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Syzygium aromaticum L.: Traditional herbal medicine against *cag*A and *vac*A 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.

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 symptomatic.[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,[3] and stimulation of the host inflammatory response.[6] CagA, is a highly immunogenic protein encoded at one end of the *cag* pathogenicity island, which injects CagA and other proteins into host cells.[10] 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.[3] 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.[3] CagA-positive *H. pylori* is also associated with the formation of gastric epithelium cell pedestals, change of the cytoskeleton, and enhancing gastric epithelium cells to produce IL-8.[11] The prevalence of CagA among *H. pylori* varies greatly from almost 100% in East Asia to less than 50% in some western countries.[12,13] 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.[3] 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.[14] 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.[15] Furthermore, to enhance vacuolation, VacA causes several cellular activities, such as membrane-channel forming, cytochrome *c* liberates from mitochondria leading to apoptosis and attaches to cell-membrane receptors, followed by induction of a proinflammatory response.[16,17] Clearly, the presence of direct *H. pylori*–host interactions result in genetic instability of the host genome.[3,9]

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*.[9] 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.[2] 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.[3,9,18] The conventional antibiotics most widely used for *H. pylori* therapy include amoxicillin, tetracycline, metronidazole, levofloxacin, clarithromycin and bismuth.[3] However, the resistance of *H. pylori* to the commonly used antibiotics is still increasing worldwide,[18] 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%.[19] The Multi-Drug Resistance (MDR) and Pan-Drug Resistance (PDR) remain an intractable challenge in public health, worldwide.[20–27] 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.[28] 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.[29–32] Antibacterial activity of numerous natural products has been recorded against *H. pylori*.[33] For centuries, several types of herbal plants and components derived from natural sources have been used in the treatment of gastric illnesses. *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.[34–36] Clove has been used as a food preservative, flavoring agent and for the treatment of gastrointestinal disorders.[37] 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.[38] 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), cefsulodin (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% O_2, 10% CO_2 and 85% N_2) 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.[39] 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.[40–43] 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× 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

Table 1
Primers and PCR amplification conditions used in this study.

Gene target	Orientation	Primer sequence (5′-3′)	PCR amplification conditions
CagA	Forward Reverse	AATACACCAACGCCTCCA 'TTGTTGCCGCTTTTGCTCTC	*Initial denaturation: 94 °C for 10 min; *Denaturation: 35 cycles of heating at 94 °C for 30 s; *Annealing: 56 °C for 30 s; *Extension: 72 °C for 50 s; *Final extension: 72 °C for 10 min.
***VacA* (*s1/s2*)**	Forward Reverse	ATGGAAATACAACAAACACAC CTGCTTGAATGCGCCAAAC	*Initial denaturation: 94 °C for 10 min; *Denaturation: 30 cycles of heating at 94 °C for 30 s; *Annealing: 50 °C for 45 s; *Extension: 72 °C for 1 min; *Final extension: 72 °C for 10 min.
***VacA* (*m1/m2*)**	Forward Reverse	CAATCTGTCCAATCAAGCGAG GCGTCTAAATAATTCCAAGG	*Initial denaturation: 94 °C for 10 min; *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** **1513R**	AGAGTTTGATCYTG GCTCAG ACGGYTACCTTGTTACGA	*Initial denaturation: 95 °C for 5min; *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.

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.[20] The cultivated plates were incubated under microaerophilic conditions for 72 h at 37 °C. The zone of inhibitions was interpreted based on the Clinical and Laboratory Standards Institute (CLSI).[45] Out of 20 *H. pylori* positive clinical strains, eight were proved to be PDR strains.

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.[46] 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 (MHBA). 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 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
Levofloxacin	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[21] 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 has 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 (100–1.5 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.[49]

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

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)	

P-value ≤ 0.05 is considered significant.
[a] Independent t-test.
[b] Chi square test.

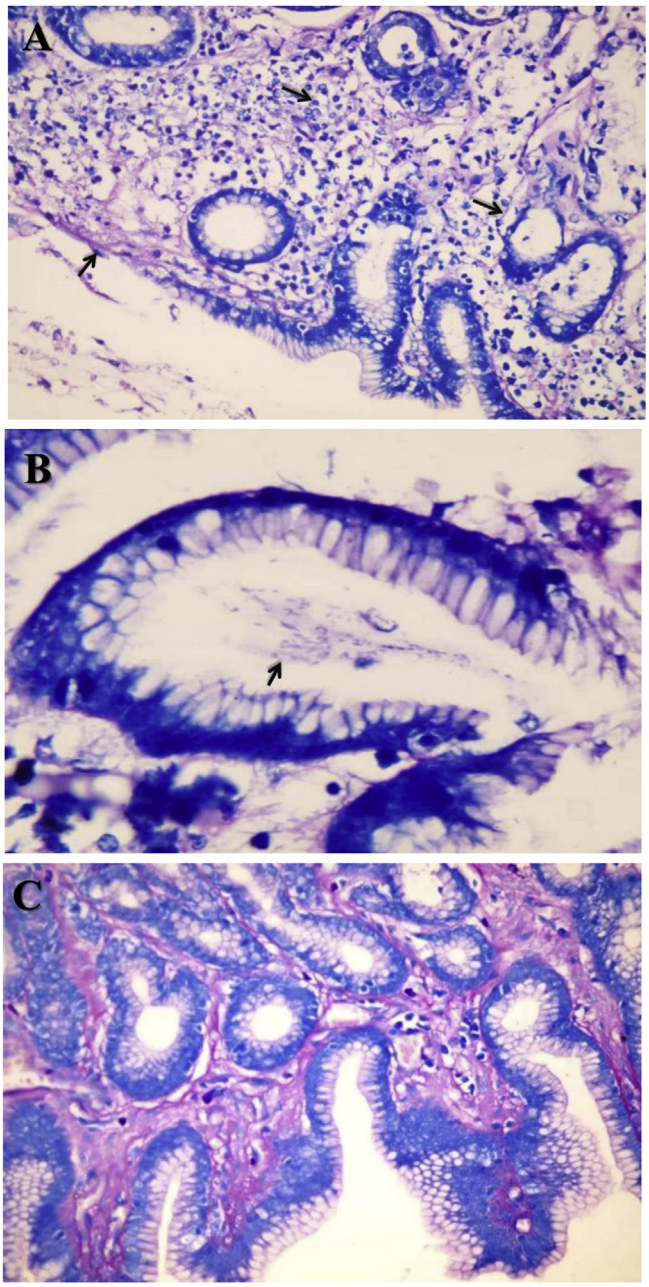

Fig. 1. Histopathological examination. (**A**) Inflammatory cells stained by Giemsa stain (400x). The vertical arrow showed ulceration of gastric mucosa (discontinuous of epithelial cells), while horizontal arrows showed atrophic gastric glands and mononuclear cellular infiltration mostly by lymphocytes cells. (**B**) Positive histopathological result stained by Giemsa stain (1000x). The arrow showed the presence of curved rods *H. pylori* colonize the lumen of the gastric glands. (**C**) Negative histopathological result stained by Giemsa stain (400x), showing normal human gastric glands, absence of *H. pylori* and no inflammatory response.

