**Syzygium aromaticum** L.: Traditional herbal medicine against cagA and vacA toxin genes-producing drug resistant *Helicobacter pylori*

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**ABSTRACT**

The Pan-Drug Resistant (PDR), *Helicobacter pylori* remains an intractable challenge in public health worldwide and this pathogenicity is mainly due to the presence of a cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA). On the other hand, plant extracts such as *Syzygium aromaticum* contain a diverse array of secondary metabolites, which could be potentially used to combat *H. pylori* pathogens. To our knowledge, this is the first report on the biomedical potential of *S. aromaticum* extract against cytotoxin-associated genes producing PDR *H. pylori*. In this investigation, out of 45 gastric antral biopsy specimens of dyspeptic patients, 20 strains were confirmed as *H. pylori*. Eight (40%) out of 20 strains were PDR *H. pylori* while the rest of the strains were Multi-Drug Resistant (MDR) strains. Genotypic analyses of PDR *H. pylori* strains showed that *cagA* and *vacA* genes were found to be 75% and 87.5%, respectively and m2s2 was the most common subtype of *vacA* gene. *S. aromaticum* showed a significant higher anti-*H. pylori* activity compared to that of *Cinnamomum zeylanicum* and *Thymus vulgaris*. Eugenol was the major phenolic compound (28.14%) detected in the methanolic extract of *S. aromaticum*. Clearly, results of the toxicological assessment confirmed the safety of *S. aromaticum* for use. Hence, these results suggest that *S. aromaticum* could be a new useful natural antimicrobial agent that could potentially combat cytotoxin genes-producing drug-resistant *H. pylori*. Moreover, these findings provide a scientific basis for the development of antimicrobial agents from traditional herbal medicines for gastroprotection against gastric ulcer.

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1. Introduction

*Helicobacter pylori* (*H. pylori*) infection is a well-known risk factor for human gastritis, gastric cancer and inflammation-associated diseases.1–3 Gastric cancer is a model for inflammation-induced cancer, which is recognized as the third most common cause of cancer related death worldwide.4 *H. pylori* which has been christened as a class 1 carcinogen by the World Health Organization (WHO), acquired the shape of an epidemic emerging as a principle cause of gastric carcinoma.5 Up to 50% of the world’s population harbors *H. pylori* in the upper gastrointestinal tract and most infected persons are asymptomatic.6 On the other hand, the prevalence of *H. pylori* infection is rising at an alarming rate in developing countries like Egypt and India.7,8

Bacterial virulence factors are often key factors in *H. pylori*–host interactions pathogenesis, where the risk of ulceration is higher with more virulent strains.9 Between 5 and 10% of *H. pylori*’s 1600 genes are thought to be *H. pylori*-specific, cytotoxin-associated (*CagA*) and vacuolating cytotoxin (*VacA*) are considered the best-described virulence determinants in *H. pylori*. These two

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virulence factors are chosen, in this study, as there is considerable evidence regarding their potential roles in disease causation, and stimulation of the host inflammatory response. CagA, is a highly immunogenic protein encoded at one end of the cag pathogenicity island, which injects CagA and other proteins into host cells. Despite the numerous in vitro studies dealing with the identification of a precise role for CagA in the pathogenesis of gastric cancer, its in vivo role remains unidentified. In one interesting study, in vivo CagA-expressing H. pylori was associated with an enhanced host inflammatory response and therefore an increased risk of peptic ulcer or gastric cancer. CagA-positive H. pylori is also associated with the formation of gastric epithelial cell pedestals, change of the cytoskeleton, and enhancing gastric epithelium cells to produce IL-8. The prevalence of CagA among H. pylori varies greatly from almost 100% in East Asia to less than 50% in some western countries. In comparison with CagA-negative strains, only CagA-positive strains were able to obtain all their necessary nutrients and iron directly from the cells and survive when nutrients were removed from the fluids overlying the cells. On the other hand, although almost all H. pylori contain the vacA gene, it remains unclear what the exact role of VacA in disease pathogenesis is. However, it is possible that VacA has a major role in disease pathogenesis through the autophagy process where VacA assists in producing vacuoles and H. pylori can survive intracellularly. Furthermore, to enhance vacuolation, VacA causes several cellular activities, such as membrane-channel forming, cytochrome c liberation from mitochondria leading to apoptosis and attaches to cell-membrane receptors, followed by induction of a proinflammatory response. Clearly, the presence of direct H. pylori-host interactions result in genetic instability of the host genome.

