Molecular Mechanisms of Natural Honey Against *H. pylori* Infection Via Suppression of NF-κB and AP-1 Activation in Gastric Epithelial Cells

Mohamed M.M. Abdel-Latif\(^a, b\) and Mekky M. Abouzied\(^c\)

\(^a\)Department of Clinical Pharmacy, Faculty of Pharmacy, Assiut University, Assiut, Egypt
\(^b\)Institute of Molecular Medicine, Trinity Centre for Health Sciences, St. James’s Hospital, Dublin 8, Ireland
\(^c\)Department of Biochemistry, Faculty of Pharmacy, Minia University, Minia, Egypt

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**Background and Aims.** Natural honey has been used as a medicine since ancient times. Honey is widely known for its antibacterial properties against *H. pylori*; however, the mechanisms of its antibacterial activity are not fully known. The present study was performed to examine the molecular mechanisms by which natural honey can inhibit *H. pylori* infection in gastric epithelial cells.

**Methods.** Electrophoretic mobility shift assay was used to measure NF-κB- and AP-1-DNA binding activity. Western blotting was used to detect IκB-α and COX-2 expression.

**Results.** *H. pylori* induced NF-κB and AP-1 DNA-binding activity in gastric epithelial cells. Manuka honey inhibited *H. pylori*-induced NF-κB and AP-1 in a time- and dose-dependent manner. Maximum inhibition of *H. pylori*-induced NF-κB and AP-1 by manuka honey was observed at concentrations of 20% at 1–2 h. Pre-treatment of AGS cells with other commercial natural honeys also inhibited *H. pylori*-induced NF-κB and AP-1 DNA-binding activity. Honey prevented *H. pylori*-induced degradation of IκB-α protein and downregulated COX-2 protein levels.

**Conclusions.** Our findings suggest that natural honey exerts its inhibitory effects against *H. pylori* by inhibiting NF-κB and AP-1 activation and downregulation of COX-2 expression. These results provide new mechanistic insights into honey effects in the suppression of *H. pylori* infection.

**Key Words:** Natural honey, *H. pylori*, NF-κB, AP-1, Gastric epithelial cells.

**Introduction**

*Helicobacter pylori* infects over half of the population worldwide (1,2). This pathogen initiates an inflammatory and immune response within the gastric mucosa, which subsequently leads to the activation of cell signaling pathways causing mucosal inflammation and cancer development (3–5). The induction of the host immune response elicits the production of pro-inflammatory proteins such as transcription factors, cytokines and adhesion molecules (6,7). *H. pylori* was reported to induce transcription factors such as NF-κB and AP-1 that regulate inflammation and signalling cascades leading to carcinogenesis (8,9).

Treatment of *H. pylori* infection has become a challenge in recent years. Successful treatment with antimicrobial agents is successful in 80–90% of patients and can lead to regression of *H. pylori*-associated diseases. However, the high rate of *H. pylori* infection in developing countries, patient incompliance and antibiotic resistance against *H. pylori* merits extensive investigations (10,11). Antibiotic resistance in *H. pylori* is a growing global concern that necessitates the search for novel therapeutic agents. Several new treatment alternatives have been introduced to overcome treatment failure. Several studies around the world have shown promising activities of natural remedies against *H. pylori* in *in vitro* and *in vivo* studies (12–17).
One of these dietary natural products is honey, a natural substance formed from nectar by honeybees. Natural honey is used extensively as a health drink worldwide since ancient times. The pharmacologically active molecules in honey are flavonoids, phenolic acids and their esters, vitamins, trace elements, amino acids and proteins as well as certain enzymes including glucose oxidase, invertase and catalase (18). The revelation in the Holy Koran and documentation in the Hadith clearly referred to the effectiveness of honey in the healing of diseases for mankind (19). Honey has been the focus of research in recent years to modulate inflammation, microbial infection and cancer (20–26).

Research studies have revealed the promising effects of honey as a non-antimicrobial approach for reducing H. pylori infection (15,17,27,28). There have been a number of reports suggesting that honey can inhibit H. pylori growth in in vitro studies (19,29–33). The exact mechanism of the inhibition of H. pylori by honey is not yet well defined. The aim of the present study was to examine the molecular mechanisms by which natural honey inhibits H. pylori infection in gastric epithelial cells.