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 *H. pylori* strains from dyspeptic Egyptian patients is considered a significant finding in this study, where the highest resistance of all 20 *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.[50] who found that 85.7% of *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.[51] reported that 54.3% of *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.[52] revealed that 27.7% of *H. pylori* isolates were resistant to AX, while 73.8 and 43.1% of *H. pylori* isolates were resistant to MTZ and CLR, respectively. In another study, all *H. pylori* isolates were sensitive to AX, while 61.1 and 22.8% of isolates were resistant to MTZ and CLR, respectively.[53] Generally, worldwide rates of *H. pylori* resistance to AX are considered low and the mechanism of resistance to β-lactam antibiotics in some *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 β-lactams.[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.[56] revealed that, 70% of *H. pylori* isolates were sensitive to LEV and CIP, while none of the isolates showed resistance to TE. Goudarzi et al.[52] found that, only 13.4% of *H. pylori* isolates were resistant to LEV and 29.2% were resistant to TE.

The prevalence of *H. pylori* antibiotic resistance varies among different geographic regions over the world, but the reported rates in developing nations including Africa are predominantly high.[57] Geographic differences linked to the existence of phylogeographic features of *H. pylori* may be a factor towards explaining the various existing antibiotic resistance.[58] As presented in Table 4, the demographic characters of patients with PDR *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 *H. pylori* (Table 4). Even though the patients who were infected with PDR *H. pylori* showed inflammatory mucosa and peptic ulcer in an endoscopic picture, 75% of those with MDR *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 *H. pylori* showed inflammatory mucosa in the histological picture, and 83.3% of those with MDR *H. pylori* showed the same picture with insignificant association of inflammation with PDR bacteria (Table 4).

3.4. Genotyping PDR H. pylori strains

Of the 20 isolated *H. pylori* strains, DNA was purified from 40% *H. pylori* isolates obtained from eight patients with a positive *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 *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 *H. pylori*, and 16/20 of *H. pylori* isolates (80%) from Vietnamese population were *cagA*.[59] Our results are in accordance with Perez-Perez et al.[39] who reported that two patients out of the eight CagA positive *H. pylori* isolates lacked the *cagA* gene.

On the other hand, seven (87.5%) PDR *H. pylori* strains were *vacA* positive. Karabiber et al.[60] reported that *cagE* and *vacA s1* correlated with CLR and MTZ resistance. Bachir et al.[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

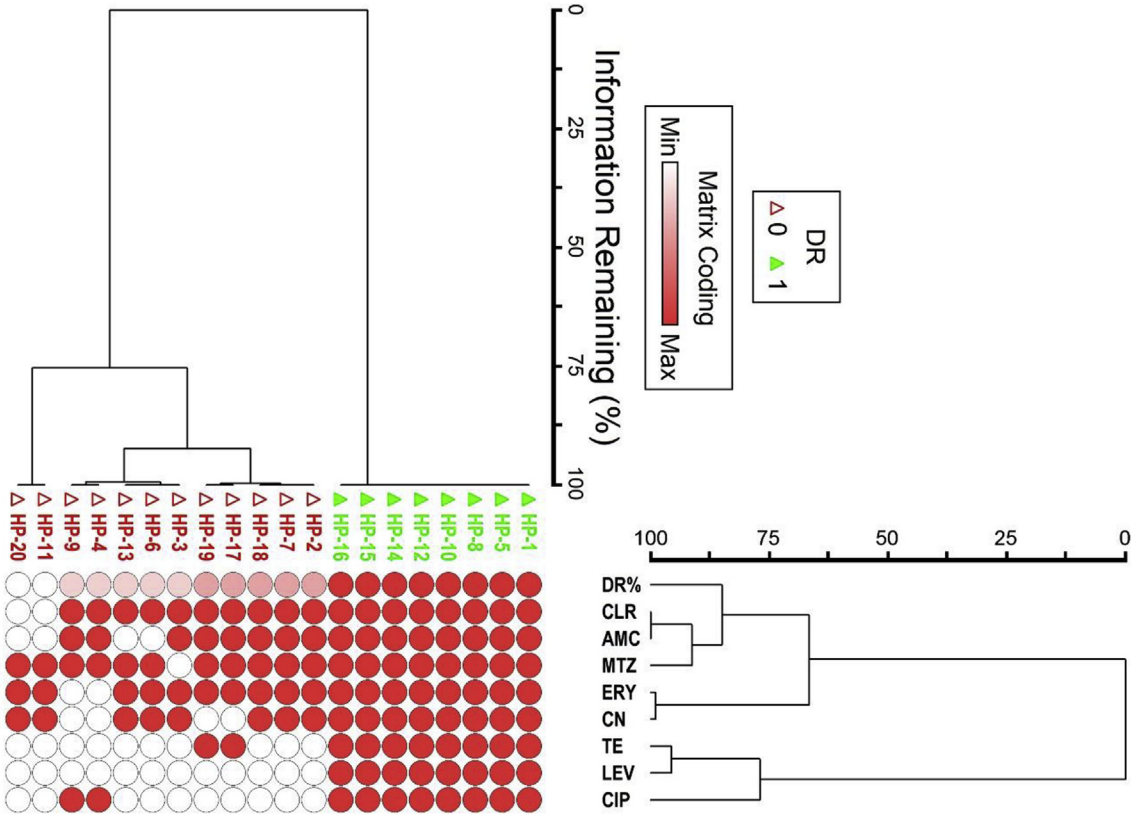

Fig. 2. Dendrogram represents clustering analysis of 20 *H. Pylori* strains (HP-1 to HP-20) based on Drug Resistance (DR). Green cluster (HP-1, HP-5, HP-8, HP-10, HP-12, HP-14, HP-15 and HP-16) represents Pan Drug Resistant (PDR) strains, while red clusters are Multi-Drug Resistant (MDR) strains. **CLR**, clarithromycin; **AMC**, amoxicillin/clavulanic acid; **MTZ**, metronidazole; **ERY**, erythromycin; **CN**, gentamicin; **TE**, tetracycline; **LEV**, levofloxacin; **CIP**, ciprofloxacin.

Table 4
Demographic and clinical characteristics of patients with drug resistant *H. pylori* (n = 20).