Gastric cancer is one of the most prevalent causes of cancer deaths worldwide. Therefore, efforts are underway to eradicate this cancer type based on the elimination of H. pylori. Eradication of H. pylori before the development of significant gastric damage, can prevent cancer and the gastric cancer in such cases is known to be an inflammation-associated malignancy. It has been reported that, eradication therapy of H. pylori usually consists of a proton pump inhibitor or bismuth compounds in combination with different antibiotics. The conventional antibiotics most widely used for H. pylori therapy include amoxicillin, tetracycline, metronidazole, levofloxacin, clarithromycin and bismuth. However, the resistance of H. pylori to the commonly used antibiotics is still increasing worldwide, and this resistance has been considered a major reason for therapy failure in the eradication of H. pylori infections, with failure rates of more than 40%. The Multi-Drug Resistance (MDR) and Pan-Drug Resistance (PDR) remain an intractable challenge in public health, worldwide. MDR strain is defined as resistant to three or more antimicrobial classes. However, resistance to all agents in all antimicrobial classes is defined as PDR. These factors, as well as others including the high cost of combination therapy, side effects of therapy and poor compliance of patients have required the search for new alternative therapies, especially from herbs that have lower side effects and can hopefully eradicate this significant human pathogen.

Medicinal plants are a source of natural phytochemical compounds that possess therapeutic properties, and play an important role in treating many human diseases. Antibacterial activity of numerous natural products has been recorded against H. pylori. For centuries, several types of herbal plants and components derived from natural sources have been used in the treatment of gastrointestinal disorders. Syzygium aromaticum is commonly known as clove and belongs to the family Myrtaceae. Clove has been known to possess various antimicrobial, antioxidant, antiviral, anticancer, anti-inflammatory and anti-nociceptive activities. Clove has been used as a food preservative, flavoring agent and for the treatment of gastrointestinal disorders. To our knowledge, this is the first report on the biomedical potential of S. aromaticum extract against cytotoxin-associated genes producing PDR H. pylori. The characterization and cytotoxicity activity of S. aromaticum were examined to evaluate the performance of this plant extract as a new leading structure in the biomedical and pharmaceutical fields.

2. Materials and methods

2.1. Processing of gastric biopsy samples

The biopsy specimens were collected from 45 dyspeptic patients who attended the Endoscopic Unit of Tanta University Hospital, after a written approval from these patients who were informed of the final results. Two gastric biopsies were taken from the antrum of each patient. Patients taking antimicrobial drugs, proton pump inhibitor, and/or bismuth salts two weeks prior to the endoscopy were excluded. The first biopsy was placed directly in a sterile tube containing 1 ml of Phosphate Buffer Saline (PBS) solution or added to 1 ml Tryptic Soy Broth (TSB) as a transport medium, then transferred to the Microbiology Unit, Faculty of Science, Tanta University and processed for culture as previously described. The second biopsy was used for histopathology examination.

2.2. Isolation and characterization of H. pylori

2.2.1. Phenotypic characterization

Specimens were processed within less than 1 h. Each biopsy specimen from each patient was separately minced within their transport medium in a tissue grinder (mortar) using a sterile pestle and rapidly inoculated onto selective Columbia Blood Agar (CBA; Oxoid, England) plates supplemented with 5% defibrinated sheep blood, including, trimethoprim (5 mg/l), cefulodin (5 mg/l), vancomycin (10 mg/l), and amphotericin B (5 mg/l). Plates were incubated at 37 °C in an anaerobic jar for 3–10 days under microaerophilic conditions (5% O2, 10% CO2 and 85% N2) by using Campygen kits (Oxoid, Basingstoke, UK). Small fragments from each grinded biopsy specimen were separately placed into Christensen’s Urea Broth (CUB) for a rapid urease test. Bacterial morphology was examined by Gram staining to verify the presence of Gram-negative spiral rod-shaped bacteria and typical colony morphology (small round colonies) as shown in Supplemental data: Fig. S1. Bacterial isolates recovered from microaerophilic conditions were confirmed phenotypically as H. pylori on the basis of positive reactions for urease, catalase and oxidase tests. In this study 20 out of 45 cultivated gastric biopsies were positive for H. pylori and were designated as HP-1 to HP-20.

