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Effect of curcumin on *Helicobacter pylori* biofilm formation

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Helicobacter pylori **is a leading etiologic agent causing peptic ulcer and gastric cancer. The alternative lifestyle as a biofilm facilitates** *H. pylori* **to survive in adverse environments. Here, we investigated effect of curcumin on** *H. pylori* **biofilm formation both qualitatively by pellicle assay and quantitatively by crystal violet staining. Three-dimensional structure of biofilm was imaged by scanning electron microscopy. The effect of curcumin on** *H. pylori* **adherence to HEp-2 cells was also investigated. Sub-inhibitory concentrations of curcumin inhibited the biofilm in dose dependent manner. However,** *H. pylori* **could restore ability to form biofilm during extended time of incubation. Scanning electron microscopy revealed less amorphous extracellular polymeric matrix, slow of morphological conversion to coccoid form with cell damage after curcumin treatment. Curcumin significantly decreased the ability of** *H. pylori* **to adhere to the HEp-2 cells. Our findings demonstrated advantages of curcumin to inhibit biofilm formation by** *H. pylori,* **making it as a potential complimentary medicine for curing of** *H. pylori-***biofilm related infections.**

Key words: *Helicobacter pylori*, biofilm, curcumin, adhesion, scanning electron microcopy, sub-inhibitory concentrations.

INTRODUCTION

Helicobacter pylori is a very common human gastro-intestinal pathogen causing peptic ulcer disease, chronic gastritis and gastric cancer. Over a half of world's population has been infected with this pathogen with estimated prevalence up to 50 and 90% in developed and developing countries, respectively (Dunn et al., 1997). The primary mode of transmission proposed for *H. pylori* is person-to-person by fecal-oral (Thomas et al., 1992), oral-oral (Madinier et al., 1997) or gastro-oral routes

(Parsonnet et al., 1999). Several recent works have demonstrated an alternate life style of *H. pylori* as a biofilm, which is likely to be important to facilitate bacterial survival in hazardous environment (Cole et al., 2004; Stark et al., 1999). The roles of water sources and associated biofilms acting as environmental transmitters of *H. pylori* have been suggested by the detection of *H. pylori* in water distribution systems (Watson et al., 2004), drinking water (Bunn et al., 2002), groundwater (Benson et al., 2004), cast iron water pipe sections (Park et al., 2001), rivers (Enroth and Engstrand, 1995) and waste water systems (Nayak and Rose, 2007). *In vivo* study has also demonstrated the existence of *H. pylori* biofilms on human gastric mucosa, which suggested involving in the pathogenicity of gastric ulcers (Carron et al., 2006). The presence of *H. pylori* in subgingival biofilm and saliva samples of chronic periodontitis subjects has supported the possibility of oral cavity being as one another possible

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reservoir of this bacterium (Souto and Colombo, 2008).

Biofilms are defined as "complex microbial ecosystems adherent to each other and/or to surface or interface" (Donlan and Costerton, 2002). The bacterial communities are embedded in an exopolysaccharide matrix expanded laterally and vertically to form microcolonies (Cole et al., 2004). *H. pylori* biofilms are exhibited in many characteristics *in vitro* including flasks, culture tubes (Cole et al., 2004) and in a glass fermenter (Stark et al., 1999). The common appearances are aggregated of bacteria not attached to surface (floc) (Hall-Stoodley et al., 2004) and form at an air-liquid interface (pellicles) (Friedman and Kolter, 2004). Scanning electron microscopy (SEM) revealed *H. pylori* primarily in coccoid form stacked several layers thick with channels for nutrient flow. The bacteria tend to enter into a viable but nonculturable state (VBNC) (Cellini et al., 2007). The biofilm can shear off, multiply, disperse and colonize as planktonic individuals causing relapse and chronic infection (Thomas and Lehman, 2006), which become a major concern for public health.

Multiple drug regimens with antibiotics have been successively used for *H. pylori* treatment. However, the growth rate of antibiotic resistance in *H. pylori* is problematic with 10 - 20% of treatment failure. The frequency of resistance to amoxicillin, clarithromycin, metronidazole and tetracycline ranges from approximately 2% to more than 45% (Romano et al., 2008). One of the important factors of eradication failure arises from the protective environment of biofilm (Lewis, 2001). The surrounded biofilms rescue bacteria from killing by antibiotics and host defense mechanisms presumably due to the differrentiation and survival of persister cells or slow growth rate (Patel, 2005; Stewart and Costerton, 2001). The natural products are alternative medicine for controlling of antibiotic resistant bacteria. The anti-biofilm activity from plant extracts has been demonstrated against *Candida albicans* (Shuford et al., 2005) and *Staphylococcus aureus* (Quave et al., 2008). However, for *H. pylori*, only antimicrobial activity from various medicinal plants has been extensively studied. *Curcuma longa* is one of those interesting plants that possesses an extensive range of beneficial pharmacological effects, including anti-inflammatory, antimutagen, antibacterial, antiviral and antioxidant effects (Araujo and Leon, 2001). Curcumin (diferuloymethane) is considered as a major active compound, which possesses the inhibitory activity against 19 strains of *H. pylori in vitro* with MICs range from 6.25-50 µg/ml (Mahady et al., 2002). It also showed combination effect with methanol extract of ginger root in inhibiting growth of *H. pylori* (Mahady et al., 2005). Although, curcumin has been prescribed for an inhibitory action against *H. pylori,* the anti-biofilm formation of this organism remains unknown.

