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Antibacterial activities of *Anisomeles indica* constituents and their inhibition effect on *Helicobacter pylori*-induced inflammation in human gastric epithelial cells

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ABSTRACT

The antibacterial activity of *Anisomeles indica* extract, and its isolated constituents against *Helicobacter pylori* growth were examined. Among tested, ethanol extract, pure constituents ovatodiolide (OVT) followed by acteoside, isoacteoside, and terniflorin showed potent antimicrobial activity. OVT demonstrated bactericide activity against *H. pylori* reference, as well as multidrug-resistant strains. On the other side, *in vitro H. pylori*-infection model revealed that OVT inhibited the *H. pylori* bacteria adhesion and invasion to human gastric epithelial (AGS) cells. In addition, OVT inhibited the *H. pylori*-induced inflammatory response by the reduced nuclear factor (NF)-κB activation and interleukin (IL)-8 expressions in *H. pylori*-infected AGS cells. Furthermore, OVT attenuated the cytotoxin-associated gene A (CagA) functions by reduced CagA translocation, phosphorylation, and caused hummingbird phenotype of AGS cells. These results indicate that OVT might be useful as food supplement or drug development for *H. pylori* complications.

1. Introduction

As a spiral, microaerophilic, Gram-negative bacteria, *Helicobacter pylori* specifically colonises gastric epithelium, and it is the most common bacterial infection worldwide (Marshall, 2002). Everyone infected by this organism develops coexisting gastritis, which typically persists for decades. However, there are biological costs incurred by such long-term relationships. *H. pylori* infection is the strongest known risk factor for malignancies that arise within the stomach and the epidemiological studies revealed that the risk for gastric cancer conferred by *H. pylori* is approximately 75% (Herrera & Parsonnet, 2009). Previous studies indicate that inhibition of *H. pylori* infection is an appropriate target for prevention of gastric cancer (Peek & Blaser, 2002). In addition, eradication of *H. pylori* significantly decreases the risk of cancer development in infected individuals without pre-malignant lesions (Wong et al., 2004), reinforce the tenet that this organism influences early stage gastric carcinogenesis.

Even though the eradication of *H. pylori* is feasible with triple therapy composed of a proton pump inhibitor, clarithromycin (CLR), and amoxicillin (AMX) or metronidazole (MTZ), however, the successful rates are approximately 70–80%. In addition, there is a risk of a vicious cycle with the emergence of a more resistant strain, or the requirement for a more aggressive antibiotic treatment (de Boer & Tytgat, 2000). As a way to solve the problems of the expense and drug resistance encountered with antibiotic based eradication, an alternative encouraging new development is the application of natural products in treatment of *H. pylori* infection (Lee, Shin, & Hahm, 2008). Natural products/medicinal plants have aroused interest as sources of fascinating variety of structurally distinct and biologically active secondary metabolites for their potential uses as remedies for the treatment of many diseases (Rishton, 2008). *Anisomeles indica* Kuntze (Labiatae) is an erect, camphor-scented, perennial woody shrub known in Taiwan as "Yu Chen Tsao", and is commonly used in traditional medicines for various conditions including gastrointestinal disease, inflammatory skin disorder and immune system deficiencies (Huang, 2003). Previously, it is reported various biological activities on *A. indica* extracts and isolated constituents which includes inhibition of inflammatory mediators as well as tumour cell proliferation (Chen et al., 2008; Hsieh, Fang, Rao, & Tzeng, 2008; Rao, Fang,

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Hsieh, Yeh, & Tzeng, 2009). Additionally, it is also reported that ethanol extract of *A. indica* exhibited inhibition activity against *H. pylori* (Wang & Huang, 2005), getting *A. indica* against *H. pylori* infection into the focus of research. However, the exact constituents and their action mechanism of *A. indica* in *H. pylori*-infected epithelial cells have not been investigated previously. As part of our ongoing search for potential inhibitors of *H. pylori*-induced inflammation (Geethangili et al., 2010; Lai, Rao, Fang, Sing, & Tzeng, 2010), herein, we selected *A. indica* which is traditionally used as a remedy for gastrointestinal diseases in Asia.

2. Materials and methods

2.1. Reagents and materials

Cell culture media (F12), phosphate buffered saline (PBS), gentamicin, vancomycin, amphotericin B, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), and phenyl methanesulfonyl fluoride (PMSF) were obtained from Sigma Chemical Company (Saint Louis, MO, USA). Antibodies against CagA, and substrate antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fetal bovine serum (FBS), L-glutamine, and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA), lysis buffer, luciferase substrate, β-galactosidase expression vector were purchased from Promega (Madison, MA, USA). All other chemicals and reagents were of the highest grade commercially available and supplied either by Sigma–Aldrich or Merck.

2.2. Plant material, extraction and isolation of pure compounds

Whole plants of *A. indica* were collected in October 2009 from the Yuli, Hualien County in Eastern Taiwan, and a botanically identified voucher specimen (YMT-09-02) was deposited in the Herbarium of the Institute of Biotechnology, Chaoyang University of Technology, Taiwan.