2.2.2. Molecular characterization

Total genomic DNA was extracted from the biopsy homogenates using the Quick- DNA™ Mini prep kit (Zymo Research, USA) following the manufacturer’s protocol. The methods applied for determining bacterial genomic DNA concentration, purification, PCR amplification, as well as DNA sequencing were actually performed according to earlier reports. Genotypic characterization of H. pylori was carried out by PCR analysis for several bacterial genes including cagA, vacA and 16S rRNA. Primers and PCR amplification conditions used in this study are given in Table 1. The PCR products were confirmed for size and purity on 2% agarose gel run with 1 X TAE buffer and stained with ethidium bromide.

2.3. Histopathologic examination

Histopathology was performed to verify the infection with H. pylori. The gastric biopsies used for histopathology were...
transported with neutral buffered formalin (10%) for at least 24 h to the Histopathology Laboratory, Faculty of Medicine, Tanta University. These biopsies were then processed.44 The biopsy sections were stained with modified Giemsa stain to determine the presence of H. pylori and for evaluation of the pathological changes to the gastric mucosa.

2.4. Antibiotic susceptibility test

In the present study antibiotic discs of different classes (Oxoid, England) were used to determine the in vitro sensitivity of H. pylori positive clinical strains to ten antimicrobial agents commonly used in the treatment of H. pylori (Table 2). The susceptibility of strains to antibiotics was performed by disk diffusion method.46 The cultivated plates were incubated under microaerophilic conditions for 72 h at 37 °C. The zone of inhibition was interpreted based on the /C14 conditions used in this study.

2.5. Plant materials

Three herbal plants namely Clove (Syzygium aromaticum L.), Cinnamon (Cinnamomum zeylanicum Nees.), and Thyme (Thymus vulgaris L.) (Supplemental data; Table S1) were used in this study and were selected based on research into their use in traditional medicine, and their utilization in popular diets especially in Egypt. The plants were purchased from the local market in Tanta, Egypt. Botanical identification of the plant’s samples was performed in the Herbarium, Botany Department, Faculty of Science, Tanta University, Egypt.

2.6. Extract preparation and antibacterial activity

The dried parts of selected plants were ground into powder using a blender. The extraction process was done as previously mentioned.27 Briefly, methanol and ethanol were used as organic solvents for the extraction. Five grams of each powdered herbal plant used in this study were soaked in 40 ml of the solvent for 3–4 days. Remain extracts were filtered and concentrated in a rotatory evaporator at 35 °C. The residual water was removed with a vacuum pump. The weighted crude extracts were suspended in the dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/ml and stored in a refrigerator. As DMSO has no antimicrobial activity, it was used as a negative control. For the preparation of aqueous extracts, the same amount of plant material was soaked in distilled water. The PDR H. pylori strains were screened for their susceptibility to different extracts of the selected plants using the agar well diffusion method.45 Briefly, 100 μl of the fresh culture of H. pylori (10^6 CFU/ml) was surface inoculated with a sterile cotton swab into Muller-Hinton blood agar (MHB). The plates lifted to dry for 10 min and wells of 9 mm diameter were made in a MHBA surface using a sterile cork borer. A fixed volume of 100 μl of each extract at 100 mg/ml was placed in the wells. The DiMethyl Sulfoxide (DMSO)

