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Original Article

Piperine treatment suppresses *Helicobacter pylori* toxin entry in to gastric epithelium and minimizes β-catenin mediated oncogenesis and IL-8 secretion *in vitro*

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Abstract: *Helicobacter pylori* related gastric cancer initiation has been studied widely. The objective of our present study was to evaluate the effect of a single compound piperine on *H. pylori* infection and its anti-inflammatory and anti-cancer effects *in vitro*. Cytotoxicity was tested by Ez-cytox cell viability assay kit. Effects of piperine on *H. pylori* toxin gene expression and IL-8 expression in mammalian cells during infection were assessed by RT-PCR. Effects of piperine on toxin entry into host cells, E-cadherin cleavage by *H. pylori*, and the changes in *H. pylori* mediated β-catenin expression and IL-8 secretion were determined by immunoblotting. Piperine treatment restrained the entry of CagA and VacA into AGS cells. Piperine administration in *H. pylori* infection reduced E-cadherin cleavage in stomach epithelium. In addition, *H. pylori* induced β-catenin up-regulation was reduced. Piperine administration impaired IL-8 secretion in *H. pylori*-infected gastric epithelial cells. As we reported previously piperine restrained *H. pylori* motility. The possible reason behind the *H. pylori* inhibition mechanism of piperine could be the dwindled motility, which weakened *H. pylori* adhesion to gastric epithelial cells. The reduced adhesion decreased the toxin entry thereby secreting less amount of IL-8. In addition, piperine treatment suppressed *H. pylori* protease led to reduction of E-cadherin cleavage and β-catenin expression resulting in diminished β-catenin translocation into the nucleus thus decreasing the risk of oncogenesis. To our knowledge, this is the preliminary report of piperine mediated *H. pylori* infection control on gastric epithelial cells *in-vitro*.

Keywords: *Helicobacter pylori*, piperine, gastric cancer, E-cadherin, β-catenin, interleukin-8

Introduction

Gastric cancer is the second major common fatal malignancy in the world [1]. Apart from general gastric cancers, research on microorganism induced gastric cancers has been not examined intensively and now researchers are concentrating on microbiologically induced oncogenesis in detail. Eighteen percent of gastric cancer cases diagnosed are thought to be induced by microbes [2]. After discovery of *Helicobacter pylori* by Marshall and Warren from a patient with gastritis, studies on gastric microbiology bloomed [3]. *H. pylori*-induced gastric cancer tops the list of bacteria induced oncogenesis hence researchers are concentrating more on gastrointestinal cancer induced by *H. pylori*. Numerous reports suggest that the onset of *H. pylori* infection in childhood occurs via blood relations such as father, mother, and siblings [4] and approximately half of the world population harbor this pathogen [5]. *H. pylori* plays an important role in patients with chronic gastritis, peptic ulcers, gastric adenocarcinomas, and gastric mucosa-associated lymphoid tissue lymphomas [6, 7]. *H. pylori* is known to produce several virulence factors. For instance CagA, VacA, peptidoglycan and several adhesion factors, which are considered to be the most important virulence factors [8]. WHO declared *H. pylori* as a carcinogen, since it is associated with gastric adenocarcinoma [9]. After a successful infection, a variety of virulence factors are translocating into gastric epi-

thelial cells [8]. Cytotoxic protein CagA is translocated into the gastric epithelial cells via type IV secretion system [10] and that is considered to be the greatest cause for the initiation of gastric cancer.

Translocated CagA is localized in the plasma membrane and is phosphorylated on a tyrosine residue of the CagA protein. Phosphorylated CagA influences various signal transduction mechanisms that lead to the initiation of gastric cancer [11]. The other major virulent toxin VacA secreted by *H. pylori* is translocated, which lead to the formation of vacuoles in gastric epithelial cells. In addition, translocated VacA interacts with mitochondria resulting in apoptosis [12]. Another major harmful role of CagA is its interaction with the E-cadherin and β-catenin complex [13]. E-cadherin is a dimer and forming the adherens junction complex. E-cadherin mediates cell-to-cell adhesion resulting in prevention of tumor invasion and metastasis. Moreover, E-cadherin is known for its tumor suppressor function and found to be suppressed in gastric cancer. In contrast, the E-cadherin associated protein, β-catenin, is also known as a transcription factor for oncogenes. Free cytoplasmic β-catenin is rapidly phosphorylated by glycogen synthase kinase-3β (GSK-3β) in the adenomatous polyposis coli (APC)/GSK-3β complex and it is degraded by the ubiquitin-proteasome pathway. Several signal transduction mechanisms can block the free β-catenin degradation in the cytoplasm of gastric epithelial cells with CagA, which injected CagA interacts with E-cadherin/β-catenin complex resulting in the release of free β-catenin. Furthermore, CagA inhibits β-catenin degradation [14]. This is one of the best established *H. pylori* induced gastric oncogenesis pathways. *H. pylori* interact with the extracellular domain of E-cadherin and induced E-cadherin cleavage, resulting in E-cadherin shedding. Loss of E-cadherin causes the detachment of adjacent cells, invasion, and metastasis of cancer cells [15]. *H. pylori* induce the secretion of IL-8 from infected gastric epithelial cells that lead to inflammation [16]. Most importantly, injection of CagA protein by the type IV secretion system provokes IL-8 secretion [17]. Based on the presence of cagPAI, *H. pylori* is divided into CagA positive and negative groups. CagA positive strains induce higher IL-8 secretion compared to the CagA negative strains [18]. Apart from the major virulent toxins, the other virulent factors such as peptidoglycans and outer membrane proteins (OMP) play a role in inducing inflammation [18, 19].

Due to changes in food and living style, people are more prone to various diseases and disorders particularly cancer. Intake of natural foods like vegetables and fruits, results in a decreased incidence of cancer [20]. Researchers are now focusing on the extraction of natural compounds for many clinical investigations. Reports suggest that intake of vegetables more than 400 g/day will reduce cancer up to 20% [20]. Food supplements containing bio-active compounds are now considered as an important substance studied extensively for anticancer activities. Hence, this study was proposed to investigate a natural compound, in the reduction of cancer risks and action against *H. pylori* infection.

