Phyto anti-biofilm elicitors as potential inhibitors of *Helicobacter pylori*

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Abstract

Helicobacter pylori (*H. pylori*) infection is a global public health concern. Due to its high adaptability in various adverse environments (temperature, pH, adhesion, phenotypic forms), targeting the bacterium is quite challenging. Moreover, due to its high persistence, decreased patient compliance and emerging antibiotic resistance, researchers have been forced to search for novel candidates with lesser or no side effects. Hence, in the current study, phyto-bioactives have been screened for its anti-biofilm attributes against *H. pylori*. Gastric biopsy samples have been screened using confirmatory techniques (microbiological, biochemical and molecular) for their virulent and non-virulent biomarkers. Physico-nutritive parameters were standardized. *H. pylori* biofilms were assessed using microtitre plate assay. Biofilms' biomass and exopolysaccharide have been evaluated using crystal violet and ruthenium red staining, respectively. Anti-biofilm screening was performed using potent aqueous phytochemicals namely *Acorus calamus*, *Colocasia esculenta* and *Vitex trifolia*. The results indicated the confluent growth of the *H. pylori* biofilms confirmed through genotyping and grew best at 37 °C for 72 h at a pH of 7.5 on polystyrene plates. Further, among the phytochemicals tested, *Acorus calamus* exhibited the highest *H. pylori* anti-biofilm activity via a dose-dependent pattern. The overall observations of the study will pave way for newer approaches to understand and combat bacterial pathogenesis and will contribute towards better health and hygiene.

Keywords *Helicobacter pylori* · Sessile bacteria · Biofilm biomass · Virulent cascade · Microtiter plate biofilm assay

Introduction

Helicobacter pylori is a microaerophilic, spiral-shaped, gram-negative bacteria found in gastric epithelium leading to complications such as gastro-duodenal ulcer and carcinoma

(Belagihalli et al. 2011). *H. pylori* infection is found to infect approximately 50% of the urban population and the rate of infection is growing exponentially (George et al. 2012). According to World Health Organization (WHO), *H. pylori* is ranked as Class I pathogen responsible for various gastric cancers, and have been documented in different epidemiological studies which highlight the pathogenicity and the ability of *H. pylori* to induce cancer. *H. pylori* affects people of all age groups, and are hence coined as young, old, pregnant and immuno-compromised patients (YOPI's). The virulence factors contributing to the pathogenesis of *H. pylori* include urease, flagella, vacuolating toxin A (*VacA*) and cytotoxin-associated gene antigen (*CagA*) (Buti et al. 2011).

Most bacterial species adhere to various biotic and abiotic surfaces under ambient conditions, and with the availability of adequate nutrients establish biofilms on respective surfaces (Dunne 2002; Jefferson 2004). Development of biofilm occurs through distinct mechanisms, viz., redistribution of cells due to surface motility, attached cells due to binary multiplication or accumulation of cells from fluids

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(Stoodley 2004). *Helicobacter pylori* has the ability to form biofilm in the gastrointestinal tract and is considered as the second most common organism to cause human infection (Cammorate et al. 2012). Various components of the pathogen have been found to aid in biofilm establishment which include flagella, *luxS* gene, *cag* and *vir B* protein (Percival and Suleman 2014). Colonization of *H. pylori* leads to gastrointestinal complications with environmental factors such as smoking, alcohol, NSAID (non-steroidal anti-inflammatory drug), PPI (proton pump inhibitor) and host factors like gene polymorphism, and immune response (Peterson et al. 1991).

Currently, three lines of antibiotic therapies are administered for the treatment of *H. pylori*-related complications, viz, concomitant and hybrid therapy, bismuth quadruple, levofloxacin therapy and culture-guided therapy, respectively (Talebi et al. 2015). Continuous use of antibiotics in treating various bacterial infections has resulted in evolution of multidrug-resistant bacteria which calls for searching therapeutic alternatives (Ashwini et al. 2014). Herbal-based new therapeutic regimes have been found to be promising in targeting the biofilm formed by *H. pylori* to prevent further complications. The plethora of natural antioxidants, polyphenols and flavonoids that are present in many phyto-bioactives aid in decreased acid mucosal secretion, inhibit pepsinogen production, increase gastric mucosa formation and decrease ulcerogenic lesions (Chatterjee et al. 2004). Extensive data mining has been done to select plants for initial screening for managing *H. pylori* and aqueous extracts of *Acorus calamus*, *Colocasia esculenta* and *Vitex trifolia* and the effect of plants' activity has been assessed on the *H. pylori* biofilms. The results of this study will help in facilitating a better understanding of biofilm model of *H. pylori* and aid in finding potent anti-biofilm drug targets of herbal origin.