| Table 1 | Primers and PCR amplification conditions used in this study. |
|---|---|---|
| Gene target | Orientation | Primer sequence (5′-3′) | PCR amplification conditions |
| VacA (s1/s2) | Forward | ATGGAATACAAACAAACAC | *Initial denaturation: 94 °C for 10 min; |
| | Reverse | CTTGTTGATGCGCCAAC | *Denaturation: 35 cycles of heating at 94 °C for 30 s; |
| | | | *Annealing: 56 °C for 30 s; |
| | | | *Extension: 72 °C for 30 s; |
| | | | *Final extension: 72 °C for 10 min. |
| VacA (m1/m2) | Forward | CAATCTCTCAATCAAGGGAG | *Initial denaturation: 94 °C for 10 min; |
| | Reverse | GCGTCTAAATAATTCCAAGG | *Denaturation: 35 cycles of heating at 94 °C for 30 s; |
| | | | *Annealing: 53 °C for 30 s; |
| | | | *Extension: 72 °C for 2 min; |
| | | | *Final extension: 72 °C for 10 min. |
| 16S rRNA | 27F | AGACTTGATCGTCGCTC | *Initial denaturation: 95 °C for 5 min; |
| | 1513R | ACGYTACCTGTTACGA | *Denaturation: 35 cycles of heating at 94 °C for 30 s; |
| | | | *Annealing: 54 °C for 30 s; |
| | | | *Extension: 72 °C for 3 min; |
| | | | *Final extension: 72 °C for 10 min. |

| Table 2 | List of antibiotics used in present study. |
|---|---|---|---|
| Antibiotic | Code | Class | Concentration (µg/disk) |
| Amoxicillin | AX | Pencillins | 25 |
| Ampicillin | AM | Pencillins | 10 |
| Amoxicillin/clavulanic acid | AMC | β-lactamase inhibitors | 30 |
| Levofoxacin | LEV | Quinolones | 5 |
| Ciprofloxacin | CIP | Quinolones | 5 |
| Metronidazole | MTZ | Nitroimidazoles | 5 |
| Clarithromycin | CLR | Macrolides | 15 |
| Erythromycin | ERY | Macrolides | 15 |
| Tetracycline | TE | Tetracyclines | 30 |
| Gentamicin | CN | Aminoglycosides | 10 |
was taken as a negative control. The experiment was determined in triplicates and results were presented as the mean ± SD. The mean inhibition zone diameters calculated and recorded in millimeters.

**2.7. Characterization of the Syzygium aromaticum extract**

The methanol extract of *S. aromaticum* was subjected to Gas Chromatography-Mass Spectrometry (GC-MS) analysis using GC-MS model Claus 580/560S, Perkin Elmer Company. The GC conditions were applied according to Safrudin et al.\(^{47}\) The name, molecular weight and compound nature of the *S. aromaticum* extract were identified based on the National Institute of Standard and Technology (NIST) library spectra data bases.

Fourier Transform InfraRed (FT-IR) analysis was carried out for determining the characteristic functional groups of the *S. aromaticum* extract using FT/IR spectrophotometer Perkin-Elmer 1430. The samples were prepared\(^{48}\) and scanned within the transmittance range of 4000-400 cm\(^{-1}\).

**2.8. Cytotoxicity assay**

Cytotoxicity assay was used to detect the treatment concentration that does not have a toxic effect on normal cells. Peripheral Blood Mononuclear Cells (PBMCs) were selected as normal cell modeling for this experiment. Potential cytotoxicity of the selected *S. aromaticum* methanol extract was performed\(^{48}\) by using different concentrations of this plant extract (100–15 mg/ml). Cell viability was calculated as follows [cell viability % = (controlled cells-treated cells)/controlled cells x 100]. This assay was determined in triplicates and results were presented as the mean ± SD.

**2.9. Statistical analysis**

In this study, PC-ORD for windows (ver.5) was used for two-way hierarchical cluster analysis using Sorensen methods for distance and beta (−0.025) for group linkage. The data was collected, tabulated and statistically analyzed using Minitab 17.1.0.0 for windows (Minitab Inc., 2013, Pennsylvania, USA). All tests were two-sided. A *p*-value < 0.05 was considered significant. Data normality was checked for using the Shapiro-Wilk test. An independent t-test was used, and a chi-square test for comparison between two or more groups of categorical data. One-way and two-way ANOVA tests were used to compare between more than two groups.