Black pepper (*Piper nigrum*) is well-known as the “king of spices” and has been reported to be having protective effects on common colds, coughs, throat diseases, intermittent fevers, colic, and dysentery. It has been used to treat various inflammations [21]. Piperine, a nitrogenous substance that is profusely present in black pepper, is thought to exert its function mainly in the gastrointestinal system [22]. Previous studies have reported that piperine has a role in anticancer activity in various cancer cell lines, such as the inhibition of lung metastasis [23], and inhibition of prostate cancer cell proliferation [24]. As we reported previously, piperine inhibits bacterial growth and prevents the adhesion to gastric epithelial cells. Based on this study, we hypothesized that, if piperine restrains bacterial growth and adhesion, it may also have a role in treating *H. pylori* infection. To the best of our knowledge, this is the first report on the inhibitory action of piperine on *H. pylori* infection *in vitro*.

Materials and methods

Bacterial and mammalian cells used in this study

H. pylori reference strains 60190, NCTC 11637, Tx30a strains from the American Type Cell Collection (ATCC, Manassas, VA, USA) were grown on Brucella agar (Becton Dickinson, Braintree, MA, USA) supplemented with 10%

fetal bovine serum (FBS; Gibco, Long Island, NY, USA) and *H. pylori* selective supplement (vancomycin - 10.0 μg/mL, cefsulodin - 5.0 μg/mL, trimethoprim - 5.0 μg/mL, amphotericin B - 5.0 μg/mL) (Oxoid, Hampshire, England) at 37°C in a 5% CO_2 humidified incubators. Strains of 60190 (ATCC 49503) and NTCC 11637 (ATCC 43504) express an intact and functional cagPaI and possess s1/m1 vacA toxin whereas the strain Tx30a (ATCC 51932) expresses s2m2 vacA toxin but does not possess the cagPaI. AGS-gastric adenocarcinoma cell lines purchased from Korea Cell Line Bank (KCLB, Seoul, Korea) and HT-29 cell line provided generously by Prof. Ki-Jong Rhee (Department of Biomedical Laboratory Science, Yonsei University) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% FBS and 100 μg/mL streptomycin, 100 U/mL penicillin (Gibco).

Cytotoxicity of piperine on AGS cell lines

A colorimetric EZ- Cytox cell viability assay kit (ITSBIO, Seoul, Korea) was used to measure the cell viability in response to piperine treatment described by the manufacturer. This assay was based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase system, which belongs to the respiratory chain of the mitochondria and active only in the viable cells. Therefore, the amount of formazan dye is directly proportional to the number of living cells. Various concentration of piperine (Sigma Aldrich, St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich) was added in a 96-well plate at a density of 2×10^4/well for 12 or 24 hours, cells were incubated with 10 μL of EZ-Cytox kit reagent for one hour. (Becton Dickinson). Absorbance was measured at 450 nm using a NanoQuant spectrophotometer (Infinite M200, Tecan Austria GmbH, Grödig, Austria) and culture medium was used as a blank. The percent reduction in mitochondrial succinate *dehydrogenase activity was calculated relative to cells treated with medium.*

Bacterial co-culture with piperine treatment

AGS-gastric adenocarcinoma cells or HT-29-human colon cancer cell lines were maintained as described before. Cells were seeded 48 hours before infection and serum-starved for 12 hours at a final density of 4×10^6. Agar grown *H. pylori* was harvested and washed with Dulbecco's phosphate buffered saline (DPBS, Gibco). Bacterial cells were resuspended in DMEM without antibiotics and serum and added to the monolayer at a multiplicity of infection of 100 in the presence or absence of 100 μM of piperine for 3, 6, 12 or 24 hours, respectively. Vehicle control maintained by equal amounts of DMSO was also added to the monolayer. After an indicated period of the infection the monolayer was washed with PBS for three times. Infected cells were lysed by 0.1% saponin in PBS mixed with protease inhibitors followed by incubation for 10 minutes at room temperature. Finally, the cell lysate was centrifuged at 10,000 rpm for 10 minutes at 4°C, and supernatant contained proteins were isolated.

Light microscopy imaging for vacuolation

AGS cells and *H. pylori* 60190 were used in this experiment. AGS cells were harvested and 2×10^6 cells were seeded in 10 cm cell culture dish for 24 hours before the experiment. Then the host cells were co-cultured with *H. pylori* at an MOI of 100 in the presence or absence of 100 μM of piperine or an equal volume of DMSO as a vehicle control. After 24 hours of infection, the cells were washed with sterile PBS thrice to remove the bacteria debris and the images were taken in under 400 × magnification.

Influence of piperine on CagA and VacA expression

Agar grown *H. pylori* cells were harvested and washed with sterile PBS, then *H. pylori* bacterial inoculum was prepared with a turbidity value equivalent to that of a 0.5 McFarland standard (1×10^8 cells) and suspended in Brucella broth supplemented with 10% FBS. We reported that 125 μM piperine was found to be minimum inhibitory concentration (MIC) on *H. pylori* growth [25]. Therefore, we used 100 μM of piperine, a sub-MIC of piperine on *H. pylori* growth. *H. pylori* broth culture was added with 100 μM of piperine and incubated for 3 days under the microaerophillic condition at 37°C. Vehicle control was maintained adding an equal amount of DMSO to the broth culture and incubated. After 3 days, *H. pylori* cells, treated with or without piperine were harvested by centrifugation at

Table 1. List of primers used in this study

Primer	Relevant sequence	Size of amplicon	Amplification cycles	Annealing temperature	References
cagA-F	5'TGGCAGTGGGTAAGTCATA3'	278	27	45	[56]
cagA-R	3'CCTGTGAGTTGGTGTTTGT5'				
*vac*A-F	5'AAACGACAAGAAGAGATCAGT3'	291	20	57	[56]
*vac*a-R	3'CCAGCAAAAGGCCCATCAA5'				
*gal*E-F	5'ATGGCATTATTATTCACAGG3'	461	20	57	[26]
*gal*E-R	3'GCTCCATAAGGATTAATGGG5'				
IL-8-F	5'ATGACTTCCAAGCTGGCCGTGGCT3'	289	27	60	[57]
IL-8-R	3'TCTCAGCCCTCTTCAAAAACTTCTC5'				
GAPDH-F	5'TATGACAACAGCCTCAAGAT3'	316	27	57	[49]
GAPDH-R	3'AGGTCCACCACTGACACGTT5'				

3,000 rpm and washed with sterile PBS, followed by cell lysis by radio immunoprecipitation assay (RIPA) buffer containing protease inhibitors. In order to lyse the cells, the mixture was incubated in ice bath for 30 minutes and sonicated for 2 minutes with 10 seconds interval (Sonicator XL-2020, Heat Systems Ultrasonics), followed by centrifugation at 14,000 rpm at 4°C for 20 minutes and after the supernatant was separated.

