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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY OF OLIVE LEAF EXTRACTS AND OLEUROPEIN AGAINST VIRULENT STRAINS OF *HELICOBACTER PYLORI*

***[1]Eman M. Halawani, [2]Hayam S. Abdelkader and [3]Roqayah Kadi**

[1]Biology Department, Faculty of Science, Taif University, KSA
[2]Microbiology and Immunology department, Faculty of Pharmacy, Modern University for Technology and Information, Egypt
[3]Biology Department, Faculty of Science, King Abdulaziz University, KSA

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ABSTRACT

Helicobacter pylori are the major cause of chronic active gastritis, peptic ulcer disease and gastric carcinoma. Currently, the eradication of *H. pylori* infection involved a triple therapy, which combines two antibiotic with a proton pump inhibitor. In this study, the aqueous and alcoholic extracts of olive leaf extracts (OLE) and Oleuropeinwere screened for their antibacterial activity against33 gastric biopsy specimens*ofH. pylori* virulent isolates. Virulent *H. pylori* isolates were detected by PCR for gene typing of virulent *CagA*/*IceA* genes and their distribution among isolates.Resistant to tetracycline and metronidazolewere detected in all isolates (100%), however no resistance was observed to amoxicillin, levofloxacin and clarithromycin. The results of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) indicated that olive leaf extract (OLE) and Oleuropein had a potent antibacterial effect against the virulent *H. pylori* isolates used in this study. The MICs of OLEs were 50 µg/mL (alcohol extract), 70 µg/mL (cold water extract), and 60 µg/mL (boiled water extract) while the MICs of Oleuropein was ranged between 20 µg/ml to 40 µg/ml. Interestingly, heat treatment of OLE water extract increased its inhibitory activity against *H. pylori*. MBC of *H. pylori* genotypes tested was ranged between 60-70 µg/ml for alcohol extract, 70-80 µg/ml for cold water extract, and 80-90 µg/ml for boiled water extract while it was ranged between 50 µg/ml to60 µg/ml for Oleuropein. These findings suggest that olive leaves extract and its active consecutions Oleuropein is an effective as the commercial antibiotics used against*H. pylori* infection.

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INTRODUCTION

*Helicobacter pylori*has been classified by the World Health Organization (WHO) as a class 1 carcinogen (WHO, 2008). The evidence supporting a role for *H. pylori* causing gastric cancer is very strong. Lifetime persistence of this organism within the host could result in a number of gastroduodenal diseases ranging from mild gastritis, atrophic gastritis, and peptic ulcer disease to malignant diseases such as gastric adenocarcinoma and Mucosa-Associated-Lymphoid-Tissue (MALT) Lymphoma (Cavaleiro *et al.,* 2011). Its prevalence is highly variable in relation to geography, ethnicity, age and socioeconomic factors which high in developing countries (WGO 2010).Unfortunately, the prevalence of *H. pylori* infection in Arabian countries was approximately high, it reached 77.5% and 79% inJordan and Bahrain (Fakhro *et al.,* 1999; Nimri *et al.,* 2006). 84% and 86%.in Kuwait and Egypt (Al Qabandi *et al.,* 2005; Mahmoud *et al.,* 2006) 87% in the Eastern region and 61.6% in Central and Western regions of Saudi Arabia (Ayoola *et al.,* 2004; BinSaeed *et al.,* 2012). The occurrence of *H. pylori* strains resistant to antibiotics would be expected to increase, and it is nowadays important to search for nonantibiotic agent that is both highlyeffective and safe could be important for the inactivationof *H. pylori*. The current eradication strategy of *H. pylori* infection by using chemotherapy regimen (clarithromycin & amoxicillin with proton pump inhibitor) sometimes causes side effects and fails to eliminate infection due to inactivation of the drug by high gastric acidity, insufficient diffusion of the drug through the gastric mucosa as well as emergence of antibiotic resistance (Godoy *et al.,* 2003; Bago *et al.,* 2010). Olive (*Olea europaea* L.), mainly originated from Mediterranean region, is a famous woody oil tree used to produce virgin olive oil. Olive oil is the cardinal characteristic of the Mediterranean diet, serving as the principal source of dietary fat. Because it is rich in bioactive compounds (vitamins, flavonoids and polyphenols), it has been associated with lower

Corresponding author: Eman M. Halawani,
Biology Department, Faculty of Science, Taif University, KSA

rates of coronary heart disease (CHD), as well as reduced breast and colon cancer risk. Olive leaves also contain a wide variety of phenolic compounds, for example, oleuropein (OE), hydroxytyrosol (HT), tyrosol (T), cumaric acid, ferulic acid, caffeic acid, *etc.* These phenolic compounds have very good biological activity, *i.e.*, antioxidative activity (Yuan *et al.,* 2015).Olive leaf extract (OLE) is known with its high antioxidant, antimicrobial and antibacterial activity. OLE is very effective activity against various diseases, such as coronary arterydisease, hypertension, high cholesterol level, arrhythmia, cancer, diabetes, overweight, osteoporosis, herpes, flu andcolds, and some bacterial, fungus and yeast infections (Erdohan *et al.,* 2011). Olive oil, in particular virgin olive oils with a high content in certain phenolic compounds, can inhibit the growth of pathogenic bacteria, this activity being higher than that reported for foods such as tea, coffee and others. Also, olive oil polyphenols, in particular the dialdehydic form of decarboxymethyloleuropein and ligstroside aglycons possess a strong bactericidal activity in vitro against *Helicobacter pylori*, which opens up the possibility of considering virgin olive oil as a chemo preventive agent for peptic ulcers or gastric cancer (Brens *et al.,* 2012).