Materials and methods

Isolation of *H. pylori* from biopsy samples

Forty biopsy samples from gastric mucosa of suspected people undergoing endoscopy were collected from KCDC, Mysore and JSS Hospital, Mysore. The transport media comprising 20% glycerol with HAM F-12 media were used and were stored at -20°C in storage vials. HAM F-12 medium has been identified as chemically defined ideal medium for cultivation of *H. pylori* (Testerman et al. 2001). The samples were then checked for the presence of *H. pylori* by preliminary studies of rapid urease test and catalase test using standard protocol (Blanchard et al. 2013). Biopsy samples were cut into small pieces and added into sterile test tubes containing HAM F-12 media

and incubated at 37°C till the color of media changed from yellow to pink (Magraud et al. 2007). Furthermore, sterile HAM F-12 media was poured onto sterile petri-plates and after solidification streaking was done from the tubes which showed a positive preliminary test for *H. pylori* in all the biopsy samples. Plates were incubated at 37°C in a carbon dioxide incubator (Thermo Fisher Scientific, Germany) at microaerobic atmosphere (5% O_2 , 10% CO_2 , and 85% N_2) for a period of 3–7 days. The colonies formed after incubation were further subjected to gram staining, catalase and rapid urease test (Blanchard et al. 2013). Positive samples were identified based on their typical morphology, gram staining and positive reaction observed in urease, oxidase and catalase test which were further sub-cultured on sterile HAM F-12 agar plates for further experiments.

Extraction of genomic DNA

Colonies were collected from positive culture plates and were subjected to PBS wash. DNA was extracted through the CTAB (hexa decyl trimethyl ammonium bromide) method using standard protocol with slight modifications (Shetty et al. 2015).

PCR amplification

PCR amplification was done with a final volume of 20 μl which contained 5 μl of DNA, 1.5 μl of Taq buffer, 0.3 μl of MgCl_2 , 1.8 μl of dNTP, 0.9 μl of primer, 0.3 μl of Taq and the volume was made up by double distilled water. PCR conditions were initially denatured at 94°C for 2 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s and extension at 72°C for 1 min with final extension at 72°C for 5 min. Rocha et al. 2005 suggested that any specimen should be designated positive of *H. pylori* where there is amplification of two different conserved target genes (Kawahara et al. 2001; Mawarasti et al. 2016). In this regard, two virulent sequences *CagA* and *VacA* (s1/s2), two avirulent sequences *atpA* and *efp* were considered along with two essential metabolic genes *frdB* and *aroK*. *frdB* gene exists in two other species of *Helicobacter*; *H. acinonychis* and is found to cause gastric infection in wild cats, e.g., cheetah, whereas *H. cetorum* is found to infect aquatic mammals, e.g., dolphins, but neither infect humans. As our biopsy samples are from humans, there is no chance of obtaining false positives due to cross-contamination by the two non-human *Helicobacter* species. The primer sequence of the same is as in Table 1. The isolates which was shown to be positive from the above aspects were considered for further experiments.

Table 1 Primer sequences of the virulent and avirulent markers selected for molecular characterization and confirmation

Primers	Forward	Reverse
Virulent markers		
<i>CagA</i>	GAT AAC AGG CAA GCT TTT GAG G	CTG CAA AAG ATT GTT TGG CAG
<i>VacA</i> (s1/s2)	ATG GAA ATA CAA CAA ACA CAC	CTG CTT GAA TGC GCC AAA C
Avirulent markers		
<i>atpA</i>	GGACTAGCGTTAAACGCACG	CTTGAAACCGACAAGCCAC
<i>efp</i>	GGCAATTTGGATGAGCGAGCTC	CTTCACCTTTTCAAGATACTC
Metabolic markers		
<i>frdB</i>	GCTAAAATTTGACCCTCAAAGC	AGCGGCTTTTACCCACTTTC
<i>aroK</i>	TTTATGGGGAGCGGTAAAAG	CACCTCTGGCGTCAATGATA

