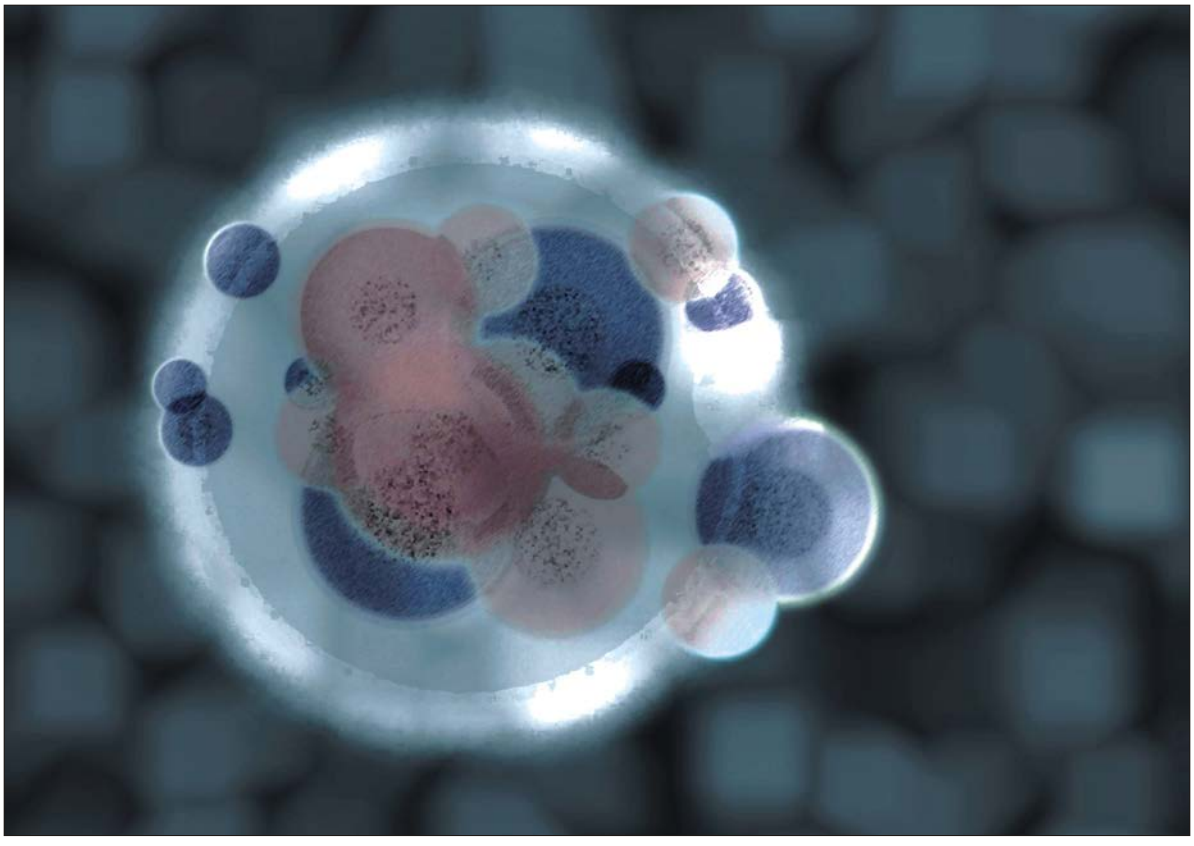

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Antimicrobial and Antiulcer Activities of Methanol Extract of *Allium sativum* on *Helicobacter pylori*

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Abstract: Methanol extract of *Allium sativum* was investigated for *in vitro* antibacterial activity against *H. pylori* using standard strain ATCC 43504 and three clinical isolates (UCH97009, UCH99041 and UCH99052) and also for anti ulcer effect in rat. All the strains except UCH99041 were inhibited by the final concentration of the tested extract (6 mg mL^{-1}) to varying degrees. The Minimum Inhibitory Concentration (MIC) against all susceptible strains ranged between 153 and 625 μg mL^{-1}. Urease activity of both UCH97009 and UCH99052 strains decreased with increase in the concentration of the extract while the cell surface hydrophobicity of two of the strains was reduced by the extract as there was no aggregation of the cell by the extract. SAT titre was >3.0 M. Methanol extract of *A. sativum* reduced mean ulcer score from 13.63±1.33 in control to 10.06±1.27 in extract treated. The anti- ulcer effect was however not significant ($p>0.05$). The acute toxicity test (intraperitonial administration) for methanol extract in mice gave LD_{50} of 8.7 g kg^{-1} body weight. The result indicates that *Allium sativum* taken at a low dose may have some therapeutics potentials against gastric ulcers associated with *H. pylori* infection.

