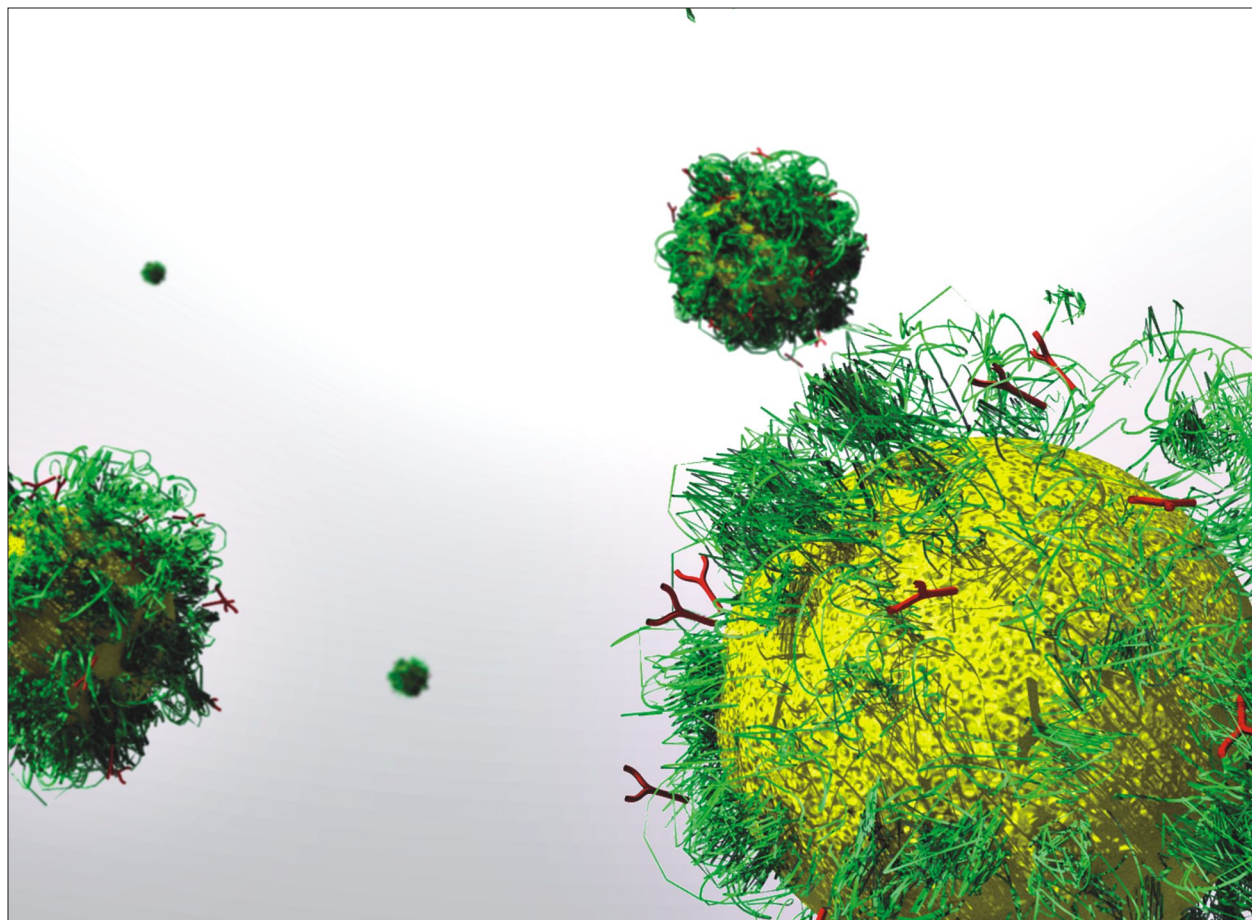




American Journal of
**Drug Discovery
and Development**

ISSN 2150-427X



Academic
Journals Inc.

www.academicjournals.com

Reduction in Mutation Frequency of Clarithromycin Resistance by Extracts from Three Iranian Herbs in *Helicobacter pylori* Isolates

