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In vitro susceptibility of *Helicobacter pylori* to urease inhibitory effects of polyphenolic extracts of local herbs from Algeria

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Abstract

Background: Gastritis and peptic ulcers are considered as major health problems worldwide with more than 80% of chronic active gastritis are due to the pathogenic bacterium *H.pylori*. Due to the emergence of antibiotic resistance among clinical strains of *H. pylori*, alternative approaches are increasingly needed.

Methods: Methanolic extracts of Algerian originated *Mentha rotundifolia, Eucalyptus globulus, Malva sylvestris, Inula viscosa, Achille aodorata and Utrica dioica* and their contents of phenolics and flavonoids were evaluated for the in vitro antimicrobial activity against *H. pylori* , in addition to its associated urease inhibition. The minimum inhibitory concentrations (MICs) of these extracts was performed using control strain of *H. pylori* and standard agar diffusion method.

Results: The highest phenolic and flavonoid contents were found in *M.rotundifolia* and *E.globulus,* while *M.sylvestris* showed the least phenolic contents. In addition, polyphenolic fractions exhibited anti-*H. pylori* activity of all of the herbal extracts with highest activity for *E.globulus* (MIC 0.094mg/ml), *I.viscosa*, (0.375mg/ml) and*U.dioica* (0.75 mg/ml), low antimicrobial activity was revealed for *A. odorata,M. rotundifolia and Malva sylvestris,* respectively (MIC >1mg/ml). For the urease activity, all extracts showed inhibitory effect at concentration of 250mg/ml. However, the range of the urease inhibitory concentrations varied significantly among the extracts with highest activity and widest range found for *E.globulus* (70-90% at concentrations 8-125 mg/ml).

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Conclusion: The presence of potential antimicrobial activities in the polyphenolic extracts of Algerian medicinal plants against *H. pylori* and their association with urease inhibition would be a potential novel agents for the control of *H. pylori* infections.

Key words: *H. pylori*, Algerian herbs, polyphenols, urease inhibition.

Introduction

Since its discovery, *Helicobacter pylori* has been increasingly emerging as a major health problem worldwide (1-3). In particular, it has been regarded as a major cause of chronic gastritis and peptic ulcer, and a major risk factor in the pathogenesis of gastric cancer and gastric mucosa-associated lymphoid tissue (MALToma) (4-7). The eradication of *H. pylori* and/or attenuating its associated virulence factors can therefore, contribute to improve clinical conditions of the patients infected with this bacterium including accelerating peptic ulcer healing [8], minimizing the recurrence of gastric cancer after resection and reduction of low-grade gastric MALToma (99, 10). Currently, effective eradication of *H. pylori* is routinely performed by treatment consisting of at least two antibiotics and a proton pump inhibitor (triple and more recently quadruple therapy). However, due to the emergence of resistant clinical strains particularly against metronidazole and clarithromycin, and increased side effects of triple therapy, new antimicrobials are needed (11-15). Therefore, a search of potential agents with high efficacy and safety against *H. pylori* infection is necessary.

Herbal preparations have long been used as a remarkable approach in encountering infections and diseases and they have been used in primary health care in several countries (16, 17). Several studies documented that various medicinal plant extracts possess a wide range of antibacterial and antioxidant activities (18-20). Phenolic extracts are considered the major antibacterial agents in most of the medicinal herbs. These fractions have been reported to contain phenolic acids such as caffeic acid and ferulic acids with diverse potent biological activities including antimicrobial, antiseptic, preservative and anti-oxidant activities (21, 22).

This study investigated the antibacterial and anti-urease activity of aqueous methanol extracts containing polyphenolic compounds of local herbs in Algeria, against survival of *H. pylori in vitro*.

Materials and methods

Plant material and extraction of polyphenolic fractions

Leaves of *Inula viscose, Mentha rotundifolia, Urtica dioica, Eucalyptus globules, Achillea odorata and Malva sylvestris* were collected in May 2011 from Jijel, Algeria. The plant material was stored at room temperature in a dry place until used. Air-dried leaves were ground with the help of an electric grinder in order to get a fine powder (23). The sifting was achieved with a sifter of which the diameter of stitches is 50µm. The plant powder was then kept in small bottles of tinted glass to ovoid the oxidization of their compounds. The extraction of polyphenols

2013
Vol. 3 No. 4:4
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was carried out at ambient temperature for 48h by maceration in methanol-water 80/20 (v/v) at a solid-liquid ratio 1/10 (w/v) with continuous stirring. The solutions were then filtered with filter paper (No. 1). The aqueous phase obtained underwent defatting with hexane as described by Yu *et al.* (24) . The covered methanol phase underwent a concentration to dryness using a rotary evaporator with a vacuum controller, at a temperature of 40°C.

