Vol.64: e21210089, 2021
https://doi.org/10.1590/1678-4324-2021210089
ISSN 1678-4324 Online Edition

Article – Human and Animal Health

Antibacterial Effects of Cinnamon Extract, Clove Oil and Antibiotics against *Helicobacter pylori* Isolated from Stomach Biopsies

Shughla Panezai[1]
https://orcid.org/0000-0002-0431-4774

Abdul Samad[2]
https://orcid.org/0000-0001-9647-2165

Muhammad Naeem[1]
https://orcid.org/0000-0002-3771-3915

Hamida Ali[2]
https://orcid.org/0000-0001-7142-3375

Muhammad Bilal Sadiq[3]
https://orcid.org/0000-0003-2487-0468

Muhammad Sadiq Achakzai[1]
http://orcid.org/0000-0003-1311-0623

Zahoor Kakar[1]
https://orcid.org/0000-0003-2692-4810

Ali Akbar[4]*
https://orcid.org/0000-0002-0376-1425

[1]University of Balochistan, Center for Advanced Studies in Vaccinology & Biotechnology, Quetta, Pakistan; [2]Sardar Bahadur Khan Women`s University, Department of Zoology, Quetta, Pakistan; [3]Forman Christian College (A Chartered University), Department of Biological Sciences, Lahore, Pakistan; [4]University of Balochistan, Department of Microbiology, Quetta, Pakistan

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Cheila Roberta Lehnen

Received: 2021.03.01; Accepted: 2021.07.21.

*Correspondence: aliakbar.uob@gmail.com; Tel.: +92-0300-7068049 (A.A.).

HIGHLIGHTS

- *Helicobacter pylori* is commonly associated with gastric ulcer.
- The bacteria are showing resistance to common antibiotics.
- Natural antimicrobial such as Clove oil, curcumin showed significant activities against *H. pylori*.

Abstract: *Helicobacter pylori* is a pathogenic bacterium causing gastric problems such as, peptic ulcers and stomach cancer. *H. pylori* were isolated from the stomach biopsy specimens (n = 100) of gastric patients by performing polymerase chain reaction (PCR) against cagA (cytotoxin associated gene A) and ureC (Urease subunit alpha) genes. Furthermore, antibiogram studies of the *H. pylori* isolates were evaluated against the common antibiotics. The overall detection rate of *H. pylori* was 71% in biopsy specimens of gastric patients. The antimicrobial susceptibility test revealed the resistance rate of *H. pylori* isolates against metronidazole (50%), clarithromycin (28.33%), tetracycline (21.66%), amoxicillin (18.33%), and ciprofloxacin (11.66%). However, the *H. pylori* isolates were completely resistant to vancomycin, erythromycin and nalidixic acid antibiotics. Clove oil showed a remarkable antimicrobial effect against *H. pylori* whereas, mild inhibition (10 mm) was observed in case of curcumin extract. Due to increase incident of resistance and high prevalence of *H. pylori* in gastric patients, natural antimicrobial like clove oil can be explored as an alternative treatment.

Keywords: *Helicobacter pylori*; Antibiotic resistance; Infections; Polymerase chain reaction.

INTRODUCTION

Helicobacter pylori is a microaerophilic and spiral-shaped Gram-negative, bacterium, found in the human gastric mucosa. *H. pylori* is 0.5-1µm in width and 2.5-5µm in length. These bacteria can change their shape according to the environment such as, they are spiral rods in fresh culture and coccoid in adverse conditions [1]. More than 30 species of *H. pylori* have been reported so far [2]. These bacteria are associated with the human diseases worldwide, that include gastritis, stomach cancer, peptic ulcer, and gastric mucosa-associated with lymphoid-tissue lymphoma [3]. More than 50% of the adult population is reported to be affected by *H. pylori* infections [4]. World Health Organization (WHO) has characterized it as a leading carcinogen linked with peptic ulcer and gastric cancer [5].