Standardization of growth conditions

Standardization of incubation duration and pH

The bacterial growth under planktonic conditions was measured at different time intervals which are 0, 6, 12, 24, 48 and 72 h by taking 1 ml of culture sample by checking OD at 600 nm, sterile media being the control. Data mining on *H. pylori* biofilms have considered 37 °C as standard temperature, which is the human body temperature and so the same was considered as optimum. pH 2, 3, 5, 6, 7, and 8 were considered for standardization for 0, 6, 12, 24, 48 and 72 h, respectively, with 1 ml sample at 600 nm OD and again media being used as control. Further, for assessing the biofilm growth patterns, the pre-culture was diluted to 5000-fold (approximately 5×10^6 CFU/mL) with PBS. Two hundred micro-liter of each suspension was added to eight wells of 96-well sterile flat bottom micro-titer plates, and the wells without inoculum were served as blank. Two plates each were incubated with respective experimental time, and the temperature and growth were determined by microtitre biofilm assay at 600 nm using a micro-plate reader using the crystal violet method (Zameer et al. 2009).

Standardization of adhesive surface

For the cultivation of biofilms on ideal surfaces, the organism was grown on different surfaces. Surfaces considered were steel, thin glass (fraction of 1 mm thickness), thick glass (1 mm thick), polystyrene and cellulose dialysis membrane. Steel, thin glass, thick glass were washed thoroughly with distilled water and swabbed with ethanol and sterilized. The sterilized polystyrene was purchased from the genuine chemical industries and the dialysis membrane is activated by a standard procedure (Zameer et al. 2010).

Activation of cellulose dialysis membranes

According to Resch et al. (2005), circular sheets of 70–75 mm diameter were cut out from 38 mm wide dialysis

tubes (Nadir, pore size 25–30 Å⁰ Roth, Germany), Membranes were pretreated with aqueous 2% sodium bicarbonate/1 mM Na-EDTA for 3 h at 60 °C, thoroughly rinsed (8–10 times) with distilled water and stored at 4 °C. Prior to use, the membranes were sterilized in 70% ethanol for 1 h and air dried aseptically and then tightly applied on HAM F-12 agar plate.

Cultivation of biofilm on steel, glass, cellulose membrane and polystyrene

Around 200 µL pre-culture was spread over the sterilized steel, glass, polystyrene and activated cellulose membrane and 100 µL pre-culture was spread over thin glass and to this 15–20 ml HAM F-12 media was added. For the development as biofilms on agar surface, the sterilized steel, thick glass, thin glass and cellulose membrane were placed on agar surface and incubated for 3 h, respectively. The 200 µL pre-culture was spread over the sterilized steel, thick glass and activated cellulose membrane and 100 µL pre-culture was spread over thin glass. Both the cultures were sealed and incubated with respective time and temperature. After the incubation period, the organism grown on the surface was quantified by crystal violet assay and ruthenium red staining assay (Borucki et al. 2003).

Cultivation of biofilm on micro-titer plates

A resuspended pre-culture was diluted to 5000-fold (approximately 5×10^6 CFU/mL) with PBS. Two hundred micro-liters of the suspension were added to eight wells of 96-well sterile polystyrene tissue culture (flat bottom) micro-titer plates, and the first row wells with medium only were served as blank. Bacterial growth was determined after incubation of 72 h at 600 nm using a micro-plate reader (Zameer et al. 2009). The fresh sterile medium was replaced by carefully aspirating the spent medium without damaging the formed bacterial biofilm, using flat bottom microtitre plates. The inoculum concentration was standardized as per Zameer et al. (2009, 2010).