Materials and Methods

Materials

NF-κB and AP-1 consensus oligonucleotides were obtained from Promega (Promega Corp., Madison, WI). Polyclonal antibody to IκB-α was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal COX-2 antibody was purchased from Cayman Chemical Company (Ann Arbor, MI). [γ32P]ATP (35 pmol, 3000 Ci/mmol) was purchased from Amersham International (Aylesbury, UK). Poly(dI-dC) was obtained from Pharmacia (Biosystems, Milton Keynes, UK).

Manuka Honey and Commercial Honey Brands

Different commercial honey brands available in the Irish market were used in this study such as manuka honey (from New Zealand), pure acacia honey, Boyne valley honey and Healy’s natural honey. Manuka honey comes from the flowers of New Zealand’s manuka bush from bees visiting Leptospermum trees. Initial experiments were done to determine the concentrations of manuka honey or other commercial honey brands to be used in our studies. Appropriate dilutions of natural honeys were made in the cell culture medium in which AGS cells are cultured just prior to use. All subsequent in vitro studies using manuka or other natural honeys were carried out at a concentration of 20%.

H. pylori Culture

H. pylori reference strain NCTC 11638 obtained from the National Collection of Type Cultures (Colindale, UK) was used in this study. Bacteria were grown in a microaerobic humidified atmosphere on 7% lysed horse blood Columbia agar at 37°C. After 48–72 h, bacteria were harvested in PBS (pH 7.4) containing 8 mmol Na2HPO4, 1.5 mmol KH2PO4, 137 mmol NaCl and 2.7 mmol KCl or RPMI 1640 medium without antibiotics and resuspended to a concentration of 6 × 10^8 colony-forming units CFU/mL using the McFarland standard kit and used immediately.

Cell Culture and Treatments

The gastric epithelial cell line AGS was obtained from the European Collection of Animal Cell Cultures, ECACC (Porton Down, Salisbury, UK). AGS cells were grown in RPMI 1640 medium supplemented with 10% filtered fetal calf serum (FCS), 100 Units/mL penicillin, 100 μg/mL streptomycin and 2 mmol L-glutamine. AGS cells were removed from flasks by trypsin/EDTA treatment and seeded at a density 5 × 10^5 cells/mL. AGS cells were removed from flasks by trypsin/EDTA treatment and seeded at a density 5 × 10^5 cells/mL for experiments. Confluent AGS cells were pre-incubated with various concentrations of manuka honey, pure acacia honey, Boyne valley honey and Healy’s natural honey ranging from 1–20% followed by stimulation with a freshly prepared suspension of H. pylori (6 × 10^8 CFU/mL) for 2 h. The ratio of H. pylori to AGS cells is 100:1 and uninfected cells were used as a control in each experiment.

Preparation of Total Cell Extracts

Cellular extract were collected by centrifugation at 1400 rpm for 5 min. The pellet of cells was resuspended in lysis buffer containing 20 mmol Tris-HCl (pH 7.5), 1% (w/v) sodium dodecyl sulfate (SDS), 150 mmol NaCl, 1 mmol EGTA, 1 mmol EDTA, 0.5 mmol phenylmethylsulfonylfluoride (PMSF) and leupeptin (10 μg/mL) and then the cells were solubilized by boiling for 5 min. The protein concentration was determined on the cell extract by the Bradford method (34).

Western Blot Analysis

Total cell extracts (50 μg protein/lane) were resolved by electrophoresis through polyacrylamide gels using 10% separating gels according to the method of Laemmlli (35). Proteins were electrotransferred onto PVDF membrane using a semidy blotting apparatus (Atto). Blots were blocked with 5% (w/v) dried skim milk in PBS for 1 h at room temperature and then incubated for 1 h at room temperature with the appropriate primary antibody (anti-IκB-α or anti-COX-2 at a dilution of 1:1000). Blots were then incubated with anti-rabbit horseradish peroxidase conjugated secondary antibody (at a dilution of 1:1000) for 1 h at room
temperature. Immunodetection was performed by enhanced chemiluminescence.