Traits (n, %)	Patients with MDR *H. Pylori* (n = 12)	Patients with PDR *H. Pylori* (n = 8)	P-value
Demography			
Age (years)	51.3 ± 11.0	55.8 ± 9.31	0.3[a]
Gender (male)	9 (75)	6 (75)	1.0[b]
Residence (Rural)	9 (75)	7 (87.5)	0.4[b]
Smoker	5 (41.6)	3 (37.5)	0.8[b]
Endoscopy			
Normal	3 (25)	0 (0.0)	0.1[b]
Peptic ulcer	7 (58.33)	6 (75)	
Inflammation	2 (16.67)	2 (25)	
Histology			
Inflamed mucosa	10 (83.3)	8 (100)	0.4[b]
Normal	2 (16.7)	0 (0.0)	

MDR, Multi-Drug Resistance; **PDR**, Pan-Drug Resistance.
P-value ≤ 0.05 is considered significant.
[a] Independent *t*-test.
[b] Chi square test.

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.[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.[39] Some of the *H. pylori* strains showed multiple alleles, despite single colonies being picked.[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.[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.[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.[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.[64] Striking genetic variability at the level of and within single genes has been noted in *vacA* and *cagA* genes.[65]

3.5. Anti- H. pylori activity of different plant extracts

In the current study, ethanol, methanol and aqueous extracts of *S. aromaticum*, *C. zeylanicum* and *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-*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- *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.[66] found that among 53 different Mexican plant extracts, *Moussonia deppeana*, *Guaiacum*

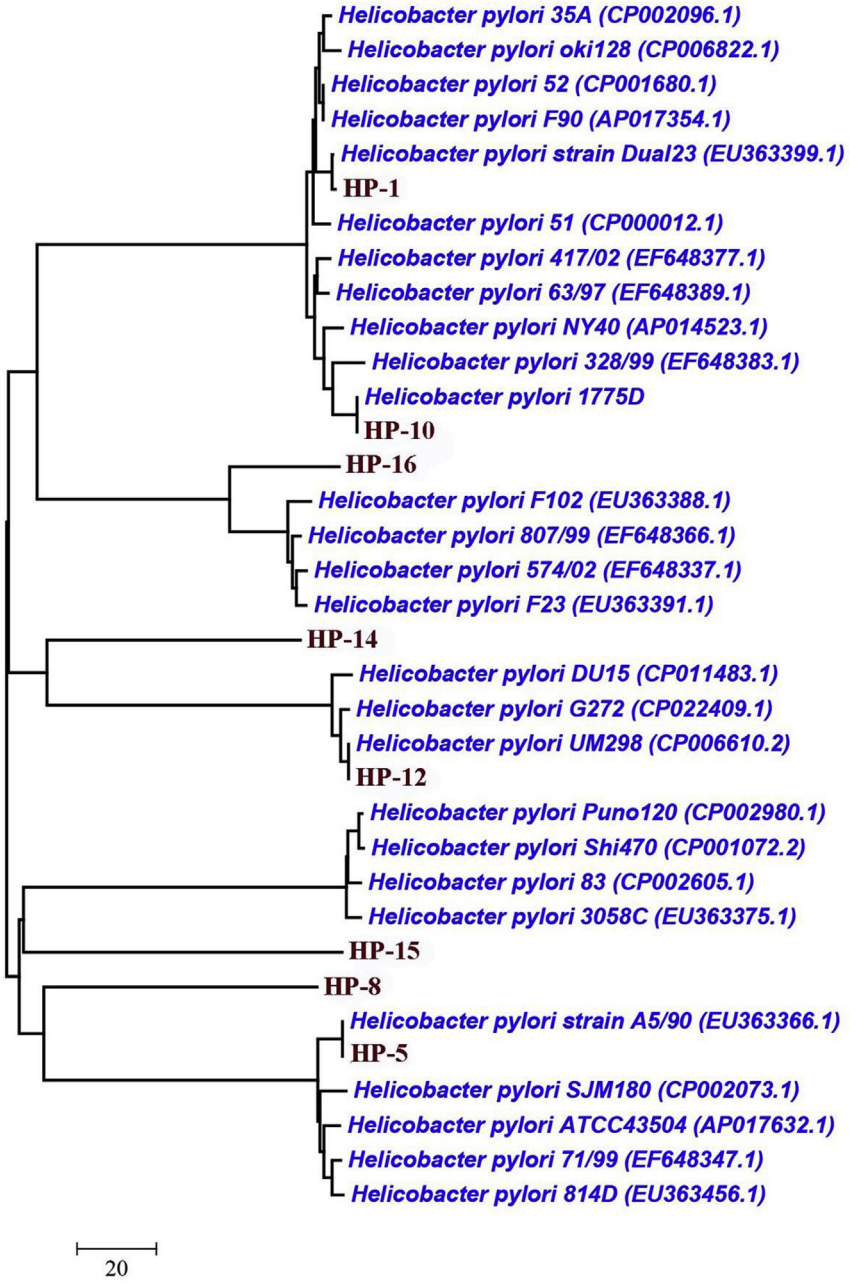

Fig. 3. Neighbour-Joining phylogenetic tree showing the placement of the PDR *H. pylori* strains (HP-1, HP-5, HP-8, HP-10, HP-12, HP-14, HP-15 and HP-16) based on 16S rRNA gene sequencing. GeneBank accession numbers are given in parentheses. The scale bar indicates the numbers of expected substitutions accumulated per site.