Measurement of toxin and IL-8 secretion in culture supernatant

AGS cells infected with *H. pylori* at an MOI of 100 in the presence or absence of 100 μM of piperine and vehicle control maintained by equal amounts of DMSO was added to the monolayer. After 12 or 24 hours, the supernatant was collected and centrifuged for 3,000 rpm at 4°C for 10 minutes to remove the host cells and cell debris. After that the supernatant was concentrated 10 fold using 3 kDa cut-off Amicon centrifugal filters (Millipore) at 3,000 rpm for 2 hours at 4°C. Samples were prepared mixing equal concentration of the protein with Lamelli sample buffer (Biorad) and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Reverse transcriptase polymerase chain reaction

H. pylori 60190 bacterial inoculums equipped with a turbidity comparable to that of a 0.5 McFarland standard (1×10^8 cells/mL) was grown in Brucella broth supplemented with 10% FBS in the presence or absence of piperine for three days at 37°C on a humidity chamber containing CO_2 incubator. The bacterial cultures were treated with 50, 70 and 100 μM piperine respectively and vehicle control was also maintained. AGS cells were used in this study to analyze the expression level of IL-8 and GAPDH. AGS cells were co-cultured with *H. pylori* at an MOI of 100 in the presence or absence of 100 μM of piperine or an equal volume of DMSO as a vehicle control. One hundred μM of piperine was administered to the host cells to determine whether it has any role on the IL-8 secretion. After 3, 6, 12 or 24 hours, the infected host cells were treated with gentamicin (200 μg/mL) for an hour to remove the adhering bacteria from the host cells. TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA and the concentration was determined by NanoQuant spectrophotometer. Twenty five nano gram of random hexamers (Invitrogen) was mixed with 2 μg of total RNA and cDNA was synthesized using 200 U of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). PCR reactions were performed in a total volume of 20 μl consisting of 2 μl of 1/10 diluted cDNA, 2 μl of 10X PCR buffer (Tris-HCl [pH 9.0], 20 mM $MgCl_2$, $[NH_4]_2SO_4$), 2.5 mM dNTPs, 20 pmole of each primer, and 0.5 U G-*Taq* DNA polymerase (Cosmo Genetech, Seoul, Korea). PCR amplifications were carried out on a PTC-200 Peltier Thermal Cycler (BioRad, Toronto, Ontario) and PCR products were analyzed by electrophoresis on a 2.0% agarose gel containing 0.5 μg/mL of ethidium bromide. Gel images were captured and analyzed using the Quantity One System (Bio-Rad, Hercules, USA). The primer sequences and PCR conditions were listed in **Table 1**.

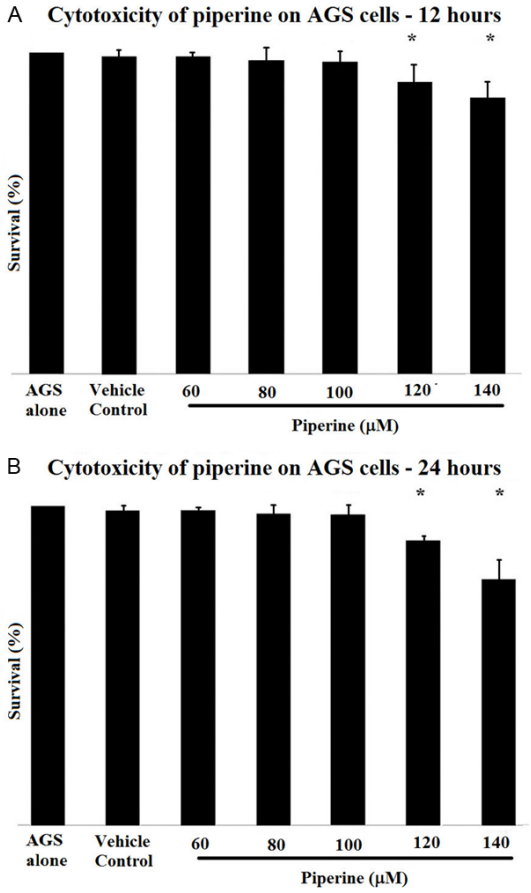

Figure 1. Cytotoxicity assay of piperine on AGS gastric adenocarcinoma cells. AGS cells were seeded in 96 well plates, and various concentrations of piperine were treated for indicated period of time. Cells were then washed with PBS and Ez-Cytox reagent was added. Cell viability of AGS cells was not significantly impaired under the concentrations of 100 µM of piperine. The represented images were from the three independent experiments and bars represent means ± SE. *$p < 0.05$.

Immunoblotting

H. pylori cells were harvested, washed with sterile PBS and lysed by RIPA buffer (Millipore, Billerica, MA, USA) for 30 minutes on ice bath and sonicated for 2 minutes with 10 second intervals (Sonicator XL-2020, Heat Systems Ultrasonics, Pittsburgh, PA, USA) followed by centrifugation at 14,000 rpm for 15 minutes at 4°C. Protein concentrations were determined based on Lowri's method (Biorad) using and NanoQuant spectrophotometer. Mammalian cell lysates were harvested and quantified. The proteins were separated by SDS-PAGE and transferred to BioTrace nitrocellulose membranes (Pall Corporation, Ann Arbor, MI, USA) by standard western blotting protocol. After protein transfer, the membranes were then blocked with 5% w/v skim milk dissolved in PBS and 0.025% Tween 20 (PBST) followed by analysis with primary antibodies as indicated and incubated overnight at 4°C. Horse-radish peroxidase tagged secondary antibody (IgG) was added and immunoreactivity was detected using EZ-Western Lumi Femto (Daeil lab service, Seoul, South Korea) and the signals were detected using a Fusion Solo Detector (Vilber Lourmat, Marne La Vallee, France).

Antibodies

Following antibodies were used to detect the target proteins; monoclonal mouse anti-CagA, polyclonal rabbit anti-VacA, monoclonal mouse anti-β-actin, monoclonal mouse anti-IL-8, horseradish peroxidase conjugated anti-rabbit IgG secondary antibody, anti-mouse IgG secondary antibody. All were purchased from Santa Cruz biotechnology. Monoclonal rabbit anti-caspase 8, monoclonal rabbit anti-caspase 9, monoclonal rabbit anti-XIAP, polyclonal rabbit anti- PARP, polyclonal rabbit anti-survivin, monoclonal mouse anti-E-cadherin, monoclonal rabbit anti-β-catenin was purchased from Cell Signalling Technology (Cell Signaling, Beverly, MA, USA). Anti-*H. pylori* whole cell antibody (rabbit polyclonal) was produced in our lab as reported previously [26].