Microtiter plate biofilm assay by crystal violet staining

Staining of the bacteria which adhered to microtitre plate was carried out according to the protocol established by Djordjevic et al. (2002) with slight modifications. Non-adherent cells were separated by rinsing thrice with 250 µl of isotonic PBS and were further dried for 30 min at 42 °C. Then, 150 µl of aqueous 1% crystal violet stain was added to each well and incubated for 30 min at 35 °C. Excess dye which was unbound was removed by rinsing thrice with 250 µl of distilled water. Further solubilization of crystal violet was done by addition of 220 µl of 95% ethanol followed by incubation at 4 °C for 45 min. 100 µl was collected from each well and transferred to fresh microtiter plates and the absorbance was recorded at 595 nm using a plate reader.

EPS quantification by ruthenium red staining

Biofilms which were grown at optimal time and temperature of 72 h and 37 °C and blanks were prepared as described above. They were subjected to wash and further were stained for matrix exopolysaccharide as per Borucki et al. (2003) with slight modifications. 200 µl of aqueous 0.1% ruthenium red was added to each well and incubated for 45 min at 35 °C. Supernatant from each well was collected and was transferred to a fresh microtiter plate and absorbance was measured for all the wells at 450 nm by subtracting from the average calculated from the blanks (Zameer et al. 2009).

Preparation of aqueous plant extracts

Initially, ten plants were collected from Mysuru regions, Karnataka, India during the months of May–September. The voucher specimen was submitted to the Faculty of Life Sciences, JSS Academy of Higher Education & Research, Mysore, Karnataka, India, and after identification, the plants were selected for screening based on the extensive data mining and their ethnobotanical influence on antibacterial activity. Further, based on the phytochemical analysis and initial antibacterial screening, three plants namely *Acorus calamus*, *Colocasia esculenta* and *Vitex trifolia* were selected for aqueous extraction (Mahmood et al. 2014; Joachim et al. 2016; Geetha et al. 2004). The plants were sterilized using tap water and further washed by 0.5% sodium hypochlorite solution and double distilled water. The samples were air dried to remove excess moisture and further dried in hot air oven overnight. The samples were powdered and sieved later and the coarse and fine particles were separated. Plant extract was prepared by adding 20 g of fine powder in 200 ml of double distilled water and was heated on a magnetic stirrer at 53 °C, 800 rpm for 3 h. The extract was then filtered using a muslin cloth and the filtrate was centrifuged

at 800 rpm for 20 min, and then the supernatant was collected, bottled and refrigerated until further use.

Anti-biofilm assay of plant extracts

Initial screening of anti-bacterial activity was tested by the anti-biofilm activity according to Duarte et al. (2005) with some modifications. Biofilm was cultivated on microtitre plate as described above, and after developing the biofilm, the supernatant was discarded and 200 µl of fresh medium was added as supplement with different concentrations of plant crude extract (5, 25, 50, 100 µg/ml) and then it was incubated for 72 h at 37 °C in a carbon dioxide incubator. A total of six plates were maintained for each plant extract, three each for crystal violet and ruthenium red assay (Zameer et al. 2016), along with the control. After the incubation period, the anti-biofilm activity was measured by crystal violet assay and ruthenium red assay.

Results and discussion

Isolation and characterization of *H. pylori*

40 gastric mucosal biopsy specimens from both male and female patients (average age of 42.6) were obtained from KCDC Mysore and ethical committee clearance was taken from JSS Academy of Higher Education & Research, Mysuru, Karnataka, India (JSS/MC/IEC/10/1964/2015-16). The specimens were checked initially using biochemical tests for presence of *H. pylori* by catalase and urease test. The positive samples for *H. pylori* were further cultured using HAM F-12 agar medium in CO₂ incubator. In the selective media used, i.e., HAM F-12 colonies grown were confirmed as *H. pylori* based on typical microscopic observation and positive tests for catalase and urease test. A total of 7 positive isolates out of 40 isolates were obtained and were further tested using molecular detection for bacterial characterization.

Studies on *H. pylori* have predicted two important virulence factors—*vacA* and *cagA* genes which are found to be involved in two distinct toxin production which are significant in confirming *H. pylori* using molecular tools (Shetty et al. 2015). In the current study along with the above mentioned genes, two avirulent genes—*atpA* and *efp* genes and two metabolic genes, *frdB* and *aroK* which code for fumarate reductase iron–sulfur protein, and shikimate kinase were also considered in molecular confirmation of the pathogen both *frdB* and *aroK* are highly conserved genes in *H. pylori* and unique to *H. pylori*. Hence, amplification of these genes would conclusively imply that the procured strains belong to *H. pylori* species. The molecular characterization of the biomarker genes has been shown in Fig. 1.

