Effects of *Punica granatum* Pericarps and *Quercus infectoria* Nutgalls on Cell Surface Hydrophobicity and Cell Survival of *Helicobacter pylori*

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The effect of ethanolic extracts of *Punica granatum* (*P. granatum*) and *Quercus infectoria* (*Q. infectoria*) on cell surface hydrophobicity of 10 clinically isolated *Helicobacter pylori* strains were investigated using salt aggregation test. Both *P. granatum* and *Q. infectoria* significantly increased the hydrophobicity of all isolates, irrespective of their antibiotic resistance patterns. These two plant species were demonstrated to produce both bacteriostatic and bactericidal activities. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were from 0.78 to 6.25 and 3.12 to 6.25 mg/ml for *P. granatum*, and from 3.12 to 6.25 and 3.12 to 12.5 mg/ml for *Q. infectoria*, respectively. Ethyl acetate and *n*-butanol fractions of both plants had values at least 10-fold lower than the MIC and MBC values of the ethanolic extracts. The results indicate no relationship of the increased cell-surface hydrophobicity with the MIC or MBC values.

Key words —— *Helicobacter pylori*, *Punica granatum*, *Quercus infectoria*, cell surface hydrophobicity, antibacterial activity, medicinal plant

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a major etiologic agent in chronic gastritis,[1)] peptic ulcer,[2)] gastric cancer,[3)] as well as gastric mucosa-associated lymphoid tissue lymphoma.[4)] Even though triple therapies consisting of two antibiotics and a proton pump inhibitor demonstrate high eradication rates,[5)] antibiotic resistance rates are now increasing.[6)] Furthermore, undesirable side effects such as nausea, vomiting, epigastric pain, abdominal discomfort, and diarrhea are often unavoidable.[7)] Therefore, finding a non-antibiotic agent that is both effective and free from side effects is of the utmost importance.

A number of medicinal plants have been reported to have antibacterial activity against *H. pylori*.[8–12)] However, there have been few detailed studies on their antibacterial mechanisms. Hydrophobic interactions appear to be commonly involved in prokaryotic and eukaryotic cell interactions.[13)] They play an important role in the physiochemical and biological behavior of numerous classes of organic compounds. The adhesion of pathogenic bacteria on host cells is required in many Gram-negative intestinal pathogenic bacteria-induced infections and can be influenced by the surface hydrophobicity of the microbial cell. The estimation of hydrophobic parameters has been carried out by many means, for example, high-performance liquid chromatography,[14)] liposome capillary electrophoresis,[15)] micellar electrokinetic chromatography,[16,17)] microemulsion electrokinetic chromatography,[18)] and salt aggregation test (SAT).[19–21)]

Recently, a series of studies has demonstrated that aqueous extracts of certain plants can affect the cell surface hydrophobicity of Gram-negative bacteria including *Escherichia coli* (*E. coli*),[20)] *Acinetobacter baumannii*,[20)] and *H. pylori*.[21)] Earlier work from our laboratories[22)] showed that two medicinal plants belonging to different families,

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Table 1. MICs of Antibiotics against Different Strains of *H. pylori*

Strain	MIC values (μg/ml)			
	Amoxicillin	Clarithomycin	Metronidazole	Tetracycline
UNSW 03-03-10-001		< 0.016 (S)	32 (R)	
UNSW 03-03-12-001		0.032 (S)	0.032 (S)	
UNSW 03-03-12-002		< 0.016 (S)	0.13 (S)	
UNSW 03-03-13-001		0.13 (S)	64 (R)	
UNSW 03-03-18-002		< 0.016 (S)	0.13 (S)	
UNSW 03-03-18-003		< 0.016 (S)	> 256 (R)	
UNSW 03-03-18-004		< 0.016 (S)	0.13 (S)	
UNSW 03-03-21-001		1 (R)	128 (R)	
UNSW 03-03-21-002		1 (R)	64 (R)	
UNSW 03-03-25-001		0.25 (S)	> 256 (R)	
ATCC 43504	0.008–0.06	0.016–0.25		0.125–1
ATCC 43579	0.008–0.06	0.016–0.125		0.06–1

R, resistant; S, sensitive.