Several species within the olive family, botanically known as *Olea europaea*, providean inhibitory activity against the growth of *H. pyloriin vitro* (Brens *et al.,* 2012; Bisignano *et al.,* 1999). Phenolicstructures similar to *oleuropein* seem to produce its antibacterial effect by damaging thebacterial membrane and/or disrupting cell peptidoglycans have usedbiophysical assays to study the interaction between oleuropein and membrane lipids; however, the exact mechanism of the antimicrobial activity of *oleuropein* is still notcompletely established (Caturla *et al*., 2005). Although(lee and lee, 2010) has proposed that it is due to thepresence of the ortho-diphenolic system (catechol) (lee and lee, 2010) Oleuropein (OE), the main polyphenol in olive leaf extract, was as high as 17%, is likely to decomposeinto hydroxytyrosol (HT) and elenolic acid under the action of light, acid, base, hightemperature (Yuan *et al.,* 2015). (lee and lee, 2010), showed the antimicrobial activity of commercial *Olea europaea* (olive) leaf extracts (abundantly oleuropein) against *Campylobacter jejuni*, *Helicobacter pylori* and methicillin-resistant *Staphylococcus aureus* (MRSA). Olive leaf extract may have a role in regulating the composition of the gastric flora by selectively reducing levels of *H. pylori* and *C. jejuni* (lee and lee, 2010). In this study, we investigated the antibacterial activity of olive leaf extracts andits active constituent, oleuropein against virulent strains of *H. pylori*in *vitro*.

MATERIAL AND METHODS

Culture and Purification of *H. pylori*

Thirty three *H. pylori* strains were isolated from the gastric biopsy specimens of 33patients attending the endoscopy clinic at three hospitals; King Faisal, AL Hada Armed Forces, and King Abdul-Aziz. None of the patients had received any antimicrobial therapy for at least 4 weeks before the study. Five different types of culture media were evaluated for isolation of *H. pylori* from clinical specimens.These media included: Modified Columbia agar containing 7% laked horse blood *H. pylori* selective agar growth media with Dent *H. pylori* antibiotic supplement), Blood sheep agar, Chocolate agar, Brucella blood agar, and brain heart infusion agar (BHIA). Immediately beforeculture, each biopsy specimen was thawed toroom temperature and processed by grindingbetween two frosted sterile microscope slides.A suspension was made with 5 ml BHI broth media and 60 µL of the suspension was streaked for isolation onto the different agar plates.The plates were incubated at 37°C for up to 3-5 days under anaerobic conditions (Goodwin, 1997). Plates were first checked for growth on day3 and daily thereafter. Typical small, roundcolonies positive for catalase, oxidase and ureasewith typical morphology on Gram stainingwere regarded as *H. pylori*. Growth, number ofcolonies, and days to appearance on the differentagar media were recorded.The typical *H. pylori* colonies were preserved in BHI broth supplemented with 20% glycerol and maintained at -80ºC until used.

Phenotypic andBiochemical identification

The growth of small, circular, smooth colonies observed after 3 to 4 days on the selective media plated with gastric biopsy specimens is an important criterion for *H. pylori* identification. No hemolytic activity is readily observed but may appear after a few days at 4°C.The clinical isolates were identified biochemically, by rapid Urease testCatalase and Oxidase tests (MacFaddin, 2000).

Molecular Identification

Genomic DNA Isolation: Genomic DNA was extracted froma 72h culture, using the QIAamp DNAminikit (QiagenGmbH, Hilden, Germany), according to (Kadi *et al*., 2014). DNA purity and quantity was determined using a GeneSys 10UV spectrophotometer (Thermo Scientific, USA).

16S rRNA gene amplification

The entire nucleotide sequence of the 16S rRNA gene of *H. pylori* has been determined (Kadi *et al*., 2014).On the basis of that sequencedesigned a set of PCR primers, Hp1/Hp2 (Hp1, 5'-CTG GAG AGA CTA AGC CCT CC-3'; Hp2, 5'-ATT ACT GAC GCT GAT TGT GC-3'), which amplify 109-bp fragment of the *H. pylori* 16S rRNA gene. The 50 µL PCR mixture contained 100ng of template DNA, PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 2.5mM $MgCl_2$, 0.001% gelatin), 0.2 mM (each) PCR primers, 0.2 mM (each) dNTPs, and 2.5 U of Taq DNA polymerase. The reactionmixture was subjected to 35 cyclesof 95ºC for 1 min, 60ºC for 1 min, and 72ºC for 2 min. The PCR products wereelectrophoresed on 1.5% agarose gels. The DNA bands were visualized byUV transillumination after the gels were stained with ethidium bromide (Kadi *et al*., 2014).

GenotypingPCR

Isolated genomic DNAs (gDNAs) were used to detect the presence of *cag*Aand *ice*A virulence genes harbored in H pylori local isolatesby PCR. Primers used and conditions for different PCR reactions are reported by (Kadi *et al*., 2014).

Antibiotic susceptibility tests

Antimicrobial sensitivity of *H. pylori*isolates were detected byconventional agar disk-diffusion procedure. The bacterial suspension (McFarland tube no. 3) of *H. pylori* was plated onMueller-Hinton agar containing 5% heparinized horse blood. The disks (6-mm diameter, Hi Media, India) of different antibiotics Clarithromycin (CLA, 15 mcg/disk), Metronidazole

(MTZ, 4 µg/mL/disk), Tetracycline (TET, 30 mcg/disk), Levofloxacin (LEV, 5 µg/mL/disk), and Amoxicillin (AMX, 25 µg/mL/disk)were placed on the plates andincubated at 37°C in a microaerophilic chamber for 72 hours andexamined for the diameter of the inhibition zone, which was measuredin millimeters, with the measuring caliper and noted. Based on the Clinical Laboratory Standards Institute (CLSI), guidelines for the fastidious organism *H. influenza* (CLSI. 2015). Zone size ≤ 18 mm was considered resistant to amoxicillin (an analog ofampicillin); ≤30 mm was considered resistant to Levofloxacin, clarithromycin, and tetracycline. For metronidazole ≤ 16-mm zonesize was considered to be resistant.

Preparation of Olive Leaf extracts (OLE)

Aqueous extracts

Olive leaves used in this study were collected in winter 2011 from Al Jouf, Hail and Tabouk, (KSA). Leaves were washed to remove impurities such as dust and then dried in an air for 5-7 days in dark. Then, they were ground by grinder and one liter water was added to 50 grams powder obtained from leaves and put on the shaker to be solved thoroughly. The extract was filtered through Whattman filter paper to remove any undissolved particles. Finally, obtained extract was sterilized through bacterial filtration (0.45 Mm) according to (Mobasher *et al.,* 2006). Another 20 gm of powdered olive leaves were soaked in 200 ml boiling water for 30 minutes. The extract was filtered through sterile filter paper and the solid parts are strained out. The filtrate was concentrated by using drying oven and the powder was dissolved into appropriate amount of ddH_2O to make stock solution (1gm/ml). The boiled extract was filtered through bacterial filter (0.45 µm) before use.