The pure constituents were separated from *A. indica* following the extraction and isolation procedures. Briefly, air-dried whole plants (2.8 kg) of *A. indica* were extracted with ethanol (10 l × 4) under reflux. After exhaustive extraction, the combined extracts were concentrated under reduced pressure to give a dark brown syrup (293 g, 9.6% w/w). The crude extract was then suspended in H_2O, defatted with *n*-hexane, and then partitioned with chloroform and *n*-butanol successively. The concentrated chloroform layer (65 g) was chromatographed on a silica gel column by eluting with hexane/ethyl acetate (EtOAc) gradient, with increasing polarity, and yielded five fractions (F1–F5). Fraction F2 was subjected to silica gel column chromatography (CC) eluted with different solvents of increasing polarity (*n*-hexane/EtOAc) to afford apigenin (**1**, 16 mg) and β-sitosterol (**2**, 55 mg). Fraction F3 (2.9 g) was purified on a silica gel column using hexane/EtOAc (8:2) to yield ovatodiolide (OVT, **3**, 120 mg). The *n*-BuOH-soluble portion (124 g) was fractionated by a silica gel column, which was eluted with $CHCl_3$/MeOH (100:10 to 30:70) to six fractions (fractions 1–6). Fractions 2 and 3 were further purified by flash CC using $CHCl_3$/MeOH (8:2), to afford β-sitosterol 3-*O*-*β*-D-glucoside (**4**, 300 mg), and apigenin 7-*O*-glucoronide (**5**, 32 mg), respectively. Fraction 4 was further separated by HPLC (CH_3CN-0.1% TFA in H_2O, 17:83, UV detection at 220 nm), and simultaneously yielded pure calceolarioside (**6**, 79 mg), cistanoside F (**7**, 17 mg), betonyoside A (**8**, 13 mg), and campneoside II (**9**, 200 mg). Fraction 5 was subjected to HPLC (MeOH-0.1% TFA in H_2O, 13:87) to afford acteoside (**10**, 245 mg), isoacteoside (**11**, 10 mg), and terniflorin (**12**, 15 mg), respectively. The chemical structures of compounds **1–12** (Fig. 1A), were unambiguously assigned on the basis of a comprehensive spectral analysis of mass spectrometry and NMR data, and a comparison with published values (Chen et al., 2008; Rao et al., 2009). In particular, the structure and relative stereochemistry of macrocyclic diterpenoid, ovatodiolide (OVT) was determined from NMR data (see Supporting Information for ^{1}H- and ^{13}C-NMR spectra), as well as from a single crystal X-ray diffraction analysis (Manchand & Blount, 1977). The oak ridge thermal ellipsoid plot (ORTEP) arising from this analysis is shown in Fig. 1B. The purity (>95%) of OVT was confirmed by an HPLC [column: RP C18 BDS, 4.6 × 150 mm, 4 μm (Thermo)]. The mobile phase consisted of acetonitrile (solvent A) and water containing 0.1% TFA (solvent B). A linear gradient programme was used as follows: 5% A at 0 min to 100% A after 40 min. The mobile phase flow rate was 1 ml/min, and the detector was monitored at 220 nm. Representative HPLC chromatogram is shown in Fig. 1C.

2.3. H. pylori strains and bacterial culture

The *H. pylori* reference strain 26695 (ATCC 700392), and multidrug-resistant strains, v633, v1254 and v1354 were obtained and cultured as described previously (Lai et al., 2010).

2.4. Cell culture and cytotoxicity assay

The human gastric carcinoma cell line, AGS, was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). They were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS at 37 °C in 5% CO_2. In the bacterial adhesion, invasion assay, and induced IL-8 secretion, the cell culture medium was not supplemented with antibiotics. The MTT assay was used to evaluate the cytotoxicity of tested agents in AGS cells as described previously (Lai et al., 2010).

2.5. Determination of anti-H. pylori activity

Anti-*H. pylori* activities of crude extract, isolated pure compounds **1**–**12** or clinical antibiotics (AMX, CLR and MTZ) were determined by disc agar diffusion method, as described previously (Lai et al., 2008a).

2.6. Determination of minimum bactericidal concentration (MBC)

H. pylori reference strain and multidrug-resistant clinical isolates were subjected to micro dilution analysis, as described previously (Lai et al., 2010), to determine the MBC for *A. indica* compound OVT and three antibiotics AMX, CLR, MTZ.

2.7. Analysis of H. pylori adhesion to and invasion into AGS cells

The assays of anti-adhesion and anti-invasion of *H. pylori* to AGS cells were performed as described previously (Geethangili et al., 2010).

2.8. Transient transfection of nuclear factor kappa B (NF-κB) reporter construct, and determination of interleukin (IL)-8

The complete protocol for determination of luciferase activity, and IL-8 analysis has been described in previous reports (Geethangili et al., 2010; Lai et al., 2008a).

