept as control. One portion from each group kept at -5°C and the other one kept under -20°C. Samples were taken to detect the count of *H. pylori* after hardening and for the first 3 days consequently, and then every week for 4 weeks. *H. pylori* was enumerated by surface plating method, using Muller Hinton agar supplemented with 7% horse blood and SR0147E. The inoculated plates were incubated at 37°C for 48 h in an atmosphere of 6% O₂, 10% CO₂ and 84% N₂, using an anaerobic jar and gas generating kits (Oxoid BR 56).

RESULTS AND DISCUSSION

According to data showed in **Table 1**, *Helicobacter* spp. could be isolated from 23.3% of the examined small scale

ice cream samples. While none of the ice cream samples belonged to large scale was harbouring *Helicobacter* spp. *Helicobacter* spp. could be isolated from 11.7% of the total examined ice cream samples. Incidence of *Helicobacter* spp. obtained by [El-Khawaga \(2006\)](#) was higher, also, [Mokhtar \(2004\)](#) found higher percent (30%) of *Helicobacter* spp. in the examined ice cream samples.

Helicobacter spp. were biochemically identified as *H. pylori*, *H. pullorum* and *H. cinaedi* with an incidence of 6.7, 13.3 and 3.3% of the examined small scale ice cream samples and 3.3, 6.7 and 1.6% of the total investigated ice cream samples, respectively (**Table 1**). Regarding incidence of *H. pylori*, it was found that [El-Khawaga \(2006\)](#) reported similar incidence. While, [Mokhtar \(2004\)](#) recovered *H. pylori* from the examined ice cream samples in lower incidence (2%). Presence of *H. pylori* in the examined samples might be due to incorporation of contaminated milk in manufacturing of ice cream or due to poor sanitation during preparation and storage of these products. *H. pylori* isolates were confirmed by PCR (**Figure 1**).

Besides this most well-known member *i.e.*, *H. pylori*, more than 20 other *Helicobacter* spp. have been identified and several of them are associated with various chronic digestive diseases ([Zhou et al., 2011](#)). *H. pullorum* has been related to diarrhea, gastroenteritis and liver disease in humans ([Ceelen et al., 2005](#)). Also, *H. cinaedi* causes bacteremia, cellulitis, septicemia and enteritis in immunocompromised, immunocompetent and healthy persons ([Taniguchi et al., 2016](#)).

H. pylori colonizes in the stomach of more than 50% of the world's population. Majority of these bacteria occur in the mucus layer of the stomach and may act as a reservoir of infection for the underlying gastric epithelium ([Dunne et al., 2014](#)). Moreover, it is widely accepted that the bacteria in contact with epithelial cells initiate disease ([Klerk et al., 2016](#)). WHO has assorted *H. pylori* as a class I carcinogen and its elimination with antibiotic combinations has been notified to be profitable in preventing gastric sickness particularly cancer ([IARC, 1994](#)) before increasing antibiotic resistance problem which generated an obstacle in treatment of *H. pylori*-associated diseases by usual therapies ([Murali et al., 2014](#)).

In this study, we investigated the anti-*H. pylori* effect of essential oil extract of mastic gum in lab prepared ice cream. First, we had to determine the anti-*H. pylori* activity of the obtained mastic oil through detecting the inhibitory zone which was found to be 19 mm, then the MIC and MBC were estimated and they were 0.4 and 0.8% v/v, respectively. It was difficult to compare the MIC and MBC with other studies as different mastic substances and methodologies have been used by different researchers yielding various results ([Tassou and Nychas, 1995](#)). [Huwez et al. \(1998\)](#) observed that crude mastic gum could kill *H. pylori* at a concentration of 0.06 mg/mL. While in another study, the mastic extracts hardly exerted any antimicrobial activity ([Thuille et al., 2003](#)). Also, [Marone et al. \(2001\)](#) who found that 50% and 90% of *H. pylori* strains were inhibited at mastic gum concentration of 125 µg/mL and 500 µg/mL, respectively. While, [Miyamoto et al. \(2014\)](#) found that from the identified 20 chemical compositions of mastic gum essential oil, an (E)-methyl isoeugenol exhibited potent antibacterial activity against four *H. pylori* strains including clarithromycin and metronidazole resistant strain. [Sharifi and Hazell \(2011\)](#) reported that the MIC values of essential oil of the mastic gum examined ranged 500-1000 mg/mL and its major constituent was α-pinene.