Statistical analysis

Data were analyzed using the Student's t-test and expressed as mean values of at least three independent replications. Differences were considered to be highly statistically significant when $p < 0.01$ and significant at $p < 0.05$.

Results

Assessment of piperine cytotoxicity on AGS cells

A colorimetric EZ-Cytox cell viability assay kit was used to determine whether piperine exhibits cytotoxicity on the AGS gastric adenocarcinoma cell line. AGS cells were treated with piperine concentration ranging from 60 to 140 µM and cytotoxicity determined after 12 or 24 hours (**Figure 1A**, **1B**). Concentrations up to 100 µM of piperine showed no cytotoxicity on AGS cells and the *p* value was not statistically

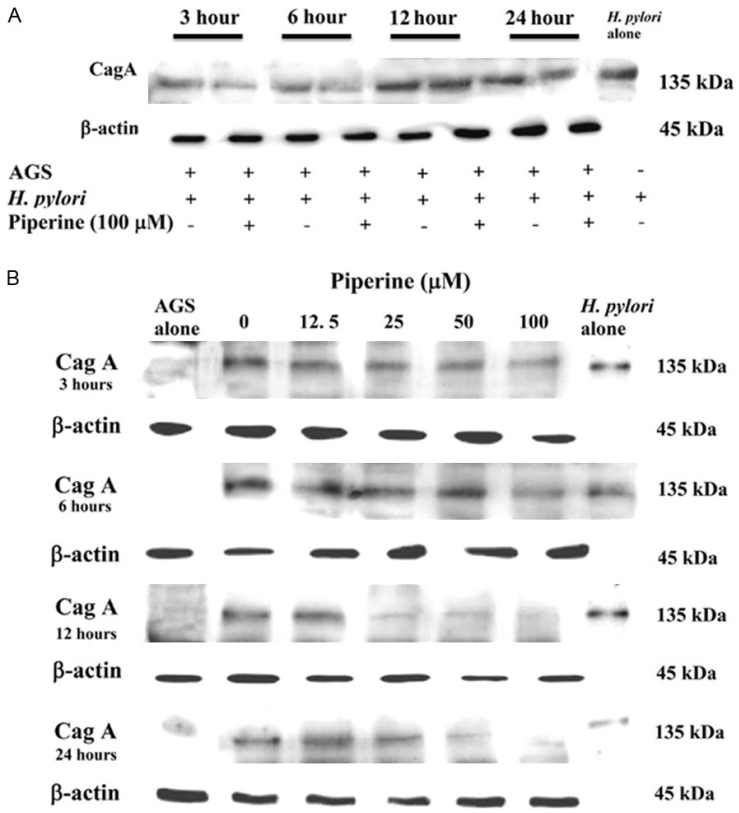

Figure 2. Effect of piperine administration in CagA injection. AGS cells were infected with *H. pylori* 60190 in the presence or absence of 100 μM of piperine for indicated period of time and harvested cell lysates were immunoblotted. A. Piperine administration (100 μM) inhibits CagA injection into AGS cells compared to piperine untreated, DMSO-treated *H. pylori* infection control. B. Piperine administration inhibits CagA injection into AGS cells dose-dependently. AGS cells were infected with *H. pylori* for indicated period of time in the presence of various concentrations of piperine (25, 50, 75 or 100 μM) or without piperine. Piperine (100 μM) treated cells have less CagA compared to untreated infection control. The CagA toxin injection was decreased from 50 μM of piperine administration and impaired completely at 100 μM. β-actin, loading control. The image is representative of three independent experiments.

significant. Concentration above 100 μM of piperine treatment caused cytotoxicity in a dose-dependent manner. Hence, 100 μM of piperine was used for all subsequent experiments.

Inhibition of CagA protein translocation

Cytotoxic protein CagA injected into host gastric epithelial cells via type IV secretion system led to a moderate numerous signal transduction pathways. Piperine inhibited bacterial growth and reduced adhesion to gastric epithelial cells as we reported previously [25]. To determine whether piperine influenced CagA toxin entry into cells, AGS cells were infected with *H. pylori* at a MOI of 100 in the presence or absence of 100 μM of piperine for 3, 6, 12 and 24 hours. As shown in **Figure 2A**, CagA protein injection was impaired, compared to piperine untreated and DMSO treated *H. pylori* infection control determined by immunoblotting. In addition, various concentrations of piperine were administered (12.5, 25, 50 or 100 μM) to evaluate the efficiency of piperine and CagA injection. The CagA injection was decreased at 25 μM and inhibited completely at 100 μM concentration of piperine (**Figure 2B**). Piperine suppressed CagA injection in AGS cells in a dose dependent manner.

Restrainment of VacA translocation and vacuolation

H. pylori secretes VacA by type V autotransporter secretion system and localized on the membrane of gastric epithelial cells. It causes internalization leading to a vacuolation characterized by accumulation of large vesicles. Immunoblotting data represented in **Figure 3A**, shows that administration of piperine restrained the toxin translocation into gastric adenocarcinoma cells, compared to piperine untreated and DMSO treated *H. pylori* infection control. To assess piperine mediated impairment of VacA entry, AGS cells were infected with *H. pylori* 60190 in the presence or absence of 100 μM piperine for 24 hours and imaged under light microscopy. DMSO treated uninfected control was also maintained (**Figure 3B**). Piperine untreated, *H. pylori* infected AGS cells exhibited severe vacuolation shown magnified under 400-X magnification (**Figure 3C**). In contrast, piperine treatment inhibited vacuolation formation in the *H. pylori* infected AGS cells (**Figure 3D**). Light microscopy substantiated the finding that piperine inhibited VacA translocation into AGS cells, from immunoblotting.