Key words: *Allium sativum*, anti-*Helicobacter pylori*, urease activity, cell surface hydrophobicity, gastric ulcer, acute toxicity

INTRODUCTION

Helicobacter pylori, a urease producing flagellated gram-negative bacterium inhibits the gastric mucosa from withstanding the stomach's hostile acidic millieu by its high urease activity (Gold, 1999).

Urease has been reported to accelerate apoptosis of the gastric epithelial cell, which plays on important role in *H. pylori* mediated pathogenesis (Keigo *et al*., 2001). *Helicobacter pylori* was isolated from the gastric mucosa of patients (Marshall and Warren, 1984). It is the major worldwide cause of bacterial gastrointestinal infections in adults and children.

Antibiotic therapy and a combination of two or three drugs have been widely used to eradicate these infections. However, development of drug resistance in bacteria calls for new sources of drugs (Narima *et al*., 2004).

In the quest for alternative drugs or compounds with therapeutic anti-ulcer potential, several plants have been tested for their inhibitory activity against *H. pylori*. Among these plants, aqueous extract of thyme showed significant inhibitory effect on *H. pylori* (Tabak *et al*., 1996), methanol extracts and hexane fractions of *Aristolochia paucinervis* rhizome and leaves exhibited inhibitory activity (Gadhi *et al*., 2001), methanol extract of the dried powered ginger rhizome (Mahady *et al*., 2003) and crude methanol extract of the leaf of *Allium ascalonicum* have some therapeutic potential against *H. pylori* (Adeniyi and Anyiam, 2004).

Allium sativum (garlic) is a common spice that is used in some part of Nigeria to treat ulcers, skin diseases and asthma by traditional herbalists. Historically, garlic has been used worldwide to fight bacterial infections since it exhibits a broad antibiotic spectrum against both gram-positive and gram-negative bacteria. Some authors have reported the *in vitro* susceptibility of *H. pylori* to garlic oil and its compounds (Chung *et al*., 1998; Graham *et al*., 1999; O'Gara *et al*., 2000).

This study is designed to investigate the *in vitro* antibacterial and antiulcer activities of methanol extract of *Allium sativum* against *Helicobacter pylori* and in rats.

MATERIALS AND METHODS

Plants material: Samples of fresh *Allium sativum* bulbs were purchased from Bodija Market, Ibadan, Nigeria and authenticated at both the Department of Botany and

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Microbiology, University of Ibadan and Forest Research Institute of Nigeria (FRIN) herbarium. Voucher specimen was deposited at FRIN with the herbarium number [FHI 104901]. They were chopped, sun-dried and pulverized for use.

Preparation of extract: Coarsely powdered sample defatted with hexane for 4 hours and weighing 573.3 g was successfully extracted using soxhlet extractor with methanol as solvent for 24 h in succession. Each extract was filtered, concentrated to dryness *in vacuo* and stored at -4°C until needed for experiment. The dried methanol extract was prepared to final concentration of 100 mg mL^{-1} in 50% methanol. About 60 μL of Tween 80 was added to enhance proper dissolution of the extract. This was then used for the various assays. The percentage yield of methanol extract is 4.6%.

Antibacterial activity

Bacterial strains: One commercially available strain of *H. pylori* (ATCC 43504) and three clinical isolates cultured from the gastric biopsy specimen of patient attending the Endoscopy unit of University College Hospital (UCH) Ibadan, Nigeria were used. The *H. pylori* bacterial cells were identified according to colony morphology, gram staining, microaerophilic growth at 37°C; oxidase+ catalase+ urease+ nitrate- H_2S- hippurate hydrolysis-and nalidixic acid. The strains (UCH97009, UCH99041 and UCH99052) were subculture in Mueller-Hinton broth supplemented with 3% sterile fetal calf serum, incubated under microaerophilic condition at 37°C for 3 days and stored in refrigerator after growth for subsequent use.