The objectives of this study aimed to investigate the effect of curcumin against *H. pylori* biofilm formation. The level of *H. pylori* biofilm formation was determined quali-tatively by pellicle assay and quantified by modified crystal violet staining assay in a presence and absence of curcumin. The cytoarchitecture in biofilm was revealed by standard SEM technique. Due to the first step of biofilm formation involves cell adhesion and colonization, the role of curcumin against *H. pylori* adhesion to HEp-2 cells was also investigated.

MATERIALS AND METHODS

Bacterial strains

H. pylori ATCC43504, ATCC43526, ATCC51932 (purchased from the American Type Culture Collection) and DMST20165 and DMST 20885 (obtained from the Department of Medical Sciences, Thailand) and clinical strains 1203, 1260, 1261, 1264, 1265, 1268, 1275, LP25 and UT142 (kindly provided by Dr. Ratha-korn Vilaichone, Faculty of medicine, Thammasat University, Thailand) were routinely cultured on brain heart infusion (BHI) agar (Oxoid, UK) supplemented with 7% (v/v) sheep blood and incubated at 37°C under microaerobic conditions (N_2, 85%; O_2, 5%; CO_2, 10%) using gas generating kit (Mitsubishi, Japan).

Screening of biofilm production

All the bacteria were screened for biofilm production using pellicle assay as previously described (Joshua et al., 2006). Briefly, all isolates were grown in 13- by 125-mm glass test tubes containing 10 ml of BHI broth supplemented with 2% (w/v) β-cyclodextrin (BCD; Sigma, USA) in the presence and absence of curcumin. The final bacterial concentration was adjusted to an OD_{600} of 0.2 (~10^8 CFU/ml). The cultures were incubated at 37°C for 7 days without shaking under microaerobic conditions. Development of pellicle and/or attached bacterial cells at interior surface of test tube at the air-liquid interface, representing as biofilm, was examined daily.

Minimum inhibitory concentration test

Minimum inhibitory concentration (MIC) was determined, in duplicate, by an agar dilution method following the Clinical and Laboratory Standards Institute (CLSI) guidelines using Mueller-Hinton agar with 5% (v/v) sheep blood. The curcumin (Sigma, USA) was dissolved in DMSO (Sigma, USA) and serially diluted 2-fold in Mueller-Hinton agar (Oxoid, UK) to give final concentrations ranging from 0.125 to 128µg/ml. A 72-h bacterial colony of *H. pylori* ATCC43504 was harvested and suspended in sterile saline solution. The bacterial final concentration was adjusted equivalently to approximately 2.0 McFarland standard (5 x 10^7 CFU/ml). Three microlitres of bacterial suspension per spot was replicated on each plate followed by incubating at 37°C for 72 h under microaerobic conditions. The MIC was defined as the lowest concentration of curcumin at which no visible growth was observed.

Examination of effect of curcumin against *H. pylori* biofilm

In order to determine the effect of curcumin concentration against *H. pylori* biofilm formation, curcumin were tested at various concentrations including 1/2, 1/4, 1/8, 1/16 and 1/32 MIC. *H. pylori* ATCC43504 was grown as described in pellicle assay for screening of biofilm production in an absence and presence of these relative

curcumin concentrations. The upmost dilution of DMSO used at 0.016% was incorporated as control. Development of biofilm was examined daily until 7 days. Subsequently, the curcumin concentrations that displayed significant effects against biofilm formation were selected for further optimum treatment-time examination by daily observation from day 1 to day 21.

Quantification of biofilm formation

A 72-h *H. pylori* ATCC43504 was grown in 13- by 125-mm glass test tubes as described in pellicle assay in an absence and presence of various curcumin concentrations (1/2, 1/4, 1/8, 1/16 and 1/ 32 MIC). BHI broths supplemented with 2% (w/v) BCD and DMSO at concentration of 0.016% were incorporated as blank and control, respectively. After 7-d incubation, all the culture medium was removed. The glass test tubes were then rinsed twice with PBS and dried for 30 min at 60°C and 10 ml of 0.1% (w/v) crystal violet was added for 5 min at room temperature. The unbound crystal violet was discarded. The glass test tubes were left to dry at 60°C for 15 min and then washed three times with PBS. The bound crystal violet was decolorized with ethanol/acetone (80:20, v/v). The level of biofilm formations were quantified by measuring the absorbance of the solution at 570 nm using a spectrophotometer (Hitachi, Japan). Triplicate absorbance reading for each concentration tested was averaged and average of the control was subtracted.