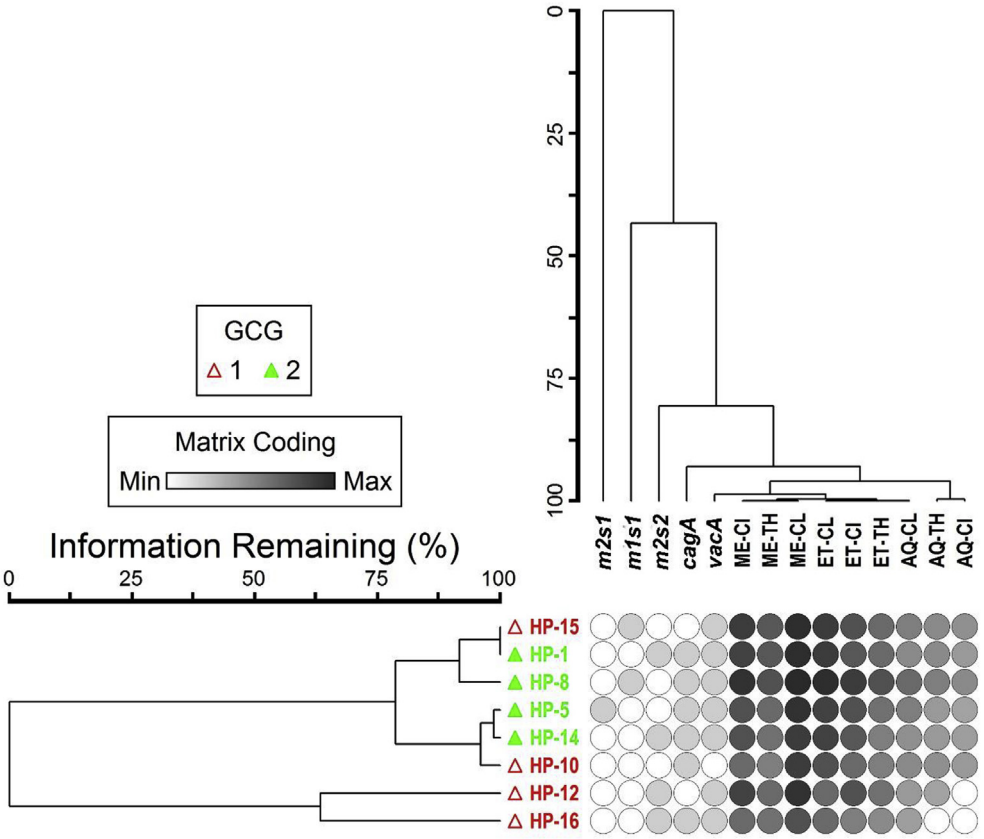

Fig. 4. Dendrogram profile of the eight PDR *H. Pylori* strains (HP-1, HP-5, HP-8, HP-10, HP-12, HP-14, HP-15 and HP-16) based on their genotyping characterization and susceptibility to different plant extracts. ***cagA*** and ***vacA***, cytotoxin genes; ***m2s1***, ***m1s1*** and ***m2s2***, subtypes of *vacA* gene; **ME**, Methanol; **ET**, Ethanol; **AQ**, Aqueous; **CL**, Clove (*Syzygium aromaticum*); **CI**, Cinnamon (*Cinnamomum zeylanicum*); **TH**, Thyme (*Thymus vulgaris*).

coulteri, *Annona cherimola*, and *Persea americana* methanol extracts showed the highest anti- *H. pylori* activity. Lawal et al.[67] found that, the methanol extract of *Theobroma cacao* Linn dried seeds had a potent inhibitory effect against *H. pylori* clinical strains compared to the n-hexane extract, that did not have any inhibitory activity *in-vitro*. In contrast, Tabak et al.[68] reported that among several plant extracts, ethanol extract of *C. zeylanicum* and aqueous extract of *T. vulgaris* had a strong inhibitory effect on *H. pylori*. Generally, all the different *S. aromaticum* extracts showed that anti-*H. pylori* activity was higher than those of *C. zeylanicum* and *T. vulgaris* (Table 5). Li et al.[69] revealed that among different extracts of 30 Chinese herbal plants, *Eugenia caryophyllata* ethanol and aqueous extracts showed remarkable inhibitory activity against all test strains of *H. pylori*. Zaidi et al.[70] found that the ethanol extract of *S. aromaticum* showed anti-inflammatory activity against *H. pylori*. Whereas, the methanol extract of *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 Syzygium aromaticum methanol extract from GC-MS and FT-IR analyses

The seven components detected in the *S. aromaticum* methanol extract by GC-MS analysis are given in Table 6 and Fig. 6. Eugenol ($C_{10}H_{12}O_2$) 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.[71] Several studies reported antifungal, antibacterial and anti-inflammatory activities of eugenol, which also possessed anti-*H. pylori* activity.[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 *S. aromaticum* methanol extract showed a presence of various functional groups (Supplemental data; Fig. S3). The peak appearing at 3372 cm^{-1} was assigned to stretching vibration of intra molecular H-bonded hydroxyl function, while the peak appearing at 2973 cm^{-1} was assigned to C–H stretching vibration (alkanes). The peak appearing at 1639 cm^{-1} was assigned to C=C stretching (alkene). The peak appearing at 838 cm^{-1} was assigned to C–H bending (aromatic), the peaks appearing in the range of 1411–1518 cm^{-1} were assigned to the presence of C=C stretching (aromatic) and the peak appearing at 1038 cm^{-1} was assigned to various C–O like ethers and phenols. Our results are in agreement with Mohammed et al.[74] who used FT-IR to analyze the alcoholic extract of *S. aromaticum* flowers.

The toxic cyano group (C≡N: 2220-2260 cm^{-1}) and acetylenic group (C≡C: 2100-2260 cm^{-1}) are absent as safety indicators of the tested *S. aromaticum* methanol extract (Supplemental data; Fig. S3). FT-IR was used in previous studies to evaluate the safety of the compounds. El-Zawawy and Ali[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.[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.[75] analyzed Rubber seed oil by using

Table 5
Antibacterial activity of different plant extracts against the eight PDR *H. pylori*strains.

Stain code	Inhibition zone diameter (mm)			$P^{\$}$
	Syzygium aromaticum (Clove)	*Cinnamomum zeylanicum* (Cinnamon)	*Thymus vulgaris* (Thyme)	
Methanol extract				
HP-1	24 ± 0.57	21 ± 0.57	19 ± 1.0	0.003
HP-5	23 ± 0.57	20 ± 0.57	18 ± 1.52	0.01
HP-8	25 ± 0.56	23 ± 0.56	20 ± 0.57	< 0.001
HP-10	22 ± 1.0	18 ± 1.0	16 ± 1.0	< 0.001
HP-12	23 ± 1.0	21 ± 1.0	18 ± 1.0	0.01
HP-14	22 ± 0.0	20 ± 0.0	17 ± 0.57	0.001
HP-15	24 ± 1.0	22 ± 1.0	19 ± 0.57	0.003
HP-16	20 ± 0.57	18 ± 1.0	17 ± 0.57	0.002
$P^{\$}$	0.001	< 0.001	0.013	
Ethanol extract				
HP-1	22 ± 0.0	19 ± 0.57	18 ± 0.57	0.005
HP-5	21 ± 1.0	20 ± 0.57	17 ± 0.0	0.005
HP-8	24 ± 0.57	22 ± 0.0	20 ± 0.57	0.005
HP-10	20 ± 1.0	18 ± 1.0	16 ± 0.57	0.005
HP-12	18 ± 0.0	20 ± 1.52	17 ± 0.57	0.05
HP-14	21 ± 1.52	19 ± 0.57	16 ± 0.0	0.01
HP-15	22 ± 1.0	20 ± 2.0	18 ± 1.0	0.01
HP-16	18 ± 0.57	16 ± 1.0	15 ± 1.52	0.005
$P^{\$}$	<0.001	0.003	0.001	
Aqueous extract				
HP-1	15 ± 0.57	13 ± 0.0	15 ± 0.56	0.02
HP-5	16 ± 2.0	11 ± 1.0	13 ± 1.0	0.001
HP-8	18 ± 0.57	15 ± 0.57	16 ± 0.57	0.005
HP-10	15 ± 1.0	13 ± 0.57	14 ± 0.57	0.005
HP-12	13 ± 1.0	0.0 ± 0.0	11 ± 0.57	< 0.001
HP-14	14 ± 0.57	12 ± 1.0	13 ± 1.15	0.005
HP-15	16 ± 0.57	14 ± 0.57	15 ± 0.0	0.02
HP-16	12 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	< 0.001
$P^{\$}$	0.001	< 0.001	< 0.001	

$: Two-way ANOVA, P-value ≤ 0.05 is considered significant.