2.9. Immunoprecipitation and immunoblot analysis

AGS cells were infected with *H. pylori* reference strain at an MOI of 100 followed by incubation at 37 °C for 6 h. The infected cells were washed with PBS three times to remove unbound *H. pylori*

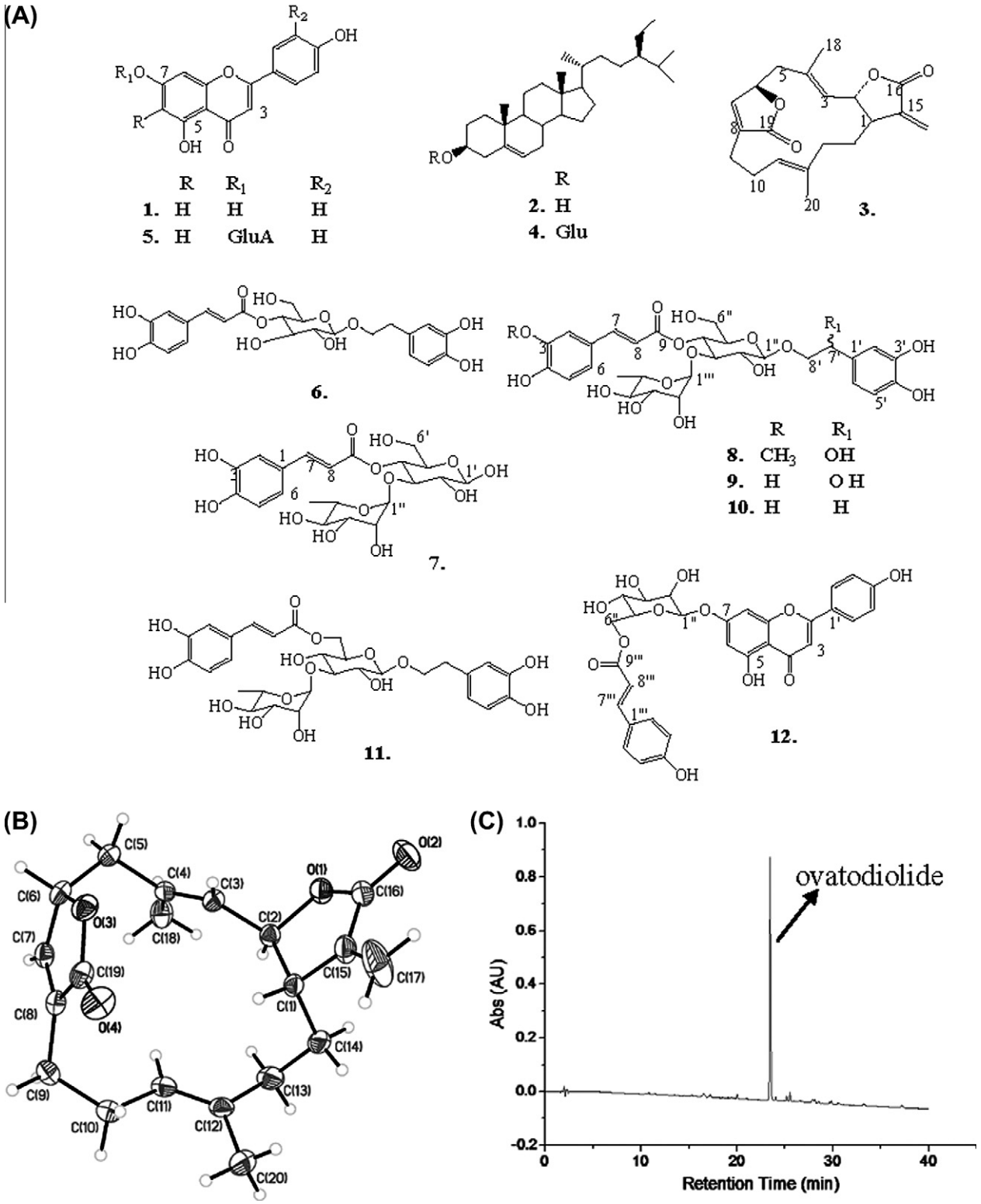

Fig. 1. (A) Chemical structures of *A. indica* constituents **1**–**12**. (B) ORTEP derived from the single crystal X-ray analysis of OVT. (C) HPLC chromatogram of OVT.

and lysed in ice-cold immunoprecipitation buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100), with 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml aprotinin, and 2 μg/ml leupeptin. Whole cell lysates were centrifuged at 16,000*g* for 30 min. The resultant supernatant, containing 1 mg of proteins, was subsequently subjected to immunoprecipitation, using 10 μg of anti-CagA antibody and 100 μl of protein A/G PLUS-agarose at 4 °C overnight. The immunoprecipitates were then subjected to 6.5% SDS–PAGE and transferred onto PMSF for immunoblot analysis. Tyrosine-phosphorylated CagA was stained using mouse anti-phosphotyrosine antibody (4G10), and CagA was stained using mouse anti-CagA antibody. β-Actin from whole-cell lysates was detected by using goat anti-actin antibody to ensure equal loading. The proteins of interest were visualised by using the

enhanced chemiluminescence assay (Amersham Pharmacia, Little Chalfont, UK) and was detected using an LAS-3000 imaging system (Fujifilm, Valhalla, NY, USA).

2.10. Analysis of H. pylori-induced AGS cell hummingbird phenotype

The quantitative analysis of *H. pylori*-induced hummingbird activity was performed, as described previously (Lai et al., 2008b). Briefly, AGS cells (1×10^6 cells/ml) were cultured in 12-well plates at 37 °C for 20 h. After one wash with PBS, cells were then infected with the *H. pylori* reference strain at a MOI of 50 for 6 h. Elongated cells were defined as cells that had thin needlelike protrusions that were >20 μm long and a typical elongated shape. All samples were determined in triplicate, in at least three independent experiments. The proportion of elongated cells was calculated the numbers of cells having the hummingbird phenotype.