Scanning electron microscopy (SEM)

The samples were prepared for SEM including pellicles collected from the untreated samples at day 4, 5, 6, 7, the pellicle from 1/4 MIC of curcumin treated at day 7 and the planktonic cells taken from 1/2 MIC of curcumin treated at day 7. Planktonic cells collected from BHI broth without 2% (w/v) BCD at day 7 and colonies grown on BHI agar supplemented with 7% (v/v) sheep blood for 3 days were also compared. SEM was performed by Scientific and Tech-nological Research Equipment Center, Chulalongkorn University, Thailand. Briefly, the pellicles and planktonic cells were collected and fixed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.2) for 1 h. Samples were washed twice by PBS, following by distilled water. All samples were then successive dehydrated through a graded series of ethanol (10 min per step; 30, 50, 70, 90, 3 x 100%; v/v), followed by coating with gold-palladium. Finally, the samples were examined with SEM (JEOL, Japan).

Adhesion assay

The HEp-2 cells (kindly provided by Dr. Pornthep Tiensiwakul, Faculty of Allied Health Sciences, Chulalongkorn University, Thailand) were grown on tissue culture plasticware in Dulbecco's modified of Eagle's medium (DMEM; Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum at 37°C in 5% CO_2 with 95% humidity. Approximately 3×10^6 cells were seeded on 6-well tissue culture plates and grown overnight to reach 80% confluence. A-72 h *H. pylori* ATCC43504 was suspended in DMEM. The bacterial suspension was added into each well at the final concentration of 3×10^8 CFU/ml followed by curcumin treatment at sub-MIC. The co-culture between HEp-2 cells and *H. pylori* without curcumin treatment was served as control. After incubation for 2 h, the cells were gently washed three times with Dulbecco's phosphate buffer saline (DPBS; Invitrogen, USA) and lysed with sterile distilled water for 15 min on the shaking platform, at 150 rpm. Cell lysate solutions were serially diluted and plated out onto brain heart infusion agar supplemented with 7% (v/v) sheep blood,

incubated at 37°C under microaerobic condition for 3-5 days to determine the number of viable bacteria. Plates containing the visible colonies range from > 25 to <250 colonies were enumerated.

Statistical analysis

All experiments were performed independently in duplicate on three separated occasions. Values were expressed as mean ± SD. The effects of curcumin at various concentrations were quantitatively evaluated by one-way analysis of variance (ANOVA). The anti-adhesion against *H. pylori* was compared under curcumin treated and untreated conditions using student's pair *t*-test. Statistical significance was accepted at the $P < 0.05$ level.

RESULTS

Screening for biofilm formation

Eleven isolates and three reference strains of *H. pylori* were preliminary screened for biofilm formation. Two characteristics of biofilm were observed on the wall of the culture test tubes at air-liquid interface in the form of pellicle and attached biofilm (Figure 1) in all tested strains. Development of these two biofilm characteristics of all *H. pylori* strains is shown in Table 1. The biofilm of three strains, including ATCC43504, ATCC43526 and 1264, could be observed on day 3 of incubation. Almost strains, except ATCC51932, DMST20165 and LP25, were able to form biofilm after 4 days of incubation. At the end of incubation period, nine strains of *H. pylori* produced extensive level of biofilms whereas 4 of the strains formed intermediate levels. Only one strain, ATCC51932, developed low biofilm level in this study.

Inhibitory effect of curcumin on *H. pylori* biofilm

Curcumin was capable to inhibit the growth of *H. pylori* ATCC43504 at an MIC value of 16 µg/ml. The concentrations of curcumin at sub-inhibitory level (1/2, 1/4, 1/8, 1/16 and 1/32 MIC) were used for biofilm assay. Their inhibitory effects on biofilm formation of *H. pylori* ATCC 43504 are shown in Table 2. In the control, both pellicle and attached biofilm was firstly detected in *H. pylori* since day 3, following by an expansion of bacterial biofilm on the continuous days. The fully mature biofilm was clearly observed on day 5 and steadily maintained up to day 7 of observation. The same characteristic of biofilm production appeared in curcumin treated at 1/8, 1/16 and 1/32 MIC. While, curcumin at 1/4 MIC markedly inhibited biofilm formation. The complete inhibition of biofilm formation was demonstrated at 1/2 MIC. DMSO at concentration of 0.016%, which was the highest concentration mixed in curcumin suspension, showed no effect against *H. pylori* biofilm production. However, both concentrations of 1/4 and 1/2 MIC were observed extensively
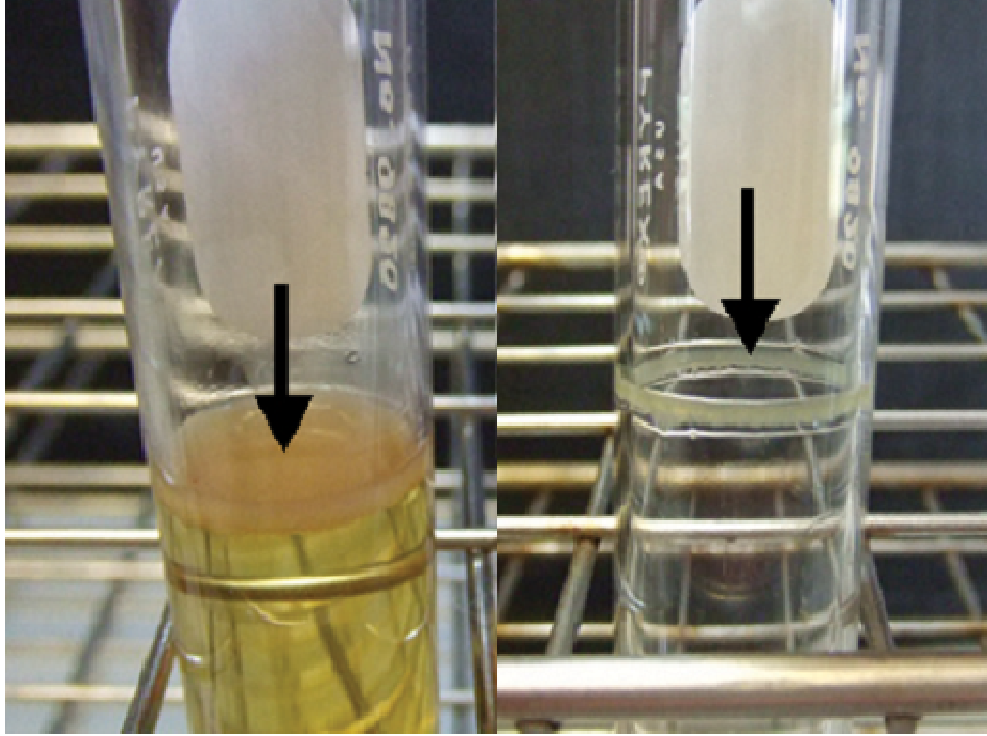
Figure 1. Photograph of pellicle assay showing 7-day biofilm formed by *H. pylori* ATCC43504. Left test tube, culture grown without curcumin demonstrating mature biofilm at air-liquid interface. Two biofilm characteristics were observed, including pellicle (left test tube; arrowed) and attached biofilm (right test tube; arrowed). Test tubes not to scale.