Alcoholic extracts

20 gm of powdered Olive leaves are covered with an 200 ml aqueous alcohol solution (70%) which remains in contact with the leaves for at least 4 hours and is then drained. This process is repeated at least two more times, and the drained extracts are combined, concentrated by distillation under vacuum, and dried by using drying oven under vacuum, to obtain a powder containing about 30-40% by weight oleuropein. The steps of the extraction are conducted at a temperature of about 20ºC to 85ºC according to (Nashman 1998). (Patent).

Oleuropein extract

20 gm commercially powdered Oleuropein obtained from XianApp Chem-Bio (Tech) were soaked in 200 ml distilled sterilized water and placed on the shaker to be solved thoroughly.for at least 30 min at room temperature to make stock solution (1gm/ml). The extract was filtered through bacterial filter (0.45 µm) before use.

Determination of MICsof (OLE) and Oleuropein by micro dilution broth method

A micro broth dilution method was performed with 96-well microplate. Each well of a 96-well microplate was coated with one-fold serial dilutions of water extract OLE, alcohol extract OLE, water boiling OLE and Oleuropein with concentrations (200–12.5 µg/mL) then air-dried. A saline suspension of each *H. pylori* strain equivalent to 3.0 McFarland standard (containing 10^6 bacteria/ml), was prepared from a 72 hour subculture of a blood agar plate. The suspension (0.5 ml) was put into 9.5 ml Mueller–Hinton broth supplemented with 5 % horse serum to a density of 10^6 bacteria/ml. A 100 µl volume of the suspension was added to each well, and the cultures were incubated at 37°C for 3 days under a microaerophilic atmosphere (10 % O_2, and 7.5 %CO_2). After incubation, the plates were examined visually, and The lowest concentration of OLE and Oleuropein that completely inhibited visible bacterial growth was recorded as the MIC for that extract. All the experiments were performed in duplicate and the results were expressed as mean of all the values.

Determination of MBCs of OLE and Oleuropein

Bactericidal activity of the OLE and Oleuropein was determined by the method described earlier by (O'Mahony *et al.,* 2005). With minor modifications (O'Mahony *et al.,* 2005). Briefly, 100 µl of each extract in the wells that showed no bacterial growth in the microtiter plate was spread onto *H. pylori* selective agar plates and incubated at 37°C for 3 days under a microaerophilic atmosphere (10 % O_2, and 7.5 % CO_2) and the colonies formed were subsequently enumerated. All experiments were performed in duplicate. The MBC was defined as the minimum concentration of each extract at which there was no visible growth of bacterial colonies at the latter cultivation.

RESULTS

Isolation of *H. pylori* pure culture from biopsy specimens

In the current study, ***H. pylori*** selective growth medium (Columbia Agar plus Dent's selective medium) proved to be the best medium; growth occurredin 9 cases out of 33, i.e., 27%. The secondmedium was Brain Heart Infusion plus sheep blood Agar; occurred in 6 out of 33 i.e. 18 %. The third medium was Chocolate Agar; growth occurred in 5 out of 33 cases, i.e., 15% of cases. The fourth and Fifth media were Brucella blood Agar and Blood Agar; growth occurred in only 3 out of 33 cases, i.e., 9%. The difference between the culture media was found to bestatistically significant ($p = <0.001$).

Figure 1(A,B,C,D and E) showed the morphological shapes of bacterial colonies on each media. *H. pylori*bacterial colonies appeared as small, rounded, and translucent, "water spray" after 3-5 days on *H. pylori* selective growth media (Fig. 1A) while the colonies appeared as white rounded brilliant large colonies after 2-3 days post incubation on Brain Heart Infusion Agar medium (Fig. 1B). Chocolate agar media showed gray/white colonies, 1 to 2 mm in size after 2 to 5 days post incubation (Fig. 1C). Brucella agar medium showed white/yellow, 3-5 mm in size bacterial colonies appeared after 3 days post incubation (Fig. 1D) while the Blood agar medium showed white/gray, 3 to 5 mm in size bacterial colonies appeared after 2-4 day (Fig 1E).All bacterial cultures were incubated for 3-5 days at 37°C in 5-9.5 % CO_2. Microscopic examination of *H. pylori*appeared as a gram-negative spiral, V or U or comma shapes.

Identification of *H. pylori* by biochemical tests

All gastric biopsy tissues gave positive results with CLO test (Fig. 2A). The *H. pylori* isolates were positive for urea broth test (Fig. 2B), catalase, and oxidasetests.

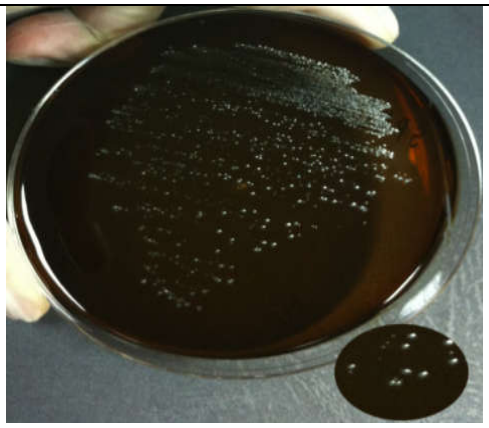

Fig. 1(1A). Rounded, and translucent "water spray" bacterial colonies appeared after 3-5 days on *H. pylori* selective growth media

Fig. 1(1B). BHIA showed white rounded brilliant large colonies after 2-3 days post incubation

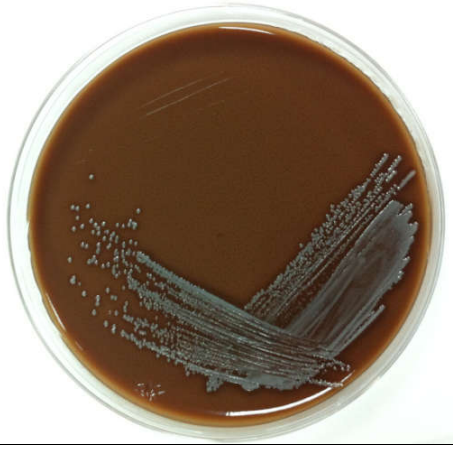

Fig. 1 (1C). Gray/white colonies 1 to 2 mm in size appeared after 2 to 5 days post incubation on Chocolate agar media