Punica granatum (*P. granatum*) Linn. and *Quercus infectoria* (*Q. infectoria*) Oliv., have remarkable antibacterial properties against this pathogen. Therefore it is interesting to investigate mechanisms involved in the modulation of bacterial cell surface hydrophobicity. In the present communication, ethanolic extracts of *P. granatum* pericarps and *Q. infectoria* nutgalls were investigated to determine their effects on the cell surface hydrophobicity of *H. pylori*. The SAT was performed as it is a feasible and simple method. Possible anti-*H. pylori* mechanisms of the two plant species were established.

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MATERIALS AND METHODS

Tested Bacterial Strains —— Ten clinical isolates of *H. pylori* obtained from peptic ulcers were cultured on Campylobacter selective agar (CSA).[23,24] *H. pylori* ATCC 43504 and ATCC 43579 were used as reference strains. Determination of the susceptibility of *H. pylori* to antimicrobials (Table 1) was performed using the disc diffusion method or E-strips (AB Biodiscs, Solna, Sweden) impregnated with exponential gradients of antibiotic as described.[25] Metronidazole, tetracyline, amoxicillin, and clarithromycin are the antibiotics most commonly used in clinical practice and hence were used for antibiotic sensitivity testing. *H. pylori* used for antibiotic sensitivity testing was in log phase (48 hr). An inoculum of 10 μl of the culture was grown on CSA plates.

Preparation of Crude Extracts —— The fruit rind of *P. granatum* and *Q. infectoria* nutgalls were cut into small pieces and dried at 60°C overnight. They were crushed in a mechanical mortar and water extracted or macerated in 95% ethanol for 7 days. The solvent was then distilled under reduced pressure in a rotary evaporator until the samples became completely dry. Aqueous extracts were dissolved in water and ethanolic extracts were dissolved in dimethyl sulfoxide (DMSO, Merck, Germany) before use in bacteriological study.

Fractionation of Active Compounds —— A modified method using solvent-solvent extraction[26] was performed. The total ethanolic extracts were concentrated in a rotational evaporator under reduced pressure and the residues were then successively partitioned between water and *n*-hexane, followed by chloroform, ethyl acetate, and *n*-butanol. The solutions were completely evaporated to give the respective fractions.

Salt Aggregation Test —— The hydrophobicity of different strains of *H. pylori* was determined using a modification of the standard procedure for the SAT.[21] Culture of *H. pylori* was suspended in sodium phosphate buffer 0.01 M according to the 5 McFarland turbidity standard to a final concentration of 1.5×10^9 cells/ml. Salt aggregation studies were performed using ammonium sulfate solutions (0.1, 0.5, 1.0, 1.5, 3.0 M) in buffer. An aliquot (20 μl) of ammonium sulfate solution and the same amount of buffer for the control were pipetted into u-shaped microtiter plate wells. To each well, 0.02 ml of stan-

dardized microbial suspension was added to give final concentrations for ammonium sulfate of 0.05, 0.25, 0.5, 0.75, and 1.5 M. The microtiter plates were gently rotated for 5 min and left for 30 min. The occurrence of aggregation was examined with light microscopy using a dark background. The strains were tested for autoaggregation using sodium phosphate buffer 0.01 M instead of ammonium sulfate. The SAT is defined as positive if bacterial aggregation is clearly visible and negative if no aggregation is observed. The SAT titer is defined as the lowest concentration at which microbes still yield clearly visible aggregaion. Strains autoaggregating in potassium phosphate buffer and/or expressing SAT titers of 0.05 and 0.25 were considered highly aggregative/hydrophobic, and strains with titers of 0.5 to 1.5 were considered low aggregative. Strain were considered nonaggregative if they did not express a positive SAT even at a concentration of ammonium sulfate of 1.5 M. The effects of *P. granatum* and *Q. infectoria* on aggregation activity were investigated by adding 20 μl of the plant extracts (250 mg/ml) to 180 μl of bacterial suspension, and left for 15 min. Thereafter, 40 μl of this mixture was added to equal volumes of ammonium sulfate (0.05 to 3.0 M), and they were then incubated at room temperature for 30 min before bacterial aggregation was estimated.

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration ——— A modified agar microdilution method in Petri dishes with a Millipore filter membrane[27] was used to determine the minimum inhibitory concentration and minimum bactericidal concentration. One microliter of each bacterial isolate containing approximately 10^4 colony forming unit (CFU) was applied onto Isosensitest agar plates supplemented with ethanolic extracts of *P. granatum* and *Q. infectoria* and their semipurified fractions. The plates were incubated at 37°C for 48 to 72 hr. Observations were performed in triplicate and results are expressed as the lowest concentration of plant extracts that produced a complete suppression of colony formation, minimal inhibitory concentration (MIC). Minimal bactericidal concentrations (MBCs) were determined with the extracts that showed significant MIC values against each bacterial strain by placing the Millipore filter membrane onto fresh Isosensitest agar. DMSO was used as control in the experiments.

