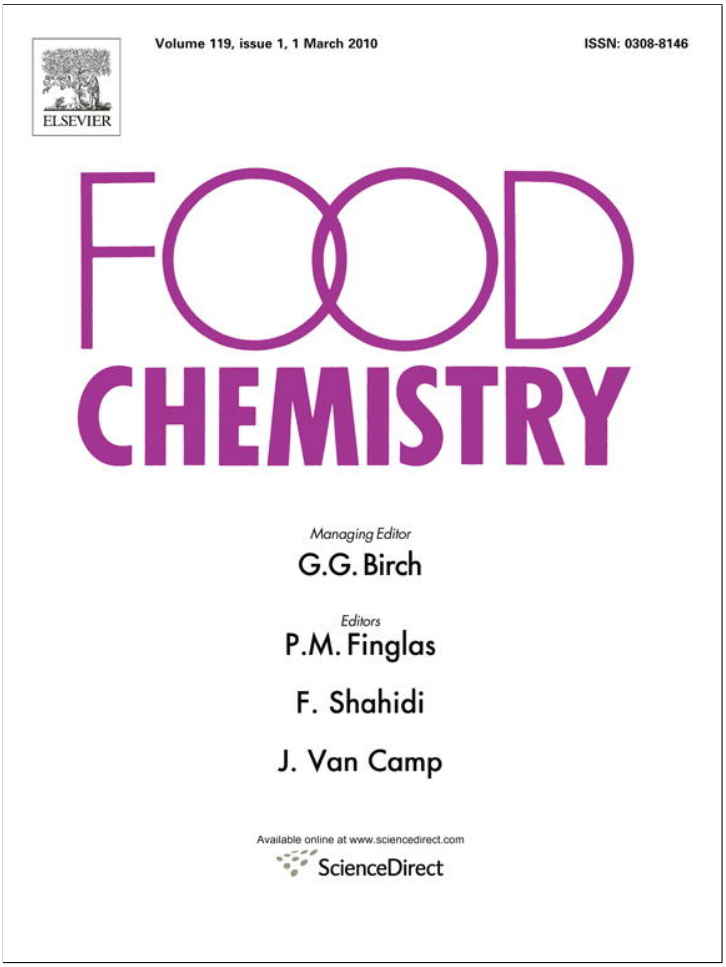

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Inhibitory effect of *Antrodia camphorata* constituents on the *Helicobacter pylori*-associated gastric inflammation

Madamanchi Geethangili [a,1], Shih-Hua Fang [b,1], Chih-Ho Lai [c], Yerra Koteswara Rao [a], Hsiu-Man Lien [d], Yew-Min Tzeng [a,*]

[a] *Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Wufeng, Taiwan, ROC*
[b] *Institute of Athletes, National Taiwan Sport University, Taichung, Taiwan, ROC*
[c] *Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan, ROC*
[d] *Department of Chemistry, Tunghai University, Taichung, Taiwan, ROC*

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ABSTRACT

Helicobacter pylori infection plays a crucial role in the pathogenesis of peptic ulcer, and gastric cancer. Among the 13 chemical constituents isolated from fruiting bodies of *Antrodia camphorata*, methyl antcinate B (**4**), antcins K (**10**) and A (**12**) displayed potential anti-*H. pylori* activity with inhibition zones of 13, 12 and 10 mm, respectively, at a concentration of 0.2 mM. The isolates **4** and **10** exhibited a dose response inhibition of *H. pylori* adhesion and invasion to AGS cells in a range of concentrations between 0.005 and 0.02 mM, while **12** has moderate effect at relatively higher concentration. Furthermore, at these concentrations (0.005–0.02 mM) the isolates **4** and **10** also inhibited the *H. pylori*-induced nuclear factor (NF)-κB activation, and the subsequent release of interleukin (IL)-8 in AGS cells. These results open the possibility of considering *A. camphorata* a chemopreventive agent for peptic ulcer or gastric cancer, but this bioactivity should be confirmed *in vivo* in the future.