^{1,2}Omid Tadjrobehkar and ¹Hamid Abdollahi

¹Department of Microbiology, Medical School, Kerman University of Medical Sciences, P.O. Box 444, Kerman, Iran

²Medical School, Zabol University of Medical Sciences, Hirmand Str, Zabol, Iran

Corresponding Author: Hamid Abdollahi, Department of Microbiology, Medical School, Kerman University of Medical Sciences, P.O. Box 444, Kerman, Iran Tel: +983413221665, +989131403106 Fax: +98-3413221665

ABSTRACT

Spontaneous point mutations are considered as major antibiotic resistance mechanism for *Helicobacter pylori*. Anti-mutagenic properties of some medicinal plants are evaluated in the present study. This could be a novel attractive approach in reducing the resistance phenomenon in *H. pylori*. The mutation prohibiting properties of ethanolic extract from *Cuminum cyminum*, *Mentha piperita* and *Camellia sinensis* were assayed by comparing mutation frequency for clarithromycin resistance in three *H. pylori* clinical isolates in the absence and in the presence of plant extracts. A-to-G transition at 2143 position of 23S rRNA gene was demonstrated as only point mutation in the all resistant mutated colonies. Furthermore, the median mutation frequency to clarithromycin resistance was detected as 27×10^{-9} for three isolates which was reduced after application of *M. piperita*, *C. cyminum* and *C. sinensis* extracts by 55.5, 68.5 and 99.5%, respectively. In the base of the results *C. sinensis* as a widely consumed beverage may be considered as potential sources of these agents.

Key words: Anti-mutagenic activity, *Camellia sinensis*, *Cuminum cyminum*, *Mentha piperita*, *Helicobacter pylori*, mutation frequency, clarithromycin resistance

INTRODUCTION

Helicobacter pylori has been known as one of the most common agent of persistent bacterial infection of human with the worldwide prevalence ranged between 7 and 87% (Malfertheiner *et al.*, 2012). This organism has been implicated to cause many major gastrointestinal diseases such as chronic gastritis, peptic ulcer and is linked to a number of more serious conditions like mucosa associated lymphoid tissue lymphoma (MALToma) and gastric carcinoma (Gerrits *et al.*, 2006).

Majority of experts recommended that infected symptomatic individuals should be treated to eradicate the organism and reduce the risk of gastric cancer (Malfertheiner *et al.*, 2012).

The recommended therapy regimens have been very diverse and changing over time.

Nowadays, conventional therapy for treatment of *H. pylori* infections is mainly combinational use of multiple drugs. First line eradication regimen is clarithromycin-based triple therapy including a proton pump inhibitor, plus one other antibiotic administrated together for 5-14 days. According to some documents, this regimen is not recommended when resistance rate reaches 15-20% (Megraud, 2007; Moder *et al.*, 2007). Eradication failure is reported in 20-30% of the cases and

adverse increasing of resistance to antibiotics especially to clarithromycin reported as the most important therapeutic problem (De Francesco *et al.*, 2012).

Apparently, *H. pylori* prefers to use DNA mutation as a strategy to adapt to changing environments rather than acquisition of new genes from related species due to transformation. One significant example of this strategy is the development of antibiotic resistance (Wang *et al.*, 1999).

Such mutations occur at a very high frequency (10^{-5} - 10^{-8}) in *H. pylori* which is much larger than many other bacteria (Kraft and Suerbaum, 2005).

Trying to find new therapeutic anti-*Helicobacter* agents is a continual growing need. There is an increasing tendency towards the replacement of synthetic drugs with natural substances from plants. Therefore, medicinal plants could be a novel source for obtaining new effective drugs.

Many studies on the antibacterial activity of a wide variety of plants from different countries have been carried out and anti *H. pylori* effect of a diverse medicinal plants have also been reported in a large body of literature (Castillo-Juarez *et al.*, 2009; Li *et al.*, 2005; Nariman *et al.*, 2009; Zaidi *et al.*, 2009). Though, to the best of our knowledge, there is no report on the clinical eradication of *H. pylori* by medicinal plant extract.