H. pylori is a pathogenic bacterium that are more prevalent in humans, but it can infect other primates as well [6]. *H. pylori* can adapt to the acidic environment and therefore it easily propagates to colonizes in the human stomach [7]. *H. pylori* have also been isolated from feces, saliva, and drinking water [8]. The mode of transmission of this infectious disease in humans is the fecal-oral route [9]. The reservoir of *H. pylori* is considered to be thriving in the contaminated water [10].

Antibiotics such as amoxicillin, tetracycline, metronidazole, and clarithromycin are commonly used for the treatment of *H. pylori* infections. However, the eradication rate of *H. pylori* is as low as 60% in various countries due to its increased antibiotic resistance to available antibiotics [4]. The documented drugs of choice for the treatment of *H. pylori* are amoxicillin, clarithromycin, metronidazole, and tetracycline to be co-administrated with proton pump inhibitor. The treatment takes 4-6 weeks usually and, in some cases, it may take longer time [11].

H. pylori exhibit genotypic variations in different geographic regions of the world. The prevalence of infection is higher in unindustrialized countries compared to the industrialized countries [9]. *H. pylori* infection can be diagnosed by different methods such as, culturing, PCR, rapid urease test, histopathology examination of gastric tissue, other tests include serological and breath tests. PCR is the most specific and sensitive test for the detection of *H. pylori* [12]. This study was designed for the isolation and identification of *H. pylori* from the biopsy samples of patients with gastrointestinal diseases through PCR using species specific primers and evaluation of their antibiotic susceptibility against available antibiotics and natural antimicrobial agents.

MATERIAL AND METHODS

Sampling

A total of n = 100 stomach biopsy samples were collected from patients, admitted to endoscopic examination at the gastroenterology department, Bolan University of Health Medical Sciences (BUHMS) hospital, Quetta. The samples were collected in sterilized tubes filled with normal saline (0.9% NaCl) and brought to the microbiology laboratory in CASVAB, University of Balochistan, Quetta. The samples were refrigerated (4 °C) till analysis.

Sample processing and culture

The biopsy samples in the normal saline solution were chopped into small pieces with the help of a sterilized scalpel blade and thoroughly homogenized with normal saline. The processed sample (1ml) was kept at -80 °C for molecular typing of *H. pylori* [4]. The remaining sample (0.5 ml) was inoculated in 4.5 ml of Columbia Blood broth (Merck) base supplemented with 10% lysed horse blood, vancomycin (10mg/l), trimethoprim (5mg/l) and amphotericin-B (5mg/l) (Sigma-Aldrich, USA). The tubes were incubated at 37°C for 48 h in microaerophilic conditions. After incubation, a loopful from tubes was streaked onto Columbia Blood agar plates supplemented with 10% lysed horse blood. The suspected *H. pylori* colonies from agar plates were verified by Gram staining (−ve), rapid urease (+ve), oxidase (+ve) and catalase (+ve) tests [13, 14].

Molecular detection of *H. pylori*

The presumptively identified *H. pylori* colonies were further subjected to a polymerase chain reaction (PCR) for the confirmation of *H. pylori* through the use of oligonucleotide primers to target the amplification of ureC and cagA genes (Table 1) [3]. Bacterial DNA was extracted by using the DNA Mini Kit (Qiagen) according to the manufacturer's instruction. The genes of interest, ureC and cagA were amplified by

automated thermocycler (Applied Biosystems, 2720). The PCR reaction mixture (20 µL) were comprised of master mix (10 µL), molecular grade water (5 µL), forward and reverse primers (1 µL) and DNA template (3 µL). Cycling conditions for ureC gene were optimized at initial denaturation at 94 °C for 4 min, followed by 35 cycles of DNA denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec, extension at 72 °C for 1 min, and the final extension at 72 °C for 7 min. cagA gene amplification was performed with the initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 30 sec and the final elongation at 72 °C for 7 min. The amplified products were then run in 2 % (w/v) agarose gel stained with ethidium bromide and PCR bands were visualized under ultra-violet light in the agarose gel. The amplified bands of interests were compared with the help of a molecular weight marker.