Fig. 1 (1D). Brucella agar medium showed off-white, 3-5 mm in size bacterial colonies appeared after 3 days post incubation

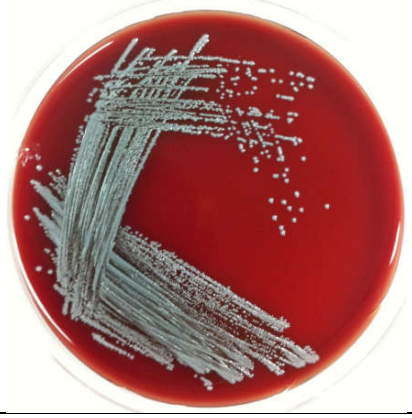

Fig. 1(1E). White, 3 to 5 mm in size colonies appeared after 2-4 days post incubation on Blood agar medium

Fig. 2 (2A). *Campylobacter*-like organism (CLOtest) (Rapid Urease) showing the positivity of *H. pylori* isolates. Pink color= Positive and Yellow color = Negative

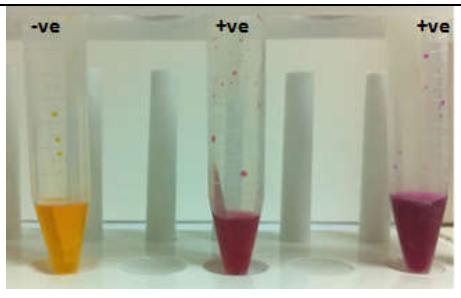

Fig. 2. (2B). Urea broth test (10 %) showing the positivity of *H. pylori* isolates.Pink color = Positive and Yellow color = Negative

Amplification of *H. pylori* 16S rDNA by PCR

Genomic DNA obtained directly from biopsies and from *H. pylori* cultures indicated that 33/33 (100 %) were found to be positive for *H. pylori* 16S rDNA. PCR products for 16SrDNA based primers gave DNA bands on agarose gel corresponding to a 109 base pair productwhen compared to the molecular ladder, thus identifying theisolates as *H. pylori*as shown in (Figure 3).

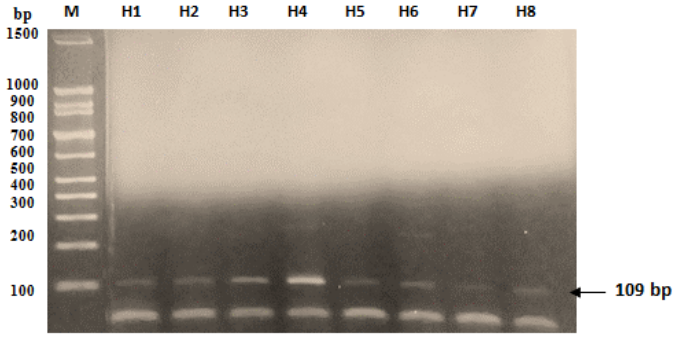

Figure 3. 1.2 % agarose gel electrophoresis showing the PCR products of 16SrDNA (109 bp) using Hp1 and Hp2PCR primers (Table 3-2). DNAs were extracted from gastric biopsies (H1 to H8) collected from AL Hada Armed Forces Hospital. M: Molecular weight DNA marker (100bp ladder)

Genotyping PCR

PCR- based geno typing of virulence genes, *cag*A and *ice*A was showed in Figures4 (A, B,C and D).The distribution of *cag*A and *ice*A1 genotypes among all *H. pylori* isolates under current studyis shown in Table 1. The *cag*A/*ice* A1 genotypewas not detected in all isolates (0%), while *cag*A/*ice*A2 genotypes were detected in 15% of *H. pylori* isolates. cagA/iceA1/iceA2 genotypes were detected in 54.5% of pylori isolates.Distribution of multiple genotypes among all isolates was detected.The cagA1/ iceA1/iceA2 genotype was found in 4 (30.7 %) PUD specimens and in 2 (10 %) gastritis cases; cagA2/iceA1/iceA2 genotype was found in 3 (23.0 %) PUD specimens and in 4 (20 %) gastritis cases; and cagA-/ iceA1/iceA2 genotype was found in only 1 (3.0 %) PUD case and not detected in gastritis specimens. The cagA1/cagA2/ iceA1/iceA2 genotype was detected in 3 (23.0 %) PUD specimens and in 2 (10 %) gastritis cases (data not shown).

Susceptibility of *H. pylori* to antibiotics and OLE

The susceptibility of *H. pylori* isolates to five conventional antibiotics currently used, Amoxicillin, Tetracyclin, Levofloxacin, Clarithromycin, and Metronidazolewere investigated. The results confirmed thatall *H. pylori* isolates were resistant to Tetracycline and Metronidazole, no resistance to Amoxicillin, Ciprofloxacin, Clarithromycin and was observed (Table 2 and Figure 5)

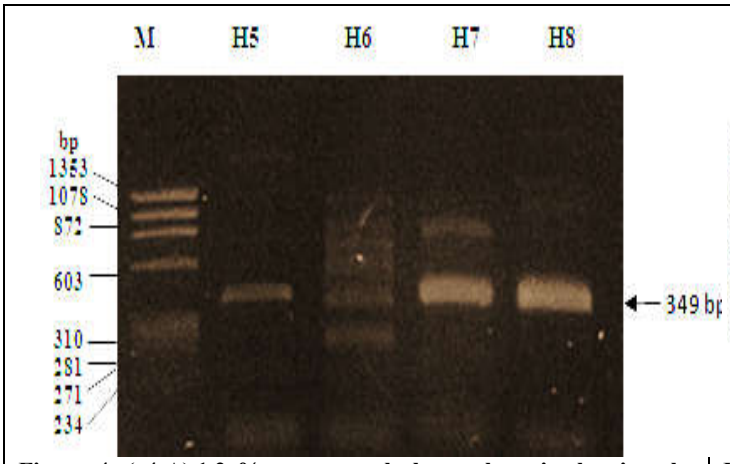

Figure 4. (4-A).1.2 % agarose gel electrophoresis showing the PCR-basedgenotyping of *cag*A1 gene(349 bp) using the primers described in Table 3-2. H5 to H8 are gastric biopsies collected from AL Hada Armed Forces Hospital. M: Molecular weight DNA marker (72-1353bp)