1. Introduction

Helicobacter pylori is a microaerophilic spiral-shaped Gram-negative bacterium that colonises the stomach for almost the entire lifetime of the host. It has been evident for over 20 years that *H. pylori* infects more than half of the world's human population, and produces gastroduodenal diseases, such as peptic ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue (MALT) lymphoma or cancer development (Peek & Blaser, 2002; Suerbaum & Michetti, 2002). In 1994, the World Health Organisation concluded that *H. pylori* is a definite or class I carcinogen in humans. The mechanism related to the persistence of *H. pylori* in infected individuals remains unclear (Uemura et al., 2001). Several short course drug regimens are prescribed for the eradication of *H. pylori* with different combinations of therapeutic agents such as antibiotics, bismuth subsalicylate, proton pump inhibitors and H_2-blockers (Shi & Klotz, 2008). However, emerging resistance to antibiotics, especially clarithromycin and metronidazole limits their use in the treatment of infections in developed and more in developing countries (Egan, Connor, & Morain, 2008). Furthermore, undesirable side effects of the drugs and the significant cost of combination therapy, require the exclusive need to search alternative approaches of eradicating or preventing infections (Cheung & Wong, 2008). Efforts to find an effective method for the non-antibiotic control of *H. pylori* infection are therefore urgently required. Thus, it is important to screen food extracts and components for promising candidates as potential *H. pylori* preventive agents (Lee, Shin, & Hahm, 2008). Furthermore, the reported results of garlic (Gail & You, 2006), turmeric (Mahady, Pendland, Yun, & Lu, 2002), red wine and green tea (Ruggiero et al., 2007) to inhibit *H. pylori*-associated gastric disorders also stimulate the significant interest in identification of potential functional food agents with anti-*H. pylori* activity.

Medicinal mushrooms have a long history of use in folk medicine. A new basidiomycete *Antrodia camphorata* in the Polyporaceae (Aphyllophorales) is a traditional medicinal mushroom which grows only on the inner heart-wood wall of an endemic tree species *Cinnamomum kanehirai* Hay (Lauraceae). *A. camphorata* is known in Taiwan as "jang-jy" and "niu-chang-chih", and is commonly used for the treatment of numerous conditions such as food and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches and liver cancer (Tsai & Liaw, 1982). Previous studies have demonstrated that the non-toxic characteristics of *A. camphorata* [oral administration of 500 mg/kg/day for 28 days in rats], which increases its potential for application in food and drug products (Lin, Kuo, & Wu, 2001).

* Corresponding author. Address: Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Wufeng 41349, Taiwan, ROC. Tel.: +886 4 23323000x4471; fax: +886 4 23395870.
E-mail address: ymtzeng@cyut.edu.tw (Y.-M. Tzeng).
[1] Contributed equally to this work.

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In spite of the large quantity of studies about the extracts of *A. camphorata*, there are as yet no published data documenting the anti-*H. pylori* effects of its constituents. Therefore, the purpose of the present study was to isolate and identify potent anti-*H. pylori* constituents from the fruiting bodies of *A. camphorata*. Concurrently, we also wish to examine the effect of potent antibacterial isolates on the inhibition of adhesion and invasion, subsequent release of nuclear factor kappa B (NF-κB) and interleukin (IL)-8 secretions from *H. pylori*-stimulated human gastric epithelial cells.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media (RPMI 1640), phosphate buffered saline (PBS), penicillin, streptomycin, gentamicin, vancomycin, amphotericin B, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma–Aldrich, St. Louis, MO, USA), fetal bovine serum (FBS), L-glutamine (Gibco BRL, Grand Island, NY, USA), lipofectamine 2000 (Invitrogen, cat. no. 12566-014), lysis buffer, luciferase substrate, β-galactosidase expression vector (Promega, Madison, MA, USA) were obtained from various suppliers. All other chemicals were of the highest grade commercially available and supplied either by Merck or Sigma.

NMR spectra were measured on a Varian Unity Inova-600 VXR-300/51 spectrometer, using TMS as the internal standard. Silica gel for column chromatography (CC), (0.063–0.200 mm), was a product of Merck Company. TLC was performed on Merck TLC plates (0.23 mm thickness), with compounds visualised by spraying with 8% (v/v) H_2SO_4 in EtOH and then heating on a hot plate.