Determination of total phenolic contents

The total phenols content was determined by Folin–Ciocalteu's method (25). In brief, 0.2 ml of sample was dissolved in 1.5 ml (1/10 dilution) of the Folin–Ciocalteu reagent. The solutions were mixed and incubated at room temperature for 5 min. 1.5 ml of 7.5% sodium carbonate (Na_2CO_3) solution were then added. After 90 min, the absorbance was measured at 725 nm. Gallic acid was used to make the calibration curve. Results were expressed as Gallic acid equivalents (GAE).

Total flavonoids content

The flavonoids content in extracts was determined spectrophotometrically using a method based on the formation of a complex flavonoid–aluminium, having the maximum absorbance at 430 nm. Quercetin was used to make the calibration curve. 1.5 ml of diluted sample was mixed with 1.5 ml of 2% aluminium chloride solution (26) . After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 430 nm with a Shimadzu UV mini 1240 spectrophotometer and the flavonoids content was expressed in mg of quercetin equivalent (QE) per gram of crude extract. The test is carried out in five replicates.

Bacterial strains and growth conditions

A standard strain *H. pylori* (NCTC 11916), was used for this investigation. The strain was isolated from human gastric biopsies and was shown to be resistant to metronidazole but susceptible to a group of different antibiotics used in the triple and quadruple therapy regiment for the treatment of this bacterium (**Table 1**). The strain was primarily cultured on Columbia blood agar (Oxoid, UK) supplemented with 7% (v/v) horse blood and Dent selective supplement (Oxoid, UK). Subcultures of the bacteria were performed using the same plates but without the Dent supplement. All of the plates were incubated at 37°C under microaerophilic conditions using CampyGen atmosphere generating system (Oxoid, UK) in anaerobic Jars for 5-7 days.

Table 1. Total phenolic and flavonoid contents of methanolic extracts of plants used in this study.

Plant	Total phenols content (mg GAE/g extract)	Total flavonoids content (mg QE/g extract)	% of flavonoids in the phenol fraction
Inula viscosa	314.55±0.95	48.34±0.87	15%
Eucalyptus globulus	643.94±0.84	153.54±1.12	21%
Achillea odorata	448.88±1.004	97.77±0.91	20%
Malva sylvestris	135.55±1.18	52. 4±0.77	39%
Urtica dioica	403.99±1.66	41.25±1.51	10%
Mentha rotundifolia	745.66±1,66	102.17±0.57	14%

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2013
Vol. 3 No. 4:4
doi: 10.3823/741

Growth of *H. pylori* was confirmed according to colony morphology, Gram staining, microaerophilic growth (at 37°C), oxidase, catalase and urease and subsequently by standard PCR of 16S rDNA test (27). *H. pylori* cultures were stored at -70°C in Trypticase soy broth (Oxoid, UK) containing 10% v/v fetal calf serum (PAA, Austria) and 15% glycerol.

Antimicrobial susceptibility testing and MIC determination

Bacterial suspensions were prepared to the 2 McFarland's standard and subsequently uniformly spread on a solid growth medium in a Petri dish. Sterile paper disks (6 mm in diameter; Oxoid, UK) were impregnated with 25 µL of the each plant extract and were placed on the surface of each agar plate. Plates were incubated for 5-7 days under appropriate cultivation conditions. Antibacterial activity was determined by the production of an inhibition zone around the impregnated disc with the extracts(28). Disks impregnated with DMSO served as negative controls and disks with standard antibiotics (ciprofloxacin, and mitronidazole, Oxoid, UK) served as positive controls. The minimal inhibitory concentrations (MICs) of each extract were determined by the two fold agar dilution method according to Klancnik *et al* (29). In brief, each fraction was serially diluted in DMSO and incorporated to molten Columbia blood agar plates supplemented with 7% (v/v) horse blood. Spot of *H. pylori* (1x 10^5CFU) was applied on the surface of each plate and the growth of visible colonies was determined after 7 days of incubation at 37°C under microaerophilic conditions.

MIC was recorded as the lowest concentration that inhibited visible growth of organisms and the Plates with the Standard antibiotics served as positive controls and plates with DMSO served as negative controls. Triplicates of each extract were performed and the average of the results was taken.