RESULTS AND DISCUSSION

The 10 clinical isolates were checked for their antibiotic resistance patterns (Table 1). Two isolates were resistant to clarithromycin and six of them were resistant to metronidazole. The cell surface hydrophobicity of the clinical isolates of *H. pylori* was determined using the SAT (Tables 2 and 3). Aggregation of eight isolates and the reference strain ATCC 43579 was estimated at ammonium sulfate concentrations of 1.5 M or greater. Only two isolates and the other reference strain, ATCC 43504, demonstrated high hydrophobic or aggregative properties (aggregated at or less than ammonium sulfate 0.05 M). The results showed an increase in the hydrophobicity of *H. pylori* cells after treatment with ethanolic extracts of both plant species.

The anti-*H. pylori* activity of the crude and semipurified fractions of the two plant species demonstrated significant antibacterial effects (Table 4). The ethanolic extracts of both *P. granatum* and *Q. infectoria* were active against all clinical isolates of *H. pylori*. The MIC and MBC values ranged from 0.78 to 6.25 and 3.12 to 6.25 mg/ml for *P. granatum*, and from 3.12 to 6.25 and 3.12 to 12.5 mg/ml for *Q. infectoria*, respectively. The ethyl acetate and *n*-butanol fractions of both plant species gave at least 10-fold lower MIC and MBC values compared with the ethanolic extracts. Aqueous, *n*-hexane, and chloroform extracts produced no antibacterial activity. DMSO controls did not have any effect on bacterial growth.

Based on our results, the two medicinal plant species have both bacteriostatic and bactericidal activity sufficient to kill *H. pylori*. This is in contrast with other studies on different medicinal plant species that reported antimicrobial activities too low to inhibit growth or kill microorganisms.[28] Modulation of *H. pylori* cell surface hydrophobicity by *P. granatum* and *Q. infectoria* may synergistically facilitate the elimination of bacterial cells from the human body. High cell surface hydrophobicity is suggested to be an important mechanism in the pathogenesis of many gastrointestinal infections.[20,21,29] Adhesive strains often possess high cell surface hydrophobicity.[30] It was demonstrated that high cell surface hydrophobicity expressed by some strains of *H. pylori*.[31] It has been demonstrated that 60% of *E. coli* strains isolated from patients with pyelonephritis were aggregative, while only 16.7% from healthy individuals showed aggregative properties.[20] In contrast, 80% of clinical isolates of *H. pylori* in our study

Table 2. Salt Aggregation Test of *H. pylori* Strains

H. pylori strain	Concentration of ammonium sulfate (M)					Pp buffer[a)]	Interpretation
	0.05	0.25	0.5	0.75	1.5		
UNSW 03-03-10-001	+	+	+	+	+	+	Highly aggregative
UNSW 03-03-12-001	+	+	+	+	+	+	Highly aggregative
UNSW 03-03-12-002	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-13-001	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-18-002	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-18-003	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-18-004	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-21-001	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-21-002	−	−	−	−	−	−	Nonaggregative
UNSW 03-03-25-001	−	−	−	−	−	−	Nonaggregative
ATCC 43504	+	+	+	+	+	−	Highly aggregative
ATCC 43579	−	−	−	−	−	−	Nonaggregative

SAT titer = the lowest concentration of ammonium sulfate solution at which bacterial cells still yield clearly visible aggregation. +, aggregation, SAT positive if bacterial aggregation was clearly visible; −, no aggregation, SAT negative if there was no aggregation or very weak. Highly aggregative or hydrophobic = strain autoaggregating in sodium phosphate buffer and/or expressing a positive SAT titer between 0.05 and 0.25 M. Nonaggregative = strain did not express a positive SAT titer even at a concentration 1.5 M. *a*) Pp buffer, potassium phosphate buffer.