until day 21. It was found that the biofilm production could restore after prolong incubation (Table 3). The mature biofilm were detected in 1/4 and 1/2 MIC after day 10 and day 17, respectively.

Quantification of biofilm formation

We quantified level of *H. pylori* biofilm treated with curcumin at subinhibitory concentrations (1/2 - 1/32 MIC). As shown in Figure 2, curcumin significantly decreased the biofilm formation in a dose dependent manner. The level of biofilm was markedly decreased when treated with curcumin at 1/2 MIC (P < 0.000) and 1/4 MIC (P = 0.001). In contrast, the biofilm levels were not significantly different between curcumin treated at 1/8, 1/16 and 1/32 MIC in comparison with the untreated one (P > 0.05).

SEM

We used SEM to document a 3-dimensional structure of *H. pylori* biofilm and compared our SEM images to the existing images from other works. The SEM images of untreated *H. pylori* ATCC43504 taken from day 4, 5, 6 and 7 (Figure 3A-D) revealed uniform biofilm architecture with dense accumulations of bacteria within an amorphous matrix. The cells connected to each other by long fibrils and seemed to form aggregates. The abundant of cross-linked fibrils were associated with mature biofilms. Moreover, the bacterial morphology changed from bacillary to coccoid form in the older culture. In contrast, the fiber-like structure was found to adhere to long rod shape bacteria in the biofilms from day 7 of curcumin treated at 1/4 MIC (Figure 3E). In addition, the amorphous substance appeared to be lesser than the untreated sample (Figure 3A) with same incubation period. While, there was no evidence of biofilm formation from 7-day planktonic bacteria of curcumin treated at 1/2 MIC (Figure 3F). Neither amorphous extracellular substance nor complex bacterial layers were observed. The bacterial cells were found in coccoid-rod mixed population and tended to produce fiber-like structure. Surprisingly, the cell destruction, represented by a pore formation over cell wall surface (Figure 3F; arrowed) occurred. While, bacteria grown without 2% (w/v) BCD have a filamentous coccoid morphology and flagellar could be observed on the surface (Figure 3G). Instead, a long bacillary shape was seen from 3-day old colonies (Figure 3H).

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Table 1. Biofilm formation of 14 strains of *H. pylori* performed by pellicle assay. Two biofilm characteristics were noted including pellicle and attached biofilm. Development of biofilm was daily observed and scored through 7 days.

Day	ATCC43504 Pellicle	ATCC43504 Attached biofilm	ATCC43526 Pellicle	ATCC43526 Attached biofilm	ATCC51932 Pellicle	ATCC51932 Attached biofilm	DMST20165 Pellicle	DMST20165 Attached biofilm	DMST20885 Pellicle	DMST20885 Attached biofilm	1203 Pellicle	1203 Attached biofilm	1260 Pellicle	1260 Attached biofilm	1261 Pellicle	1261 Attached biofilm	1264 Pellicle	1264 Attached biofilm	1265 Pellicle	1265 Attached biofilm	1268 Pellicle	1268 Attached biofilm	1275 Pellicle	1275 Attached biofilm	LP25 Pellicle	LP25 Attached biofilm	UT142 Pellicle	UT142 Attached biofilm
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	++	-	-	-	-	-	-	-	-	-	-
4	++	++	+++	+++	-	-	-	-	++	++	+	+	+	+	+	+	+++	+++	+	+	+	+	+	+	-	-	++	++
5	+++	+++	+++	+++	-	-	++	+	+++	+++	++	++	+	+	++	++	+++	+++	++	++	+++	+++	++	++	+	+	+++	+++
6	+++	+++	+++	+++	-	-	++	++	+++	+++	++	++	++	++	++	++	+++	+++	++	++	+++	+++	++	++	++	++	+++	+++
7	+++	+++	+++	+++	+	+	+++	+++	+++	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++	++	+++	+++