Fig. 1 Molecular confirmation through amplification of virulent (a). *CagA* and (b). *VacA*, non-virulent. (c) *atpA* and d *efp* e *aroK*, f *frdB* markers in seven positive isolates. In the lane, M refers to DNA ladder of 1 KB and they (lane 1–7) are the positive isolates. e *aroK*, f

frdB markers in seven positive isolates. In the lane, M refers to DNA ladder of 1 KB, lane 1 refers to negative control and they (lane 2–8) are the positive isolates

As per the results obtained, it was observed that the detection of *H. pylori* is not solely sufficient to observe a thorough development of mucosal damage, but the presence of marker genes was critical in providing such information. The present study though suffices a limitation of small number of samples but certainly highlights the significance of detection of pathogen using biomarkers to treat the disease. Further, *cagA* and *vacA* gene can be targeted as risk biomarkers for the infection prognosis of gastric mucosa, *frdB* and *aroK* could be further used as a diagnostic marker for *H. pylori* infection.

***H. pylori* biofilm standardization, development and quantification**

Helicobacter pylori growth was monitored in planktonic form for standardization of optimal time duration and pH. Initial tests for the biofilm forming capacity of *H. pylori* in HAM F-12 were performed after incubation for 72 h at 37 °C in polystyrene microtiter plates in CO₂ chamber and further stained with crystal violet for visual inspection using inverted microscope (Zameer et al. 2009). There was significant formation of biofilm and was used for further

Fig. 2 **a** Growth curve of *H. pylori* in planktonic state. **b** Growth curve of *H. pylori* in biofilm form (Biofilm biomass)

Fig. 3 Standardization of pH for growth assessment on *H. pylori* biofilms

experimentation. Growth of *H. pylori* in static culture and as biofilms on microtitre plates with HAM F-12 was quantified after an incubation period of 72 h. A comparison of growth curve of planktonic and biofilm of *H. pylori* in terms of different time duration was observed according to the representation in Fig. 2. Through this study, it was found that *H. pylori* planktonic growth optimum was 48 h and for biofilm it was 72 h. Optimum temperature of 37 °C was considered which was justified in previous works done on *H. pylori*. The *H. pylori* biofilm was developed at pH 7.5.

Further, different pH ranges of 2, 3, 4, 5, 6, and 7 were standardized at different time intervals of 6, 12, 24, 48 and 72 h at 37 °C and it was found that growth of *H. pylori* was optimum at pH 3 as shown in Fig. 3.

H. pylori biofilm formation on glass, polystyrene and steel surface with HAM F-12 agar was quantified after incubation for 72 h at 37 °C. Figure 4 shows the EPS strength (OD 495 nm) and biofilm biomass (OD 595 nm) on dialysis membrane broth (DB), dialysis membrane agar (DA), thick glass broth (TGB), think glass agar (TGA), thin glass broth (TB), thin glass agar (TA), steel broth (SB), steel Agar (SA), polystyrene broth (PB) and polystyrene agar (PA), respectively.

After the incubation period of 72 h, the number of adherent cells and EPS concentration were highest on polystyrene in comparison to glass and steel surfaces, which showed a significantly higher capacity of biofilm formation.

Analysis of anti-biofilm activity by plant extracts

Among the ten plants which were selected for initial screening, three plants, i.e., *Acorus calamus*, *Vitex trifolia* and *Colocasia esculenta* exhibited higher antibacterial activity on planktonic culture and hence were selected for their anti-biofilm activity on *H. pylori*. Table 2 summarizes the ten plants which were selected on basis of ethno-botanical