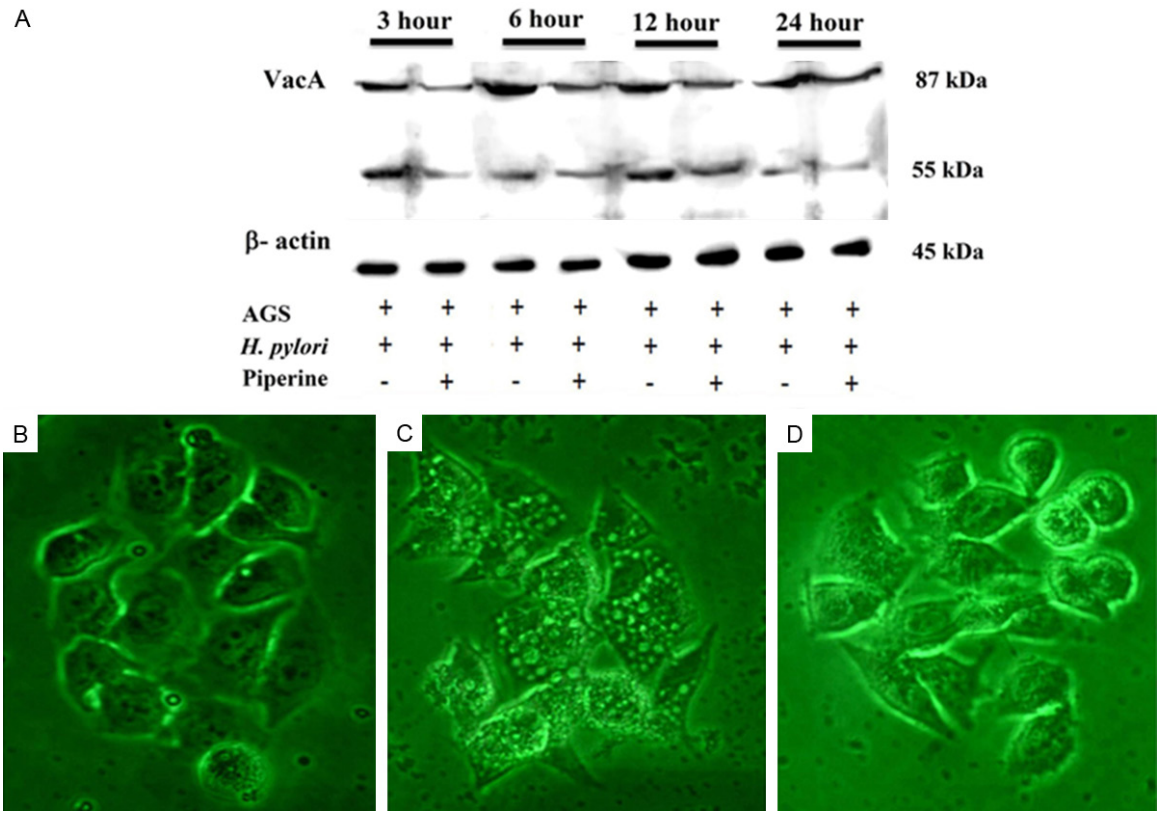

Figure 3. Effect of piperine administration in VacA translocation and vacuolation. AGS cells were infected with *H. pylori* 60190 in the presence or absence of 100 μM of piperine for indicated period of time and harvested cell lysates were immunoblotted. A. Effect of piperine on VacA translocation. Piperine administration (100 μM) inhibits VacA translocation into AGS cells compared to piperine untreated, DMSO-treated *H. pylori* infection control. B-D. Effect of piperine on vacuolation. AGS cells were infected with *H. pylori* for 24 hours in the presence or absence of 100 μM of piperine and the cells were observed (400 ×) under the light microscope imaging. Piperine untreated *H. pylori*-infected cells have shown intense vacuoles inside of gastric adenocarcinoma cells, but 100 μM piperine administration restrains vacuolation completely. B. Uninfected. C. AGS cells infected with *H. pylori* 60190. D. AGS cells infected with *H. pylori* 60190 in the presence of 100 μM of piperine. The image is representative of three independent experiments.

CagA or VacA protein synthesis

Piperine diminished CagA injection and VacA translocation into the AGS cells. Kim SH et al., [26] reported that anthocyanin down-regulated synthesis and secretion of *H. pylori* toxins. Thus, we tested the possibility that piperine may exhibit similar effects on toxin synthesis and secretion. Therefore, *H. pylori* were treated with 100 μM of piperine and RT-PCR, and immunoblotting were performed to determine whether it had a role on toxin biosynthesis. RT-PCR (**Figure 4A**) and immunoblotting (**Figure 4B**) data revealed that piperine did not influence toxin expression or synthesis as compared to the DMSO treated vehicle control. The *cag*A, *vac*A mRNA were normalized to those of the house keeping gene *gal*E and CagA, VacA protein levels were normalized to those of the whole cell antibody as described before [26]. Thus, piperine does not impair toxin synthesis.

Inhibition of E-cadherin cleavage and β-catenin expression

H. pylori infection led to ectodomain cleavage of E-cadherin. E-cadherin formed the adherence junction and was reported as a tumor suppressor protein. Ectodomain cleavage of E-cadherin resulted in bacterial pass through to the intercellular space. At the same time, *H. pylori* infection induced a nuclear accumulation of β-catenin. To determine whether piperine had an inhibitory role on *H. pylori* mediated E-cadherin cleavage or *H. pylori* mediated β-catenin induction, HT-29 and Caco2 cells

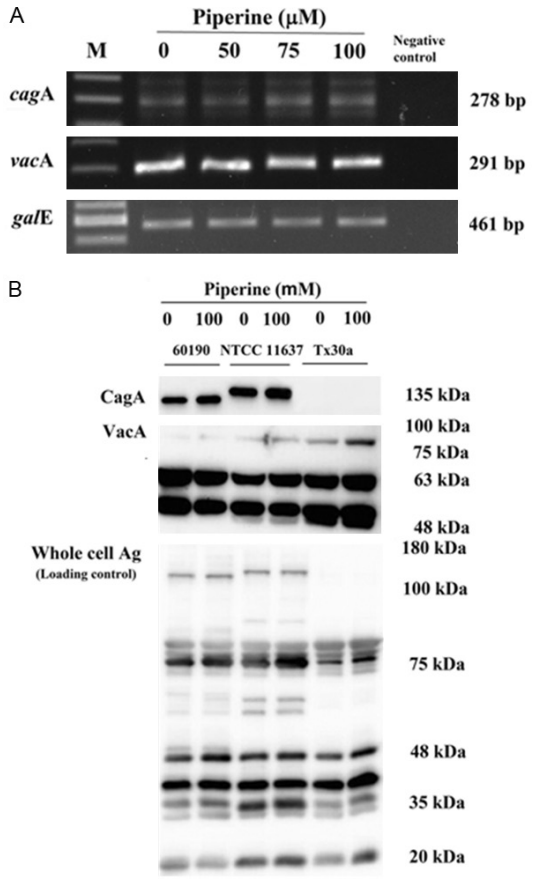

Figure 4. Effect of piperine on *H. pylori* toxin transcription and translation. *H. pylori* 60190 was treated with 100 μM of piperine for 3 days, total RNA and proteins were isolated. A. Influence of piperine on *H. pylori* toxin gene expression. RT-PCR was performed for *cag*A, *vac*A expression to determine the influence of piperine on *H. pylori* mRNA level. *gal*E was used as a loading control. B. Influence of piperine on *H. pylori* toxin synthesis. *H. pylori* 60190, NTCC 11637, Tx30a were treated with 100 μM of piperine for 3 days and harvested cell lysates were immunoblotting. Piperine (100 μM) had no influence on both CagA and VacA synthesis. *H. pylori* 60190 whole cell antigen was used as a loading control. The image is representative of three independent experiments.