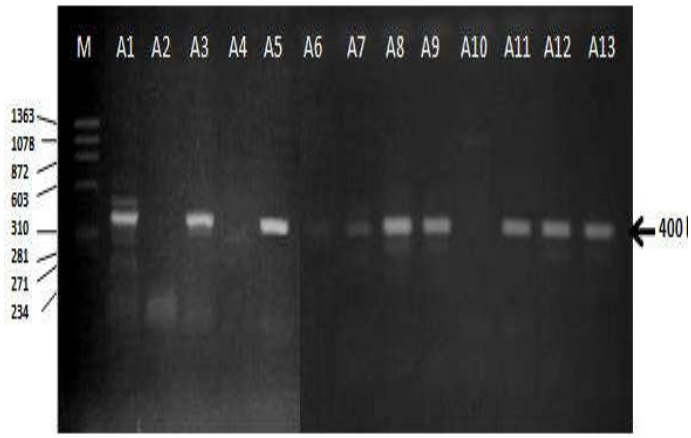

Figure 4. (4-B). 1.2 % agarose gel electrophoresis showing the PCR-based genotyping of *cag*A2 gene (400 bp) using the primers described in Table 3-2. A1 to A13 are gastric biopsies collected from King Abdul Aziz Hospital. M: Molecular weight DNA marker (72-1353 bp)

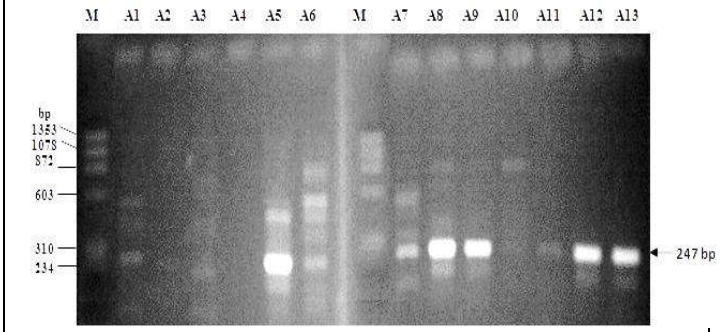

Figure4. (4-C).1.2 % agarose gel electrophoresis showing the PCR-basedgenotyping of *ice*A1 gene(247 bp) using the primers described in Table 3-2. A1 to A13 are gastric biopsies collected from King Abdul Aziz Hospital. M: Molecular weight DNA marker (72-1353 bp)

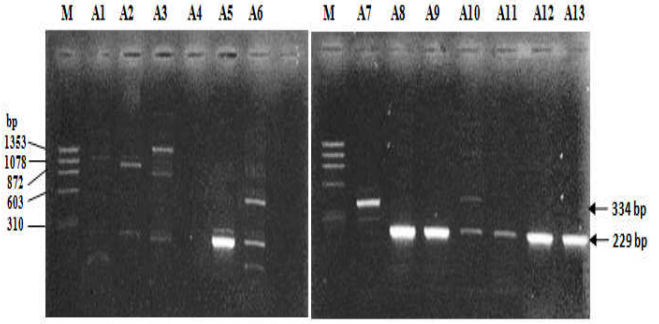

Figure 4. (4-D).1.2 % agarose gel electrophoresis showing the PCR-basedgenotyping of *ice*A2 gene (229 or 334 bp) using the primers described in Table 3-2. A1 to A13 are gastric biopsies collected from King Abdul Aziz Hospital. M: Molecular weight DNA marker (72-1353 bp)

Table 1. Occurrence of *cag*A+ and *iceA*1/*iceA*2 double positives among allcases studied of 13 peptic ulcer duodenal (PUD) and 20 gastritis

PUD and Gastritis cases (n = 33):			
	cagA+	*cagA*-	Total
*iceA*1	0	1 (3%)	1 (3%)
*iceA*2	5 (15%)	2 (6.0%)	7 (21%)
*iceA*1/*iceA*2	18 (54.5%)	1 (3%)	19 (57.5%)
iceA-	4 (12%)	2 (6%)	6 (18%)
Total	27 (81.8%)	6 (18%)	33 (100%)

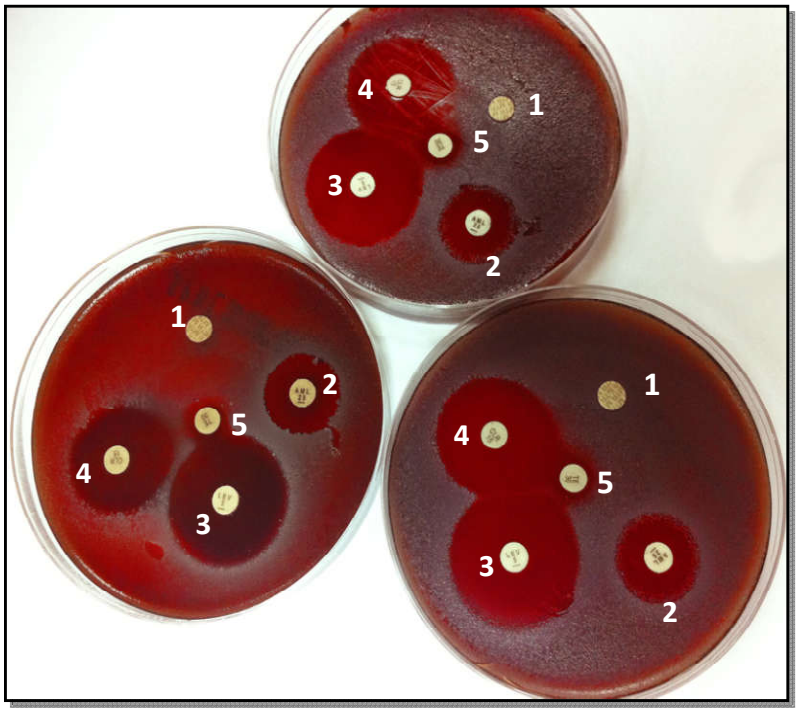

Figure 5. A disk diffusion test with virulent *H. pylori* culture genotype *cag*A1/ *cag*A2/*ice*A1/*ice*A2. The diameters of all zones of inhibition were measured and those values translated to categories of susceptible or resistant. Antibiotics used were: 1: metronidazole, 2: amoxicillin, 3: levofloxacin, 4: clarithromycin, 5: tetracycline

