Anti-*Helicobacter Pylori* Activities of Shoya Powder and Essential Oils of *Thymus Vulgaris* and *Eucalyptus Globulus*

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Abstract: *Background: Helicobacter pylori*, an infective agent of more than 50% of the world population is prominent to be the main causative factor in the etiologies of chronic, active or type B gastritis, peptic and duodenal ulcer, gastric carcinoma, and mucosa-associated lymphoid tumors. A high prevalence of this bacterium in dental plaque is always reported. Pharmacological treatment of *H. pylori* infections includes administration of 3-fold therapeutic regimens which are typically used to suppress *H. pylori* activity. However, antibiotic resistance frequently develops as a consequence of such treatment. Thus, searching for alternative therapies for *H. pylori* infections is of special interest.

Materials and Methods: In this study, anti *H. pylori* activities of a traditional antimicrobial drug so-called Shoya and also essential oils of *Thymus vulgaris* and *Eucalyptus globulus* were investigated using antimicrobial analysis and serological screening methods.

Results: The agar dilution method results revealed the Shoya with the highest inhibitory effect against *H. pylori*. Also serological screening on tested mice showed a significant effect of this drug in lowering the sera amount of anti *H. pylori* specific IgA and IgG titers. Both of the essential oils showed different degrees of antibacterial effect against *H. pylori*.

Conclusion: The obtained results showed the antibacterial effect of Shoya powder and Essential oils from *Thymus vulgaris* and *Eucalyptus globulus* and purposes new therapeutical alternatives to control the *H. pylori* infection. Additional studies and clinical trials are necessary to approve the use of these data in health care and pharmacopeia systems.

Keywords: *Helicobacter pylori,* Shoya, Thymus vulgaris, Eucalyptus globulus.

INTRODUCTION

Helicobacter pylori is an extracellular gram-negative, spiral bacterium, which typically infects 40% of the adult population in developed countries and up to 90% in some developing countries [1, 2]. Chronic gastritis is seen in nearly all individuals, 10-15% of whom will develop peptic ulcer disease or gastric cancer, the second most common cause of cancer mortality worldwide [3]. There is a high prevalence of *H. pylori* related gastric infections and dental plaque colonization in developing countries [4, 5]. Current therapies for *H. pylori* are typically based on combination of a proton pump inhibitor and two antibiotics, but drawbacks include patient compliance, antibiotic resistance, and recurrence of infection. Since infection can cause life threatening diseases and therapy is neither 100% effective nor universally available, development of new therapies may be critically necessary [6].

The Shoya powder is a compound of five substances and acts as a strong antimicrobial drug which can be used for treatment of severe and mild infections. Essential oils (EOs) have been shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties [7-9]. Some oils have been used in food preservation [10], aromatherapy [11] and fragrance industries [12, 13]. Therefore, it is reasonable to expect a variety of plant compounds in these oils with specific as well as general antimicrobial activity and antibiotic potential [14].

Thyme *(Thymus vulgaris* L.), a member of the Labiateae family, is an aromatic and medicinal plant of increasing importance in horticulture [15, 16]. *T. vulgaris,* also known as *common thyme*, has long been used as a source of the essential oil (thyme oil) and other compounds (e.g. thymol, flavanoid, caffeic acid and labiatic acid) derived from the different parts of the plant [17, 18]. The oil was reported to have antimicrobial effect on bacteria and fungi [19-21] carminative and expectorant [17] activities, most of which are mediated by thymol and carvacrol, as the phenolic components of the oil [22].

Eucalyptus is native to Australia. The genus Eucalyptus contains about 600 species. Of all the species, *Eucalyptus globulus* is the most widely cultivated in subtropical and Mediterranean regions [23]. Essential oils from *Eucalyptus* species are used in folk medicine and also widely used in modern cosmetics, food, and pharmaceutical industries [24, 25].

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The present study was aimed to investigate the anti *H. pylori* activities of Shoya and also essential oils of *Thymus vulgaris* and *Eucalyptus globulus*.