**3. Results and discussion**

**3.1. Demographic and clinical characteristics of patients**

The current study was carried out on 45 dyspeptic patients clinically expected to have an infection caused by *H. pylori*. The mean age of patients with positive *H. pylori* was 53 years, which was older than those with a negative *H. pylori* state but with insignificant statistical difference. The majority of patients were males (31/45; 68.9%) and from rural areas (36/45; 80%) with insignificant statistical association of specific gender type or residence area with positive *H. pylori* state (Table 3). Even though 40% of positive *H. pylori* patients were smokers but with insignificant effect, so there is no association between smoking and infection by *H. pylori*. The endoscopic findings were different in patients (Table 3), the majority (65%) of patients with positive *H. pylori* showed peptic ulcer (duodenal or gastric), inflammatory mucosa was present in 20% of cases, with insignificant association of peptic ulcer or inflammation with positive *H. pylori* state. In addition, 20 (44.4%) out of 45 gastric antral biopsy samples were positive for *H. pylori* culture. Antrum is site of gastric biopsy that used by the majority of endoscopists with highly specificity and sensitivity (up to 90%). Gastric Antral biopsy specimens have been recorded to be more sensitive in *H. pylori* detection when compared to the specimens of corpus.\(^{59}\)

**3.2. Histopathologic examination**

Positive *H. pylori* gastric biopsies showed the pathological changes to the gastric mucosa and indicated the presence of *H. pylori* colonized in the lumen of the gastric glands, with chronic inflammatory infiltrate in lamina propria (Fig. 1A and B). On the other hand, negative *H. pylori* gastric biopsies showed normal human gastric glands and an absence of *H. pylori* bacteria from the lumen of the gastric mucosa (Fig. 1 C). The histopathology’s advantages include its capability of confirming the infection by *H. pylori* with high specificity and can explain the degree of inflammation. In this study *H. pylori* was determined by histopathology in 44.4% of the total cases studied. Inflamed mucosa was the most prominent histological picture of endoscopic biopsy in both positive and negative *H. pylori* patients, with insignificant association to a particular group of them (Table 3).

**3.3. Antibiotic susceptibility testing**

The clustering analyses of drug resistant-producing *H. pylori* presented in Fig. 2 revealed that green color clusters, consisting of

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**Table 3** Demographic and clinical characteristics of patients (n = 45).

| Traits (n, %) | Negative to *H. Pylori* (n = 25) | Positive to *H. Pylori* (n = 20) | *P*-value |
|--------------|----------------------------------|---------------------------------|-----------|
| **Demography** |                                  |                                 |           |
| Age (years)  | 46.7 ± 12.4                      | 53.1 ± 10.3                     | 0.06\(^{a}\) |
| Gender (male) | 16 (64)                          | 15 (75)                         | 0.4\(^{b}\) |
| Residence (Rural) | 20 (80)                  | 16 (80)                         | 1.0\(^{b}\) |
| Smoker       | 13 (52)                          | 8 (40)                          | 0.5\(^{b}\) |
| **Endoscopy** |                                  |                                 |           |
| Normal       | 5 (20)                           | 3 (15)                          | 0.4\(^{b}\) |
| Peptic ulcer | 10 (40)                          | 13 (65)                         |           |
| Inflammation | 10 (40)                          | 4 (20)                          |           |
| **Histology** |                                  |                                 |           |
| Inflamed mucosa | 22 (88)                      | 18 (90)                         | 0.4\(^{b}\) |
| Normal       | 3 (12)                           | 2 (10)                          |           |

\(^{a}\) Independent t-test.  
\(^{b}\) Chi square test.  