The present study was designed to focus on the preventive properties of some plant extracts on the appearance of resistant mutants in *H. pylori*. This is a relatively new approach in the use of medicinal plants for their anti-mutagenic properties which could be beneficial in reducing the antibiotic resistant problem in this bacterium.

MATERIALS AND METHODS

Plant collection and preparation of extracts: *Cuminum cyminum* (cumin), *Mentha piperita* (mint) and *Camellia sinensis* (green tea) which are used to cure gastrointestinal tract disorders in traditional Iranian medicine were purchased from a decent local medicinal herb shop. They were kindly identified by Professor G. H. Shahidi (Department of Botany, Faculty of Agriculture, Bahonar University, Kerman, Iran). Voucher numbers are kept at the Kerman University of Medical Sciences.

The plant parts (seeds of *C. cyminum* and the leaves of *M. piperita* and *C. sinensis*) were washed with distilled water, dried at 60°C over night and grounded with a mechanical blender. Each 100 g of powdered plant was soaked in 500 mL of extra pure ethanol (Merck) at room temperature for 5 days (solvent to sample ratio of 5:1 (v/w) and then filtered through whatman filter paper No. 5. The filtrates were concentrated, under reduced pressure then were evaporated to leave a solid pellet. The solid extracts were stored in dark dishes at -20°C until use. They were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) before use in experiments (final concentration of DMSO was = 1% in mixture).

***Helicobacter* strains and culture conditions:** In this study local clinical isolates of *H. pylori* were used. They were obtained from twelve antral biopsies of patients referred to endoscopy unit of medical University Hospital (Afzalipour, Kerman, Iran).

All biopsies were placed in brucella broth (BBL, USA) enriched with 10% fetal bovine serum (Biochrom AG, Berlin, Germany) and taken to microbiology laboratory immediately. Biopsy samples were crushed and 500 µL of suspension were streaked onto brucella agar containing 10% defibrinated sheep blood, supplemented with 8 µg vancomycin mL⁻¹, 10 µg amphotricin B mL⁻¹ and 5 µg trimetoprim mL⁻¹. Inoculated plates were incubated in a microaerobic atmosphere for 3 to 5 days at 37°C. The suspected colonies were confirmed as *H. pylori* by Gram staining and

positive urease, oxidase and catalase test. Isolates were maintained in brucella broth containing 30% glycerol and 10% fetal bovine serum at -80°C.

Clarithromycin susceptible isolates were selected by inoculating a suspension of bacteria adjusted to a McFarland turbidity of 3 (approximately 9.0×10^8 CFU mL⁻¹) on Muller-Hinton agar (Merck) plates containing 10% fetal bovine serum and 1 µg clarithromycin mL⁻¹.

Seven *H. pylori* strains were isolated from 12 biopsy samples. There were five clarithromycin sensitive isolates that did not grow in 1 µg mL⁻¹ concentration of clarithromycin. In all experiment, we used three clarithromycin sensitive isolates (K1, K7, K11) which were chosen randomly from the above collection.

Minimum inhibitory concentration determination (MIC): Dried extracts were solved in minimal volume of DMSO. The prepared plant extracts were added to melted Muller-Hinton agar (Merck) enriched with 10% fetal bovine serum to give final concentration of 62.5, 125, 250, 500 and 1000 µg mL⁻¹. Plates were dried at 37°C for 12 h. Equal volume (2 µL) of a bacterial suspension adjusted to a McFarland turbidity of 3 from each isolate, was spot inoculated on the plates. Similar plates containing 0.5% of DMSO and without extract were used as negative control. Clarithromycin were applied as positive control for verification of the results. The plates were incubated in a microaerobic atmosphere for 3 days at 37°C.

The MIC of clarithromycin was determined for each of the three isolates by a similar method. The final concentration of 0.03, 0.06, 0.125, 0.25, 0.5 and 1 µg clarithromycin mL⁻¹ in the same medium were made and inoculated as above. The MIC values were obtained after 3 days of incubation.