Figure 6. A disk diffusion test with *H. pylori* culture genotype *cag*A2/*ice*A1/*ice*A2. A combined multidrug effect was shown between the five antibiotics tested. Antibiotics used were: 1: amoxicillin 2: tetracyclin 3: metronidazole 4: clarithromycin 5: levofloxacin

*H. pylori*isolates showed to beresistant totetracycline and metronidazole as monodrug *in vitro* however, when combined with any of the tested antibiotics, a strong effect on the eradication of *H. pylori* may be noticed (Figure 6).

Minimum Inhibitory Concentration (MIC) and MBC of OLE and Oleuropein

The results from the MIC determination confirm that OLE and oleuropein have antibacterial effect against the three virulent genotypes strains of *H. pylori*used in this study.

The MIC of OLE was 25 µg/mL (alcohol extract), 200 µg/mL (cold extract), and 25 µg/mL (boiled extract) (Tables 3-A& 3-B and 4). The MIC of oleuropein was 30 µg/ml, 40 µg /ml and 20 µg /ml for*cag*A1/*ice*A1/*ice*A2, *cag*A2/*ice*A1/*ice*A2 and*cag*A1/*cag*A2/*ice*A1/*ice*A2, respectively. OLE also showed antibacterial effect against the same *H. pylori* strains. Heat treatment of OLE water extracts didn't reduce the inhibitory activity against *H. pylori.* Results from minimum bactericidal concentrations (MBC) after incubation with the oleuropein for 72 hresulted in killing of all three *H. pylori* isolates that were formally tested, at 60 µg/mL and 50 µg/mL as shown inTable (5 and 6).

Table 2. Critical inhibition zone (Mean) diameters (mm) of susceptibilities of *H. pylori* strains to selected antimicrobial agents

Antimicrobial Agent	Inhibition zone (Mean) Diameters (mm)					
	*cag*A1 /*ice*A1/*ice*A2		*cag*A2/*ice*A1/*ice*A2		*cag*A1/ *cag*A2/*ice*A1/*ice*A2	
	R	S	R	S	R	S
Tetracycline (TE) (30µg)	R(10 mm)		R(11mm)		R13(mm)	
Amoxicillin (AML) (25µg)	S(20 mm)		S(20 mm)		S(23 mm)	
Levofloxacin ((5µg)	S(35 mm)		S(33 mm)		S(45 mm)	
Clarithromycin (CLR) (15µg)	S(30 mm)		S(28 mm)		S(33 mm)	
Metronidazole (LZ) (5 µg)	R(0 mm)		R(0 mm)		R(0 mm)	

Table 3A. Minimum Inhibitory Concentration (MICs) of OLEs against different *H.pylori* genotypes by microdilution broth assay

*H. pylori*genotypes	MICs of Olive leaves Extracts (Alcohol extract) (µg /ml)										
	500	400	300	200	100	50	25	12.5	6.25	Ctrl	MIC
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50 µg/ml
cagA2/iceA1/iceA2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50 µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50 µg/ml
	MICs of Olive leaves Extracts (coldwater extract) (µg /ml)										
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	100 µg/ml
cagA2/iceA1/iceA2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50µg/ml
	MICs of Olive leaves Extracts (boiled water extract) (µg /ml)										
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50µg/ml
cagA2/iceA1/iceA2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50 µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	50 µg/ml

OLE: Olive Leaf Extract +ve: Positive bacterial growth -ve: Negative bacterial growth Ctrl: Control (bacterial culture without OLE)
Each *H. pylori*isolate was repeated three times.

Table 3B. Minimum Inhibitory Concentration (MICs) of OLEs against some *H.pylori* genotypes by microdilution broth assay

	MICs of Olive leaves Extracts (OLE)(µg/ml)							
	90	80	70	60	50	40	Ctrl	MIC
Alcohol extract	-ve	-ve	-ve	-ve	-ve	+ve	+ve	50 µg/ml
Water extract (cold)	-ve	-ve	-ve	+ve	+ve	+ve	+ve	70 µg/ml
Water extract (Boiled)	-ve	-ve	-ve	-ve	+ve	+ve	+ve	60 µg/ml

OLE: Olive Leaf Extract +ve: Positive bacterial growth -ve: Negative bacterial growth
Ctrl: Control (bacterial culture without OLE)Each *H. pylori*isolate was repeated three times.

Table 4. Minimum bactericidal concentrations (MBCs) of OLEs against tested *H.pylori* genotypes

*H. pylori*genotypes	MBCs of Olive leaves Extracts (Alcohol extract) (µg /ml)										
	100	90	80	70	60	50	40	30	20	Ctrl	MBC
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	60 µg/ml
cagA2/iceA1/iceA2	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	70 µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	60 µg/ml
	MBCs of Olive leaves Extracts (coldwater extract) (µg /ml)										
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	80µg/ml
cagA2/iceA1/iceA2	-ve	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	70µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	80µg/ml
	MBCs of Olive leaves Extracts (boiled water extract) (µg /ml)										
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	80µg/ml
cagA2/iceA1/iceA2	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	90µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	90µg/ml

OLE: Olive Leaf Extract +ve: Positive bacterial growth -ve: Negative bacterial growth Ctrl: Control (bacterial culture without OLE)
Each ***H. pylori*** isolate was repeated three times.