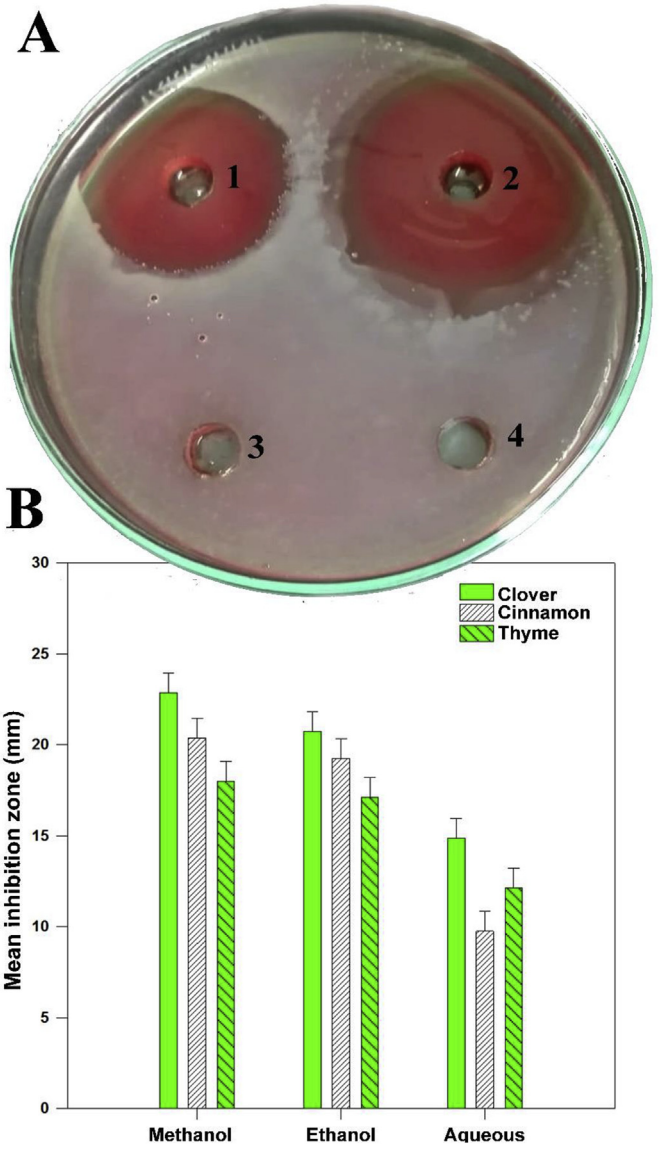

Fig. 5. Anti- *H. pylori* activity of different plant extracts. (**A**) Representative blood agar plate showing the effects of *Syzygium aromaticum* ethanol extract (well no. **1**), *S. aromaticum* methanol extract (well no. **2**), negative controls (wells **3** and **4**) containing DiMethyl SulfOxide (DMSO) against PDR HP-8 strain. (**B**) Mean inhibition zone diameters of methanol, ethanol and aqueous plant extracts (Clove, Cinnamon and Thyme).

FT-IR spectroscopy and the results showed that, the toxic cyanide group was absent as an indicator of the safety of this oil. The presence of the hydroxyl group in the *S. aromaticum* extract might be the reason for its inhibitory effect, this was supported by Ultee et al.[76] who reported that, the presence of the hydroxyl group is important for the antimicrobial activity of carvacrol against food-borne pathogen *Bacillus cereus*. Also, Maddox et al.[77] revealed that, the presence of hydroxyl groups in the structure of phenolic acids enhance their inhibitory activity against the phytopathogenic bacterium *Xylella fastidiosa*.

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 IC_{50} of the experiment, and it showed an inhibition rate of 46.73%. These results are in agreement with Hamad et al.[50] 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 IC_{50} 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 cytotoxin-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 cytotoxin-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*. In addition, further studies are needed to reveal the gastrointestinal protective mechanism of *S. aromaticum*.

Table 6
Gas chromatography-mass spectrometry (GC-MS) of *S. aromaticum* methanol extract.

Peak	RT (min)	Peak area (%)	Compound name
2	2.318	1.887	Carophyllene
9	3.054	5.000	4-Hydroxy-4-methyl-2-pentanone
11	10.587	12.430	Eugenol acetate
12	11.052	1.181	Cinnamic acid, p-(trimethylsiloxy)-, methyl ester
13	12.593	28.140	Eugenol
16	21.726	1.363	Sulfurous acid, 2-propyl tridecyl ester
26	27.619	1.552	2-Phenyl-1-(benzimidazolyl) acetic acid

RT, Retention Time.