Dual concentrations (500 and 1000 µg mL⁻¹) of the plant extracts in combination with sub-inhibitory concentration (0.06 µg mL⁻¹) of clarithromycin in culture were prepared. They were used in screening of the possible synergistic anti-*Helicobacter* properties of clarithromycin and plant extracts. Same media containing 0.06 µg clarithromycin mL⁻¹ and without extract was used as control.

Determination of mutation frequency: In *H. pylori*, clarithromycin resistance caused chiefly by point mutations in 23S rRNA which was used as a mutational marker in our experiments.

A fluctuation assay developed with 20 parallel cultures. Because the mutation frequency were obtained from median of 20 repeated cultures of each isolate, 25 independent tube containing 1 mL brucella broth supplemented with 10% fetal bovine serum were inoculated with 10 µL of a preculture (10^4 - 10^5 CFU mL⁻¹) and were incubated in a microaerobic atmosphere for 3 days at 37°C. A sample (50 µL) of preculture were plated in Muller-Hinton agar containing 10% fetal bovine serum and 1 µg clarithromycin mL⁻¹ to assure that there is no preexisting resistant mutants.

A viable count was performed for 5 of the above tubes. For assessment of mutation frequency, 1 mL of each of the remaining 20 tubes was spread on Muller-Hinton agar plates containing 10% fetal bovine serum and 1 µg clarithromycin mL⁻¹ separately and observed colonies were counted after 3 days of incubation.

Mutation frequency was calculated from the median number of counted colony in 20 tubes divided by the total viable count. A colony of resistant mutants assigned to each of isolates was picked up and confirmed by PCR sequencing method (Rosche and Foster, 2000; Wang *et al.*, 2001).

Determination of anti-mutagenic properties of plant extracts: Mutation frequency was evaluated in the presence of five sub-inhibitory concentration of plant extracts (62.5, 125, 250, 500

Table 1: DNA sequence of oligonucleotide primers used in PCR-sequencing process

Primers	Sequences (5'→3')	Position
HP-F	TAAGGTGTGCCACAGCGAT	1811-1830
HP-R	CTAACAGAAACATCAAGGG	2279-2260

and 1000 µg mL⁻¹) by the previously mentioned method. Anti-mutagenic properties of plant extract were estimated by comparing the calculated mutation frequency in the presence of plant extracts and their absence.

All the experiments were performed in triplicate.

DNA extraction: All 3 clarithromycin susceptible *H. pylori* isolates and one resistant mutant colony incorporated to each isolate (picked up in mutation frequency assessment experiments) were used in DNA extraction process.

DNA was extracted using Bioneer genomics kit for DNA extraction (Bioneer, South Korea).

PCR amplification and nucleotide sequencing: To detect point mutations in the 23S rRNA gene responsible for clarithromycin resistance, oligonucleotide primers (Table 1) were derived from the known sequences of 23S rRNA gene from wild type strain UA802 (GenBank accession no. U27270). These primers used to amplify a 468 bp fragment corresponding to position 1811-2279 of the 23S rRNA gene. Amplification was carried out in a gradient thermal cycler (Biometra, Germany). PCR reaction consisted of 35 cycles initiated with a 7 min denaturation at 94°C followed by 1 min denaturation at 94°C, 1 min annealing at 57°C and 1 min extension at 72°C. A final extension step for 5 min at 72°C at the end of amplification.

The amplified fragment was sequenced by Macrogen company (Gasan-dong, Geumchen-gu, Seoul, Korea). The base pairs sequences of the mutants (resistant colonies) were compared with the sequences of the same fragment extracted from preliminary non-resistant colonies as well as 23S rRNA gene sequences from a clarithromycin sensitive *H. pylori* strain in the GenBank (Taylor *et al.*, 1997).

RESULTS

Extraction yield: Extraction yield (% w/w) was calculated from the weight of final solid extract divided by the weight of the primary powdered plant parts.