Preparation of Shoya Powder and Essential Oils

The Shoya powder suspensions were prepared in 6 dilutions (10^{-1}–10^{-6} mg/ ml) using distilled water as the solvent. The herbs of *T. vulgaris* (garden Thyme) and the leaves of *E. globulus* collected and harvested at full flowering state and were authenticated by Dr. R. Omidbaigi, (Professor of botany at the college of agriculture, Tarbiat Modares University, Tehran, Iran). To isolate the oils, the collected materials of every treatment (90g three times) were subjected to hydrodistillation using a Clevenger type apparatus for 6 hours to produce essential oil according to the method recommended by the European Pharmacia [26]. The oils were dried over anhydrous sodium sulfate and stored in sealed vials at low temperature (4°C) before analysis.

Bacterial Strain and Culturing

Helicobacter pylori ATCC 700392 and *Helicobacter pylori* clinical isolates were cultured in brucella agar [QUELAB incorporations, Canada. containing: peptone, yeast extract, dextrose, sodium chloride, agar and PH of 7.5 in each liter], plus 5% (v/v) defibrinated sheep blood and 10% (v/v) fetal calf serum.

Antimicrobial Analysis

The agar dilution method was used as approved by the NCCLS [27] with minor modifications: a series of two fold dilutions was prepared for each of the *Thymus* and *Eucalyptus* essential oils as the following: 0.03% (v/v), 0.05% (v/v), 0.07% (v/v) and 0.09% (v/v) in the enriched brucella agar medium. To enhance the essential oil solubility, 0.5% (v/v) of tween-20% was added into agar. The Shoya powder suspensions were prepared in serial dilutions and spread into mediums. Inoculated plates with 5µl of *H. pylori* containing about 5×10^5 of the microorganism were incubated in a microaerophlic jar system (5% O_2, 10% CO_2 and 85% N_2) at 37°C for 72 h and then visible colonies formed on the plates were enumerated. The MIC values were defined as the lowest concentration of Shoya and EOs at which no colony of the test bacteria was detected. Finally, the agar disk diffusion method was used to assess the anti *H. pylori* activity of Shoya suspensions. 100µl of each suspension containing about 10^8 cells/ ml was spread on enriched brucella agar mediums. Six mm filter papers containing 10-12 µl from any of suspensions were placed on agar surface. Inoculated plates were incubated for 72 h as described above and then the inhibition zones were measured in diameters. Each test was repeated as duplicate.

Immunization Assay

In order to analyze the immunological effect of Shoya powder on antibody production, a group of 20 mice showing very low titers of IgA and IgG (T0) against *H. pylori* were first challenged orally with *H. pylori* and evaluated after two weeks to analyze their specific anti *H. pylori* IgA and IgG titers (T1) in sera using a mouse anti *H. pylori* IgA and IgG serotyping kit (Roche bichemicals, Germany). These were then treated orally with the Shoya solution for two weeks and were tested for the final titers (T2) of IgA and IgG to assess the potential therapeutic and eradicative effect of Shoya powder against *H. pylori*.

Analyzing Gastric Tissue

Finally, the mice stomachs were dissected by gastrectomy and divided into longitudinal strips to assess the presence of *H. pylori* using a rapid urease broth test kit (Chemy Enzyme chemicals, Iran).

$T1^a$: Titers of specific anti-*H. pylori* IgA and IgG in tested mice sera before challenging with *H. pylori*. $T2^b$; Titers of specific anti-*H. pylori* IgA and IgG in mice sera two weeks after challenging with *H. pylori*. $T3^c$; final titers of IgA and IgG in mice infected with *H. pylori* and treated two weeks with Shoya suspension. Antibody levels are shown as Mean ± SD of ODR per microgram of protein units for anti *H.pylori* IgG and IgA.

RESULTS

Antimicrobial Assay

The MIC results for three tested compounds are shown in Tables **1** and **2** which show the antibacterial effect of Shoya powder and essential oils of T. vulgaris and E. globulus against H. pylori ATCC 700392 using agar dilution method. Shoya powder exhibited relatively a very high anti *H. pylori* activity (10^{-5} mg ml^{-1}) (Table **2**). Anti *H. pylori* activity in *T. vulgaris* and *E. globulus* were 10.8 and 46.4 (µg/ml) respectively.