Table 3. Effects of Ethanolic Extracts of *P. granatum* and *Q. infectoria* (5 μg) on Cell Surface Hydrophobicity of *H. pylori*

H. pylori	SAT titer (M)					
	P. granatum		*Q. infectoria*		DMSO	
UNSW 03-03-10-001	0.05	H/A	0.05	H/A	0.05	H
UNSW 03-03-12-001	0.05	H/A	0.05	H/A	0.05	H
UNSW 03-03-12-002	0.05	H	0.05	H	>1.5	N
UNSW 03-03-13-001	0.05	H	0.05	H	>1.5	N
UNSW 03-03-18-002	0.05	H	0.05	H	>1.5	N
UNSW 03-03-18-003	0.05	H	0.05	H	>1.5	N
UNSW 03-03-18-004	0.05	H	0.05	H	>1.5	N
UNSW 03-03-21-001	0.05	H	0.05	H	>1.5	N
UNSW 03-03-21-002	0.05	H	0.05	H	>1.5	N
UNSW 03-03-25-001	0.05	H	0.05	H	>1.5	N
ATCC 43504	0.05	H/A	0.05	H/A	0.05	H
ATCC 43579	0.05	H	0.05	H	>1.5	N

SAT titer = the lowest concentration of ammonium sulfate solution at which bacterial cells still yield clearly visible aggregation. H/A, strain autoaggregating in sodium phosphate buffer; H, highly aggregative or hydrophobic (SAT titer between 0.05 and 0.25 M); N, nonaggregative (SAT titer $>$ 1.5 M).

did not show high cell surface hydrophobicity, *i.e.*, were nonaggregative.

Various plant species worldwide are used in traditional medicine as treatments for bacterial infections. The antibacterial activity of *P. granatum* and *Q. infectoria* against all strains of *H. pylori* could allow their use in the treatment of *H. pylori* infection. Ethanolic extracts of *P. granatum* and *Q. infectoria* were strongly effective against *H. pylori*. Resistant isolates to commonly prescribed antibiotics were included in this study to determine whether there is any resistance pattern among these isolates occur with the use of medicinal plants. There was no resistance to the natural substances from the two plant species found in any isolates, irrespective of their antibiotic patterns. Although natural products are not necessarily safer than antibiotic therapy, some patients prefer to use herbal medicine. Our finding tentatively suggests possible benefits from these herbal preparations with antibacterial activity. Semipurified substances from the plants should be further investigated as they might provide new com-

Table 4. MIC and MBC of *P. granatum* and *Q. infectoria* against *H. pylori*

H. pylori	MIC/MBC values (mg/ml)					
	P. granatum			*Q. infectoria*		
	Ethanolic extract	Ethyl acetate fraction	*n*-Butanol fraction	Ethanolic extract	Ethyl acetate fraction	*n*-Butanol fraction
UNSW 03-03-10-001	1.56/6.25	0.16/0.16	0.62/0.62	3.12/6.25	0.31/0.31	0.31/0.31
UNSW 03-03-12-001	3.12/6.25	0.16/0.16	0.62/0.62	6.25/6.25	0.16/0.16	0.16/0.16
UNSW 03-03-12-002	3.12/6.25	0.16/0.16	0.31/0.31	6.25/6.25	0.31/0.31	0.16/0.16
UNSW 03-03-13-001	0.78/3.12	0.16/0.16	0.16/0.16	3.12/6.25	0.16/0.16	0.31/0.31
UNSW 03-03-18-002	3.12/3.12	0.31/0.62	0.16/0.16	6.25/6.25	0.31/0.31	0.16/0.16
UNSW 03-03-18-003	3.12/6.25	0.31/0.31	0.16/0.16	6.25/6.25	0.16/0.16	0.31/0.31
UNSW 03-03-18-004	1.56/3.12	0.16/0.16	0.16/0.16	3.12/6.25	0.31/0.31	0.16/0.16
UNSW 03-03-21-001	6.25/6.25	0.16/0.16	0.16/0.16	6.25/12.5	0.16/0.16	0.31/0.31
UNSW 03-03-21-002	3.12/6.25	0.16/0.16	0.31/0.31	6.25/6.25	0.16/0.16	0.31/0.31
UNSW 03-03-25-001	3.12/3.12	0.16/0.16	0.31/0.31	3.12/6.25	0.31/0.31	0.31/0.31
ATCC 43504	3.12/6.25	0.31/0.62	0.16/0.16	6.25/12.5	0.62/0.62	0.62/0.62
ATCC 43579	1.56/3.12	0.31/0.31	0.31/0.31	3.12/3.12	0.62/0.62	0.62/0.62

pounds that could minimize problems of drug resistance in *H. pylori* infections.

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