Table 1. Sequence of oligonucleotide primers used for the detection of *H. pylori.*

Primers	Sequence (5′ – 3′)	Amplicon size	Reference
UreC	F:AAGCTTTTAGGGGTGTTAGGGGTTT R: AAGCTTACTTTCTAACACTAACGC	294-bp	[28]
CagA	F: AATACACCAACGCCTCCAAG R: TTGTTGCCGCTTTTGCTCTC	400-bp	[29]

Preparation of curcumin extract and clove oil

Dry curcumin (*Curcuma longa*) was purchased from the local herbal market of Quetta, Pakistan, and grounded to fine powder with the help of mechanical grinder. The powdered curcumin (30 g) was extracted by maceration for 24 h using ethanol (300 mL) as extraction solvent. The extract was obtained by filtration followed by the removal of solvent and the extract was freeze dried to get fine powder. The stock solution (10 mg/mL) of curcumin was prepared in 20% dimethyl sulfoxide (DMSO). The analytical grade clove oil (Sigma Aldrich, USA) was used for evaluation of antimicrobial potential against *H. pylori*.

Antibiotic susceptibility of *H. pylori*

The *H. pylori* isolates were evaluated for their antibiogram on Mueller-Hinton Agar (Sigma Aldrich, USA) supplemented with 10% lysed horse blood. Overnight grown culture of *H. pylori* was adjusted to 0.5 McFarland standard and the inoculum was spread over the surface of plate aseptically using a sterile cotton swab. Antibiotic discs were placed over the surface of agar with the help of sterile forceps and incubated in the microaerophilic environment for 24-48 h at 37 °C. Diameter of inhibition zones were measured in millimeters following incubation time.

RESULTS

A total of 100 patients were examined clinically and their endoscopic biopsy samples were analyzed for the presence of *H. pylori* using PCR and biochemical tests. Age of the patients included in the study was between 18 to 60 years including both males and females.

Out of 100 patients, 41(41%) patients had gastritis followed by 29 (29%) pyloric ulcer, 12 (12%) duodenal ulcer, 7 (7%) fundus ulcer, 6 (6%) erythema and 4 (4%) of the patients had no lesion in their stomach and 1 (1%) patient had a foreign body in the stomach (Table 2). The results of PCR (Figure 1) showed that *H. pylori* are present in all the duodenal ulcer biopsy specimens (12/12). Out of 29 pyloric ulcer patients 27 (93.10%) were positive for *H. pylori* followed by 71.43% (5/7) in fundus ulcer, 58.53% (24/41) in gastritis patients, 33.33% (2/6) in patients with the erythema. Out of four patients with no gastric lesion, one patient (25%) was found to be positive for *H. pylori*. The overall detection rate of *H. pylori* was 71% through PCR.

Table 2. Prevalence of *H. pylori* in gastric patients (n =100)

Specimens	Nº of samples	Prevalence of *H. pylori,* n (%)
Gastritis	41	24 (58.53)
Pyloric Ulcer	29	27 (93.1)
Erythema	6	2 (33.33)
Foreign Bodies	1	0 (0)
Gastric Ulcer	7	5 (71.43)
Duodenal Ulcer	12	12 (100)
No Lesions	4	1 (25)
Total	100	71 (71)