Level of individual biofilm type represent as (-, absent); not any form of biofilm seen, (+, just visible); thin pellicle film or a fine attached biofilm, (++, intermediate); accumulated pellicle of liquid surface or a thin attached biofilm, (+++, extensive); a mature pellicle covering whole liquid surface or a dense attached biofilm. Experiments were performed in duplicate on three separated occasions. Representative scores were the major scores derived from three separated experiments. Biofilm grading was based on the earlier work (Joshua et al., 2006).

Table 2. Effects of curcumin at subinhibitory concentrations (1/2-1/32 MIC) on *H. pylori* ATCC43504 biofilm formation. Development of pellicle and/or attached biofilm was daily observed for 7 days.

Day	Curcumin concentrations													
	0		1/32 MIC		1/16 MIC		1/8 MIC		1/4 MIC		1/2 MIC		0.016% DMSO	
	Pellicle	Attached biofilm	Pellicle	Attached biofilm	Pellicle	Attached biofilm	Pellicle	Attached biofilm	Pellicle	Attached biofilm	Pellicle	Attached biofilm	Pellicle	Attached biofilm
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	-	+	-	+	-	-	-	-	+	+
4	++	++	++	++	+	+	+	+	-	-	-	-	++	+
5	+++	+++	+++	+++	++	+++	++	+++	-	+	-	-	+++	++
6	+++	+++	+++	+++	+++	+++	++	+++	+	+	-	-	+++	+++
7	+++	+++	+++	+++	+++	+++	+++	+++	++	++	-	-	+++	+++

Level of individual biofilm type represent as -; absent, +; just visible, ++; intermediate, +++; extensive, as described previously in Table 1. Experiments were performed in duplicate on three separated occasions. Representative scores were the major scores derived from three separated experiments.

Table 3. Effects of curcumin at various incubation times on *H. pylori* ATCC43504 biofilm formation. Biofilm developments were daily observed through 21 days in a presence of curcumin at 1/2 and 1/4 MIC.

Day	Curcumin concentration					
	0		1/4 MIC		1/2 MIC	
	Pellicle	Attached biofilm	Pellicle	Attached biofilm	Pellicle	Attached biofilm
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	+	-	-	-	-
4	+	+	-	+	-	-
5	++	+++	-	+	-	-
6	+++	+++	+	+	-	-
7	+++	+++	+	+	-	-
8	+++	+++	++	++	-	-
9	+++	+++	++	+++	-	-
10	+++	+++	+++	+++	-	-
11	+++	+++	+++	+++	-	-
12	+++	+++	+++	+++	-	-
13	+++	+++	+++	+++	-	-
14	+++	+++	+++	+++	-	-
15	+++	+++	+++	+++	+	+
16	+++	+++	+++	+++	++	++
17	+++	+++	+++	+++	+++	+++
18	+++	+++	+++	+++	+++	+++
19	+++	+++	+++	+++	+++	+++
20	+++	+++	+++	+++	+++	+++
21	+++	+++	+++	+++	+++	+++

Level of individual biofilm type represent as -; absent, +; just visible, ++; intermediate, +++; extensive, as described previously in Table 1. Experiments were performed in duplicate on three separated occasions. Representative scores were the major scores derived from three separated experiments.

Anti-adhesive activity of curcumin against *H. pylori*

In order to understand the mechanism of the anti-biofilm action of curcumin, the ability of *H. pylori* to adhere, which is likely to be the initial step of biofilm formation, was investigated. The anti-adhesive activity of curcumin was assessed against *H. pylori* ATCC43504 on HEp-2 cells. The curcumin at 1/2 MIC, which completely inhibitted biofilm formation of *H. pylori* on day 7 of observation, was tested. A significant inhibition of *H. pylori* adherence was observed with a reduction of 83.2% compared to the untreated control (P = 0.009) (Figure 4).