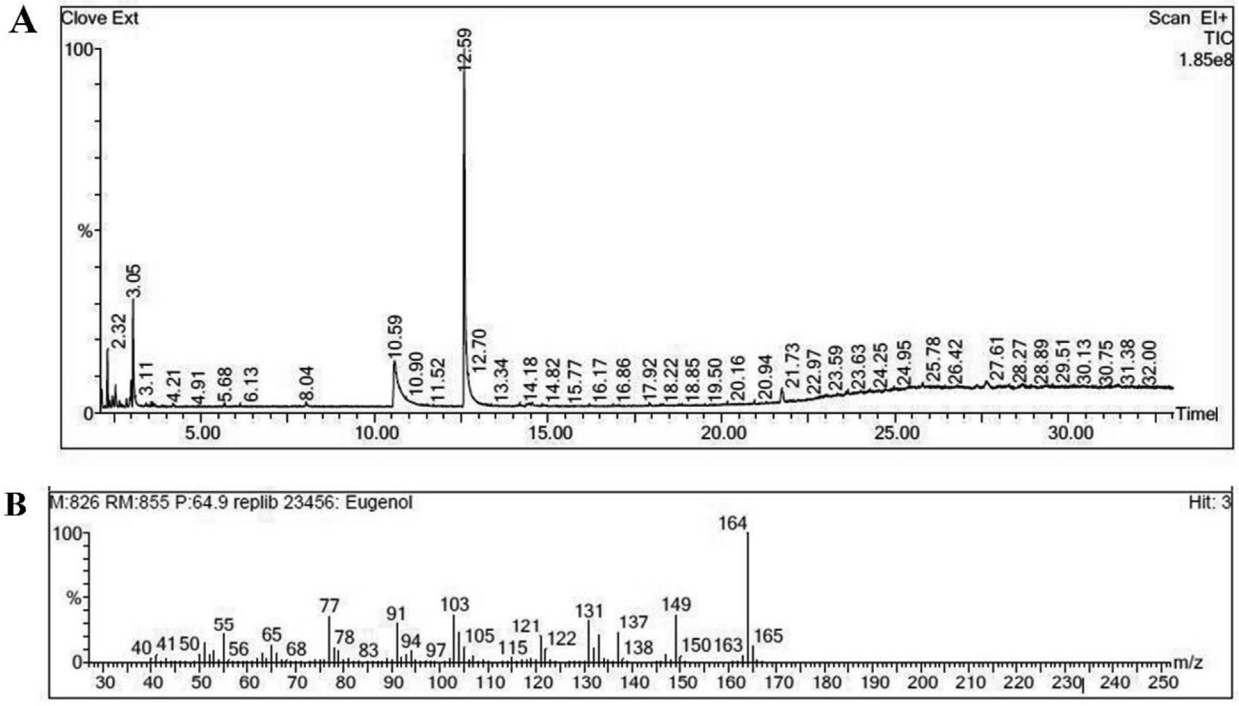

Fig. 6. *Syzygium aromaticum* (Clove) methanol extract from GC-MS. (**A**) Chromatogram of *S. aromaticum*; (**B**) Mass Spectrum chromatogram of Eugenol.

Table 7
Cytotoxicity of the *S. aromaticum* methanol extract.

Extract concentration (mg/ml)	Inhibition rate (%)
100	46.73.± 0.15
50	41.93 ± 0.12
25	36.76 ± 0.25
12.5	32.86 ± 0.15
6.25	27.83 ± 0.21
3.125	20.66 ± 0.57
1.5	9.83 ± 0.35
P$	<0.001

$: One way ANOVA, P considered significant if < 0.05.

Conflicts 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.

References

1. Suzuki K, Tanaka KSH, Yano T, et al. Deficiency of stomach-type claudin-18 in mice induces gastric tumor formation independent of *H. Pylori* infection. *Cell Mol Gastroenterol Hepatol*. 2019. https://doi.org/10.1016/j.cmgh.2019.03.003.
2. Graham DY, Dore MP. *Helicobacter pylori* therapy: a paradigm shift. *Expert Rev Anti Infect Ther*. 2016;14(6):577–585.
3. Yamaoka Y, Graham DY. *Helicobacter pylori* virulence and cancer pathogenesis. *Future Oncol*. 2014;10(8):1487–1500.
4. Correa P. Gastric cancer: overview. *Gastroenterol Clin North Am*. 2013;42(2):211–217.
5. Ghosh P, Bodhankar SL. Determination of risk factors and transmission pathways of *Helicobacter pylori* in asymptomatic subjects in Western India using polymerase chain reaction. *Asian Pac J Trop Dis*. 2012:12–17.
6. Majumdar D, Bebb J. *Helicobacter pylori* infection and peptic ulcers. *Medicine*. 2019;47(5):292–300.
7. Suzuki H, Iwasaki E, Hibi T. *Helicobacter pylori* and gastric cancer. *Gastric Cancer*. 2009;12(2):79–87.
8. Rana R, Wang SL, Li J, Wang YX, Rao QW, Yang CQ. *Helicobacter pylori* infection:

a recent approach to diagnosis and management. *J Biomed*. 2017;2(1):45–56.
9. Graham DY. Treating *Helicobacter pylori* effectively while minimizing misuse of antibiotics. *Clevel Clin J Med*. 2017;84(4):311.
10. Tegtmeyer N, Wessler S, Backert S. Role of the *cag*-pathogenicity island encoded type IV secretion system in *Helicobacter pylori* pathogenesis. *FEBS J*. 2011;278(8):1190–1202.
11. Boonyanugomol W, Chomvarin C, Hahnvajanawong C, Sripa B, Kaparakis-Liaskos M, Ferrero RL. *Helicobacter pylori cag* pathogenicity island (*cag*PAI) involved in bacterial internalization and IL-8 induced responses via NOD1- and MyD88-dependent mechanisms in human biliary epithelial cells. *PLoS One*. 2013;8(10), e77358.
12. Yamaoka Y. Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat Rev Gastroenterol Hepatol*. 2010;7(11):629–641.
13. Yamaoka Y, Kodama T, Kashima K, Graham DY, Sepulveda AR. Variants of the 3' region of the *cag*A gene in *Helicobacter pylori* isolates from patients with different *H. pylori*-associated diseases. *J Clin Microbiol*. 1998;36(8):2258–2263.
14. Graham DY, Lu H, Yamaoka Y. African, Asian or Indian enigma, the East Asian *Helicobacter pylori*: facts or medical myths. *J Dig Dis*. 2009;10(2):77–84.
15. Raju D, Hussey S, Ang M, et al. Vacuolating cytotoxin and variants in *Atg16L1* that disrupt autophagy promote *Helicobacter pylori* infection in humans. *Gastroenterology*. 2012;142(5):1160–1171.
16. Akazawa Y, Isomoto H, Matsushima K, et al. Endoplasmic reticulum stress contributes to *Helicobacter pylori* VacA-induced apoptosis. *PLoS One*. 2013;8(12), e82322.
17. Amieva MR, El-Omar EM. Host-bacterial interactions in *Helicobacter pylori* infection. *Gastroenterology*. 2008;134(1):306–323.
18. Abadi ATB, Yamaoka Y. Helicobacter pylori therapy and clinical perspective. *J Glob Antimicrob Resist*. 2018;14:111–117.
19. Lai CH, Kuo CH, Chen PY, Poon SK, Chang CS, Wang WC. Association of antibiotic resistance and higher internalization activity in resistant *Helicobacter pylori* isolates. *J Antimicrob Chemother*. 2006;57(3):466–471.
20. Ali SS, Morsy R, El-Zawawy NA, Fareed MF, Bedaiwy MY. Synthesized zinc peroxide nanoparticles (ZnO_2-NPs): a novel antimicrobial, anti-elastase, anti-keratinase, and anti-inflammatory approach toward polymicrobial burn wounds. *Int J Nanomed*. 2017;12:6059–6073.
21. Kenawy E, Ali SS, Al-Etewy M, Sun J, Wu J, El-Zawawy N. Synthesis, characterization and biomedical applications of a novel Schiff base on methyl acrylate-functionalized chitosan bearing *p*-nitrobenzaldehyde groups. *Int J Biol Macromol*. 2019;122:833–843.
22. Ali SS, Kenawy E, Sonbol FI, et al. Pharmaceutical potential of a novel chitosan derivative Schiff base with special reference to antibacterial, anti-Biofilm, antioxidant, anti-Inflammatory, hemocompatibility and cytotoxic activities. *Pharm Res*. 2019;36(1):5.
23. El-Zawawy NA, Ali SS. Pyocyanin as anti-tyrosinase and anti tinea corporis: a novel treatment study. *Microb Pathog*. 2016;100:213–220.
24. Ali SS, Shaaban MT, Abomohra A, El-Safity K. Macroalgal activity against multiple drug resistant *Aeromonas hydrophila*: a novel treatment study towards enhancement of fish growth performance. *Microb Pathog*. 2016;101:89–95.
25. Morsy R, Ali SS, El-Shetehy M. Development of hydroxyapatite-chitosan gel sunscreen combating clinical multidrug-resistant bacteria. *J Mol Struct*. 2017;1143:251–258.
26. El Zawawy NA, Ali SS. Anti-proteolytic activity of *Ganoderma lucidum* methanol extract against *Pseudomonas aeruginosa*. *J Infect Dev Countr*. 2016;10(9):1020–1024.
27. El Shafay SM, Ali SS, El-Sheekh MM. Antimicrobial activity of some seaweeds species from Red sea, against multidrug resistant bacteria. *Egypt J Aquat Res*. 2016;42:65–74.
28. Ali SS, El-Zawawy NA, Al-Tohamy R, El-Sapagh S, Mustafa AM, Sun J. *Lycium shawii* Roem. & Schult.: a new bioactive antimicrobial and antioxidant agent to combat multi-drug/pan-drug resistant pathogens of wound burn infections. *J Trad Complement Med*. 2019. https://doi.org/10.1016/j.jtcme.2019.01.004.
29. Al-Tohamy R, Ali SS, Saad-Allah K, et al. Phytochemical analysis and assessment of antioxidant and antimicrobial activities of some medicinal plant species from Egyptian flora. *J Appl Biomed*. 2018;16(4):289–300.
30. El-Shouny WA, Ali SS, Fayed SF. The antibacterial activity of *Nigella sativa* against multidrug resistant *Pseudomonas aeruginosa* isolated from diabetic wound infections. *MATTER: Int J Sci Technol*. 2016;2(1):113–134.
31. El-Shouny WA, Ali SS, Alnabarawy AM. *In vitro* antibacterial potential of *Rosa Damascena* and *Terminalia Chebula* against bacterial peritonitis. *GJBAHS*. 2016;5(2):40–49.
32. El-Shouny WA, Ali SS, Sun J, Samy SM, Ali A. Drug resistance profile and molecular characterization of extended spectrum beta-lactamase (ESβL)-producing *Pseudomonas aeruginosa* isolated from burn wound infections. Essential oils and their potential for utilization. *Microb Pathog*. 2018;116:301–312.
33. Nostro A, Cellini L, Bartolomeo SD, et al. Antibacterial effect of plant extracts against *Helicobacter pylori*. *Phytother Res: J Pharmacol Toxicol*. 2005;19(3):198–202.
34. Fu Y, Zu Y, Chen L, et al. Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother Res*. 2007;21(10):989–994.
35. Cortés-Rojas DF, de Souza CRF, Oliveira WP. Clove (*Syzygium aromaticum*): a precious spice. *Asian Pac J Trop Biomed*. 2014;4(2):90–96.
36. Han X, Parker TL. Anti-inflammatory activity of clove (*Eugenia caryophyllata*) essential oil in human dermal fibroblasts. *Pharm Biol*. 2017;55(1):1619–1622.
37. Nassan MA, Mohamed EH, Abdelhafez S, Ismail TA. Effect of clove and cinnamon extracts on experimental model of acute hematogenous pyelonephritis in albino rats: immunopathological and antimicrobial study. *Int J Immunopathol Pharmacol*. 2015;28(1):60–68.
38. Peek Jr RM, Miller GG, Tham KT, Pérez-Pérez GI, Cover TL, Atherton JC. Detection of *Helicobacter pylori* gene expression in human gastric mucosa. *J Clin Microbiol*. 1995;33:28–32.
39. Perez-Perez GI, Van TN, Huong DT, et al. Isolation and characterization of *Helicobacter pylori* recovered from gastric biopsies under anaerobic conditions. *Diagn Microbiol Infect Dis*. 2016;86:136–140.
40. Ali SS, Abomohra A, Sun J. Effective bio-pretreatment of sawdust waste with a novel microbial consortium for enhanced biomethanation. *Bioresour Technol*. 2017;238:425–432.
41. Ali SS, Wu J, Xie R, Zhou F, Sun J, Huang M. Screening and characterizing of xylanolytic and xylose-fermenting yeasts isolated from the wood-feeding termite, *Reticulitermes chinenesis*. *PLoS One*. 2017;12(7), e0181141.
42. Ali SS, Sun J. Effective thermal pretreatment of water hyacinth (*Eichhornia crassipes*) for the enhancement of biomethanation: VIT® gene probe technology for microbial community analysis with special reference to methanogenic *Archaea*. *J Environ Chem Eng*. 2019;7(1):102853.
43. Ali SS, Al-Tohamy R, Sun J, Wu J, Huizi L. Screening and construction of a novel microbial consortium SSA-6 enriched from the gut symbionts of wood-feeding termite, *Coptotermes formosanus* and its biomass-based biorefineries. *Fuel*. 2019;236:1128–1145.
44. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis: the updated Sydney system. *Am J Surg Pathol*. 1996;20(10):1161–1181.
45. Clinical and Laboratory Standard Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing: 24th Informational Supplement M 100–S24*. Wayne, PA, USA: CLSI; 2014.
46. Njume C, Afolayan AJ, Ndip RN. Preliminary phytochemical screening and *in vitro* anti-Helicobacter pylori activity of acetone and aqueous extracts of the stem bark of *Sclerocarya birrea* (Anacardiaceae). *Arch Med Res*. 2011;42(3):252–257.
47. Safrudin I, Maimulyanti A, Prihadi AR. Effect of crushing of clove bud (*Syzygium aromaticum*) and distillation rate on main constituents of the essential oil. *Am J Essent Oil Nat Prod*. 2015;2(3):12–15.
48. EL-Adawi HI, Khalil MA, EL-Sheekh MM, El-Deeb NM, Hussein MZ. Cytotoxicity assay and antioxidant activities of the lactic acid bacterial strains. *Afr J Microbiol Res*. 2012;6(8):1700–1712.
49. Dursun M, Yilmaz S, Yukselen V, Kilinç N, Canoruç F, Tuzcu A. Evaluation of optimal gastric mucosal biopsy site and number for identification of *Helicobacter pylori*, gastric atrophy and intestinal metaplasia. *Hepato-Gastroenterology*. 2004;51(60):1732–1735.
50. Hamad GM, Taha TH, El-Deeb NM, Alshehri AM. Advanced trends in controlling *Helicobacter pylori* infections using functional and therapeutically supplements in baby milk. *J Food Sci Technol*. 2015;52(12):8156–8163.
51. Rasheed F, Campbell BJ, Alfizah H, et al. Analysis of clinical isolates of *Helicobacter pylori* in Pakistan reveals high degrees of pathogenicity and high frequencies of antibiotic resistance. *Helicobacter*. 2014;19(5):387–399.
52. Goudarzi M, Heidary M, Azad M, Fazeli M, Goudarzi H. Evaluation of antimicrobial susceptibility and integron carriage in *Helicobacter pylori* isolates from patients. *Gastroenterol Hepatol Bed Bench*. 2016;9(1):S47–S52.
53. Bachir M, Allem R, Tifrit A, et al. Primary antibiotic resistance and its relationship with *cagA* and *vacA* genes in *Helicobacter pylori* isolates from Algerian patients. *Braz J Microbiol*. 2018;49(3):544–551.
54. Qureshi NN, Gallaher B, Schiller NL. Evolution of amoxicillin resistance of *Helicobacter pylori in vitro*: characterization of resistance mechanisms. *Microb Drug Resist*. 2014;20(6):509–516.
55. Kwon DH, Dore MP, Kim JJ, et al. High-level β-lactam resistance associated with acquired multidrug resistance in *Helicobacter pylori*. *Antimicrob Agents Chemother*. 2003;47(7):2169–2178.
56. Eng NF, Ybazeta G, Chapman K, et al. Antimicrobial susceptibility of Canadian isolates of *Helicobacter pylori* in northeastern ontario. *Can J Infect Dis Med Microbiol*. 2015;26(3):137–144.
57. Jaka H, Rhee JA, Östlundh L, et al. The magnitude of antibiotic resistance to *Helicobacter pylori* in Africa and identified mutations which confer resistance to antibiotics: systematic review and meta-analysis. *BMC Infect Dis*. 2018;18(1):193.
58. Wu W, Yang Y, Sun G. Recent insights into antibiotic resistance in *Helicobacter pylori* eradication. *Gastroenterol Res Pract*. 2012;2012.
59. Yamaoka Y, Kato M, Asaka M. Geographic differences in gastric cancer incidence can be explained by differences between *Helicobacter pylori* strains. *Intern Med*. 2008;47:1077–1083.
60. Karabiber H, Selimoglu MA, Otlu B, Yildirim O, Ozer A. Virulence factors and antibiotic resistance in children with *Helicobacter pylori* gastritis. *J Pediatr Gastroenterol Nutr*. 2014;58(5):608–612.
61. Boukhris SA, Amarti A, El Rhazi K, et al. *Helicobacter pylori* genotypes associated with gastric histo-pathological damages in a Moroccan population. *PLoS One*. 2013;8(12), e82646.
62. El-Shenawy A, Diab M, Shemis M, et al. Detection of *Helicobacter pylori* vacA, cagA and iceA1 virulence genes associated with gastric diseases in Egyptian patients. *EJMHG*. 2017;18(4):365–371.
63. Nejati S, Karkhah A, Darvish H, Validi M, Ebrahimpour S, Nouri HR. Influence of *Helicobacter pylori* virulence factors CagA and VacA on pathogenesis of gastrointestinal disorders. *Microb Pathog*. 2018;117:43–48.