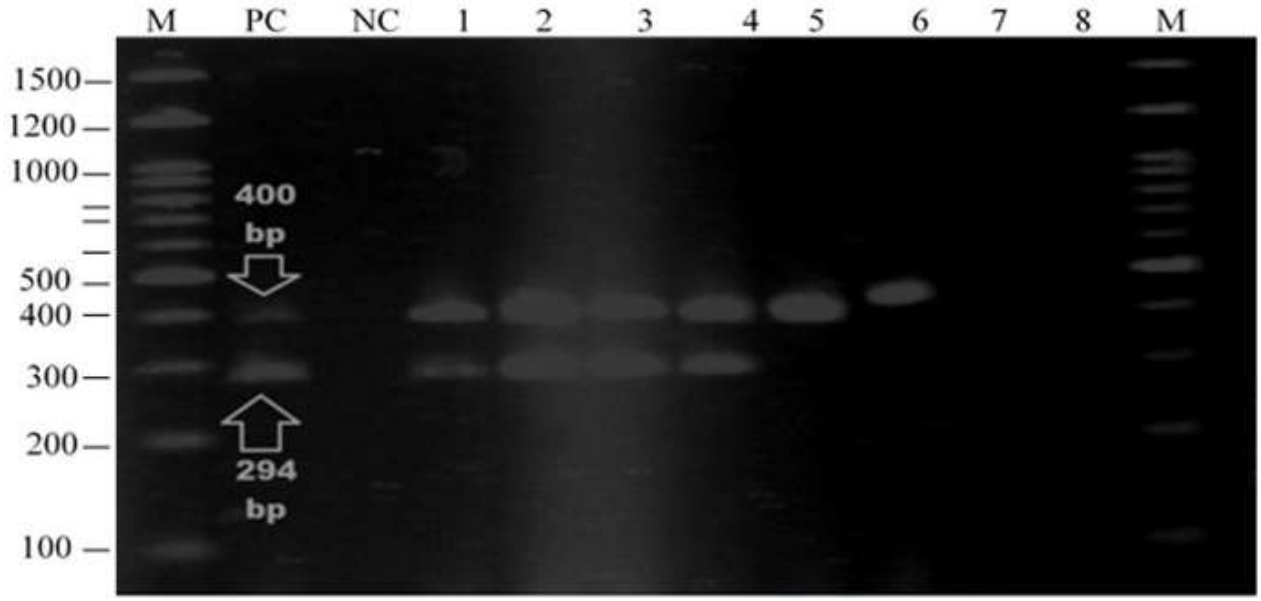

Figure 1. Agarose gel (2%), lane's 1-6 = positive for gene cagA (400bp); lane's 1-4 = positive for ureC (294bp) gene. M = 100bp DNA marker; PC = positive control; NC = negative control.

Antibiogram of *H. pylori*

Disc diffusion method following the guideline of EUCAST (European Committee on Antimicrobial Susceptibility testing) were used for the antibiogram determination of *H. pylori* isolates. Antibiotic sensitivity test results showed that the *H. pylori* isolates were susceptible to metronidazole, tetracycline, clarithromycin, ciprofloxacin and amoxicillin. Whereas all the tested isolates were found resistant to vancomycin, erythromycin, and nalidixic acid. The isolates were found susceptible to curcumin extract with a 10 mm inhibition zone recorded, while the clove oil showed a remarkable inhibition zone (20 mm) (Table 3 and Figure 2).

Table 3. Antibiogram of *H. pylori* isolates

S. Nº	Antibiotic	Susceptibility/Resistance
1	Metronidazole	Susceptible
2	Clarithromycin	Susceptible
3	Amoxicillin	Susceptible
4	Tetracycline	Susceptible
5	Ciprofloxacin	Susceptible
6	Vancomycin	Resistant
7	Erythromycin	Resistant
8	Nalidixic acid	Resistant
9	Curcumin	10 ± 1 mm
10	Clove Oil	20 ± 0.5 mm