**Nuclear Extract Preparation**

Nuclear extracts were prepared from AGS cells as described previously (36). Briefly, AGS cells were washed twice in ice-cold PBS. The cells were pelleted by centrifugation at 1400 rpm for 5 min and washed once in (1 mL) buffer A 10 mmol Hepes (pH 7.9), 1.5 mmol MgCl₂, 10 mmol KCl, 0.5 mmol PMSF and 0.5 mmol dithiothreitol (DTT) and centrifuged at 10000 rpm for 10 min. The pellet of cells was then resuspended in buffer A (20 µl) containing 0.1% (v/v) NP-40 for 10 min on ice and lysed cells were centrifuged at 10000 rpm for 10 min. The supernatant was discarded and the nuclear pellet was extracted with (15 µl) buffer C (20 mmol Hepes (pH 7.9), 420 mmol NaCl, 1.5 mmol MgCl₂, 0.2 mmol EDTA, 25% (w/v) glycerol and 0.5 mmol PMSF) for 15 min on ice. After incubation, the nuclei were centrifuged at 10000 rpm for 10 min and the supernatant was diluted with 4 volumes of buffer D (10 mmol Hepes (pH 7.9), 50 mmol KCl, 0.2 mmol EDTA, 25% (w/v) glycerol and 0.5 mmol PMSF). The nuclear extracts were used immediately or stored at −70°C until required. The protein concentration was determined on nuclear extracts by the Bradford method (34).

**Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts (4 µg of protein) were incubated with 10000 cpm of the 32P-labeled NF-κB or AP-1 oligonucleotide [that had been previously labeled with (γ32P) ATP (10 mCi/mm) at the 5'-ends with T4 polynucleotide kinase]. The assay was performed in 20 µL of binding buffer [10 mmol Tris (pH 7.5), 4% (w/v) glycerol, 5 mmol DTT, 1 mmol EDTA, 100 mmol NaCl and 0.1 mg/mL nuclease-free BSA] in the presence of 2 µg poly(dI–dC) as non-specific competitor. The reaction mixture was then incubated for 30 min at room temperature after the addition of the probe DNA. The binding reaction was terminated using a loading dye (0.25% bromophenol, 0.25% xylene cyanol, 30% (w/v) glycerol in deionized water) prior to electrophoretic separation of the DNA-protein complexes on 5% polyacrylamide gels that had been pre-electrophoresed for 30 min at 80 V. Gels were run at 150 V for 1–2 h at room temperature. After electrophoresis, the gels were dried and autoradiographed at −70°C for 24–36 h with intensifying screens. Each experiment

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**Figure 1.** (A) Effect of different concentrations of manuka honey on cell viability of AGS cells. AGS cells were treated with varying concentrations of manuka honey (1, 5, 10 and 20%) for 24. (B) Effect of different commercial honeys on cell viability of AGS cells. Cells were treated with 20% manuka honey, 20% pure acacia honey, 20% Boyne valley honey or 20% Healy’s natural honey for 1 h or 24 h. Cell viability was quantitated by PMS/MTS assay. The absorbance was read at 492 nm using an ELISA plate reader. Each experiment was repeated three times in triplicate per treatment group with similar results. Results are presented as mean ± SD. *statistical significance (p < 0.05) of honey treatment vs. untreated cells. (C) Effect of manuka honey on cell morphology. AGS cells were treated with 20% manuka honey for 1 h or 24 h. At the end of treatment, the cells were examined under the inverted microscope for morphological changes. Representative images are shown.
was performed three times with similar results and one representative result is presented.

**Cell Viability and Cytotoxicity Studies**

TAGS cells (1 × 10⁵ cells/mL) were incubated with different concentrations of manuka honey ranging from 1–20% for 24 h. In other experiments, AGS cells (1 × 10⁵ cells/mL) were incubated with 20% of manuka honey, pure acacia honey, Boyne valley honey or Healy’s natural honey for 1 h or 24 h. At the end of incubation, 20 μL of freshly prepared PMS (phenazine methosulfate)/MTS (3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) 2H-tetrazolium, inner salt; MTS) solution was added to each well of the cultured cells and the plates were incubated for 4 h at 37°C. The absorbance was read at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. AGS cells (5 × 10⁴ cells/mL) treated with manuka honey, pure acacia honey, Boyne valley honey or Healy’s natural honey at concentration of 20% for 1 h or 24 h were also examined using the normal inverted phase contrast microscope for cytotoxicity changes.