DISCUSSION

The ability of *H. pylori* to produce biofilm is thought to be important in facilitating bacteria to survive in adverse conditions (Cole et al., 2004; Stark et al., 1999). Despite the environmental limitations of *H. pylori* isolates, their survival in biofilms may play an important role in the transmission of the pathogen to humans. *H. pylori* has been conferred as another pathogen that considered to have an evidence for a waterborne route of infection (Watson et al., 2004). Here, we demonstrated that *H. pylori* could form biofilm in liquid monoculture with two distinct characteristics. It can form a pellicle and attach to a glass surface at air-liquid interface indicating the most presumable its microaerobic character (Cole et al., 2004). Similar forms of biofilms have been observed previously in other bacteria such as *Campylobacter jejuni* (Joshua et al., 2006). Although, all the tested *H. pylori* strains here enabled to produce biofilm, the dramatically different biofilm levels have been observed both in reference and clinical isolates. *H. pylori* ATCC51932, which is *cagA* negative strain, exhibited the lowest biofilm level. This effecter protein, CagA, has been suggested to have a role in *H. pylori* colonization (Torres and Backert, 2008).
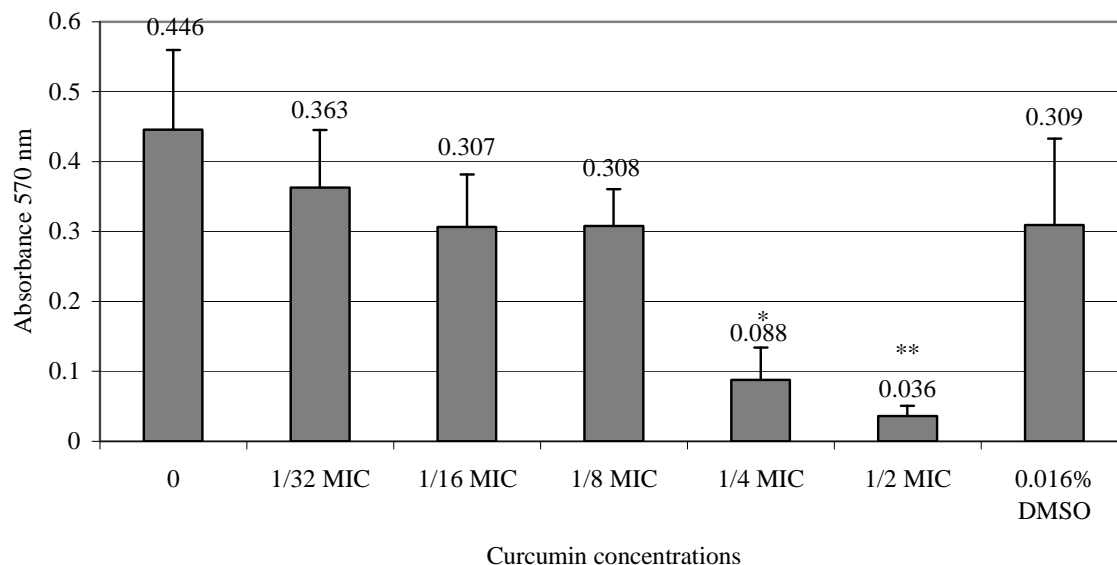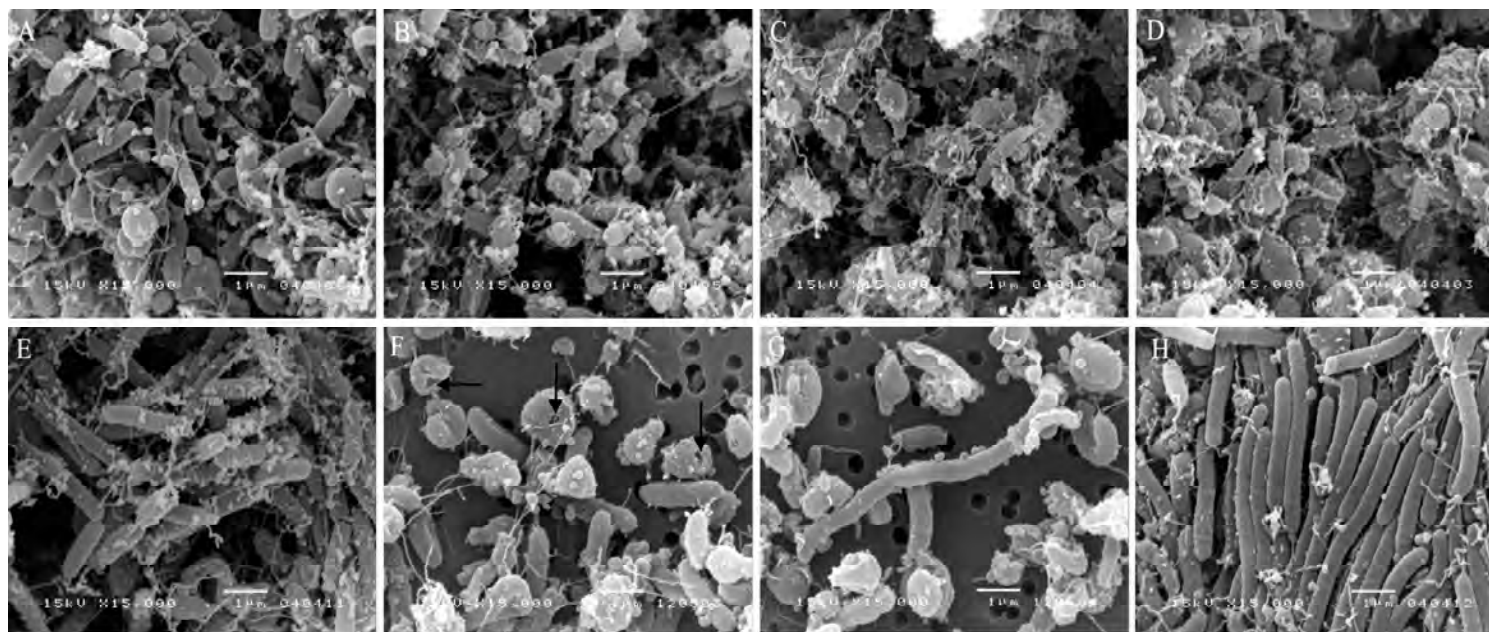
Figure 2. Biofilm quantification of *H. pylori* ATCC43504 at subinhibitory concentrations of curcumin. The level of biofilm production was quantified by crystal violet staining and subsequently measuring absorbance at 570 nm at 7 day. Asterisks (*) and (**) indicate significant differences from control at the $P = 0.001$ and $P < 0.000$ levels, respectively. Experiments were performed in duplicate on three separated occasions. Error bars represent ± standard deviation from the mean.