Table 5. Minimum Inhibiting Concentration (MICs) of oleuropein against tested *H.pylori* genotypes by microdilutionbroth assay

*H. pylori*genotype	MICs of oleuropein extract (µg/ml)							
	50	40	30	20	10	5	Control	MIC
*cag*A1/*ice*A1/*ice*A2	-ve	-ve	-ve	+ve	+ve	+ve	+ve	30 µg/ml
cagA2/iceA1/iceA2	-ve	-ve	+ve	+ve	+ve	+ve	+ve	40 µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	+ve	-ve	+ve	+ve	+ve	20 µg/ml

Table 6. Minimum bactericidal concentrations (MBCs) of oleuropeine against tested *H.pylori* genotypes

*H. pylori*genotype	oleuropeine dilutions (µg/ml)				
	60	50	40	Control	MBC
*cag*A1/*ice*A1/*ice*A2	-ve	+ve	+ve	+ve	60 µg/ml
cagA2/iceA1/iceA2	-ve	+ve	+ve	+ve	60 µg/ml
*cag*A1/ *cag*A2/*ice*A1/*ice*A2	-ve	-ve	+ve	+ve	50 µg/ml

+ve or –ve indicates ***H. pylori*** survival or loss of viability after 72 hof co-incubation. Control: bacterial culture without oleuropein.

It is clear from the results that OLE and Oleuropein provided a significant antibacterial effect against *H. pylori* (Antihelicobacter pylori), and it can be usedas alternative bactericidal agents to antibiotics.

DISCUSSION

The current study attempted to determine the antibacterial activity of OLE and oleuropein against virulent isolates of H. pyloriisolated from a group of patients attending the endoscopy clinic at Al-Hada Armed Forces Hospital, King Faisal Hospital, and King Abdul Aziz Hospital at Taif region, Saudi Arabia. The detection of. H. pylori infections were inves tigated by both culture and PCR of DNA obtained directly from biopsies. Our data experienced great difficulty in obtaining pure cultures of H. pylorifrom gastric biopsies with a consequently higher failure rate as also reported by (Farhat Rizvi and Abdul Hannan, 2005). The reasons for this problem are not immediately apparent as reported by (Secka *et al.,* 2011). However, great care is needed in the collection, transport and culture of this microorganism. Collection procedure and transport of the biopsy specimens has been found to be a very important factor in the successful growth of this microorganism.In this study, the rate of isolation from the two transport media (BHI and Brucell broth) was nearly the same.
In this study, H. pylori selective media (commercial culture) was the most effective culture medium despite the low positivity rate. This was because of the rich medium and antibiotic supplement used to suppress contaminants. A higher rate as reported previouslywas not obtained most probably due to patient selection (Goodwin, 1997; Dore *et al*., 2000). Also for maximum detection the patients must be off treatment when biopsy is taken (Suzuk *et al*., 2012). In our study the patients' biopsies were taken without any prior instructions to stop medication.

H. pylori was detected by PCR directly from clinical specimens and from culture, since the assessment of gene transcription and their levels of transcription, will reflect the actual events occurring in the human host (in vivo) rather than in culture, and genotyping from bacterial cultures depends on a homogenous population of either slow bacterial growth, that may be missed in culture, or fast growing bacteria, while genotyping directly from clinical specimens depends on a heterogeneous population of slow and fast growers. In addition, culture proved tobe cumbersome and time consuming (Dunn *et al*., 199; Kavermann, *et al*., 2003), and its use prior to PCR of the virulence genes will delay molecular studies. Moreover, PCR is sensitiveand can detect as few as 10 CFU of bacteria in clinical specimens (Mégraud *et al*., 2014; Pacheco *et al*., 2008; Megraud *et al*., 2007). In the current study, since 91% of H. pylori positive specimens had at least one or more virulence genes cagA and/or iceA suggests that the majority of H. pylori recovered had virulence potential. The cagA gene was detected in 81.8 % (27/33) of recovered H. pylori specimens which is relatively similar to other countries where its prevalence is around 61.8% in Saudi strains (Momenah *et al.,* 2008; BinSaeed 2012; Kadi *et al.,* 2014). 70 % in European strains, 85 % in Estonian and Russian strains, and 90 % in east Asian strains (Ashour *et al.,* 2003). These results could reflect the genetic variations particular to this area of the world. Anotherexplanation for these results is that some cases could have been missed because it was shown recently, that the cagA gene could be subject to partial deletions without the loss ofthe whole cag pathogenicity island (PAI) (Godoy *et al.,* 2003).

Overall, iceA1 was detected in one case studied (0.3 %) of all33 cases and iceA2 was found in 7 cases (21.2 %). Inthe present study, the iceA2 amplification yielded boththe 229 bp and 334 bp fragments, this differencein the fragment size is due to the presence of a 105 bpin - frame amplicon present in the 334 bp fragment thatis absent in the 229 bp fragment (Rizzato *et al*., 2012). The iceA gene detected in (n=12/13) 92.3 % of H. pylori positive cases occurring more frequently in peptic ulcer disease (PUD) group than in the gastritis n= 15/20 (75 %) patients. These results are agreed with the previous report by (Dadashzadeh *et al*., 2015).who foundan association between the iceA1 allele and peptic ulcer disease also Momenah and Tayeb,found that 100 % of ulcer cases were infected with iceA1 with statistically significant correlation (p=0.0001), while iceA1 allele was found in 94.6% of gastritis cases (Momenah *et al.,*2007). Other studies from Asia were suggesting that vacA, cagA and iceA genotypes were not associated with peptic ulcer disease (Arévalo-Galvis *et al.,* 2015). These findings may reflect important geographic differences between H. pylori strains and patients. As reported by (Al Qabandi *et al*., 2006). That H. pylori genotypes are not uniformly distributed over the world (Al Qabandi *et al.,* 2006). CagA-positive H. pylori detected in our specimens were almost equally distributed among PUD and gastritis patients (85 and 76.9 %, respectively). Similarly, in other western countries, cagA had a high prevalence almost equally distributed among PUD and gastritis patients (95 and 80 %, respectively) (Correa *et al.,* 2011). Similar patterns observed in eastern countries (Omar *et al*., 2010). Our results are disconcordant with studies in other countries where the cagA gene is moreprevalent and it occurs as frequently in PUD as in gastritis patients. For instance, in Saudi Arabia, cagA gene showed high percentage (70 %) in peptic ulcer cases compared to gastritis cases (Marie 2012). In Brazil, the cagA gene was detected in 90.5% of patients with duodenal ulcer and 60% in gastritis patients (Brito *et al.,* 2003). Detection of cag PAI in asymptomatic carriers was also reported in some eastern countries such as Bangladesh and India but not in western countries (Rahman *et al.,* 2003). Our data have shown that the cagA genotype was high with gastritis cases (85%) versus 92% for the iceA genotype which was high with PUD (n= 12). However, Fisher's exact test showed that there were no association revealed between cagA (or iceA) genotypes and the two clinical outcomes. However, these percentages were highly significant when examined within each clinical outcome ($P < 0.001$). Although H. pylori is susceptible to many antibiotics in vitro, only a few antibiotics can be used in vivo to cure the infection (Nishizawa *et al.,* 2011). The frequent indication for anti-H pylori therapy, together with the limited choice of antibiotics, has resulted in the development of antibiotic resistance in H pylori, which substantially impairs the treatment of H. pylori-associated disorders. Successful treatment of an H. pylori infection therefore requires a combination of drugs, consisting of one or more antibiotics in combination with an acid-suppressive drug (proton pump inhibitors [PPIs] or H28-receptor antagonists) or a bismuth component (Nishizawa *et al.,* 2011; Moghaddam *et al.,* 2016). The susceptibility results of the studied H. pylori isolates against five antibiotics commercially used showed that threevirulent groups ofH. Pyloriisolates, were susceptible to amoxicillin, Levofloxacin, clarithromycin antibiotics and resistant to tetracyclineand metronidazole. These H. pyloriisolates were shown to be composed of multiple genotypes as revealed by PCR-based genotyping (Kadi *et al.,* 2014). Resistances to tetracyclineand metronidazole have also been held responsible for therapy failure with these drugs;