**Results**

**Effect of Natural Honey on Cell Viability**

We tested the effect of manuka honey on the growth and cell viability of at varying concentrations of 1, 5, 10 and 20% using PMS/MTS assay. Treatment of AGS cells with manuka honey showed a marked growth inhibition in a dose-dependent manner after 24 h. There was little or no growth inhibition at low concentration of manuka honey at concentrations of 1 and 5% after 24 h (Figure 1A). However, there were significant decreases in cell viability at concentrations of 10 and 20% compared with untreated cells. Incubation of AGS cells with different honeys (manuka honey, pure acacia honey, Boyne valley honey and Healy’s natural honey) for 1 h had no effect on growth or cell viability of AGS cells, whereas treatment of AGS cells with different honeys for 24 h caused a significant decrease in the number of viable cells; p < 0.05 (Figure 1B). Furthermore, incubation of AGS cells with 20% manuka honey for 1 h had no notable effect on cell morphology (Figure 1C), whereas increasing incubation time AGS cells with 20% honey for 24 h induced morphological changes.
such as abnormal nuclear morphology, apoptotic body formation and cell shrinkage, indicative of cytotoxicity, compared with untreated AGS cells. Similarly, cells treated with 20% of other commercial honey brands for 24 h showed marked morphological changes (data not shown), which is consistent with cell viability results. Therefore, pre-treatment of AGS cells with 20% honey for 1 h, the time point used in our experiments, had no effect on cell viability or morphology.

**H. pylori Induces NF-κB and AP-1**

Exposure of the gastric epithelial cell line AGS to *H. pylori* (6 × 10⁸ CFU mL⁻¹) for 2 h induces NF-κB DNA-binding activity (Figure 2A). Competition assays with × 100 fold molar excess of cold NF-κB oligonucleotide completely abolished NF-κB DNA-complex formation induced by *H. pylori* and confirmed the specificity of NF-κB DNA complex. Nuclear extract from Hut78 cells, which contain high levels of constitutive NF-κB, was used as a positive control. On the other hand, exposure of AGS cells to *H. pylori* for 2 h also induced AP-1 DNA-binding activity (Figure 2B). Competition assays with ×100-fold molar excess of cold AP-1 oligonucleotide completely abolished AP-1 DNA complex induced by *H. pylori* and confirmed the specificity of AP-1 DNA complex. Cells treated with PMA (10 ng/mL) were used as a positive control.

**Effect of Natural Honey on NF-κB Activation by H. pylori**

The potential mechanisms of the antibacterial activity of natural honey in gastric epithelial cells were investigated. Subsequent experiments were undertaken to investigate the effects of co-treatment with manuka honey or other natural honeys and *H. pylori* on NF-κB activation in gastric cells. Time-course experiments showed that the inhibition of NF-κB by manuka honey was detected as early as 30 min with a maximal inhibition at 1–2 h (Figure 3A). In dose-response experiments, AGS cells were pre-treated with varying concentrations of manuka honey ranging from 1–20% for 1 h. The medium was removed and replaced with medium containing *H. pylori* (6 × 10⁸ CFU/mL) for 2 h. Manuka honey at increasing concentrations inhibited *H. pylori*-induced NF-κB DNA-binding activity (Figure 3B). Maximal inhibition of NF-κB activation was noted at concentrations of 20% of manuka honey.

Next, we investigated the effect of commercial honeys (pure acacia honey, Boyne valley honey and Healy’s natural honey) on NF-κB activity. Pre-treatment of AGS cells with 20% commercial honey brands inhibited *H. pylori*-induced NF-κB activation (Figure 3C). Furthermore, exposure of AGS cells to 20%...
natural honeys of manuka honey, pure acacia honey, Boyne valley honey or Healy’s natural honey resulted in inhibition of IκB-α degradation induced by *H. pylori* treatment (Figure 4). Treatment of AGS cells with manuka honey or other commercial brand honeys had no effect on cell viability at tested concentrations over time exposure used.

### Effect of Natural Honey on AP-1 Activation by *H. pylori*

Further, we have examined whether honey could affect AP-1 DNA-binding activity in response to *H. pylori*. AGS cells were pre-incubated with various concentrations of manuka honey ranging from 1% to 20% for different periods of time. Manuka honey inhibited AP-1 DNA-binding activity in a time- and dose-dependent manner (Figure 5A and 5B). Maximal inhibition of *H. pylori*-induced AP-1 activation was noted at concentrations of 20%. Similarly, pure acacia honey, Boyne valley honey and Healy’s natural honey inhibited *H. pylori*-induced AP-1 binding activity (Figure 5C).