64. Cover TL, Blaser MJ. *Helicobacter pylori* in health and disease. *Gastroenterology*. 2009;136(6):1863–1873.
65. Göttke MU, Fallone CA, Barkun AN, et al. Genetic variability determinants of *Helicobacter pylori*: influence of clinical background and geographic origin of isolates. *J Infect Dis*. 2000;181(5):1674–1681.
66. Castillo-Juárez I, González V, Jaime-Aguilar H, et al. Anti-*Helicobacter pylori* activity of plants used in Mexican traditional medicine for gastrointestinal disorders. *J Ethnopharmacol*. 2009;122(2):402–405.
67. Lawal TO, Olorunnipa TA, Adeniyi BA. Susceptibility testing and bactericidal activities of *Theobroma cacao* Linn. (cocoa) on *Helicobacter pylori* in an *in vitro* study. *J Herb Med*. 2014;4(4):201–207.
68. Tabak M, Armon R, Potasman I, Neeman I. *In vitro* inhibition of *Helicobacter pylori* by extracts of thyme. *J Appl Microbiol*. 1996;80(6):667–672.
69. Li Y, Xu C, Zhang Q, Liu JY, Tan RX. *In vitro* anti-*Helicobacter pylori* action of 30 Chinese herbal medicines used to treat ulcer diseases. *J Ethnopharmacol*. 2005;98(3):329–333.
70. Zaidi SF, Muhammad JS, Shahryar S, et al. Anti-inflammatory and cytoprotective effects of selected Pakistani medicinal plants in *Helicobacter pylori*-infected gastric epithelial cells. *J Ethnopharmacol*. 2012;141(1):403–410.
71. Gülçin İ. Antioxidant activity of eugenol: a structure–activity relationship study. *J Med Food*. 2011;14(9):975–985.
72. Chaieb K, Hajlaoui H, Zmantar T, et al. The chemical composition and biological activity of clove essential oil, *Eugenia caryophyllata* (*Syzigium aromaticum* L. Myrtaceae): a short review. *Phytother Res*. 2007;21(6):501–506.
73. Chami F, Chami N, Bennis S, Trouillas J, Remmal A. Evaluation of carvacrol and eugenol as prophylaxis and treatment of vaginal candidiasis in an immunosuppressed rat model. *J Antimicrob Chemother*. 2004;54(5):909–914.
74. Mohammed KAK, Abdulkadhim HM, Noori SI. Chemical composition and antibacterial effects of clove (*Syzygium aromaticum*) flowers. *Int J Curr Microbiol App Sci*. 2016;5(2):483–489.
75. Salimon J, Abdullah BM, Salih N. Rubber (*Hevea brasiliensis*) seed oil toxicity effect and Linamarin compound analysis. *Lipids Health Dis*. 2012;11(1):74.
76. Ultee A, Bennik MHJ, Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol*. 2002;68(4):1561–1568.
77. Maddox CE, Laur LM, Tian L. Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. *Curr Microbiol*. 2010;60(1):53.