2.11. Statistical analysis

The data are presented as mean ± standard deviation of triplicate experiments. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple-comparison test. The symbol (*) indicates $p < 0.05$ when compared with untreated controls.

3. Results

3.1. Growth inhibition of H. pylori

Initial attention focused on the investigation of relative inhibitory potency of *A. indica* ethanol crude extract and its isolated pure compounds **1**–**12** (Fig. 1A) against *H. pylori* reference strain Hp 26695 growth, using the disk agar diffusion assay (Lai et al., 2008b). Unless otherwise stated, pure compounds **1**–**12**, and crude ethanol extract were first tested at a single maximum concentration of 0.5 mg/ml. For referencing, inhibitory effects of standard antimicrobial agents for treatment of *H. pylori* infection clarithromycin (CLR), amoxicillin (AMX) both at 0.05 mg/ml, and metronidazole (MTZ) at 0.8 mg/ml, were determined as well. To quantitative the inhibitory effect of *H. pylori*, the diameter of growth inhibition area was measured and expressed in millimeters and presented in Table 1. As shown in Table 1, tested agents showed a wide range of inhibitory effect against *H. pylori* growth with inhibition zone ranging from 0 to 21 mm. The antibiotics CLR, AMX and MTZ inhibited the bacterial growth with inhibition zone of 21, 14 and 7 mm, respectively. *A. indica* whole plants ethanol extract showed an inhibition zone value of 19 mm. Among the isolated pure compounds **1**–**12**, macrocyclic diterpenoid OVT (**3**) showed the most potent inhibitory effect with inhibition zone of 20 mm. The strong antibacterial efficacy of OVT became significant as compared with AMX, CLR and MTZ (Table 1). Among the tested phenyl propanoids at 0.5 mg/ml, acteoside (**10**) followed by isoacteoside (**11**), cistanoside F (**7**), and campneoside II (**9**) with inhibition zones of 12, 11, 8 and 7 mm, respectively, showed higher *H. pylori* growth inhibition, as compared with MTZ at 0.8 mg/ml (Table 1). A similar inhibitory trend was observed with the tested flavonoids apigenin (**1**), apigenin 7-*O*-glucoronide (**5**), terniflorin (**12**), and sterol compounds β-sitosterol (**2**), β-sitosterol 3-*O*-*β*-D-glucoside (**4**) with inhibition zones of 7, 8, 11, 8 and 11 mm, respectively. Although phenyl propanoids and flavonoids were less active than OVT, their inhibition zone values were comparable with MTZ (Table 1). In contrast, for phenyl propanoids calceolarioside (**6**) and betonyoside A (**8**) no inhibition could be detected at a concentration of 0.5 mg/ml.

Table 1
Growth inhibitory effects of *A. indica* ethanol crude extract and isolated pure compounds **1**–**12** against *H. pylori* determined by disc agar diffusion assay.

Tested sample[a]	Inhibition zone (mm)[b]
1	7 ± 2
2	8 ± 2
3	20 ± 4
4	11 ± 3
5	9 ± 1
6	0
7	8 ± 1
8	0
9	7 ± 1
10	12 ± 3
11	11 ± 2
12	11 ± 3
Ethanol extract	19 ± 2
AMX	14 ± 3
CLR	21 ± 4
MTZ	7 ± 1

AMX, amoxicillin; CLR, clarithromycin; and MTZ, metronidazole.
[a] Concentrations of ethanol extract and pure compounds **1**–**12** were at 0.5 mg/ml and, both AMX and CLR at 0.05 mg/ml, and MTZ at 0.8 mg/ml.
[b] Data are related to DMSO control and shown as the mean ± SD from three independent experiments.

Table 2
Minimum bactericidal concentration (MBC) of ovatodiolide, crude extract and standard antibiotics against *H. pylori* reference strain and multidrug-resistant isolates.

Tested sample	MBC (μM)			
	Hp reference strain	v633	v1254	v1354
Ovatodiolide	100 ± 7	50 ± 3	100 ± 8	50 ± 5
Ethanol extract	400 ± 16	400 ± 21	400 ± 14	400 ± 12
AMX	12.5 ± 3	12.5 ± 4	12.5 ± 4	6.25 ± 1
CLR	1.6 ± 0.8	50 ± 6	25 ± 4	100 ± 7
MTZ	25 ± 4	400 ± 11	800 ± 32	1600 ± 100

AMX, amoxicillin; CLR, clarithromycin; and MTZ, metronidazole. Hp 26695 was a reference strain. Strains v663, v1254 and v1354 were clinical isolates, which show resistant to both metronidazole and clarithromycin. Average molecular weight of ethanol extract was approximately 500 Da. Data are related to DMSO control and shown as the mean ± SD from three independent experiments.