*P*-value ≤ 0.05 is considered significant.
eight strains; HP-1, HP-5, HP-8, HP-10, HP-12, HP-14, HP-15 and HP-16 showed 100% resistance to the examined antibiotics and were proven to be PDR strains, while the remaining 12 strains (red color clusters) were classified as MDR strains (Fig. 2). The high rate of antibiotic resistance among the isolated \textit{H. pylori} strains from dyspeptic Egyptian patients is a significant finding in this study, where the highest resistance of all 20 \textit{H. pylori} clinical strains recorded by amoxicillin (AX) and ampicillin (AM) was found to be 100%, followed by metronidazole (MTZ; 95%), clarithromycin (CLR; 90%) and erythromycin (ERY; 90%). Our results are in agreement with Hamada et al.\textsuperscript{50} who found that 85.7% of \textit{H. pylori} isolates were resistant to AX, 71.4% of the isolates were resistant to AM, while 57.1% were resistant to ERY. Rasheed et al.\textsuperscript{23} reported that 54.3% of \textit{H. pylori} isolates were resistant to AX, while 73.9% and 47.8% of the isolates were resistant to MTZ and ERY, respectively. Goudarzi et al.\textsuperscript{24} revealed that 27.7% of \textit{H. pylori} isolates were resistant to AX, while 73.8 and 43.1% of \textit{H. pylori} isolates were resistant to MTZ and CLR, respectively. In another study, all \textit{H. pylori} isolates were sensitive to AX, while 61.1 and 22.8% of isolates were resistant to MTZ and CLR, respectively.\textsuperscript{24} Generally, worldwide rates of \textit{H. pylori} resistance to AX are considered low and the mechanism of resistance to \(\beta\)-lactam antibiotics in some \textit{H. pylori} strains is due to a change in the Penicillin Binding Protein (PBP) resulting in a decrease of the affinity of PBP for AX and decreased permeation and accumulation of penicillin into the bacterial cell. Active efflux pumps can also confer resistance to \(\beta\)-lactams.\textsuperscript{54,55} In contrast, in this study the lowest resistance was recorded by levofloxacin (LEV; 40%), tetracycline (TE; 50%) and ciprofloxacin (CIP; 50%). Eng et al.\textsuperscript{31} revealed that, 70% of \textit{H. pylori} isolates were sensitive to LEV and CIP, while none of the isolates showed resistance to TE. Goudarzi et al.\textsuperscript{25} found that, only 13.4% of \textit{H. pylori} isolates were resistant to LEV and 29.2% were resistant to TE.

The prevalence of \textit{H. pylori} antibiotic resistance varies among different geographic regions over the world, but the reported rates in developing nations including Africa are predominantly high.\textsuperscript{57} Geographic differences linked to the existence of phylogeographic features of \textit{H. pylori} may be a factor towards explaining the various existing antibiotic resistance.\textsuperscript{58} As presented in Table 4, the demographic characters of patients with PDR \textit{H. pylori} showed that, the mean age was 55 years. In addition, 75 and 87.5% were male and from rural areas, respectively. Only 37.5% of them were smokers, with insignificant impact of age, male gender, rural residence or smoking habits on PDR ability of \textit{H. pylori} (Table 4). Even though the patients who were infected with PDR \textit{H. pylori} showed inflammatory mucosa and peptic ulcer in an endoscopic picture, 75% of those with MDR \textit{H. pylori} also showed the same picture with insignificant association of either inflammation nor peptic ulcer with PDR bacteria (Table 4). Also, the patients who were infected with PDR \textit{H. pylori} showed inflammatory mucosa in the histological picture, and 83.3% of those with MDR \textit{H. pylori} showed the same picture with insignificant association of inflammation with PDR bacteria (Table 4).

3.4. Genotyping PDR \textit{H. pylori} strains

Of the 20 isolated \textit{H. pylori} strains, DNA was purified from 40% \textit{H. pylori} isolates obtained from eight patients with a positive \textit{H. pylori} status. Each strain of these PDR strains was genotyped by 16S rRNA (Fig. 3), cagA and vacA (Fig. 4 and Supplemental data; Fig. S2). In this study, the presence of CagA positive strains was in 75% of the PDR \textit{H. pylori} strains tested. In addition, two strains (HP-12 and HP-15) lacked the cagA gene according to PCR analysis. The presence of CagA positive strains was nearly universal among patients from East Asian countries colonized with \textit{H. pylori}, and 16/20 of \textit{H. pylori} isolates (80%) from Vietnamese population were cagA.\textsuperscript{59} Our results are in accordance with Perez-Perez et al.\textsuperscript{29} who reported that two patients out of the eight CagA positive \textit{H. pylori} isolates lacked the cagA gene.