were co-cultured with *H. pylori* in the presence or absence of piperine (100 μM) for 3, 6, 12 and 24 hours respectively. Full-length 120 kDa E-cadherin was cleaved by *H. pylori* generating 80 kDa fragments [27] that lessened the adhesion to the neighbor cells. Piperine administration diminished E-cadherin cleavage which decreased 80 kDa fragments and increased full length 120 kDa E-cadherin protein, as determined by immunoblotting (**Figure 5A**), compared to piperine untreated, DMSO treated *H. pylori* infected control. As stated before *H. pylori* infected cells express high level of β-catenin, compared to uninfected cells. Piperine administration reduces β-catenin expression compared to the piperine untreated *H. pylori* infected AGS cells (**Figure 5B**). Hoy *et al.*, [28] reported that *H. pylori* induced E-cadherin cleavage was mediated by the *H. pylori* secreted protease called HtrA and piperine administration inhibited the E-cadherin cleavage and suppressed induced β-catenin expression in infected host cells. We suggest that inhibition of E-cadherin cleavage resulted in the inhibition of disruption of E-cadherin/β-catenin complex, which would prevent gastric cancer initiation.

Inhibition of H. pylori infection mediated IL-8 secretion

H. pylori infection in gastric epithelial cells led to a high level of IL-8 secretion. To determine whether piperine had a role on *H. pylori* mediated IL-8 expression or synthesis, AGS cells and HT-29 cells were co-cultured with *H. pylori* 60190 ($CagA^+/VacA^+$) and *H. pylori* Tx30a ($CagA^-/VacA^+$) in the presence or absence of 100 μM of piperine for 3, 6, 12 and 24 hours. As depicted in **Figure 6**, piperine suppressed IL-8 mRNA expression determined by RT-PCR (**Figure 6A**) and IL-8 protein synthesis determined by immunoblotting (**Figure 6B**) in *H. pylori* infected host cells. In addition, IL-8 expression and secretion were elevated more in the CagA positive *H. pylori* infected cells than CagA negative *H. pylori* infected cells. Piperine impaired IL-8 expression and synthesis in cell lysate. To determine whether piperine had a role on IL-8 secretion in supernatant, host cells were co-cultured with *H. pylori* 60190 for 12 or 24 hours in the presence or absence of 100 μM of piperine. Then the supernatant was collected and concentrated 10 fold. Piperine treated host cells secreted less IL-8 (**Figure 7C**) compared to the untreated cells, which was confirmed by immunoblotting. These observations were similar to that of observed in synthesis of IL-8 in the cell lysate. In addition, *H. pylori* toxin secretion (CagA, VacA) was also diminished (**Figure 7A**, **7B**) in the supernatant. Piperine administration inhibited IL-8 secretion in cell lysate and supernatant and reduced *H. pylori* toxin secretion in supernatant.

Discussion

Numerous chemotherapeutic agents are available for cancer treatment though these agents possess cytotoxic effects on normal cells [29].

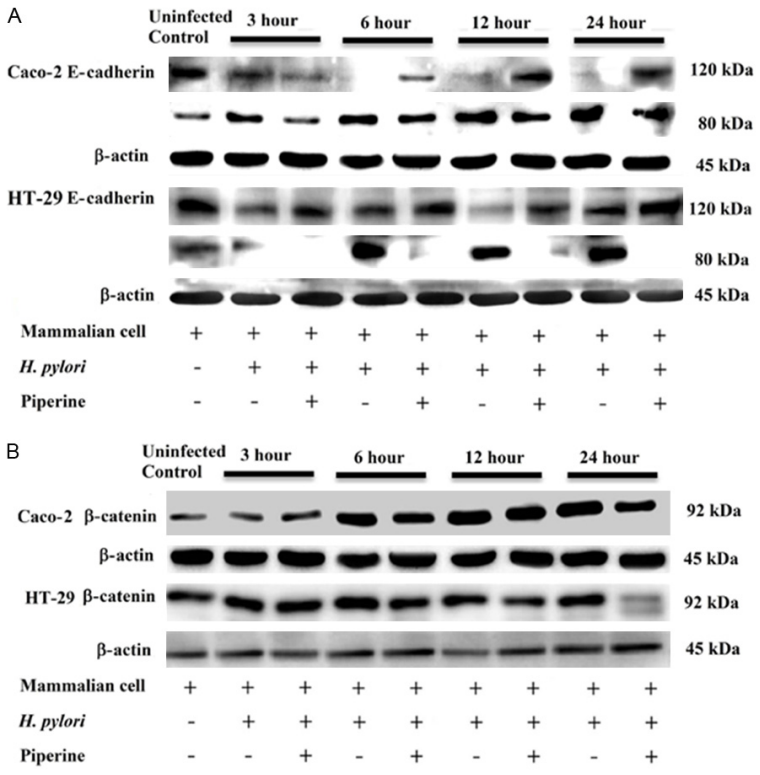

Figure 5. Effect of piperine on *H. pylori* mediated E-cadherin cleavage and β-catenin level. Caco-2 or HT-29 cells were co-cultured with *H. pylori* 60190 in the presence or absence of 100 μM of piperine for indicated period of time and harvested cell lysates were immunoblotted. A. Effect of piperine on *H. pylori* mediated E-cadherin cleavage. Piperine inhibited E-cadherin cleavage and prevented 80 kDa shedding *H. pylori* infected host cells compared to the piperine untreated DMSO-treated *H. pylori* infected host cells. B. Effect of piperine on *H. pylori* mediated β-catenin upregulation. Piperine inhibited increased level of β-catenin in *H. pylori* infected host cells compared to the piperine untreated DMSO-treated *H. pylori* infected host cells. β-actin was used as a loading control. The image is representative of three independent experiments.