Urease inhibition assay

Assay of urease activity was determined by measuring the release of ammonia in both agar and broth containing urea and phenol red indicator(Oxoid CM0053)as described by Nagata et al., 1992 (30) with some modifications; in agar base method, 10 ul of each phytochemical extract was mixed with equal volume of 10^8CFU/ml *H. pylori* suspension and incubated at 37°C for 30 min. Subsequently, a loopful inoculum of the mixture was then streaked on the standard urea plates and incubated at 37°C up to 24 hrs to monitor the colour change of the media. Each experiment was repeated three times. Controls consisted of suspended bacteria in PBS, Bacteria in DMSO and DMSO alone. In the broth method, 10 ul of 10^8 CFU/ml *H. pylori* suspension was incubated with equal amount of serially diluted phytochemical extracts in 96 well microplates for 30 min at 37°C. Subsequently, 200 ul of detection reagent composed of 50mM phosphate buffer, pH 6.8 containing 500mM Urea and 0.02% phenol red was added to each well. The colour development was monitored by measuring the OD at 555 nm in 5 min intervals. Controls included bacteria with the reagent, and reagent with phytochemical mixtures. A high absorption value indicated high urease activity. The percentage of inhibition was calculated by the equation % inhibition = [(activity without phytochemical extract - activity with phytochemical extract)/ (activity without phytochemical extract)] x 100. The activity was compared to a reference urease inhibitor acetohdyroxamic acid.

Results

Total Phenols and flavonoids contents

The contents of total phenolic and flavonoid compounds in the aqueous methanloic extracts of each plant material, were reported as gallic acid

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2013
Vol. 3 No. 4:4
doi: 10.3823/741

and quercetine equivalents, respectively by reference standard curve (y = 0.0024x + 0.0886, r2= 0.9142). The phenolic contents of the extracts were in the range of 135.55±1.18-745.66±1.66 with highest contents found in *M.rotundifolia* and *E.globulus* while *M.sylvestris* showed the least amount of total phenolic contents (**Table 1**). The flavonoid contents were in the range of 41.25±1.51-153.54±1.12 with high contents found in *E. globulus.*

Antimicrobial activity testing and MIC

Screening and evaluation of antimicrobial activity of six methanolic plant extracts against the metronidazole resistant *H. pylori* strain (**Table 2**) was carried out using disc diffusion method. All extracts showed antimicrobial activity with concentrations of 500 mg/ml with zones of inhibitions ranging from 14-45 mm (**Table 3**). The highest antimicrobial activity was reported for *E.globulus* with MIC value of 0.09375 mg/ml followed by *I.viscosa*and *U.dioica*(0.375 mg/ml). The least activity has been reported for both *A.odorata* and *M.sylvestris* with MIC of 3.0 mg/ml.

Inhibition of urease activity

Determination of the urease inhibitory potential of the herbal extracts has been performed by firstly mixing equal amounts of 500 mg/ml and 1x 10^9 CFU/

Table 2. Antimicrobial susceptibility testing of standard antimicrobial agents used in the treatment of *H. pylori* infections.

Antibiotic	Stock Concentration (ug/ml)	Antimicrobial effect		
		Inhibition zone (mm)	MIC (ug/ml)	susceptibility
Amoxicillin	100	50	0.015	S
Clarithromycin	100	45	0.12	S
Ciprofloxacin	100	20	0.25	S
Levofloxacin	100	36	0.12	S
Metronidazole	100	No inhibition	128	R

S= sensitive, R= resistant

Table 3. Antimicrobial activity of methanolic extracts of studied plants against *H. pylori.*

Plant	Stock Concentration (mg/ml)	Antimicrobial effect	
		Inhibition zone (mm)	MIC (mg/ml)
Achillea odorata	500	23	3.0
Urtica dioica	500	30	0.375
Mentha rotundifolia	500	20	1.5
Malva sylvestris	500	14	3.0
Inula viscosa	500	30	0.375
Eucalyptus globulus	500	45	0.09375

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2013
Vol. 3 No. 4:4
doi: 10.3823/741

Table 4. Results of urease inhibition activities of the mathanolic extracts of the studied plants.