Also, the *cag*PAI encodes a type IV secretion system (T4SS), has been noted to be associated in attachment and invasion of *H. pylori* into hepatocytes (Ito et al., 2008). Although postulate of the relevant of this virulent factor with biofilm formation has not been elucidated, its role in colonization and adhesion may be associated.

Production of biofilm by *H. pylori* may be important for enabling its resistance to antimicrobials and host defense factors (Houben et al., 1999). Treatments with standard antibiotics are ineffective at eradicating biofilm-related infections. Moreover, the evolution of bacterial resistance is very rapid which restricts the use of newer generations of antibiotics (Levy, 2002). Therefore, the new strategies to overcome biofilm infections have been proposed, for example the use of carbohydrate-based therapeutic as anti-adhesive (Tenke et al., 2004), the use of phytomedicine to prevent biofilm formation (Limsuwan et al., 2008; Rasooli et al., 2008) and the use of bacteriophage to hydrolyze biofilm extracellular polymers (Donlan, 2009). The anti-biofilm activities have been demonstrated in a number of medicinal plants (Limsong et al., 2004; Duarte et al., 2006; Cartagena et al., 2007; Kuzma et al., 2007). In the traditional medicine, turmeric is one of the well-known herbs used for gastric ailment (Araujo and Leon, 2001). Curcumin, a major compound derived from the rhizomes of turmeric, possesses a board pharmacological effect (Negi et al., 1999) and confers anti-*H. pylori* activity (Mahady et al., 2002). In the present study, its anti-biofilm activity against *H. pylori* was observed, with dose dependent manner. Although, cur-

cumin effectively inhibited *H. pylori* biofilm formation, the ability could restore in the later period. However, in our point of view, curcumin could delay biofilm formation making most of bacterial cells being as planktonic, which is more sensitive to antimicrobial agents rather than in biofilm state (Mah and O'Toole, 2001). Curcumin has been shown to down-regulated virulence factors, quorum sensing and biofilm initiation genes of *Pseudomonas aeruginosa* (Rudrappa and Bais, 2008). However, the precise mechanism of this agent against *H. pylori* biofilm requires further investigation.

The progression of biofilm formation by *H. pylori* mimics previous reports, beginning with individual bacteria adhering to the abiotic surface, expansion into colonies and formation of a 3-D structure (Cole et al., 2004). The architecture of *H. pylori* biofilms was observed by SEM revealed that the bacteria aggregated together surrounded by extracellular polymeric matrix and fibrillar network. This observation associates with the mature biofilms explained previously (Watnick and Kolter, 2000). The ultrastructure of biofilm is comprised of bacterial cells encased in biopolymer matrix. The matrix mainly contains exopolysaccharide, proteins and DNA (Thomas and Lehman, 2006). The polymeric matrix may be responsible for the biofilm volume and the protection of biofilm community (Cvitkovitch et al., 2003). Moreover, most of the bacterial cell shape embedded in extracellular polymeric matrix changed from rod to coccoid in the older culture. This morphological conversion may lead bacteria to a dormant stage and a loss of culturability (Cellini et al.,

Figure 3. SEM photomicrograph of biofilm development of *H. pylori* ATCC43504 at a magnification of X 15,000. A-D; pellicle cells taken from bacterial culture without curcumin at day 4, 5, 6 and 7, respectively. E; pellicle cells taken from culture with 1/4 MIC of curcumin on day 7. F; planktonic cells taken from culture with 1/2 MIC of curcumin on day 7 show perforations on the cell surface (arrowed). G: bacterial cells taken from culture without 2% (w/v) BCD on day 7. H; Bacteria from 3-day direct colonies. Bars, 1 µm.

2007). The bacterium tends to enter the VBNC state, which represents a non-growing population of cells in microbial biofilms in order to ensure firmly adhesion to the surfaces (Lewis, 2005). In response to sub-optimum conditions such as sub-inhibitory drug concentrations, nutrient depletion or adverse atmospheres, most of the human pathogen (*Campylobacter* spp., *Escherichia coli*, *Francisella tularensis*, *Legionella pneumophila*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *P. aeruginosa*, *Salmonella* spp., *Shigella* spp., *Vibrio cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *H. pylori*) are able to convert to the VBNC form. This bacterial state has been sug-

gested to play a role in recurrent and drug resistant infections (Ozcakir, 2007). Noteworthy, in the curcumin treated conditions, less amorphous substance with retardation of morphological change was investigated. Moreover, curcumin at 1/2 MIC absolutely inhibited the biofilm formation and perforation of cells was revealed in the planktonic cells from day 7. This could be associated with cell wall and cell membrane damage similar in *L. monocytogenes*, *Salmonella typhimurium* and *E. coli* exposed to bacteriocins or hydrostatic pressures (Kalchayanand et al., 2004). The evidence of cell injury has been observed with planktonic and sessile *Bacillus subtilis*, *Pseudomonas*

fluorescens, *P. aeruginosa* after treatment with sanitizer against biofilms (Lindsay and von Holy, 1999; DeQueiroz and Day, 2007).