Several studies report that compounds from natural resources and nutritional supplements have bioactivity against cancer [30]. Piperine is a natural compound present in black pepper used as a food enhancer and reported as an anticancer compound [31, 32]. This study demonstrated the inhibitory effects of piperine on *H. pylori* infection and their pathogenesis, especially on toxin entry into host cells and vacuolation, inhibition of *H. pylori* mediated E-cadherin cleavage and β-catenin expression, and down-regulation of IL-8 secretion. As reported, the drug resistance of *H. pylori* has been increasing steadily over time [33]. Based on this view, we aimed to explore the natural compound, which might act against *H. pylori*, as piperine has been reported as a protective agent against gastric ulcers [34].

It has been reported that various natural compounds present in food supplements hinder *H. pylori* growth [35], adhesion [36], or toxin secretion [37]. Before piperine administration in mammalian cells during infection, it is neccesary to ensure the cytotoxicity of piperine on mammalian cells. One hundred μM of piperine found to be non-cytotoxic and was used for consequent experiments. Immunoblot revealed that administrated piperine inhibited CagA injection and VacA translocation into the AGS cells. As reported previously toxic protein CagA was injected by type IV secretion system [37], and then CagA was phosphorylated by host Src kinase, interacted with the host SHP2, and involved in various signal transduction mechanisms, which resulted in the gastric cancer initiation [38]. Data from this study strongly recommended that administered piperine inhibited CagA injection in a dose dependent manner and it might have a role on inhibition of initiation of gastric cancer by CagA injection impairment. The other major virulent toxin VacA is secreted by *H. pylori* type V secretion system and translocated into the gastric epithelial cells. Translocated VacA toxin is internalized to form endosome, which interact with the host mitochondria resulting in the release of cytochrome C that causes cell death [39]. *H. pylori* infection is also known for its vacuolation in the infected gastric epithelial cells [40]. One hundred μM of piperine inhibited VacA translocation into the gastric epithelial cells and was determined by the immunoblotting. This showed that piperine might have blocked the entry of VacA toxin thereby preventing the cell death and gastritis. Light microscopy imaging and immunoblotting data strongly implied that piperine has an inhibitory role on VacA translocation into the gastric epithelial cells. To better understand the piperine influenced toxin entry

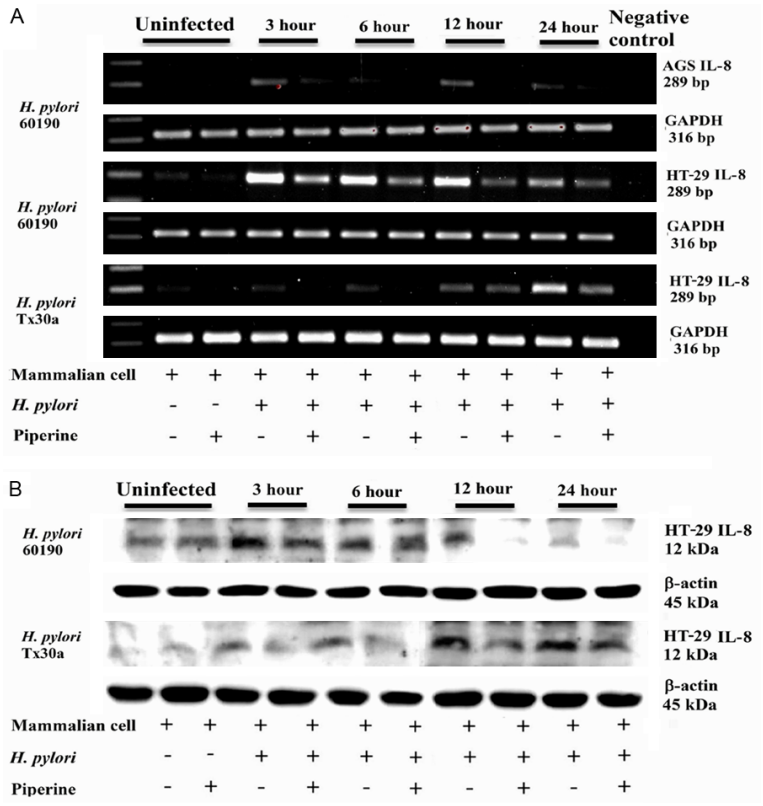

Figure 6. Effect of piperine on IL-8 expression and synthesis. AGS or HT-29 cells were infected with *H. pylori* for indicated period of time in the presence or absence of 100 μM piperine. A. Effect of piperine on IL-8 gene expression in host cells in response to *H. pylori* infection. RT-PCR was performed for IL-8 mRNA expression and piperine suppressed IL-8 expression in *H. pylori* infected host cells compared to the piperine untreated DMSO-treated *H. pylori* infected host cells. GADPH was used as a loading control. B. Effect of piperine on IL-8 synthesis in host cells in response to *H. pylori* infection. Total proteins were isolated and immunoblot was performed to determine IL-8 expression. Piperine suppressed IL-8 synthesis in *H. pylori* infected host cells compared to the piperine untreated DMSO-treated *H. pylori* infected host cells. β-actin was used as a loading control. The image is representative of three independent experiments.

impairment, we hypothesized that piperine might have had a direct role on expression or synthesis of both toxins upon piperine treatment. However, toxin entry impairment was independent of the direct action of piperine on toxin gene expression and synthesis. The possible reason behind the inhibition of toxin entry might be the interaction of piperine with secretion apparatus or gene expression, which was the crucial for toxin injection into the host cell. It has been reported that natural compounds or synthesized compounds have influenced type III secretion system and its regulators [41, 42].