Antimicrobial agents: Pylorid (25 mg mL^{-1}) and metronidazole (100 μg mL^{-1}) each were used in this study as positive controls while few drops of Tween 80 was added to 50% methanol and used as the negative control for the susceptibility testing.

Experimental studies

Phytochemical screening: A quantitative chemical analysis of the presence of various secondary metabolites such as alkaloids, anthraquinones, tannins, cynogenetic glycosides and steroidal nucleus were examined using standard phytochemical screening procedures.

Acute toxicity study in mice: A total of thirty albino swiss mice with an average weight of 13.5±1.5 were used for the study. The mice were obtained from the Central Animal House, College of medicine, University of Ibadan, Ibadan where they were bred and kept until the time for experiment. The animals were well ventilated and maintained at room temperature of about 27°C. These animals were allocated to six groups; Five mice served as control and classified as groupA, while the other twenty five mice were put into groups B, C, D, E and F with five mice per group. Each mouse in group A received intraperitoneal injection of 0.2 mL distilled water, while graded doses of 20, 40, 80, 110 and 150 mg of methanol extract of *Allium sativum* was given to each mouse in groups B to F, respectively. All injections were given after allowing the animals to acclimatize to the environment for one week and starving them for 24 h. After 24 h, the number of deaths and survivors were recorded and put into a plot of percentage mortality against log of doses used where the LD_{50} was extrapolated.

Susceptibility: The agar cup diffusion technique was used to determine the susceptibility of *H. pylori* to the extract in a method similar to the previous procedures described by Diker and Hascelik (1994) and Adeniyi (1996). However, plates were incubated at 37°C in an automatic CO_2-O_2 incubator under microaerophilic conditions (85% N_2, 10%CO_2 and 5%O_2) for 2-3 days after which diameters of zones of inhibition were measured. Since the extract was reconstituted in 50% methanol with traces of polysorbate (fatty acid-free) Tween 80 before being tested, this diluent was included in each plate as a solvent control besides the chemotherapeutic agents included as positive controls. The antimicrobial studies were done in triplicates and diameters of zones of inhibition (mm) are expressed as mean and standard errors of means.

Determination of Minimum Inhibitory Concentration (MICs): MICs were measured for biologically active extract using broth micro dilution method. Ten microliters of each test dilution was added to wells of 96-well plate and each well was inoculated with 190 μL of a logarithmic phase test culture (Optical density equivalent to 10^8 CFU mL^{-1}). Extract was tested at various concentrations. The control agent included were pylorid and metronidazole. The MICs were read after 3-5 days of incubation at 37°C under microaerophilic conditions. The MIC was regarded as the least concentration of the extract that gave no visible growth from a triplicate experiment (Lajubutu *et al*., 1995).

Urease activity assay: The effects of the methanol extract of *A. sativium* on the urease activity of two of the *H. pylori* (UCH 97009 and UCH 99052) strains were investigated using the alkalimetric method (Hamilton-muller and Gargan, 1979; Mobley *et al*., 1988).

Hydrophobicity assay: For the determination of microbial cell surface hydrophobicity (CSH), the Salt Aggregation Test (SAT) was used as described by Ljungh *et al*. (1985).

To examine the aggregation activity of *H. pylori,* equal volumes (0.5 mL) of bacterial suspension (10^9 CFU) and 6 mg mL^{-1} final concentration of *A. sativum* methanol extract was mixed and left at room temperature for 15 min. Thereafter, 0.05 mL of the mixture was added to 0.05 mL of 0.10-3.0 M-ammonium sulphate diluted with 0.02 M-phosphate buffer in flat-shaped 96 wells microplate. After incubation for 3 h at room temperature, microbial aggregation was estimated visually as described in the control experiment.