In separate experiments, we determined the minimum bactericidal concentration (MBC) for the potent antibacterial pure compound OVT and ethanol extract of *A. indica* against *H. pylori* reference (Hp26695), as well as multidrug–resistant strains v633, v1254, and v1354 (Lai et al., 2010). MBC values were defined as the lowest concentration of the tested agent that completely inhibited visible *H. pylori* growth on brucella blood agar plate. As shown in Table 2, the MBC of OVT was 100, 50, 100 and 50 μM against *H. pylori* strains Hp 26695, v633, v1254, and v1354, respectively. It was interesting to note that the MBC of OVT against multidrug–resistant strains v633 and v1354 were lower (50 μM) than that of reference strain Hp26695 (100 μM). The crude ethanol extract showed less potential with MBC of 400 μM against all the tested bacterial strains, as compared with the pure compound OVT and antibiotics AMX and CLR (Table 2).

3.2. Effect of OVT on the adhesion and invasion of H. pylori to AGS cells

Our next intention focused on examination of OVT effect on the proliferation of human gastric carcinoma (AGS) cells by MTT assay (Geethangili et al., 2010). We did not observe any considerable growth inhibition in AGS cells following 48 h of OVT treatment up to 60 μM doses (data not shown). Thus, therefore OVT with a maximal concentration of 60 μM was chosen to examine its effect on *H. pylori*-induced inflammation in AGS cells.

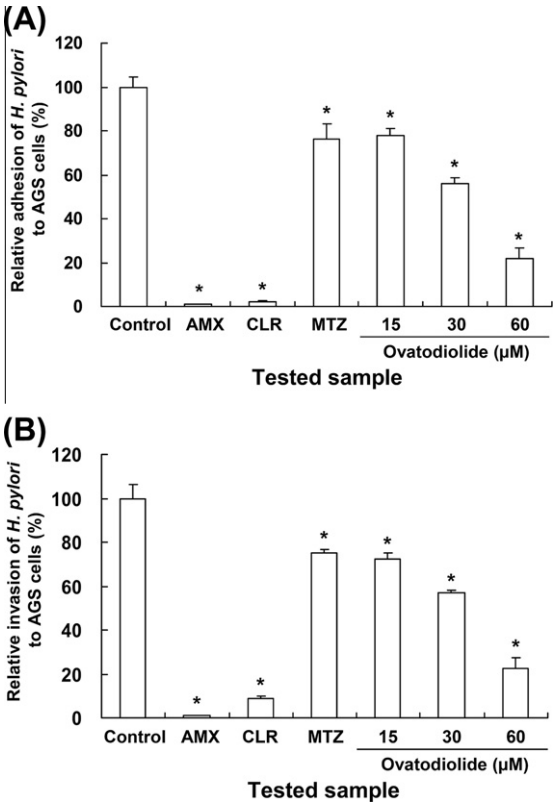

Fig. 2. Effect of ovatodiolide (OVT) on *H. pylori* adhesion (A), and invasion (B) to AGS cells. AGS cells were treated with various doses of OVT prior infection with *H. pylori*, at a MOI of 50 for 6 h. The antibiotics amoxicillin (AMX), clarithromycin (CLR), and metronidazole (MTZ) were used as positive control at a concentration of 15 μM. Reported values were the means ± SD of six independent experiments. The significant difference was set at $^{*}p < 0.05$ when compared to DMSO treated cells.

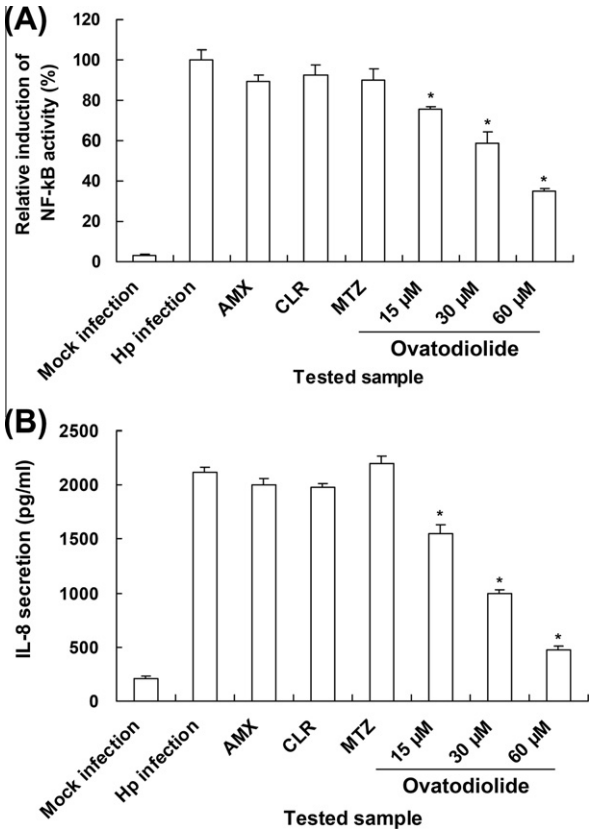

Fig. 3. Effect of ovatodiolide (OVT) on NF-κB activation (A) and IL-8 secretion (B) in *H. pylori*-infected AGS cells. The antibiotics amoxicillin (AMX), clarithromycin (CLR), and metronidazole (MTZ) at a concentration of 15 μM were used as positive control. Reported values were the means ± SD of six independent experiments. The significant difference was set at $^{*}p < 0.05$ when compared with *H. pylori*-infected AGS cells.