however, there are not enough data available yet to make an accurate estimate of the effect of these resistance on treatment success (Dore *et al.,* 2000; Ecclissato *et al.,* 2002; Mégraud *et al.,* 2011). In a study for antibiotic resistance among H. pylori isolates from Western region of Saudi Arabia by Momenah and Asghar, found that 13 isolates were resistant to tetracycline in comparison to a single isolate collected from other region of Saudi Arabia during the period 1990-1996 indicating a gradual tetracycline emerging resistance among H. pylori clinical isolates (Momenah and Asghar, 2008). Our antibiotic susceptibility results of H. pylori isolates showed a di-drug resistant against tetracyclineand metronidazole which indicating a warning sign of emerging resistance to these antibiotic as reported by (Momenah and Asghar 2008). Also in study done in Saudi Arabia about prevalence of antibiotic resistance of H. pylori from gastritis patients, revealed that 72% of H. pylori strains were resistant to metronidazole and 8% for clarithromycin (AL-Omar *et al*., 2013).Similar to other developing countries, a high level of metronidazole resistance 40 isolates (72.7%) were detected, suggest that this medication may not be beneficial for first- line therapy in Iran (Moghaddam *et al.,* 2016)

When the five antibiotics combined together were used in vitro, a synergistic antibacterial effect was shown indicating that these combined antibiotics may affect the H. pylori by helping each other. However, in vivo metronidazoleinhibits the proton motive force of the bacterium, and destabilizes its site of colonization in the stomach. Clarithromycinbinds 23S rRNA ribosomal subunit, resulting in inhibition of protein synthesis. Amoxicillin bind to beta-lactam antibiotic to penicillin-binding proteins (PBP) inhibits cell division. Tetracyclin bind to ribosome prevents association with aminoacyl tRNA and subsequent protein synthesis. In H pylori, the antibiotic resistance mechanisms are mainly based on point mutations located on the bacterial chromosome,thus the antibiotic resistance easily develops de novo, although horizontal gene transfer via natural transformation among susceptible and resistant strains cannot be excluded (Smeets *et al*., 2003; Kekill *et al.,* 2016).

In the current study, three H. pylori virulent isolates had at least one or more virulence genes cagA and/or iceA were selected for studying the antibacterial activity of OLE and Oleuropein. Olea europaea leaf extract had antimicrobial activities and might had valuable bioactive source, and would seem to be applicable in both the health and medical foodas reported byOk-Hwan Lee and Boo-Yong Lee. Our results of minimum inhibitory concentration (MIC) indicated that olive leaf extract (OLE) and oleuropein had antibacterial effect against the studied isolates of H. pylori. The MIC for OLE was 50 µg/mL (alcohol extract), 70µg/mL (water extract), and 60 µg/mL (boiled extract). Interestingly, heat treatment of OLE water extract increased its inhibitory activity against H. pylori. The MIC for oleuropein was rang from 30 µg/ml to 40 µg /ml and 20 µg /ml respectively. Our data was in agreement with the data obtained by (Sudjanaet *et al*., 2009), who reported that the a commercial extract derived from the leaves of Olea europaea (olive) had an antibacterial activity against Helicobacter pylori using agar dilution and broth microdilution techniques with as low as 0.31–0.78% (v/v) (Lee and Lee, 2010).Oleuropein has been shown to have strong antimicrobial activity against both Gram- negative and Gram-positive bacteria (Omar, 2010; Marie, 2012). Phenolic structures similar to oleuropein seem to produce its antibacterial effect by damaging the bacterial membrane and/or disrupting cell peptidoglycans. Different authors have used biophysical assays to study the interaction between oleuropein and membrane lipids (Caturla *et al*., 2005). However, the exact mechanism of the antimicrobial activity of oleuropein is still not completely established, although some authors have proposed that it is due to the presence of the ortho-diphenolic system (catechol) (Ok-Hwan *et al*., 2010). In 2009, Vaki, proposed that the glycoside group modifies the ability to penetrate the cell membrane and get to the target site. Effective interference with the production procedures of certain amino acids necessary for the growth of specific microorganisms has also been suggested. Another mechanism proposed is the direct stimulation of phagocytosis as a response ofthe immune system to microbes of all types (Yuan *et al*., 2015).

Conclusion

The use of crude leaf of olives water extracts (Olea europaea) or active- constitute Oleuropein can be used as natural drugs for H. pylori eradication and promote the human health. Oleuropein water extracts completely inhibitedthe growth of H. pyloriwith MBC value at 50 -60 µg/ml in all tested virulent strains. We, can conclude that Oleuropein exhibited potent anti-H. pylori activity.

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