Indomethacin- induced gastric ulceration: Twenty four adult male rats obtained from Pre-clinical animal house of the University of Ibadan and weighing between 200 and 250 g were used for the study. The animals were divided into three groups of eight animals per group. Group I animals which served as control received 0.2 mL of distilled water for 15 days, each rat in Group II received 100 mg kg^{-1} of methanol extract of *A. sativum* subcutaneously for 15 days, the rats in Group III each received 10 μg kg^{-1} of misoprostol for 15 days.

Throughout the treatment period, the rats were freely fed on rat's cubes and water given ad libitum. On the 16th day, indomethacin suspended in 0.9% normal saline was administered orally at 40 mg kg^{-1} using an oro-gastric tube. The induction of ulcer by this method is rapid only requiring 4 h.

After 4 h, a blow to the head of each rat later killed the rats and their stomachs were surgically removed and opened up by an incision along the lesser curvature. Each stomach was examined macroscopically for the presence and scoring of gastric ulceration using the Alphin and Ward (1967) method.

The 'scoring' technique used for the indomethacin-induced gastric ulceration was assessed with the following criteria:

Criteria	Ulcer score
Normal stomach	0.0
Punctuate hemorrhage or pin point ulcers	0.5
Two or more small hemorrhagic ulcers	1.0
Ulcers greater than 3 mm in diameter	2.0

Statistical analysis: The results were expressed as Mean±SEM. Comparisons were made using student's t-test. $p<0.05$ was regarded as significant.

RESULT

The percentage yield of methanol extract was 4.6%. Phytochemical screening result showed that *Allium sativum* contains saponins, cyanogenetic glycoside, flavonoids and steroids in low concentration while alkaloids, tannins and anthraquinones were not present.

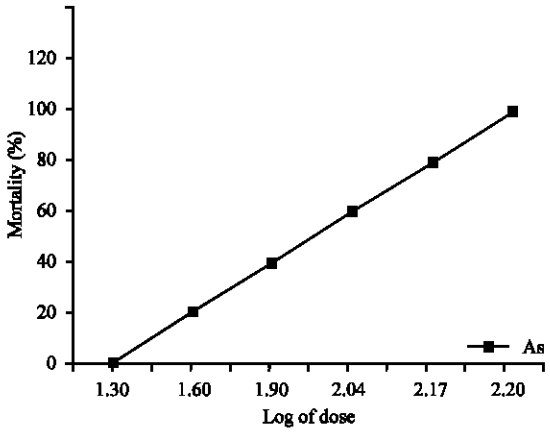

Fig. 1: Acute toxicity profile of *Allium sativum* (As) methanol extract in mice

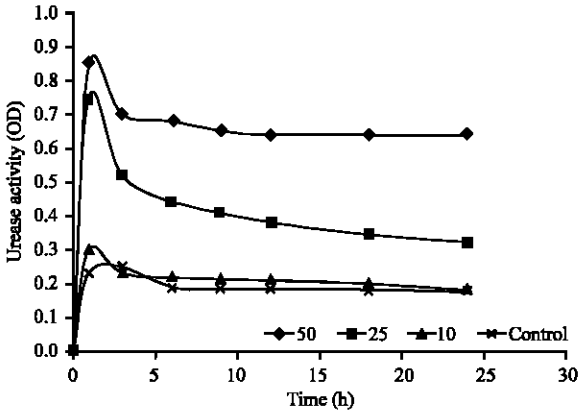

Fig. 2: Effect of different concentrations of methanol extract of *sativum* on urease activity of *Helicobacter pylori* (UCH97009) strain

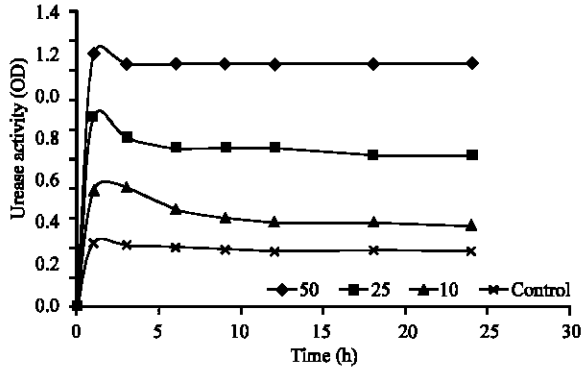

Fig. 3: Effect of different concentrations of methanol extract of *sativum* on urease activity of *Helicobacter pylori* (UCH99052) strain