Herbal extract	Concentration range of urease inhibition (mg/ml)	% of urease inhibition
Achillea odorata	125-250	0-10%
Urtica dioica	64-250	25-45%
Mentha rotundifolia	64-250	45-50%
Malva sylvestris	125-250	50-75%
Inula viscosa	125-250	60-70%
Eucalyptus globulus	16-125	70-90%

ml of *H. pylori* and testing the mixture in the standard urease based agar media and subsequently,the percentage of urease inhibition of each extract (concentration range of 2.5-250 mg/ml) has been determined in the broth method mentioned above. All of the extracts showed preliminary urease inhibitory effect of concentration 250 mg/ml. The highest urease inhibitory activity has been reported for *E.globulus*(90%) in comparison to *M.sylvestris* and *I. viscosa* (60-70%). The least inhibitory effect has been reported for *A.odorata* (10%).The range of the concentrations of significant urease inhibitory effect (> 50%) of each extract showed that the widest range was for *E.globulus*(16-250 mg/ml) and *M.rotundifolia*(64-250 mg/ml) (**Table 4**). In comparison, *I. viscosa*, *M.sylvestris* and *A.odorata* showed very narrow range of concentrations required for urease inhibition (125-250 mg/ml).

Discussion

Natural products have been increasingly considered as an important source of innovative therapeutic agents which could be adjunctive or even alternatives to the currently used antimicrobial agents (16,17,31). It has been commonly used in traditional medicine by Arabic communities since a wide variety of herbals exhibit wide spectrum antimicrobial and anti-fungal properties. In Algeria, there are a plenty of endemic herbs that have been widely used in traditional medicine for a variety of diseases including gastrointestinal disorders. However, few studies have evaluated the actual potential antimicrobial activity of these plants against the gastric pathogenic bacterium *H. pylori* and its associated virulence factors.

The present study, investigated the antimicrobial activity of polyphenolic extracts of six local Algerian herbs including *Inula, Malva, Achillea, Eucalyptus, Utrica and Mentha spp*. against *H. pylori* in addition to evaluate the potential inhibiting effect on the virulence factor of urease enzyme which is secreted by this organism. Our preliminary screening showed that the polyphenolic fractions of the all of tested extracts possess considerable antimicrobial activity against *H. pylori* . In addition, significant urease inhibition has been observed to some of the tested herbs. The results also showed significant variations in the total phenolic and flavonoid contents among the extracts of the tested plants. In particular, *E.globulus* showed both potent antimicrobial (MIC < 100ug/ml) and anti-urease activities (more than 70% at concentrations > 60 mg/ml). Both *U.dioica and I.viscosa* showed strong antimicrobial activities in comparison to *M.sylvestris* and *A.odorata* (MIC 3 mg/ml).Our results are in agreement with most

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2013
Vol. 3 No. 4:4
doi: 10.3823/741

of the studies on the effect of *Eucalyptus* and *Utricas spp* against *H. pylori*. In a study conducted on *E.grandis,* the methanol extract of the stem bark showed strong antimicrobial and antiurease activities against 11 eamined *H. pylori* strains (MIC range 0.39 and 1.56 µg/ml) (32). In contrast to our results, a stronger effect of *Achillea millefolium* against *H. pylori* strains with MIC of 50 ug/ml has been reported(33). Similar findings were reported regarding the effect of *Mentha longifolia* on clinical strains of *H. pylori* in Iran with MIC range of 31.25 to 500µg/ml (19). The variation of MIC values between different studies is mostly related to the plant species, the extract nature and the variation of the *H. pylori* strains and their susceptibility or resistance to antimicrobial agents(34).

On the basis of this study, the presence of anti-*H.pylori* specific phenolic compound/compounds among the examined plants is not the same. The total polyphenolic and the flavonoid content have not been shown to be the only factor that is responsible for the antimicrobial activity against *H. pylori*. Several studies showed that the variation of the content and chemical modifications of numerous natural phenolic compounds like rosmarinic acid,ferruginol, and kaempferol do significantly affect the results of the antimicrobial activity of the fractions(34-36). In addition, the antimicrobial activity of the extracts could have synergistic effects with various phenolic compounds, many of which are still unidentified. Moreover, other compounds in plant extracts, for example, like anti-oxidants including ascorbic acid and ferulic acid, alone or in combination with other phenols, might be responsible for the antimicrobial effects as reported by various studies (18, 21, 35, 37). This can also be postulated for the urease inhibition activity of the plants, since the activity was independent and not linked to the antimicrobial activity of the extracts or its total polyphenolic and flavonoid contents.

In conclusion, the presented work demonstrated that plant extracts used in this study had considerable in vitro activities against *H. pylori*. In addition, urease inhibition has been demonstrated for some of the plants. Our results suggest that these herbs should be further examined as adjunct to or as an alternative treatment of *H. pylori* infections.

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2013
Vol. 3 No. 4:4
doi: 10.3823/741

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