H. pylori adhesion to AGS cells is an important initial event in the pathogenesis of gastric malignancies (Amieva & El-Omar, 2008). We next performed the ability of OVT to inhibit the adhesion of *H. pylori* to AGS cells. AGS cells were infected with *H. pylori*, at a multiplicity of infection 50 for 6 h, in the presence of antibiotics AMX, CLR, MTZ at 15 μM, or one of varying concentrations of OVT. The number of adhesion *H. pylori* cells to AGS cells was counted by colony-forming units (CFU). It was observed that OVT displayed potent anti-adhesion effect with a reduction of 21.9%, 44.2% and 78.3% at 15, 30 and 60 μM, respectively; as compared with vehicle DMSO treated control cells (Fig. 2A). Next, we also assessed the effect of OVT on the invasion of *H. pylori* to AGS cells using the similar experimental procedures (Geethangili et al., 2010). Compared with vehicle-treated cells, culture medium increased the invasion capability of *H. pylori* into AGS cells. However, OVT treatment attenuated cell invasion by 27.5%, 43.3% and 77.3% at 15, 30 and 60 μM, respectively (Fig. 2B). During these anti-adhesion and anti-invasion assays the inhibitory effects of antibiotics AMX and CLR were more than 90%, while only slight inhibitory effect (around 20%) of MTZ was observed (Fig. 2).

3.3. Effect of OVT on H. pylori induced inflammation in AGS cells

Next, we examined the effects of OVT on *H. pylori*-induced inflammation by determining the production of inflammatory mediators, such as NF-κB activation and IL-8 secretions from *H. pylori*-infected AGS cells. AGS cells were transfected with a NF-κB reporter construct (NF-κB-luc), and used to examine luciferase expression following treatment with OVT or antibiotics AMX, CLR, MTZ. As shown in Fig. 3A, pretreatment of cells with OVT prior to *H. pylori* infection led to a dose-dependent reduction in induction of NF-κB activity. The three antibiotics AMX, CLR, MTZ had no effect on induction of NF-κB activity at a concentration of 15 μM, as compared with *H. pylori*-infected AGS cells. However, OVT showed a marked response, it was able to decrease the NF-κB activity by 17.8%, 41.5%, and 64.9% at concentrations of 15, 30 and 60 μM, respectively. Since NF-κB was found to mediate *H. pylori*-induced IL-8 secretion of AGS cells (Wroblewski, Peek, & Wilson, 2010). We next examined the IL-8 production in AGS cells infected with *H. pylori* with, or without, preincubation of OVT raging from 15 to 60 μM. IL-8 production in *H. pylori*-infected AGS cells was increased, however, pretreatment with 15, 30, and 60 μM of OVT for 24 h noticeably decreased the IL-8 production by 27.5%, 61.7%, and 81.5%, respectively, as compared with *H. pylori*-infected AGS cells (Fig. 3B). During this assay the results of antibiotics were in parallel to that of NF-κB activity assay.

3.4. Effect of OVT on the expression levels of cytotoxin-associated gene A (CagA) translocation and phosphorylation in H. pylori-infected AGS cells

We further examined the OVT effect on *H. pylori* induced virulence factor CagA by analysing the expression levels of CagA translocation and phosphorylation in *H. pylori*-infected AGS cells. CagA was immunoprecipitated from *H. pylori*-infected AGS cells and analysed by immunoblot, to quantify the amount of CagA protein delivered into AGS cells (Lai et al., 2008a). We found that upon