On the other hand, seven (87.5%) PDR \textit{H. pylori} strains were vacA positive. Karabiber et al.\textsuperscript{60} reported that cagE and vacA s1 correlated with CLR and MTZ resistance. Bachir et al.\textsuperscript{53} revealed that, no statically significant relationship was found between cagA and vacA genotypes and antibiotic resistance except for the MTZ, which had a presence similar to the cagA genotype. In this study, we found that...
the most common subtype of the vacA gene among PDR H. pylori strains was m2s2 (50%), followed by m1s2 (25%), m2s1 (12.5%), while the subtype m1s2 was not detected in any of the tested PDR strains (Fig. 4). Boukhris et al.\textsuperscript{61} found that m2s2 was represented the most predominant genotype of vacA gene. Of 20 H. pylori strains, 12 (75%) showed the s1/m2 genotype and four showed the s1/m1 genotype.\textsuperscript{39} Some of the H. pylori strains showed multiple alleles, despite single colonies being picked.\textsuperscript{39}

As a general rule, all H. pylori strains are considered to be vacA positive. The fact that in this study the HP-10 strain was detected to be vacA negative. These results are in agreement with El-Shenawy et al.\textsuperscript{62} who found that vacA gene was detected in only 61.6% of H. pylori positive patients, while cagA gene was detected in 26.6%. Similarly, Boukhris et al.\textsuperscript{61} found that 84.3% of H. pylori positive patients had vacA gene and 59.6% had cagA gene. The variations in frequencies of vacA and cagA genes among H. pylori strains over the world may be due to the genetic heterogeneity and the ability of H. pylori to change the expression of vacA and cagA genes with geographic diversity.\textsuperscript{63} Various H. pylori genes are more highly diverse in a nucleotide sequence. In addition to variation in the nucleotide sequences of individual genes among H. pylori strains, there is considerable variation in gene content.\textsuperscript{64} Striking genetic variability at the level of and within single genes has been noted in vacA and cagA genes.\textsuperscript{65}

3.5. Anti-\textit{H. pylori} activity of different plant extracts

In the current study, ethanol, methanol and aqueous extracts of \textit{S. aromaticum}, \textit{C. zeylanicum} and \textit{T. vulgaris} were screened in vitro for their inhibitory activity against PDR H. pylori strains using the agar well diffusion method (Table 5 and Fig. 5). The results revealed that all the tested plant extracts showed anti-\textit{H. pylori} activity with an inhibition zone diameter of between zero and 25 ± 0.57 mm while, methanol extracts of these tested plants showed considerable anti-\textit{H. pylori} activity compared to the ethanol and aqueous extracts. In addition, the DMSO has no antibacterial activity as shown in Fig. 5A. Castillo-Juárez et al.\textsuperscript{66} found that among 53 different Mexican plant extracts, \textit{Moussonia deppeana}, \textit{Guaiacum...
Fig. 3. Neighbour-joining phylogenetic tree showing the placement of the PDR H. pylori strains (HP-1, HP-5, HP-8, HP-10, HP-12, HP-14, HP-15 and HP-16) based on 16S rRNA gene sequencing. GenBank accession numbers are given in parentheses. The scale bar indicates the numbers of expected substitutions accumulated per site.
coulteri, Annona cherimola, and Persea americana methanol extracts showed the highest anti- \textit{H. pylori} activity. Lawal et al.\textsuperscript{67} found that, the methanol extract of \textit{Theobroma cacao} Linn dried seeds had a potent inhibitory effect against \textit{H. pylori} clinical strains compared to the n-hexane extract, that did not have any inhibitory activity in vitro. In contrast, Tabak et al.\textsuperscript{68} reported that among several plant extracts, ethanol extract of \textit{C. zeylanicum} and aqueous extract of \textit{T. vulgaris} had a strong inhibitory effect on \textit{H. pylori}. Generally, all the different \textit{S. aromaticum} extracts showed that anti-\textit{H. pylori} activity was higher than those of \textit{C. zeylanicum} and \textit{T. vulgaris} (Table 5). Li et al.\textsuperscript{69} revealed that among different extracts of 30 Chinese herbal plants, \textit{Eugenia caryophyllata} ethanol and aqueous extracts showed remarkable inhibitory activity against all test strains of \textit{H. pylori}. Zaidi et al.\textsuperscript{70} found that the ethanol extract of \textit{S. aromaticum} showed anti-inflammatory activity against \textit{H. pylori}. Whereas, the methanol extract of \textit{S. aromaticum} showed the highest activity with a mean inhibition zone of 22.87 mm (Fig. 5B). The low activity reported by ethanol and aqueous extracts in this current study may be due to the fact that not enough active constituents were extracted by these solvents.