Extraction method had a yield about 5% for *Cuminum cyminum* to 10% for *Mentha piperita* and *Camellia sinensis*.

MIC values: MIC of clarithromycin for all three isolates was determined separately. There was not any observed colony in concentration 0.125 µg clarithromycin mL⁻¹ and above it. The exhibited results were the same for all three isolates.

None of the evaluated herbs in the applied concentration (62.5, 125, 250, 500 and 1000 µg mL⁻¹) were active against bacterial isolates.

Point mutations associated with clarithromycin resistance: The sequences of 468 bp fragment of DNA from preliminary susceptible isolates were completely identical to sequences associated with clarithromycin sensitive wild type strain UA802. Therefore they validated as clarithromycin susceptible.

Major point mutation detected in the sequences associated with the resistant mutant colonies was A-to-G transition at 2143 position (A2143G).

Mutation frequency values: The mean mutation frequency of clarithromycin resistance was calculated from values obtained from triplicate assays for three isolates. The median mutation frequency to clarithromycin resistance was 27×10^{-9} for three isolates.

Effect of plant extracts on mutation frequency values: Results obtained from the application of various concentrations of plant extracts showed that, the effective concentrations in reduction of mutation frequency were 250, 500 and 1000 $\mu\text{g mL}^{-1}$.

The effects of these concentrations were very similar and increased concentration had only slightly higher effect (data not showed), therefore, we used the minimal effective concentration of 250 $\mu\text{g mL}^{-1}$ in the rest of experiments.

Some reduction in the mean mutation frequency value was observed after application of all three extracts. The mean mutation frequency value was reduced to 12×10^{-9} and 8.5×10^{-9} respectively after application of *M. piperita* and *C. sinensis* extracts.

A considerable loss of mutation frequency value was happened when Cs extract was applied and mean of mutation frequency value shifted from 27×10^{-9} to zero (no mutant colony was observed). This finding suggests that a bacterial suspension with higher concentration have to be used in this case. Therefore, similar experiments were performed with a heavier bacterial suspension and mean mutation frequency value was calculated as 0.11×10^{-9} .

DISCUSSION

In recent years, huge bodies of literature from different countries have reported a worldwide increase in antibiotic resistance of *H. pylori* strains towards different groups of antibiotics (Wu *et al.*, 2012).

Point mutations in various chromosomal genes are described as the main resistance mechanism in *H. pylori* (De Francesco *et al.*, 2012; Gerrits *et al.*, 2006). Mutation as a complex process affects the genotype, but not necessarily phenotype.

Different mutation events can affect different genes, however they can result in similar antibiotic resistance phenotypes. Therefore, the reported prevalence of phenotypic antibiotic resistance is significantly lower than the genotypic resistance (De Francesco *et al.*, 2010). On the contrary, a mutation event is important, if it result in production of an observable resistant phenotype (Martinez and Baquero, 2000).

In this study we evaluated the occurrence of mutation events by the relevant variable of "Mutation Frequency" that recorded the number of mutant cells instead of mutation events. This value expresses the real consequent of a complex mutation process including its favorability and its costs in the bacterial population. Therefore only the favorable mutational events which resulted to an antibiotic resistant phenotype are detected.

Application of resistance modifying factors including anti-mutagenic agents is an attractive alternative approach in targeting and blocking the resistance phenomenon. Medicinal plants may be considered as potential sources of these agents (Sibanda and Okoh, 2007).

All three plant species applied in this study (Cumin, Mint and Green tea) are used in Iranian traditional medicine in the treatment of gastrointestinal disorders.