The results obtained from toxicity test of methanolic extract of *Allium sativum* are presented in Fig. 1. The intraperitoneal LD50 for the extract was 0.87 g/100 g body weight of mice. Antimicrobial susceptibility screening

Table 1: Preliminary *in vitro* anti-*Helicobacter pylori* activity of *Allium sativum*

	Diameters zone of inhibition*			
Extracts/control	ATCC 43504	UCH 97009	UCH 99041	UCH 99052
Methanol (6 mg mL^{-1})	19±0.3	16±0.5	R	15±0.5
Pylorid(R) 25 mg mL^{-1}	24±0.4	22±0.2	11±0.2	17±0.5
Metronidazole 100 (μg mL^{-1})	19±0.5	16±0.3	R	13±0.2

*Result are average of triplicate experiment

Table 2: Minimum Inhibitory concentrations of *Allium sativum* extracts against *Helicobacter pylori*

	Minimum inhibitory concentration*			
Extracts/control	ATCC 43504	UCH 97009	UCH 99041	UCH 99052
Methanol Extract (mg mL^{-1})	0.153	0.153	>6	0.625
Pylorid (mg mL^{-1})	3.12	6.25	12.5	6.25
Metronidazole (μg mL^{-1})	25	50	>100	50

*Result are average of triplicate experiment

Table 3: Effect of methanolic extract of *Allium sativum* on cell surface hydrophobicity of *Helicobacter pylori*

$(NH_4)_2SO_4$ concentration	ATCC 43504	UCH 97009	UCH 99041	UCH 99052
0.10M	-	-	-	-
0.20M	-	-	-	-
0.25M	-	-	-	-
0.30M	-	-	-	-
0.40M	-	-	-	-
1.00M	-	-	-	-
1.50M	+	-	-	-
2.00M	+	+	-	-
3.00M	+	+	-	-
Sat titre	≤1.5	≤2	>3	>3

Key: + = Aggregation (Positive SAT); - = No Aggregation (Negative SAT), NT = Not Tested, Results is average of triplicate experiments

Table 4: The mean ulcer scores in rats pretreated with misoprostol and methanol extracts of *Allium sativum*

Group	Treatment	Mean ulcer score±SEM
I	Control (given distilled water)	13.63±1.33
II	Rats pre-treated with methanol extract (15)	10.06±1.27
III	Rats pre-treated with misoprostol (15)	6.50±1.11*

*Significantly different from control values ($p<0.05$)

revealed that all the strains were susceptible to the extract with little or no difference (Table 1). However, strain UCH 97009 was more susceptible. The inhibitory concentration of the extract against susceptible strains of *H. pylori* ranged between 153 and 625 ug mL^{-1}, which compared favorably with the positive control (Table 2). All *H. pylori* isolates, exhibited an initial increase in activity at various concentrations of methanolic extract of *Allium sativum* added (Fig. 2 and 3). Shortly after that, at about one hour duration of incubation, there was sharp significant decrease in urease activity. The control however showed steady insignificant decrease in urease activity.

None of the tested *H. pylori isolate* aggregated either at or below 1M ammonium sulphate or in the case of sodium phosphate buffer, for all strains there was no aggregation at all in any of the $(NH_4)_2SO_4$ concentrations. SAT titre for all the strains was > 3.0. The addition of the methanol extract of *Allium sativum* enhanced cell aggregation of two of the *H. pylori* strains tested which implied that the CSH of two *H. pylori* strains was decreased by extract (Table 3).

Table 4 shows the results obtained with experimental model of indomethacin-induced acute gastric ulceration in rats. Methanol extract of *Allium sativum* at a dose of 100 mg kg^{-1} for 15 days-demonstrated reduction in mean ulcer score when compared to the animals not treated with extract (Control). However, a dose of 10 μg kg^{-1} misoprostol for 15 days significantly reduced the mean ulcer score with respect to the control ($p<0.05$).