Table 2 Antibacterial activity of ten selected plants against *H. pylori*

S. no	Name of the plant	Anti-bacterial activity
1	<i>Couroupitaguianesis</i>	—
2	<i>Delonix regia</i>	—
3	<i>Saccharum officinarum</i>	—
4	<i>Acorus calamus</i>	+++
5	<i>Vitex trifolia</i>	++
6	<i>Punicagranatum</i>	—
7	<i>Cymbopogon citrates</i>	—
8	<i>Colocasia esculenta</i>	+
9	<i>Alterantherasessilis</i>	—
10	<i>Musa paradisiacal</i>	—

+++ Strongly positive (20 mm), ++ moderately positive (15 mm), + mild positive (12 mm), — negative (less than 10 mm)

literature. The hydroethanolic extracts were prepared with the leaves' sample and disc diffusion assay on the HAM F-12 agar plates was performed to assess the antibacterial potency. After incubation of 24 h, the zone of inhibition (diameter) was measured in millimeters (mm), and the plants of sl. no. 4, 5 and 8 in the table exhibited antibacterial activity (planktonic forms) and hence were further evaluated for their anti-biofilm efficacy (sessile forms). A comparative detail of their activity has been summarized in Fig. 5. Adhesion and colonization were used as prerequisites for the establishment of bacterial pathogenesis. The prevention of adhesion was used as a potential target for the development of new therapies in the prevention of infection. *H. pylori* has developed a multiplicity of adhesion mechanisms commonly targeting surface carbohydrate structures, but our ability to rationally design effective anti-biofilm was limited due to lack of detailed information regarding human 'glycome' and of the bacterial function in relation to it. The potential for the future development of carbohydrate-based anti-phytoquorum has to been demonstrated by a significant number of in vitro and in vivo studies. Such therapies will be particularly relevant for infections of mucosal surfaces (stomach) where topical application or delivery is possible.

Acorus calamus L. also vernacularly called as Baje, is one of the plants with an ancient background. It has been used in Indian culture for generations. It is also known for its ability of treating amnesia, insomnia, bronchitis, asthma, counter acidity and to ease heartburn (Pattanaik et al. 2013). In the present study, *Acorus calamus* proved to be the most potent herbal preparation with a good inhibition of biofilm followed by *Vitex trifolia* and *Colocasia esculenta*, respectively. In conclusion, a better understanding of *H. pylori* biofilms can be very helpful in understanding the dynamics of development of the pathogen on food processing surfaces and the phytomolecule cocktails could be used as a potent

Fig. 5 Anti-biofilm potential of phytochemicals from *Acorus calamus*, *Colocasia esculenta* and *Vitex trifolia* on *H. pylori* biofilms. **a** Crystal violet assay for assessing biofilm biomass expressed in terms of percentage inhibition. **b** Ruthenium red assay for exopolymers expressed in terms of percentage inhibition

sanitizer against the *H. pylori* infection and other similar-related organisms, ultimately paving newer insights for food safety and hygiene.

Conclusion

The current study significantly establishes the usage of phytochemicals as potentially a safe and effective alternative for treatment of *H. pylori* contaminations and infection through anti-biofilm property. The *frdB* and *aroK* genes used in this study could be further used as a rapid diagnostic tool for diagnosis of *H. pylori* thereby, preventing the ambiguity in diagnosis, which in turn leads to specific targeted administration of treatment regimen, which helps in preventing abuse of antibiotics. The phytochemicals of the above-mentioned plants can be further elucidated to check the exact mechanism through which the extracts exhibit anti-biofilm property, and phytochemicals can be further purified and used for treatment of multiple drug-resistant strains, thereby

preventing chronic progression and manifestation of infection to cancers.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