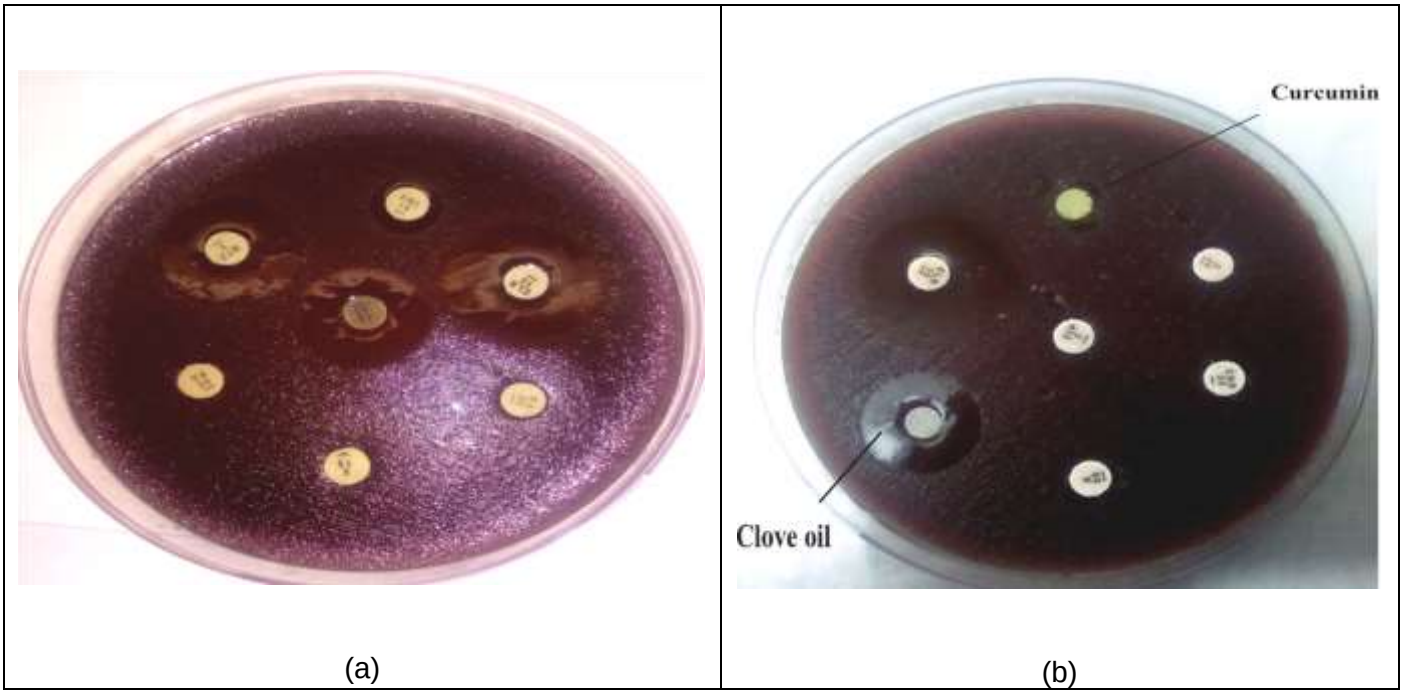

Figure 2. (a) Antibiotic susceptibility of H. pylori to commercially available antibiotics, (b)curcumin extract and clove oil.

DISCUSSION

The fecal-oral transmission route seems to be contributing substantially to the prevalence of *H. pylori* infection and making this disease a serious public health issue equally important to both developing and developed countries [15]. PCR is highly specific and sensitive molecular technique for the diagnosis of *H. pylori* infection [6]. In this study we analyzed the effectiveness of PCR for the detection of *H. pylori* in patients with gastric problems. Findings of this study are in line with the results of a similar study done in India [16], they used the *ureC* gene amplification through PCR for culture positive specimens. Espinoza and coauthors [17] found the detection rate of pathogen as high as 100% by ureC gene amplification in Mexico. As compared to findings of this study low detection rate for *H. pylori* was observed in Egypt (41.4%) [18] and Poland (46%) [19].

An increase resistance of *H. pylori* infection to available antibiotics is a major challenge for health practitioners and patients [20]. It has been reported that chromosomal mutations are the cause of drug resistance development in *H. pylori*. The drug resistance in *H. pylori* has been reported to vary with the geographical origin, it also depends on the variability of drugs used [21].

Abu-Sbeih and coauthors [22] reported 100% resistance to vancomycin in a similar study conducted in Jordon, these results are in compliance with the results of our study. Whereas another study reported 100 % *H. pylori* resistance to erythromycin [23] following the same pattern of results reported in our study.