DISCUSSION

This study has further proved the *in vitro* susceptibility of *H. pylori* to extract of *Allium sativum* as reported by various authors on the *A. sativum* oil. Compared with the positive controls, *H. pylori* showed a higher susceptility pattern in some strains and a very little difference in other strains. They may be due to the presence of metabolites like saponin as revealed from its phytochemical screening result. Urease activity of the strains tested was decreased on addition of increasing concentrations of the extracts. This activity of the Hp is of great therapeutic significance because of the special role urease plays in the pathogenesis of *Helicobacter pylori*. It helps the organism to survive and colonize the hostile gastric mucosa by hydrolyzing urea into ammonium creating a conducive environment for the pathogen to flourish (Keigo *et al*., 2001).

Antimicrobial substance or factor that has the potential to reduce the potent urease activity of *H. pylori* will therefore affect the survival and growth of the organism in the gastric mucosa. Infact most of the proton-pump inhibitors such as lansoprazole and omeprazole are equally potent urease inhibitors. This was as confirmed that garlic could readily reduce urease

activity of *H. pylori*. It's therapeutic potential against Hp is therefore not in doubt (O'Gara *et al*., 2000).

For many bacterial infections, different pathogenetic phases can be distinguished, such as mucosal adhesion; tissue colonization and subsequent invasion. Adhesive strains often possess high CSH as determined by SAT and other methods. High CSH appears to be important in the pathogenesis of several microorganisms associated with gastrointestinal infection. The Hydrophobicity assay result showed that methanol extract of *Allium sativum* blocked the aggregation two of the *H. pylori* strains. The inability of the methanol garlic extracts to decrease the CSH of the other *H. pylori* strains by blocking cell aggregation may be due to absence of tannins and alkalrids in the plant as revealed from our phytochemical result. Annuk *et al.* (1999) have shown that the tannins content of bearberry and cowberry plant extract was responsible for the enhanced aggregation *H. pylori* strains with these plants and consequent decrease in their CSH (Annuk *et al*., 1999). However, tannins are lacking in garlic going by our findings.

Other medicinal plants they worked with; wild camomile and pineapple weed blocked cell aggregation of the *H. pylori* strains and had a reduced activity to decrease CSH because of their low tannin content. However, they related enhancement of cell aggregation (i.e., decrease in CSH) to increase antibacterial activity potentiated by high tannin content. This work has shown that increased antibacterial activity may not be dependent on decrease in CSH. This is because the *H. pylori* strains were all highly susceptible to *Allium sativum* methanol extract yet the extract did not decrease their CSH. Incidence of stomach cancer in populations with high *Allium* vegetable intake as reported by Sivam *et al.* (1997) lends credence to the above assertion.

The results of experimentally induced ulceration with indomethacin showed that methanol extract of garlic caused decrease in ulcer scores when compared with the control. This was not significant. This suggests that garlic anti-ulcer effect is likely mediated which might be similar to that of misoprostol which equally reduced the severity of gastric lesions developed by indomethacin in this study. Misoprostol is a known stable analogue of PGE_1. This drug inhibits gastric acid secretion, both basal and that occurring in response to food and also increases the secretion of mucus and bicarbonate.

The use of decoctions of garlic in the treatment of ulcer and related gastroduodenal diseases as practiced by some traditional herbalists in some parts of Northern Nigeria may be justified by the high and plausible susceptibility pattern of *H. pylori* to garlic as revealed from this work coupled with decrease of *H. pylori* urease activity by garlic. *Allium sativum* may possess anti-*H. pylori* potentials that may be explored for the development of therapeutic agents against this pathogen. The toxicity of this plant should be taken into consideration when it is used. *In vivo* studies is still progress on the anti- *H. pylori* activity of this plant.

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REFERENCES

Adeniyi, B.A., 1996. A study of antimicrobial properties of extracts from *Diospyros mespiliformis* HOESTH (EBENACEAE) Ph.D Thesis, University of Ibadan.

Adeniyi, B.A. and F.M. Anyiam, 2004. *In vitro* anti-*Helicobacter pylori* potential of methanol extract of *Allium ascalonicum* leaf: Susceptibility and effect on urease activity. Phytother. Res., 18: 358-361.

Alphin, R.S. and J.W. Ward, 1967. Actions of hexapyronium bromide on gastric secretion in dogs and ulceration in rats. Arch Inter de Pharmacol. Therap., 168: 82-100.