### Effect of Natural Honey on COX-2 Expression by *H. pylori*

*H. pylori* infection is associated with upregulation of COX-2 expression, which is an important key enzyme in inflammation and carcinogenesis. Exposure of AGS cells to *H. pylori* resulted in increased COX-2 protein levels. Pre-treatment of AGS cells with manuka honey, pure acacia honey, Boyne valley honey or Healy’s natural honey at concentrations of 20% resulted in downregulation of COX-2 induction by *H. pylori* (Figure 6).

### Discussion

Natural honey has been used since ancient times as a food and a medicinal remedy worldwide. Honey has received great attention recently in the medical field for its antimicrobial, anti-inflammatory and anticancer effects; however, the exact mechanism for its actions is unknown. The present study was designed to examine the activity of manuka honey and some commercial honey brands against *H. pylori* and to determine the molecular mechanisms of their antibacterial activities. We show here for the first time the mechanisms of how natural honey could inhibit *H. pylori* infection in gastric cells. Our findings demonstrated that natural honey inhibited *H. pylori*-induced NF-κB and AP-1 activities in gastric epithelial cells. The inhibition of NF-κB and AP-1 was observed at concentrations of 5% of each honey used with a maximal inhibition at concentrations of 20% honey. Little or no inhibition of NF-κB and AP-1 was seen at honey concentrations of 1%. Our findings clearly indicate that the greatest inhibition of the pro-inflammatory transcription factors NF-κB and AP-1 was achieved at higher concentrations of honey, which correlated with its *in vitro* antibacterial activity.

The inhibition of NF-κB and AP-1 transcription occurred at comparable concentrations of different kinds of honey at similar concentrations approximating 10–20% that caused inhibition of the growth of *H. pylori in vitro* (19,29–32), but these studies did not address the molecular mechanisms of such inhibition. In our study a maximum inhibition of NF-κB and AP-1 activity was observed at concentrations of 20% of natural honey tested. Ali et al. (19) reported that natural honey had an inhibitory effect on *H. pylori in vitro* at solutions of both 10 and 20% honey. al Somal et al. (29) found that manuka honey at concentrations as low as 5% completely inhibited the growth of *H. pylori*. Osato et al. (31) compared manuka honey to honeys obtained commercially from Texas and Iowa and found that all honeys at concentrations of 15% inhibited growth of all *H. pylori* isolates tested.

In this study, incubation of AGS cells for 1 h with different types of commercial honeys had no notable effect on growth or cell viability of AGS cells and upon treatment of AGS cells with honey for 24 h caused a significant reduction in cell growth (*p* < 0.05). Furthermore, manuka honey caused a decrease in cell growth in a dose-dependent manner. Notably, 10–20% of manuka produced a significant (*p* < 0.05) decrease in the growth of gastric
incubation of cultured AGS cells with honey for 24 h caused a marked alteration in cell appearance and morphology. However, no sign of cytotoxicity at concentrations of 20% of honey compared to the untreated control was observed at 1 h exposure.

Our findings clearly demonstrate that manuka honey attenuated NF-κB and AP-1 DNA binding activities in a dose- and time-dependent manner. This inhibition was coincident with the inhibition of IκB-α degradation.

Interestingly, other commercial honey brands (Shaw’s acacia honey, Boyne valley honey and Healy’s natural honey) were as efficacious as manuka honey in inhibiting NF-κB and AP-1 activities at similar concentrations approximating 10–20%. The antibacterial activity of honey varies widely among different types of honey (37,38). It has been postulated that the antimicrobial activity of honey could be attributed to several factors like the osmotic effect of honey, acidity, hydrogen peroxide content, and the phytochemical components (21,39).