Spices and herbs are used in food preparation since ancient times, which is not just a flavoring agent, but also act as traditional food preservatives and medicine. In addition to improvement in the flavors, certain herbs and spices extended the shelf life of foods because of their antioxidant and antimicrobial potential. Essential oils and spices such as garlic, ginger, cinnamon, mustard, and clove oil were already reported for various health remedies [24].

In this study, curcumin extract showed anti-Helicobacter activities, whereas the clove oil exhibited promising results. Akbar and coauthors [25] reported the antimicrobial activity of curcumin against clinical isolates. Curcumin has been reported to have the ability, to reduce the adhesions of *H. pylori* in the stomach [26]. Judaki and coauthors [27] described, that triple therapy group combined with curcumin significantly decreased the inflammation.

CONCLUSION

Due to the increase prevalence and antibiotic resistance of *H. pylori*, there is a dire need to explore natural antimicrobials sources to control the *H. pylori* infections. Curcumin and clove oils showed inhibition against antibiotic resistant strains and *H. pylori*, therefore traditional herbs should be explored further as a remedy for *H. pylori* infections. Moreover, the available antibiotics can be combined with curcumin or clove oil to enhance the antibacterial spectrum against antibiotic resistant pathogens.

Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Krzyżek P, Biernat MM, Gościniak G. Intensive formation of coccoid forms as a feature strongly associated with highly pathogenic Helicobacter pylori strains. Folia Microbiol. 2019;64:273-81.
2. Tanih N, Dube C, Green E, et al. An African perspective on Helicobacter pylori: prevalence of human infection, drug resistance, and alternative approaches to treatment. Ann Trop Med Parasitol. 2009;103:189-204.
3. Shafaie S, Kaboosi H, Ghadikolaii FP. Prevalence of non Helicobacter pylori gastric Helicobacters in Iranian dyspeptic patients. BMC Gastroenterol. 2020;20:1-7.
4. Hamidi S, Badmasti F, Sadeghpour Heravi F, et al. Antibiotic resistance and clonal relatedness of Helicobacter pylori strains isolated from stomach biopsy specimens in northeast of Iran. *Helicobacter*. 2020;25: e12684.
5. Jones KR, Cha J-H, Merrell DS. Who's winning the war? Molecular mechanisms of antibiotic resistance in Helicobacter pylori. Curr Drug Therapy. 2008;3:190-203.
6. Van Doorn LJ, Figueiredo C, Mégraud F, et al. Geographic distribution of vacA allelic types of Helicobacter pylori. Gastroenterol. 1999;116:823-30.
7. Zullo A, Hassan C, De Francesco V, et al. Helicobacter pylori and functional dyspepsia: an unsolved issue? World J Gastroenterol*: WJG*. 2014;20:8957.
8. Kim N, Lim SH, Lee KH, et al. Helicobacter pylori in dental plaque and saliva. Korean J Internal Med. 2000;15:187.
9. Samra ZQ, Javaid U, Ghafoor S, Batool A, Dar N, Athar MA. PCR assay targeting virulence genes of Helicobacter pylori isolated from drinking water and clinical samples in Lahore metropolitan, Pakistan. J Water Health. 2011;9:208-16.
10. Giao MS, Azevedo N, Wilks SA, Vieira M, Keevil CW. Persistence of Helicobacter pylori in heterotrophic drinking-water biofilms. Appl Environ Microbiol. 2008;74:5898-904.
11. Suerbaum S and Michetti P. Helicobacter pylori infection. New England J Med. 2002; 347: 1175-86.
12. Liu H, Rahman A, Semino-Mora C, Doi SQ, Dubois A. Specific and sensitive detection of H. pylori in biological specimens by real-time RT-PCR and in situ hybridization. PloS One. 2008; 3: e2689.
13. Ducournau A, Bénéjat L, Sifré E, Bessède E, Lehours P, Mégraud F. Helicobacter pylori resistance to antibiotics in 2014 in France detected by phenotypic and genotypic methods. Clin Microbiol Infect. 2016; 22: 715-8.
14. Selgrad M, Meile J, Bornschein J, et al. Antibiotic susceptibility of Helicobacter pylori in central Germany and its relationship with the number of eradication therapies. European J Gastroenterol Hepatol. 2013; 25: 1257-60.
15. Stefano K, Marco M, Federica G, Laura B, Barbara B, Gioacchino L. Helicobacter pylori, transmission routes and recurrence of infection: state of the art. Acta Bio Medica*: Atenei Parmensis*. 2018;89:72.
16. Singh V, Mishra S, Rao G, et al. Evaluation of nested PCR in detection of Helicobacter pylori targeting a highly conserved gene: HSP60. Helicobacter. 2008;13:30-4.
17. Espinoza MGC, Vazquez RG, Mendez IM, Vargas CR and Cerezo SG. Detection of the glmM gene in Helicobacter pylori isolates with a novel primer by PCR. J Clin Microbiol. 2011;49:1650-2.