Annuk, H., S. Hirmo, E. Turi, M. Mikelsaar, E. Ara and T. Wadstrom, 1999. Effect on cell surface hydrophobicity and susceptibility of *Helicobacter pylori* to medicinal plant extracts. FEMS Microbiol. Lett., 172: 41-45.

Chuag, J.G., G.W. Chem, L.T. Chang, J.G. Lin, C.C. Yeh and J.F. Wang, 1998. Effects of garlic compounds-diallyl sulphide and diallyl disulphite on arylamine N-acetyltransferase activity in strains of *Helicobacter pylori* from peptic ulcer patients. Am. J. Chin. Med., 26: 353-364.

Diker, K.S. and G. Hascelik, 1994. The Bactericidal activity of tea against *Helicobacter pylori*. Letter in Applied Microbiol., 19: 299-300.

Gadhi, C.A, A. Benharref, M. Jana and A. Lozniewski, 2001. Anti-*Helicobacter pylori* activity of Aristolochia paucinervis pomel extracts. J. Ethnopharmacol., 75: 203-205.

Gold, B.D., 1999. Current therapy for *Helicobacter pylori* infection in children and adolescents. Can. J. Gastroenterol., 13: 571-579.

Graham, D.Y., S.Y. Anderson and T. Lang, 1999. Garlic of jalapeno peppers for treatment of *Helicobacter pylori* infections. Am. J. Gastroterol., 94: 1200-1202.

Hamilton-miller, J.M.T. and R.A. Gargan, 1979. Rapid screening of urease inhibitors. Investigative Urol., 16: 327-328.

Keigo, S., D. Yohei, S. Naohiro, Y. Tetsuya, N. Toshi, I. Yoshitsugu and A. Yosichika, 2001. Apoptotic signaling pathways 'activated by *Helicobacter pylori* infection and increase of Apoptosis-inducing Activity under serum-starved conditions. Infect. Immun., 9: 3181-3189.

Lajubutu, B.A., R.J. Pinney, M.F. Roberts, H.A. Odelola and B.A. Oso, 1995. Antibacterial Activity of Diosquinone and Plumbagin from the Root of *Diospyros mespiliformis* (Hostch) [Ebenaceae]. Phytotherapy Res., 9: 346-350.

Ljungh, A.I., A. Brown and T. Wadstroem, 1985. Surface Hydrophobicity of Coagulase Negative Staphylococci Determined by Salt Aggregation Test (SAT) In: The Staphylococci (Jeljaszewicz, J., Ed.). Gustav Fischer Verlag Stuttgart, New York, pp: 157-161.

Mahady, G.B., S.L. Pendland, G.S. Yun, Z.Z. Lu and A. Stonia, 2003. Ginger (Zingiber officinale Roscoe) and the gingerols inhibit the growth of Cag A+ strains of *Helicobacter pylori*. Anticancer Res., 23: 3699-3702.

Marshall, B.J. and J.R. Warren, 1984. Unidentified curved bacilli: in the stomach of patients with gastritis and peptic ulceration. Lancet, 11: 1311-1315.

Mobley, H.L.T., M.J. Coresia, L.E. Rosenthal and B.D. Jones, 1988. Characterization of urease from *Campylobacter pylori*. J. Clin. Microbiol., 26: 831-836.

Nariman, F., F. Eftekhar, Z. Habibi and T. Falsafi, 2004. Anti-*Helicobacter pylori* activities of six Iranian plants. *Helicobacter*., 9: 146.

O'Gara, E.A.O., D.J. H.H and D.J. Maslin, 2000. Activities of garlic oil, garlic powder and their diallyl constituents against *Helicobacter pylori*. Applied Environ. Microbiol., 66: 2269-2273.

Sivam, G.P., J.W. Lampe, B.S. Ulness, S.R. Wanzy and J.O. Potter, 1997. *Helicobacter pylori in vitro* susceptibility to garlic (*Allium sativum*) extracts. Nutr. Cancer, 27: 118-121.

Tabak, M., R. Armon, I. Potasman and I. Neeman, 1996. *In vitro* inhibition of *Helicobacter pylori* by extracts of thyme. J. Applied Bacteriol., 80: 667-672.