References

- Ashwini P, Sumana MN, Shilpa U, Mamatha P, Manasa P, Dhananjaya BL, Prasad MN (2014) A review on *Helicobacter pylori*: its biology, complications and management. *Int J Pharm Pharm Sci* 7(1):14–20
- Belagihalli M, Mysore A, Harish Nayaka SM, Dharmesh (2011) Inhibition of *Helicobacter pylori* growth and its cytotoxicity by 2-hydroxy 4-methoxy benzaldehyde of *Decalepishamiltonii* (Wight & Arn); a new functional attributes. *Biochimie* 93:678–688. <https://doi.org/10.1016/j.biochi.2010.12.009>
- Beulah KC, Aishwarya T, Waseem K, Meghashri S, Hedgekatte R, Nagendra Prasad MN, Dhananjaya BL, Zameer F (2015) Phyto-antiquorumones: an herbal approach for blocking bacterial trafficking and pathogenesis. *Int J Pharm Pharm Sci* 7(1):29–34
- Blanchard TG, Czinn SJ, Correa P, Nakazawa T, Keelan M, Morningstar L et al (2013) Genome sequences of 65 *Helicobacter pylori* strains isolated from asymptomatic individuals and patients with gastric cancer, peptic ulcer disease, or gastritis. *Pathog Dis* 68:39–43. <https://doi.org/10.1111/2049-632X.12045>
- Borucki MK, Peppin JD, White D, Loge F, Call DR (2003) Variation in biofilm formation among strains of *Listeria monocytogenes*. *Appl Environ Microbiol* 69:7336–7342
- Buti L et al (2011) *Helicobacter pylori* cytotoxin-associated gene A (CagA) subverts the apoptosis-stimulating protein of p53 (ASPP2) tumor suppressor pathway of the host. *Proc Natl Acad Sci USA* 108:9238–9243. <https://doi.org/10.1073/pnas.1106200108>
- Cammarota G, Sanguinetti M, Gallo A, Posteraro B (2012) Biofilm formation by *Helicobacter pylori* as a target for eradication of resistant infection. *Aliment Pharmacol Ther* 36(3):222–230. <https://doi.org/10.1111/j.1365-2036.2012.05165.x>
- Chatterjee A, Yasmin T, Bagchi S et al (2004) Inhibition of *Helicobacter pylori* in vitro by various berry extracts, with enhanced susceptibility to clarithromycin. *Mol Cell Biochem* 265:19–26
- Chauhan MS, Syed AA (2011) Effect of *Ocimum tenuiflorum* leaf extract against infective endocarditis. *Int J Phytomed* 3(4):470–474
- Djordjevic D, Wiedmann M, McLandsborough LA (2002) Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl Environ Microbiol* 68:2950–2958
- Duarte S, Gregoire S, Singh AP, Vorsa N, Schaich K, Bowen WH, Koo H (2006) Inhibitory effects of cranberry polyphenols on formation and acidogenicity of *Streptococcus mutans* biofilms. *FEMS Microbiol Lett* 257:50–56. <https://doi.org/10.1111/j.1574-6968.2006.00147.x>
- Dunne WM Jr (2002) Bacterial adhesion: seen any good biofilms lately? *Clin Microbiol Rev*
- Fletcher M, Marshall KC (1982) Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. *Appl Environ Microbiol* 44:184–192
- Geetha V, Doss A, Pichai AAD (2004) Antimicrobial potential of *Vitex trifolia* Linn. *Anc Sci Life* 23(4):30–32
- Hall-Stoodley L, Costerton JW, Stoodley P (2004) Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* 2:95–108. <https://doi.org/10.1038/nrmicro821>
- Jefferson KK (2004) What drives bacteria to produce a biofilm? *FEMS Microbiol Lett* 236:163–173. <https://doi.org/10.1016/j.femsle.2004.06.005>
- Joachim KD, Francesco KT, Victor K (2016) Antibacterial activities of the methanol extracts of *Canarium schweinfurthii* and four other Cameroonian dietary plants against multi-drug resistant Gram-negative bacteria. *Saudi J Biol Sci* 23(5):565–570. <https://doi.org/10.1016/j.sjbs.2015.06.006>
- Kawahara T, Teshima S, Oka A, Sugiyama T, Kishi K, Rokutan K (2001) Type I *Helicobacter pylori* lipopolysaccharide stimulated toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. *69:4382–9*. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC98510/>
- Lyczak JB, Cannon CL, Pier GB (2000) Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* 2, 1051–1060