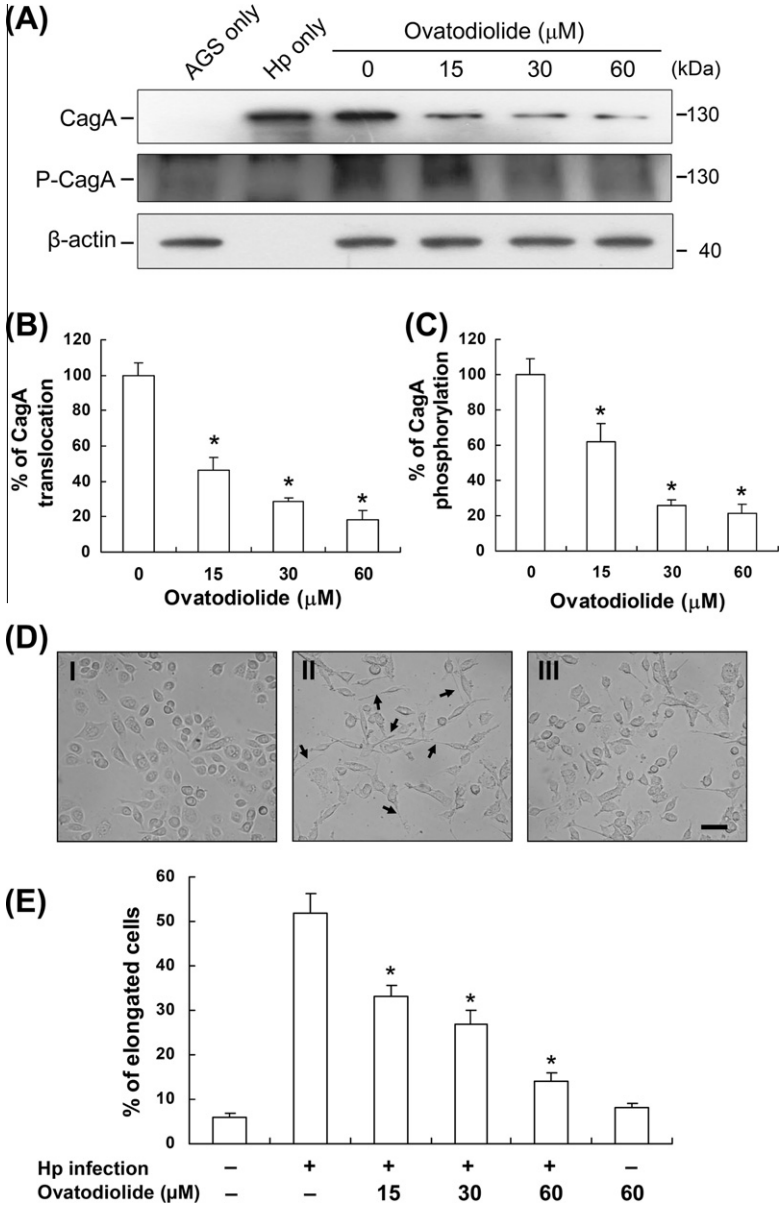

Fig. 4. Effect of ovatodiolide (OVT) on *H. pylori* CagA functions. (A) AGS cells were treated with indicated concentrations of OVT followed by infection of *H. pylori* for 6 h. The level of CagA translocation (B) and CagA phosphorylation (C) was determined by densitometric analysis. (D) I. Mock infection. II. *H. pylori*-infected AGS cells. III. Pretreatment of *H. pylori*-infected AGS cells with 60 μM OVT, the cells were cultured for 6 h, (E) the proportion of elongated (hummingbird phenotype) cells was evaluated. For details see materials methods Sections 2.9 and 2.10. Arrows in (B) denoted elongated hummingbird phenotypes of AGS cells. Scale bar, 20 μm.

H. pylori infection the translocation and the phosphorylated CagA were increased to levels higher than those in non-infected cells (Fig. 4A and B). However, with the OVT concentrations of 15, 30 and 60 μM, the levels of translocated and phosphorylated CagA were decreased in the range 55.8–79.0%, and 37.6–76.0%, respectively (Fig. 4A and B). These results indicate that OVT may play a role in the reduction of *H. pylori* CagA biological functions in AGS cells.

We next explored whether OVT that inhibited CagA translocation and phosphorylation also specifically attenuated CagA-induced responses by evaluating the hummingbird phenotype of AGS cells. Upon infection of AGS cells with *H. pylori*, nearly 52% of cells exhibited the elongated cells (hummingbird phenotype), compared with untreated cells (Fig. 4D and E). However, pretreatment of *H. pylori*-infected AGS cells with OVT, the proportion of elongated cells was reduced in a dose-dependent manner. Specifically, the elongated *H. pylori* infected AGS cells were reduced from 52% to 15% when pretreated with 60 μM of OVT (Fig. 4E).

4. Discussion

H. pylori highly colonise the human gastric mucosa and perfectly adapted to that environment. Concerning pathology, *H. pylori* causes gastritis and is classified as a major primary risk factor for the development of gastric or peptic ulcers, gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (Wroblewski et al., 2010). It has been reported that the ethanol extraction of *A. indica* exerted potent growth inhibition of *H. pylori* (Wang & Huang, 2005), however the precise active compounds responsible for anti-*H. pylori* activity of this plant have not been clearly identified. The present study demonstrated that the isolated pure compounds **1**–**5**, **7**, and **9**–**12** from *A. indica* showed potent growth inhibition against *H. pylori* (Table 1). In particular, OVT (**3**) showed superior *H. pylori* growth inhibition, as compared with *A. indica* ethanol extract (Table 1). Based on the results of this study, it may conclude that the observed anti-*H. pylori* growth of *A. indica* ethanol extract was due to the presence of compounds **1**–**5**,

7, and **9**–**12**. It is known that OVT inhibit the growth of human cancer cells, HIV-1 infection, and enhanced production of inflammatory mediators (Chen et al., 2008; Hou et al., 2009; Rao et al., 2009; Shahidul Alam, Quader, & Rashid, 2000), however, its effects against *H. pylori* growth has not been determined. To the best of our knowledge, this was the first report of *A. indica* pure compounds toward *H. pylori* growth inhibition. Thus, our results demonstrated that *A. indica* constituents in particular OVT may be useful to develop potential growth inhibitors against *H. pylori*.