H. pylori infection can colonize the gastric epithelium causing mucosal inflammation and stomach cancer via several mechanisms. One of these mechanisms is the activation of transcription factors such as NF-κB and AP-1 activities that regulate multiple cellular processes during inflammation and carcinogenesis (8,9). We have previously shown that treatment of gastric epithelial cells with CAPE inhibited NF-κB and AP-1 activation by H. pylori. Pretreatment of gastric epithelial cells with CAPE also upregulated IκB-α levels in comparison with control AGS cells (36). Similarly, Natarajan et al. (40) demonstrated that CAPE inhibited the activation of NF-κB induced by a wide variety of agents. Wu et al. (41) demonstrated that the activity of NF-κB and the expression of matrix metalloproteinase-9, IL-1β, and IL-8 in gastric cancer cells by H. pylori were reversed by CAPE treatment. In

Figure 5. Effect of natural honey on H. pylori-induced AP-1 DNA-binding activity in gastric epithelial cells. (A) Time-course of AP-1 inhibition by manuka honey. AGS cells were pre-treated with 20% manuka honey for different periods of time ranging from 15 min to 2 h followed by stimulation with H. pylori (6 × 10⁸ CFU/mL) for an additional 2 h. (B) Dose-response of AP-1 inhibition by manuka honey. AGS cells were pre-treated with varying concentrations of manuka honey ranging from 1–20% for 1 h followed by co-culture with H. pylori (6 × 10⁸ CFU/mL) for an additional 2 h. (C) Effect of commercial honey brands on AP-1 DNA-binding activity. AGS cells were pre-treated with Shaw’s acacia honey, Boyne valley honey and Healy’s natural honey at 20% or CAPE (10 μg/mL) for 1 h followed by 2 h co-culture with H. pylori (6 × 10⁸ CFU/mL). Nuclear extracts were prepared and assayed for AP-1 binding activity in an EMSA reaction. HB1, Shaw’s acacia honey; HB2, Boyne valley honey; HB3, Healy’s natural honey. Each experiment was repeated three times and a representative gel is shown.

Figure 6. Effect of natural honey on H. pylori-induced COX-2 expression. AGS cells were pre-treated with 20% of commercial honey brands [Shaw’s acacia honey (HB1), Boyne valley honey (HB2), Healy’s natural honey (HB3) or Manuka honey (HB4)] for 1 h followed by 2 h co-culture with H. pylori (6 × 10⁸ CFU/mL). Total cell extracts were prepared, separated by 10% polyacrylamide gels, blotted onto PVDF membrane and probed with anti-COX-2 antibody. Each experiment was repeated three times with similar results and a representative gel is shown.
Monogolian gerbils, CAPE significantly inhibited H. pylori-induced NF-κB activation and prevented degradation of IκB-α and phosphorylation of p65 in gastric cancer cells (42).

Various natural compounds have been shown to exhibit anti-inflammatory and anticancer activities through inactivation of NF-κB (43). Honey or its derivatives has been reported to suppress inflammation via inactivation of NF-κB and inhibition of transcription of genes for pro-inflammatory mediators such as COX-2, TNF-α, IL-6 and iNOS (36,41,43,44). No study has addressed the molecular mechanism by which honey exhibits its antimicrobial activity against *H. pylori* infection. In this study we have shown a possible molecular mechanism for NF-κB and AP-1 pathways in the preventive effects of honey against *H. pylori*. Additionally, manuka honey and commercial honey brands were capable of downregulating *H. pylori*-induced COX-2 proteins levels. In agreement with our findings, Hussein et al. (45) reported that Gelam honey inhibited the nuclear translocation and activation of NF-κB and decreased the cytosolic degradation of IκB-α with subsequent decrease of inflammatory mediators COX-2 and TNF-α in an acute inflammation rat model. Clinical intervention by natural honey may provide an approach for suppression of *H. pylori* infection. In a recent study, the consumption of honey was associated with reduced prevalence of *H. pylori* infection in 150 dyspeptic patients examined endoscopically and by the urea breath test. Positivity rate was lower by 50.6% in patients consuming honey ≥1 day weekly compared with 70.8% of the remainder (46).

In conclusion, our findings suggest that natural honey exhibited its inhibitory effects against *H. pylori* via inhibition of NF-κB and AP-1 activation, blocking IκB-α degradation and suppression of the pro-inflammatory mediator COX-2. Our data offer a novel insight into the molecular mechanisms for natural honey in reducing *H. pylori* infection. These results merit further investigation into honey effects as a promising dietary substance for amelioration of *H. pylori* infection.

**Conflict of Interest**

The authors declare no conflicts of interest.

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