- Mahmood B, Hessameddin S, Kowsar B, Massoud A, Faraz M (2014) Screening of 20 commonly used Iranian traditional medicinal plants against urease. *Iran J Pharm Res Winter* 13(Suppl):195–198
- Marshall KC, Stout R, Mitchell R (1971) Mechanism of the initial events in the sorption of marine bacteria to surfaces. *J Gen Microbiol* 68:337–348
- Mawarasti B, Purbayu H, Alimsardjono L (2016) CagA and VacA gene expression in *Helicobacter pylori* infected patients in Dr. Soetomo. *General Hospital* 17(3):181–187. <http://www.ina-jghe.com/journal/index.php/jghe/article/view/440>
- Megraud F, Lehours P (2007) *Helicobacter pylori* detection and antimicrobial susceptibility testing. *Clin Microbiol Rev* 20:280–322. <https://doi.org/10.1128/CMR.00033-06>
- Pattanaik J, Kumar Y, Khatri RS (2013) *Acorus calamus* Linn. A herbal tonic for central nervous system. *J Sci Innov Res* 2(5):950–954
- Percival SL, Suleman L (2014) Biofilms and *Helicobacter pylori*: dissemination and persistence within the environment and host. *World J Gast Pathophysiol* 5:122–132. <https://doi.org/10.4291/wjgp.v5.i3.122>
- Peterson WL (1991) *Helicobacter pylori* and peptic ulcer disease. *N Engl J Med* 324:1043–1048. <https://doi.org/10.1056/NEJM199104113241507>
- Resch A, Rosenstein R, Nerz C, Gotz F (2005) Differential gene expression profiling of *Staphylococcus aureus* cultivated under biofilm and planktonic conditions. *Appl Environ Microbiol* 71:2663–2676. <https://doi.org/10.1128/AEM.71.5.2663-2676.2005>
- Rocha GA, Queiroz DM, Mendes EN et al (1992) Indirect immunofluorescence determination of the frequency of anti-H. pylori antibodies in Brazilian blood donors. *Braz J Med Biol Res* 25(7):683–689
- Shetty V, Ballal M, Lingadakai R, Mukhopadhyay AK (2015) Determination of *Helicobacter pylori* virulence genes in clinical isolates of symptomatic patients from South Coastal region of Karnataka—a preliminary work. *Austin J Gastroenterol* 2:1031
- Smith AW (2005) Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems? *Adv Drug Deliv Rev* 57:1539–1550. <https://doi.org/10.1016/j.addr.2005.04.007>
- Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Hickey MJ, Brinkman FS, Hufnagle WO, Kowalik DJ, Lagrou M, Garber RL, Goltry L, Tolentino E, Westbrook-Wadman S, Yuan Y, Brody LL, Coulter SN, Folger KR, Kas A, Larbig K, Lim R, Smith K, Spencer D, Wong GK, Wu Z, Paulsen IT, Reizer J, Saier MH, Hancock RE, Lory S, Olson MV (2000) Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* 406:959–964. <https://doi.org/10.1038/35023079>
- Talebi A, Ierardi E, Lee J (2015) Why do we still have *Helicobacter pylori* in our Stomachs? *Malays J Med Sci* 22:70–75
- Truchadoa P, Larrosaa M, Castro-Ibaneza I, Allende A (2015) Plant food extracts and phytochemicals: their role as quorum sensing

- inhibitors. Trends Food Sci Technol 43:189–204. <https://doi.org/10.1016/j.tifs.2015.02.009>
- Zameer F, Gopal S (2010) Transcriptome analysis of thiol disulfide redox metabolism genes in *Listeria monocytogenes* in biofilm and planktonic forms. Int J Pure Appl Sci 4(1):21–27
- Zameer F, Gopal S, Krohne G, Kreft J (2009) Development of a biofilm model for *Listeria monocytogenes*. World J Microbiol Biotechnol 26(6): 1143–1147. <https://doi.org/10.1007/s11274-009-0271-4>
- Zameer F, Kreft J, Gopal S (2010) Interaction of the dual species biofilms of *Listeria monocytogenes* and *Staphylococcus epidermidis*. J Food Saf 30(4):954–968. <https://doi.org/10.1016/j.ijfoodmicro.2008.05.006>
- Zameer F, Rukmangada MS, Chauhan JB, Khanum SA, Kumar P, Devi AT, Prasad N, Dhananjaya MN BL (2016) Evaluation of adhesive and anti-adhesive properties of *Pseudomonas aeruginosa* biofilms and its inhibition by herbal plants. Iran J Microbiol 8(2):108–119