According to our knowledge, there is a study concerning antimicrobial effect of mastic oil against *E. coli* O157 in yoghurt ([Pagiataki et al., 2013](#)). However, this is the first study discussing the anti-*H. pylori* effect of essential oil extracted from mastic gum in ice cream. In ice cream samples stored at domestic freezer temperature (-5°C), the added essential mastic gum oil (0.8 and 1.6% v/v) suppressed quickly the growth of the inoculated *H. pylori* (approximately 1-log and 1.2 log, respectively) within the hours of hardening. The most bactericidal action was observed after third day of storage causing about 4-logs decrease in the load of *H. pylori* with the reduction of 99.2% for the sample treated with 0.8% v/v of mastic essential oil, while it was at the second day of storage with reduction of 99.7% in ice cream sample treated with 1.6% v/v of mastic gum essential oil. *H. pylori* couldn't be counted (reaching 100% reduction) or isolated from samples at the first week versus the third day of storage for samples treated with mastic essential oil at concentration of 0.8 and 1.6 % v/v, respectively, while at

Table 1. Incidence of different *Helicobacter* spp. in the examined ice cream samples

Samples	No. of ice cream samples	Positive samples No. (%)	<i>Helicobacter</i> spp.		
			<i>H. pylori</i> No. (%)	<i>H. pullorum</i> No. (%)	<i>H. cinaedi</i> No. (%)
Small Scale ice cream	30	7 (23.3)	2 (6.7)	4 (13.3%)	1 (3.3)
Large scale ice cream	30	0 (0)	0 (0)	0 (0)	0 (0)
Total	60	7 (11.7)	2 (3.3)	4 (6.7)	1 (1.6)

Table 2. Effect of different concentrations of essential oil extracted from mastic gum on *H. pylori* in lab prepared ice cream stored at freezing temperature (-5°C)

Storage period	Control group	Essential oil of mastic gum (% v/v)			
		0.8%	Red%	1.6%	Red. %
Initial count	1.5x 10 ⁶	1.5x10 ⁶		1.5 x 10 ⁶	
After hardening	7.8x10 ⁵	1.8x10 ⁵	76.9	9.8x10 ⁴	86.3
1 st day	3x 10 ⁵	1.9x10 ⁴	93.7	6.6x10 ³	97.8
2 nd day	1x10 ⁵	6.4x10 ³	93.6	3x10 ²	99.7
3 rd day	7.9x10 ⁴	6x10 ²	99.2	N.D	100
1 st week	8.5x10 ³	N.D	100	-	-
2 nd week	-	-	-	-	-
3 rd week	-	-	-	-	-
4 th week	-	-	-	-	-

Table 3. Effect of different concentrations of essential oil extracted from mastic gum on *H. pylori* in lab prepared ice cream stored at domestic deep freezing temperature (-20°C)

Storage period	Control group	Essential oil of mastic gum (% v/v)			
		0.8%	Red. %	1.6%	Red. %
Initial count	1.5x 10 ⁶	1.5x10 ⁶		1.5 x 10 ⁶	
After hardening	2x10 ⁵	2.3x10 ⁴	88.5	8.5x10 ³	95.7
1 st day	7x 10 ⁴	8.5x10 ²	98.7	5.7x10 ²	99.2
2 nd day	2.6x10 ⁴	2.5x10 ²	99	100	99.6
3 rd day	4x10 ³	N.D	100	N.D	-100
1 st week	-	-	-	-	-
2 nd week	-	-	-	-	-
3 rd week	-	-	-	-	-
4 th week	-	-	-	-	-