There are comparable reports in some literatures about similar capacity of these medicinal plants. Moreover, cancer-preventative effects, anti-mutagenic activity, anti-aging activity, free radical-scavenging, antioxidant and anti-inflammatory activity from *Mentha piperita* (Ahmad *et al.*, 2012; Tajkarimi *et al.*, 2010), *Cuminum cyminum* (Allahghadri *et al.*, 2010; Tajkarimi *et al.*, 2010) and *Camellia sinensis* (Kada *et al.*, 1985; Tajkarimi *et al.*, 2010) have been

expressed repeatedly in literature. Even, better anti-oxidative activity of medicinal plants in compare with that of synthetic antioxidants was reported (Allahghadri *et al.*, 2010). All three extract used in the present study were effective in reduction of mutation occurrences. The mean mutation frequency of clarithromycin resistance reduced after application of extract from *Cuminum cyminum* and *Mentha piperita* by 68.5 and 55.5%, respectively in compare to normal condition (absence of plant extracts).

Camellia sinensis showed most potent anti-mutagenic activity which reduced mutation frequency by 99.5%. This is supported by previous studies that were described *Camellia sinensis* as one of the most effective anti-mutagenic and anti-oxidant medicinal plants. The higher total phenolic content of the *Camellia sinensis* suggested as dominant factor accounted for higher total antioxidant capacity of this herb (Cai *et al.*, 2004; Wang *et al.*, 1989).

These finding suggested, beneficial usage of the tested medicinal plants in combination with antibiotics or sidelong antibiotic therapy. This idea could result in retardation in appearance of antibiotic resistant strains of *H. pylori* which is a usual consequence of antibiotic therapy.

CONCLUSION

Amazing capability of medicinal plant in prohibition of clarithromycin resistance in *H. pylori* was showed by the results obtained from the present study. In this respect, *Camellia sinensis* is best recommended choice.

We suggest further similar investigations to be carried out sidelong antibiotic therapy in animal models. An accumulative analysis of these investigations could be used as a key in designing of a perfect and pervasive eradication therapy protocol.

In addition, there is an increasing tendency towards replacing the chemical drugs with natural ones which could obtain more patients compliance.

ACKNOWLEDGMENTS

This study was funded by a Grant (No. 90/246) from research committee of Kerman University of Medical Sciences, Kerman, Iran. The authors also thank Dr. Shahidi for his kindly cooperation in identifying the plants used in this study. The authors have not any conflict of interests to clarity.

REFERENCES

- Ahmad, N., H. Fazal, I. Ahmad and B.H. Abbasi, 2012. Free radical scavenging (DPPH) potential in nine *Mentha* species. *Toxicol. Ind. Health*, 28: 83-89.
- Allahghadri, T., I. Rasooli, P. Owlia, M.J. Nadooshan, T. Ghazanfari, M. Taghizadeh and S.D.A. Astaneh, 2010. Antimicrobial property, antioxidant capacity and cytotoxicity of essential oil from cumin produced in Iran. *J. Food. Sci.*, 75: H54-H61.
- Cai, Y., Q. Luo, M. Sun and H. Corke, 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.*, 74: 2157-2184.
- Castillo-Juarez, I., V. Gonzalez, H. Jaime-Aguilar, G. Martinez, E. Linares, R. Bye and I. Romero, 2009. Anti-*Helicobacter pylori* activity of plants used in mexican traditional medicine for gastrointestinal disorders. *J. Ethnopharmacol.*, 122: 402-405.
- De Francesco, V., A. Zullo, E. Ierardi, F. Giorgio and F. Perna *et al.*, 2010. Phenotypic and genotypic *Helicobacter pylori* clarithromycin resistance and therapeutic outcome: Benefits and limits. *J. Antimicrob. Chemother.*, 65: 327-332.
- De Francesco, V., E. Ierardi, C. Hassan and A. Zullo, 2012. *Helicobacter pylori* therapy: Present and future. *World J. Gastrointest Pharmacol. Ther.*, 3: 68-73.