Table 1. Antimicrobial Activity of Thymus Vulgaris and Eucalyptus Globulus Essential Oils Serial Dilutions Against H. Pylori ATCC 700392

H. Pylori Growth	Eucalyptus Globules (µg/ml)	H. pylori Growth	Thymus Vulgaris (µg/ml)
+	5.8	+	5.4
+	11.6	–	10.8
+	23.2	–	21.2
–	46.4	–	42.4
–	92.8	–	84.8

Table 2. Antimicrobial Activity of Shoya Powder Suspensions Against *H. Pylori* ATCC 700392

Inhibition Zone (mm)	Visible Growth	Suspension Dilutions (mg/ml)
16	–	$1/10(10^{-1})$
16	–	$1/100(10^{-2})$
15.5	–	$1/1000(10^{-3})$
15	–	$1/10000(10^{-4})$
14	–	$1/100000(10^{-5})$
12	+	$1/1000000(10^{-6})$

MATERIALS AND METHODS

Immunization Assay

The Shoya powder suspension treatments against challenged mice could apparently reduce the specific anti *H. pylori* IgA and IgG. Table **3** shows the tested mice immunization analysis results obtained during 4 months of screening.

Rapid Urease Broth Test

This test detects *Helicobacter pylori* (*H. pylori*) by finding the presence of urease. Urease is an enzyme produced by *H. pylori*. Urease broth is a differential medium that tests the ability of an organism to produce an exoenzyme, called urease that hydrolyzes urea to ammonia and carbon dioxide. The broth contains two pH buffers, urea, a very small amount of nutrients for the bacteria, and the pH indicator phenol red. Phenol red turns yellow in an acidic environment and fuchsia in an alkaline environment. If the urea in the broth is degraded and ammonia is produced, an alkaline environment is created, and the media turns pink.

DISCUSSION

There are problems with current antibacterial treatments against *H. pylori* such as multidrug resistance, high expenses, drug interventions, poor satisfaction, side effects and their impact on the normal intestinal flora [6] which together highlight the need for alternative therapeutic methods such as traditional medicine. Yuan-Chuen Wang *et al* reported anti *H. pylori* activity of *Plumbago zylanica* L. with MIC of 0.32 to 1.28 mg ml^{-1} [28]. Cellini *et al*., reported that the phosphate extract of garlic possesses anti *H. pylori* activity against 19 strains of *H. pylori* with MIC ranging from 2-5 mg ml^{-1} [29]. The anti *H. pylori* activity of the methanol extract of *Myroxylon peruiferum*, a medicinal plant of Brazil was 62.5 mg ml^{-1} [30]. The anti *H. pylori* effect of 22 micromyctes was studied against one standard strain and 11 clinical isolates of *H. pylori. Penicillium ochlochloron* and *Penicillium funiculosum* have been proven as the most active fungi against this microorganism (MIC 3.9 mg ml^{-1}) [31]. Our findings through this research significantly indicate Shoya powder as a potential lead compound of a novel class of *H. pylori* inhibitors where it shows a very high anti *H. pylori* effect (MIC 10^{-6}mg ml^{-1}). More ever, it is not toxic, and is widely available as a low price traditional drug compound.

Determine the antibodies against *H. pylori* yields in a relatively simple diagnosis, especially with kits that can be used to perform this method and are now being widely and commercially available [32].

Table 3. Serological Analysis of Variable Titers of IgA and IgG in Mice Challenged with *H. Pylori* and Treated with Shoya During 4 Months