The severity of *H. pylori* infection depends on multiple factors like bacterial strain(s), age of acquisition and immune response. Disease progression may also be influenced by additional factors, such as host environment and diet (Herrera & Parsonnet, 2009). On the other hand, *H. pylori* isolates show considerable genetic diversity, even within isolates from the same patient (Covacci, Telford, Del Giudice, Parsonnet, & Rappuoli, 1999). Whole genome sequences of certain isolates reveal differences in sequence, as well as gene content. This is a result of frequent recombination, which makes *H. pylori* one of the most genetically diverse bacteria (Covacci et al., 1999). In the present study, OVT inhibited the growth of reference Hp26695, as well as multidrug–resistant strains v633, v1254 and v1354 (Table 2). Notably, OVT with lower MBC values showed superior growth inhibition as compared with crude ethanol extract and antibiotics MTZ against multidrug–resistant strains. In addition, OVT showed comparable MBC value with that of CLR (Table 2).

Adherence of *H. pylori* to the gastric epithelium is an important virulence factor for the initial and persistent colonisation, as well as for the bacterium-to-cell signalling. Intimate contact of *H. pylori* to the gastric epithelium can lead to the exertion of virulence factors, which includes CagA and the type IV secretion system encoded in the *cag* pathogenicity island, and the vacuolating cytotoxin A (Polk & Peek, 2010). On the other side, in our previous study we have reported that pretreatment of *H. pylori* isolates from the failure group of a clinical trial had a higher invasion than those which were successfully eradicated (Lai et al., 2006). This evidence suggested that *H. pylori* strains with high antibiotic resistance and/or elevated invading activity are not eradicated during antibiotic treatment, and thus leading to persist intracellularly in the human stomach (Lai et al., 2006). It is therefore an urgent need to develop new agents that prevents (eradicate) the invasion of antibiotic resistance *H. pylori* strains. The results of this study demonstrated that OVT dose-dependently reduced the *H. pylori* bacteria adhesion and invasion to AGS cells (Fig. 2). Thus, the present study indicates that the mode of action of OVT was related to its antibacterial activity, in addition to anti-adhesion and anti-invasion activities against *H. pylori*.

It is generally accepted that the attachment of *H. pylori* to AGS cells leads to the efficient delivery of CagA into AGS cells (Wroblewski et al., 2010). It is known that CagA is a key determinant for the induction of pro-inflammatory gene programme, which include the up-regulation of NF-κB activation and IL-8 secretions during *H. pylori* infection into AGS cells (Covacci et al., 1999; Wroblewski et al., 2010). An important function of the NF-κB pathway is the initiation and amplification of the innate immune system in mounting a fulminant reaction to combat infections (Covacci et al., 1999). Therefore, inhibition of NF-κB activation, as well as IL-8 secretion, in *H. pylori*-infected AGS cells might be a useful strategy for the management of chronic gastritis. In this study, treatment with OVT dose-dependently inhibited the *H. pylori*-induced NF-κB activation and subsequently IL-8 secretion from AGS cells (Fig. 3). These results conclude that OVT suppressed the *H. pylori*-induced inflammation through the inhibition of components NF-κB and IL-8 of pro-inflammatory signalling pathway.

The differences in clinical outcomes among *H. pylori*-infected individuals have also been linked to different virulence factors in infecting strains (Covacci et al., 1999). CagA is produced by most strains of *H. pylori* species (Hatakeyama, 2008). Within the AGS cells, some CagA molecules are tyrosine-phosphorylated by the Src/Abl kinase on tyrosine motifs. Furthermore, phosphorylated CagA (pCagA) is known to disrupt tight cell junctions, resulting in cell elongation and the formation of the so-called 'hummingbird phenotype' (Hatakeyama, 2008). Previous studies strongly indicate that pCagA contributes to the development of the *H. pylori*-associated gastric illnesses, including gastric cancer (Polk & Peek, 2010). The present study demonstrated that OVT attenuated the CagA functions through the reduced expression levels of *H. pylori* CagA translocation and phosphorylation into AGS cells (Fig. 4A–C). Furthermore, OVT also reduced the hummingbird phenotype in *H. pylori*-infected AGS cells (Fig. 4D and E). Correlated with the above observations, OVT inhibited the *H. pylori*-induced NF-κB activation and IL-8 secretion (Fig. 3), which was rescued by the CagA translocation and phosphorylation, suggesting that OVT was likely to inhibit the *H. pylori*-induced inflammation by attenuating CagA functions. To the best of our knowledge, we demonstrate here for the first time on OVT inhibited *H. pylori*-associated inflammation in AGS cells. Further studies are needed to validate the tested concentrations of OVT and the molecular mechanisms for against *H. pylori*-induced host carcinogenesis. Therefore, the next step might be that we will check whether OVT can exert similar anti-inflammatory activities in a *H. pylori*-infected animal model, to extend our current findings into clinical implication.

5. Conclusions

In conclusion, we have demonstrated for the first time that *A. indica* isolates inhibited *H. pylori* growth. In particular, ovatodiolide potently suppressed the growth of *H. pylori* reference as well as multidrug–resistant strains, and reduced the adhesion and invasion of *H. pylori* to AGS cells. Ovatodiolide attenuated the *H. pylori* induced inflammatory response by the reduced NF-kB activation and IL-8 expression in AGS cells. Furthermore, ovatodiolide also found has potent effects in inhibiting pathogenesis of cells through attenuation of *H. pylori* CagA functions. Thus, this study demonstrated that ovatodiolide might be useful as a functional food additive, or to the development of new therapeutic drugs for protecting against infections such as *H. pylori*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.11.037.

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