Piperine impairment action on flagellar regulatory synthetic gene *flh*A and flagellar hook gene *flg*E caused dwindled motility on *H. pylori* [25]. FlhA involved in *H. pylori* flagellum assembly, the secretion of flagellum compounds and the diverse role of non-flagellum secretion system apparatus [43]. Published reports supported our observations that piperine might have an influence on secretion system apparatus. Either the interaction of piperine with the secretion system apparatus or its influence on gene expression of secretion system might be the reason for the impaired toxin entry. *H. pylori* targets on dimer protein E-cadherin and *H. pylori* HtrA protease cleave the ectodomain part of E-cadherin, as a consequence of disruption of intracellular adhesion [28]. Piperine actively involved in inhibiting *H. pylori* mediated E-cadherin cleavage and reduced in E-cadherin shedding. The possible reason behind this phenomenon was that *H. pylori* HtrA is reported as an enzyme protease that cleaves ectodomain part of E-cadherin, which results in detachment from neighbor cells [44]. Piperine administration conceivably involved in the inhibition of secreted protease enzyme and inhibited E-cadherin shedding. *H. pylori* infection has been reported to initiate gastric cancer via activation of transcription factor β-catenin. Injected oncogenic CagA protein interacted and disturbed E-cadherin/β-catenin multiprotein complex leading to the activation of free β-catenin, which was translocated into nucleus to initiate oncogenes [13]. Piperine administration suppressed β-catenin protein level, which was usually up-regulated in *H. pylori*-infected cells. As stated, piperine inhibited CagA injection, led to inhibition of disturbance of the E-cadherin/β-catenin multiprotein complex and reduced free β-catenin level. It was understood that reduced β-catenin level leads to an impaired translocation into the nucleus so as to thwart oncogene initiation. In other

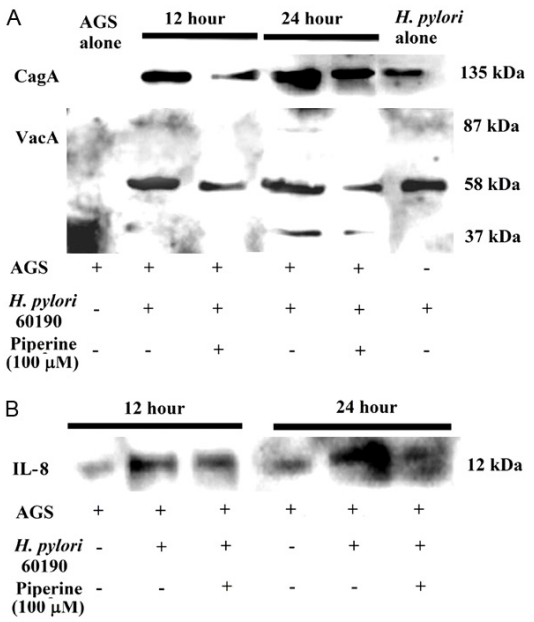

Figure 7. Effect of piperine on *H. pylori* toxin (CagA and VacA) and IL-8 secretion in the supernatant during infection. AGS cells were infected with *H. pylori* for indicated period of time in the presence or absence of 100 μM piperine and supernatant were collected then, concentrated and subjected to immunoblot. A. Effect of piperine on toxin (CagA and VacA) secretion in *H. pylori* infection. Piperine treatment impaired toxin secretion. B. Effect of piperine on IL-8 secretion. One hundred μM of piperine administration restrains IL-8 secretion. The image is representative of three independent experiments.

words, piperine supplement plausibly inhibits gastric cancer initiation by inhibiting injection of CagA.

It has been hypothesized that if piperine inhibits CagA injection then it might be involved in the inhibition of IL-8 secretion because, *H. pylori* provokes IL-8 secretion in infected gastric epithelial cells [45]. Piperine treated *H. pylori* infected host cells had shown less IL-8 secretion compared to untreated *H. pylori* infected control. In contrast to CagA positive stain (*H. pylori* 60190), AGS cells infected with CagA negative strains (*H. pylori* Tx30a) expressed relatively less IL-8 in the absence of piperine. Meanwhile, piperine treatment impaired IL-8 expression or synthesis in host cells infected with CagA positive or CagA negative strain. Numerous reports have been published that CagA injection induces IL-8 secretion. However, piperine suppressed interleukin secretion in various cells lines and reported as an anti-inflammatory agent [46-49]. Our results supported the previous observations that inhibition of CagA injection led to the impairment of IL-8 expression or synthesis. These observations supported the hypothesis that piperine actively inhibited IL-8 secretion and perhaps it could be one of the best natural compounds to treat the *H. pylori* infection. Based on these observations, our model inhibition mechanism pathway behind piperine mediated *H. pylori* infection control as followed. Piperine actively inhibited *H. pylori* growth and adhesion to gastric epithelial cells and the restraint of the biosynthetic regulator gene *flhA* (integral membrane component of the export apparatus) and flagellar hook gene *flgE* by piperine treatment might have led to the reduction of motility as we reported previously. Due to this, *H. pylori,* might have less attraction towards the gastric epithelial cells. Inhibition of adhesion led to an impaired CagA toxin injection and VacA translocation. The restrained CagA injection resulted in the inhibition of E-cadherin/β-catenin complex disruption and IL-8 secretion. Piperine has previously been reported to play a role in anticancer activities [50, 51].

Piperine has an anti-bacterial, anti-cancer and anti-inflammatory role which was critically discussed in this study. Piperine has a synergistic role on antibacterial function when it combines with ciprofloxacin. Piperine acts as an inhibitor of bacterial drug efflux pump and inhibits bacterial growth even in the multi-drug resistant strains [52-54]. In addition with food enhancer and health protective agents piperine act as an anti-bacterial agent that inhibited *H. pylori* growth and adhesion [25]. It was reported that piperine actively involved against cancer initiation [23, 29, 31]. Suppressive effects of piperine on β-catenin level in the *H. pylori*-infected cells supported the rationale that it may be one of the active agents that suppressed cancer initiation. Anti-inflammatory role of piperine decreased interleukin secretion [46-48] and also administration of piperine impaired IL-8 secretion in *H. pylori*-infected cells, resulting in inhibition of the inflammation so as to prove its ability as an anti-inflammatory agent. It is believed that daily consumption of black pepper reduces one's chance of infection and gastric cancer caused by *H. pylori*. Black pepper comprised of 5-9% piperine [55]. Roughly, about 600 mg of black pepper daily intake will prevent *H. pylori* mediated infection and gastric carcinogenesis.

In summary, the effect of piperine on *H. pylori* infection was elucidated. Based on the results observed in this study, piperine treatment reduces toxin secretion and toxin entry. Pipe rine administration during *H. pylori* infection decreased E-cadherin cleavage and down-regulated level of β-catenin. Reduction in CagA injection resulted in reduced IL-8 secretion. Piperine administration reduces *H. pylori* adhesion and motility that we reported previously. We assumed that diminished *H. pylori* adhesion to gastric cells led to an impairment of toxin entry, reduced E-cadherin cleavage, level of β-catenin and IL-8 influenced inflammation. To the best of my knowledge, this is the first report on the effect of piperine on *H. pylori* infection and against its infection with gastric adenocarcinoma cells.

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Disclosure of conflict of interest

None.

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