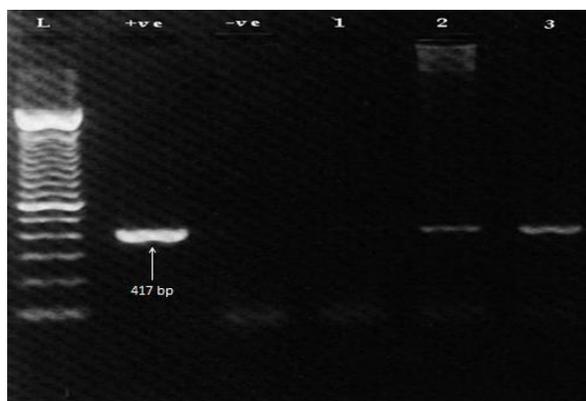


Figure 1. Confirmation of *H. pylori* isolates by PCR reaction. Lane L: Ladder, Lane +ve: positive control, Lane -ve: negative control. Lane 1, 2, 3: *Helicobacter* isolates obtained from ice cream samples, Lane 2 and 3 are *H. pylori* isolates and amplified at 417-bp fragment of the flanking region of *H. pylori ureC* gene.

the same time, its count was 8.5x10³ and 7.9x10⁴ CFU/mL in control samples, respectively (Table 2).

From data showed in Table 3, it was found that the high initial population of *H. pylori* (inoculum 10⁶ CFU/gm) in samples stored at -20 °C was reduced by about 2-logs and more than 2-logs in samples treated with 0.8 and 1.6% v/v essential oil of mastic gum, respectively during the

hours of hardening. The most drastic effect on *H. pylori* was observed in ice cream samples held at -20°C at the second day of storage for both concentrations 0.8 and 1.6% v/v (about 99 and 99.6 reduction %, respectively). We failed to count or isolate *H. pylori* at the third day of storage from samples contained 0.8 and 1.6% v/v mastic oil, respectively, where it was found in control sample with a count of 4x10³ CFU/mL at the same time. Also in these samples, it was noticed that there is slower dimension rate of *H. pylori* load as compared to those stored at -8°C also 100% reduction in *H. pylori* couldn't be reached in samples with 1.6% v/v mastic oil before samples with 0.8% v/v mastic oil and stored at -20°C as expected. We couldn't find an explanation clarifying this behavior of mastic essential oil under -20°C. The pronounced effect of mastic oil against microarobic bacteria could be related to its enhanced activity under lower oxygen tensions (Gkogka et al., 2013).

From food safety point of view, Solnick et al. (2001) stated that the minimum infectious dose of *H. pylori* is 10⁴ CFU and it was found that 0.8 and 1.6% mastic oil concentrations achieved reduction in *H. pylori* under 10⁴ CFU at the second and first day of storage of ice cream samples stored at -5°C, respectively, while in samples stored at -20°C, this reduction under 10⁴ CFU was

accomplished after the first day of storage and after hardening time for the two concentrations, respectively. From the therapeutic point of view, ice cream with mastic gum essential oil can play role as a functional food as its inclusion in the diet can be helpful, not only in patients with clinical symptoms but also in case of asymptomatic patients as it could reduce the risk, as well as the development of an unfavourable outcome of the infection.

CONCLUSION

Food safety regulations as well as quality standards including good manufacturing practices (GMPs) and hazard analysis and critical control points (HACCP) should be more concerned in Egyptian food in order to control contamination and proliferation of pathogenic bacteria. The present study shows that mastic oil at the tested concentrations has spectacular bactericidal effect against *H. pylori* inoculated in ice cream. Ice cream being so delicious food could be modified to a functional food by addition of essential oil of mastic gum and used as a therapy and introduced to the diet of patients to help in eradication of *H. pylori* from patients' stomach.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest with any other people or organizations in any financial or personal relationship.

AUTHORS' CONTRIBUTION

NMS and MAE contributed equally. Both the authors read and approved the final manuscript.

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