The attachment is prescribed as the necessary initial step in the formation of a biofilm (Joshua et al., 2006; Palmer et al., 2007). The ability to adhere to stratum could subsequently promote biofilm establishment. Thus, stopping bacterial adhesion is thought to be one advantage strategy to overcome biofilm-associated infections (Bavington and Page, 2005). As well as looking at the anti-biofilm activity, the ability of curcumin to reduce adherence of *H. pylori* to the HEp-2 cells was also demonstrated here. Consistently, turmeric
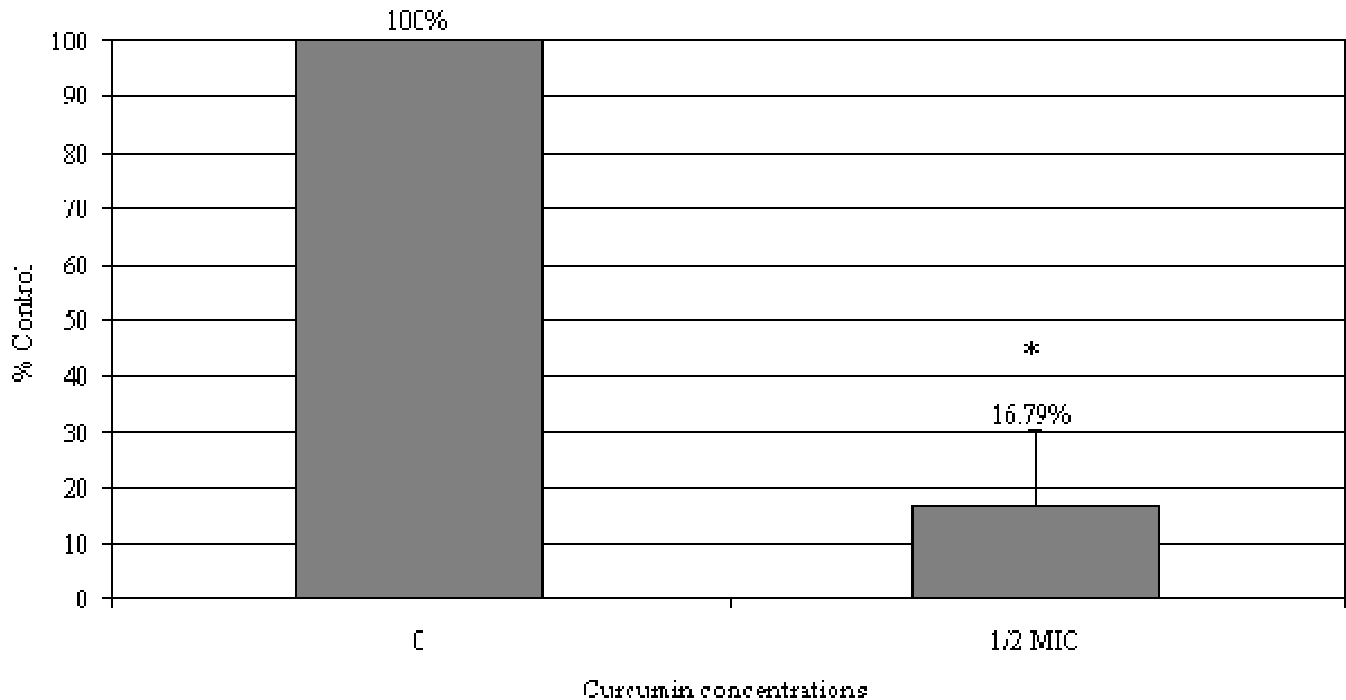
Figure 4. Effect of curcumin against *H. pylori* ATCC43504 adherence to the HEp-2 cells. *H. pylori* was co-cultured with HEp-2 cells for 2 h in a presence of 1/2 MIC of curcumin. Results expressed as the proportion of adherence bacteria versus control. Proportion of adherence bacteria was calculated as the rate of the number of adherence bacteria divided by the bacterial inoculum. Asterisk (*) indicates significant difference from control at the $P = 0.009$ level by pair *t*-test. Experiments were performed in duplicate on three separated occasions. Error bars represent ± standard deviation from the mean.

is one out of 3 plants showing to inhibit adhesion of *H. pylori* to the stomach sections previously (O'Mahony et al., 2005). And this may results in preventing biofilm formation and/or leading to biofilm detachment on human gastric mucosa.

Conclusion

Curcumin exhibited inhibition of biofilm formation and adherence to the human epithelial against *H. pylori*. The virtue of anti-*H. pylori* property of curcumin could be crucial in inhibition of pathogenesis caused by this organism. Hence, curcumin may be a potential supplemental material for the treatment of biofilm-involved *H. pylori* infection making a new approach for effective therapy.

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