18. Helaly G, El-Afandy N, Hassan A, Dowidar N, Sharaf S. Diagnostic value of housekeeping [glmM] gene expression in antral biopsies in comparison to rapid urease test and histological detection of Helicobacter Pylori infection. Egypt J Med Microbiol. 2009;18:119-30.
19. Ciesielska U, Dzięgiel P, Jagoda E, Podhorska-Okołów M, Zabel M. The detection of Helicobacter pylori in paraffin sections using the PCR technique and various primers as compared to histological techniques. Folia Morphol. 2004;63:229-31.
20. Datta S, Chattopadhyay S, Patra R, et al. Most Helicobacter pylori strains of Kolkata in India are resistant to metronidazole but susceptible to other drugs commonly used for eradication and ulcer therapy. Alimentary Pharmacol Therapeutics. 2005;22:51-7.
21. Torres-Debat M, Pérez-Pérez G, Olivares A, et al. Antimicrobial susceptibility of Helicobacter pylori and mechanisms of clarithromycin resistance in strains isolated from patients in Uruguay. Rev Esp Enferm Dig. 2009;101:757-62.
22. Abu-Sbeih RS, Hawari AD, Hassawi DS and Al-Daghistani HI. Isolation and detection of Helicobacter pylori from patients suffering from peptic ulcer using biochemical tests and molecular techniques. American J Biochem Biotech. 2014;10:58.
23. Al-Eraky DM, Helmy OM, Ragab YM, Abdul-Khalek Z, El-Seidi EA, Ramadan MA. Prevalence of CagA and antimicrobial sensitivity of H. pylori isolates of patients with gastric cancer in Egypt. Infect Agents Cancer. 2018;13:1-7.
24. Tajkarimi M, Ibrahim SA, Cliver D. Antimicrobial herb and spice compounds in food. Food Cont. 2010; 21:1199-218.
25. Akbar A, Ali I, Samiullah, N Ullah, Khan SA, Rehman Z, Rehman SU. Functional, antioxidant, antimicrobial potential and food safety applications of curcuma longa and cuminum cyminum. Pak J Bot. 2019;51:1129-35.
26. O'Mahony R, Al-Khtheeri H, Weerasekera D, et al. Bactericidal and anti-adhesive properties of culinary and medicinal plants against Helicobacter pylori. World J Gastroenterol. 2005;11:7499.
27. Judaki A, Rahmani A, Feizi J, Asadollahi K, Hafezi Ahmadi MR. Curcumin in combination with triple therapy regimes ameliorates oxidative stress and histopathologic changes in chronic gastritis-associated Helicobacter pylori infection. Arquivos Gastroenterol. 2017;54:177-82.
28. Labigne A, Cussac V, Courcoux P. Shuttle cloning and nucleotide sequences of Helicobacter pylori genes responsible for urease activity. J Bacteriol. 1991;173:1920-31.
29. Tummuru M, Cover T, Blaser M. Cloning and expression of a high-molecular-mass major antigen of Helicobacter pylori: evidence of linkage to cytotoxin production. Infect Immu. 1993;61:1799-809.