$T3^c$(µg/ml)		$T2^b$(µg/ml)		$T1^a$(µg/ml)		Tested Mice
$T3^c$		$T2^b$		$T1^a$		
IgG	IgA	IgG	IgA	IgG	IgA	
4±0.23	5±0.27	21±0.88	30±0.12	3±1.23	6 ±1.34	1
2±1.21	3±1.61	18±0.97	27±0.39	2±1.11	5 ± 1.29	2
2±2.13	8±1.81	7±2.11	35±0.99	3±0.78	7.5 ±1.2	3
2±1.33	4±2.20	7±1.21	15±1.11	4±0.93	8 ± 1.02	4
2±0.87	3±1.23	8±1.29	12±1.21	2±1.24	3.5 ±1.48	5
2±1.12	3±2.48	7±2.11	13±1.43	2.5±1.78	4 ± 1.11	6
2±0.89	3.5±1.11	6±0.22	17±1.65	3±2.13	5± 1.42	7
3±0.79	9±7.40	15±1.15	45±0.34	4±2.53	9 ± 1.63	8
3±1.26	4±2.01	15±1.10	19±0.84	5±2.36	6.5 ±.12	9
2.5±2.01	3.5±1.22	16±1.17	23.5±0.38	6±0.79	7 ± 1.43	10
3±0.96	4±2.43	16±2.11	28.5±1.41	5±0.63	9 ± 2.18	11
3±1.24	3±0.75	17±1.19	29±1.32	3±2.16	4 ± 1.21	12
3.5± 1.09	4±1.88	11±1.32	12±1.65	2.5±2.32	± 2.423	13
2.5±2.11	4±1.37	12±0.86	14±1.67	2±.94	3.5 ±0.89	14
2.5±1.31	3±2.17	12±1.53	17±2.22	2±1.37	3 ±0.96	15

$T1^a$: Titers of specific anti-*H. pylori* IgA and IgG in tested mice sera before challenging with *H. pylori*. $T2^b$; Titers of specific anti-*H. pylori* IgA and IgG in mice sera two weeks after challenging with *H. pylori*. $T3^c$; final titers of IgA and IgG in mice infected with *H. pylori* and treated two weeks with Shoya suspension. Antibody levels are shown as Mean ± SD of ODR per microgram of protein units for anti *H.pylori* IgG and IgA.

Evaluating the effect of Shoya powder suspensions on specific antibody production in human and mice cases clearly resulted in a meaningful decrease in titers of specific anti *H. pylori* IgA and IgG which can be referred to the therapeutic effect of this traditional drug. Essential oils are considered as possible sources of new antimicrobial agents especially against bacterial pathogens [33]. Many studies have investigated the antibacterial activity of essential oils from *T. vulgaris* and *E. globulus* against different pathogens [34]. Their antimicrobial activity is mainly attributed to the presence of some active constituents in their EOs together with their hydrophobicity which enables them for rupturing cell membranes and intrastructures [35]. In this study, EOs of *T. vulgaris* and *E. globulus* were used to assess their antibacterial activity against *H. pylori* ATCC 700392 by inserting some minor changes to the NCCLS recommended agar dilution method that have been originally developed for analyzing the conventional antimicrobial agents activity, so it could be used to analyze plant extracts and essential oils for their antimicrobial activity [36]. The obtained results confirm that EO from *T. vulgaris* showed better inhibitory effect against *H. pylori* than EO from *E. globulus*. Previous studies performed in Pakistan [37, 38] India [39], Nigeria [40] and Venezuela [41] indicate positive correlation between oral and gastric *H. pylori* colonization. It is implicated that oral cavity may be the first colonization site which then infects the gastric mucosa.

According to difficulties for eradication of *H. pylori* Due to the disadvantages of antibacterial treatments and presence of *H. pylori* in mouth as a secondary reservoir [42] and also the obtained results of this research, it is recommended to combine the triple drug treatment regime with Shoya as a mouth washing solution or as a tooth paste ingredient or together with EOs of *T. vulgaris* and *E. globulus* in order to control the *H. pylori* presence specially for eradication of *H. pylori* in dental plaques and related diseases. Additional clinical research and trials are necessary to completely confirm the above results for medical purposes. As mentioned above, dental plaques play a critical role as important reservoirs for *H. pylori*, therefore this bacteria will be able for colonization in dental plaque and inside oral yeasts where is protected from antibacterial drugs effects. In this study using Shoya powder against *H. pylori* resulted in complete eradication of this bacterium which can be effective enough to reduce the rate of infection transmission from mouth to gastric.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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