- Gerrits, M.M., A.H.M. van Vliet, E.J. Kuipers and J.G. Kusters, 2006. *Helicobacter pylori* and antimicrobial resistance: Molecular mechanisms and clinical implications. *Lancet Infect. Dis.*, 6: 699-709.
- Kada, T., K. Kaneko, S. Matsuzaki, T. Matsuzaki and Y. Hora, 1985. Detection and chemical identification of natural bio-antimutagens: A case of the green tea factor. *Mol. Mech. Mutagenesis*, 150: 127-132.
- Kraft, C. and S. Suerbaum, 2005. Mutation and recombination in *Helicobacter pylori*: Mechanisms and role in generating strain diversity. *Int. J. Med. Microbiol.*, 295: 299-305.
- Li, Y., C. Xu, Q. Zhang, J.Y. Liu and R.X. Tan, 2005. *In vitro* anti-*Helicobacter pylori* action of 30 Chinese herbal medicines used to treat ulcer diseases. *J. Ethnopharmacol.*, 98: 329-333.
- Malferttheiner, P., F. Megraud, C.A. O'Morain, J. Atherton and A.T.R. Axon *et al.*, 2012. Management of *Helicobacter pylori* infection-the Maastricht IV/ Florence consensus report. *Gut*, 61: 646-664.
- Martinez, J.L. and F. Baquero, 2000. Mutation frequencies and antibiotic resistance. *Antimicrob. Agents Chemother.*, 44: 1771-1777.
- Megraud, F., 2007. Detection of *Helicobacter pylori* and its sensitivity to antibiotics. A step forward in the use of molecular methods. *Gastroenterol. Clin. Biol.*, 31: 790-791.
- Moder, K.A., F. Layer, W. Konig and B. Konig, 2007. Rapid screening of clarithromycin resistance in *Helicobacter pylori* by pyrosequencing. *J. Med. Microbiol.*, 56: 1370-1376.
- Nariman, F., F. Eftekhari, Z. Habibi, S. Massarrat and R. Malekzadeh, 2009. Antibacterial activity of twenty Iranian plant extracts against clinical isolates of *helicobacter pylori*. *Iran. J. Basic Med. Sci.*, 12: 105-111.
- Rosche, W.A. and P.L. Foster, 2000. Determining mutation rates in bacterial populations. *Methods*, 20: 4-17.
- Sibanda, T. and A.I. Okoh, 2007. The challenges of overcoming antibiotic resistance: Plant extracts as potential sources of antimicrobial and resistance modifying agents. *Afr. J. Biotechnol.*, 6: 2886-2896.
- Tajkarimi, M.M., S.A. Ibrahim and D.O. Cliver, 2010. Antimicrobial herb and spice compounds in food. *Food Control*, 21: 1199-1218.
- Taylor, D.E., Z. Ge, D. Purych, T. Lo and K. Hiratsuka, 1997. Cloning and sequence analysis of two copies of a 23S rRNA gene from *Helicobacter pylori* and association of clarithromycin resistance with 23S rRNA mutations. *Antimicrob. Agents Chemother.*, 41: 2621-2628.
- Wang, Z.Y., S.J. Cheng, Z.C. Zhou, M. Athar, W.A. Khan, D.R. Bickers and H. Mukhtar, 1989. Antimutagenic activity of green tea polyphenols. *Mutat. Res. Genet. Toxicol.*, 223: 273-285.
- Wang, G., M.Z. Humayun and D.E. Taylor, 1999. Mutation as an origin of genetic variability in *Helicobacter pylori*. *Trends Microbiol.*, 7: 488-493.
- Wang, G., T.J.M. Wilson, Q. Jiang and D.E. Taylor, 2001. Spontaneous mutations that confer antibiotic resistance in *Helicobacter pylori*. *Antimicrob. Agents Chemother.*, 45: 727-733.
- Wu, W., Y. Yang and G. Sun, 2012. Recent insights into antibiotic resistance in *Helicobacter pylori* eradication. *Gastroenterol. Res. Pract.*, Vol. 2012. 10.1155/2012/723183
- Zaidi, S.F., K. Yamada, M. Kadowaki, K. Usmanghani and T. Sugiyama, 2009. Bactericidal activity of medicinal plants, employed for the treatment of gastrointestinal ailments, against *Helicobacter pylori*. *J. Ethnopharmacol.*, 121: 286-291.