3.6. Characterization of the \textit{Syzygium aromaticum} methanol extract from GC-MS and FT-IR analyses

The seven components detected in the \textit{S. aromaticum} methanol extract by GC-MS analysis are given in Table 6 and Fig. 6. Eugenol (C\textsubscript{10}H\textsubscript{12}O\textsubscript{2}) is the major phenolic compound present in the methanol extract of clove (28.14), followed by eugenol acetate (12.43\%) and 4-hydroxy-4-methyl-2-pentanone (5\%) (Fig. 6A). The high level of eugenol in the clove is responsible for its strong biological activities.\textsuperscript{71,72} Several studies reported antifungal, antibacterial and anti-inflammatory activities of eugenol, which also possessed anti-\textit{H. pylori} activity.\textsuperscript{72,73} GC-MS data showed eugenol as the major constituent of clove methanol extract having a molecular ion peak at 164 m/z (Fig. 6B).

In this study, FT-IR spectrum of \textit{S. aromaticum} methanol extract showed a presence of various functional groups (Supplemental data; Fig. S3). The peak appearing at 3372 cm\textsuperscript{-1} was assigned to stretching vibration of intra molecular H-bonded hydroxyl function, while the peak appearing at 2973 cm\textsuperscript{-1} was assigned to C\textsubscript{e}-H stretching vibration (alkanes). The peak appearing at 1639 cm\textsuperscript{-1} was assigned to C\textsubscript{e}\textsubscript{C} stretching (alkene). The peak appearing at 838 cm\textsuperscript{-1} was assigned to C\textsubscript{e}-H bending (aromatic), the peaks appearing in the range of 1411–1518 cm\textsuperscript{-1} were assigned to the presence of C=C stretching (aromatic) and the peak appearing at 1038 cm\textsuperscript{-1} was assigned to various C=O like ethers and phenols.

Our results are in agreement with Mohammed et al.\textsuperscript{74} who used FT-IR to analyze the alcoholic extract of \textit{S. aromaticum} flowers. The toxic cyano group (C=N: 2220-2260 cm\textsuperscript{-1}) and acetylenic group (C=C: 2100-2260 cm\textsuperscript{-1}) are absent as safety indicators of the tested \textit{S. aromaticum} methanol extract (Supplemental data; Fig. S5). FT-IR was used in previous studies to evaluate the safety of the compounds. El-Zawawy and Ali\textsuperscript{26} used FT-IR to analyze pyocyanin pigment and its safety was confirmed by the absence of the toxic cyano group and acetylenic group in this pigment. El-Shouny et al.\textsuperscript{32} used FT-IR to analyze ginger oil and the safety of the oil was also determined by the absence of the toxic cyanide and acetylene groups. Salimon et al.\textsuperscript{75} analyzed Rubber seed oil by using...
3.7. Cytotoxicity assay

The results of cytotoxicity (Table 7) showed that the clove methanol extract utilization was safe even up to the maximum concentration (100 mg/ml) that was tested on PBMCs. Clearly, the inhibition rate increases with the increasing of the concentration of the tested S. aromaticum extract, while maximum concentration of clove extract did not reach IC₅₀ of the experiment, and it showed an inhibition rate of 46.73%. These results are in agreement with Hamad et al. who studied the cytotoxicity of different herbal extracts and reported that even up to the maximum concentration (20 mg/ml), the extracts did not reach IC₅₀ and showed an inhibition rate of 22.51%.

4. Conclusion

To the best of our knowledge, this study may be the first to investigate the biomedical potential of S. aromaticum extract against cytotoxic-associated genes producing drug-resistant H. pylori. This study has shown that the methanol extract of S. aromaticum exhibited as promising for use in the biomedical application fields, since it allied excellent and significant antibacterial activity against PDR H. pylori. The efficiency of S. aromaticum might be due to the presence of eugenol as the major phenolic compound. Therefore, S. aromaticum methanolic extract may be recommended for treating cytotoxic-associated genes producing PDR, with the potential of enhancing the efficacy for combating drug-resistant strains. This study may serve as a fruitful platform to explore novel derivatives as a new leading structure valued for biomedical therapeutics against H. pylori.
Conflits of interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2019.05.002.

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