2.2. Fungal material, extraction and isolation

The fruiting bodies of *A. camphorata* were collected from the Yuli, Hualien County, Taiwan, in December 2007. The species was identified by Dr. Yew-Min Tzeng at the Bioprocess Engineering laboratory, Chaoyang University of Technology. A voucher specimen (YMT 7002) was deposited in the Herbarium of the Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Taiwan, ROC.

The constituents **1**–**13** were separated from *A. camphorata* following the extraction and isolation procedures as described previously (Shen et al., 2003). Briefly, dried powder of *A. camphorata* (102 g) was extracted successively with *n*-hexane and $CHCl_3$ using a Soxhlet extractor. After solvent evaporation, the *n*-hexane extract (3.1 g) was subjected to silica gel CC eluted with different solvents of increasing polarity (*n*-hexane/EtOAc) to give oleic acid (**1**, 10 mg), 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bi-methylenedioxy-6,6′-dimethylbiphenyl (**2**, 36 mg), and ergosterol (**3**, 50 mg). The $CHCl_3$ extract (37 g) was subjected to silica gel CC and eluted with increasing polarity using mixtures of *n*-hexane/EtOAc. Following the TLC analysis, eluates of similar profiles were combined to give six fractions (A–F). Fraction B was purified by CC to afford methyl antcinate B (**4**, 7 mg), dehydroeburicoic acid (**5**, 750 mg) and 15α-acetyl dehydrosulphurenic acid (**6**, 150 mg), respectively. From the fraction C, compounds 3β, 15α-dihydroxy lanosta-7,9 (11), 24-triene-21-oic acid (**7**, 30 mg), and zhankuic acid A (**8**, 1.1 g) were obtained. Fraction D was further separated using a silica gel column eluting with a gradient of *n*-hexane/EtOAc, to afford five subfractions. Dehydrosulphurenic acid (**9**, 350 mg) and antcin K (**10**, 7 mg) were obtained from subfractions D–3 and D–4, respectively. Zhankuic acid C (**11**, 410 mg) was obtained from fractions E. Fraction F was further purified by silica gel CC using $CHCl_3$/MeOH from 100% $CHCl_3$ to 20% MeOH to yield antcin A (**12**, 125 mg) and a mixture of triterpenes that was further separated by CC using $CHCl_3$/MeOH elution (9:1) to give sulphurenic acid (**13**, 450 mg). The structures of compounds **1**–**13** were determined by ^1H and ^{13}C NMR spectroscopy and by comparison of the spectral data with those published values (Male et al., 2008; Shen et al., 2003).

2.3. Cell lines and growth conditions

H. pylori was grown in Brucella agar plates (Becton Dickinson) supplemented with 10% sheep blood, 6 μg/ml of vancomycin and 2 μg/ml of amphotericin B, and incubated for 48 h at 37 °C under a microaerophilic atmosphere. Bacterial density was estimated by the absorbance measurement at 600 nm. Heat-killed *H. pylori* obtained by boiling during 30 min at 56 °C followed by an incubation for 10 min at 80 °C. Unless otherwise stated, experiments were performed with *H. pylori* strain 26695 (ATCC 700392), obtained from the American Type Culture Collection (ATCC).

Human gastric cancer AGS cells (gastric adenocarcinoma, ATCC CRL 1739) were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in RPMI-1640 medium (pH 7.4; Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum, 4 mM glutamine (Gibco BRL, Grand Island, NY, USA), antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin). The MTT assay was used to detect the cytoxicity of *A. camphorata* isolates in AGS cells as described previously (Rao, Geethangili, Fang, & Tzeng, 2007).

2.4. Anti-H. pylori activity

The disc-diffusion method was used to screen *A. camphorata* isolates against *H. pylori* strain 26695 as described previously (Lai et al., 2008).

2.5. Inhibition of H. pylori adhesion to and invasion into AGS cells

Prior to infection, 80% confluent AGS cells were washed twice in PBS and incubated overnight in serum and antibiotic free medium (Invitrogen). For infection, 48 h colonies of *H. pylori* were collected and added to cells at, unless otherwise stated, a multiplicity of infection (MOI) of 100. Cultures were maintained at 37 °C under a 5% CO_2 humidified atmosphere. *H. pylori* adhesion to and invasion of cultured epithelial cells was done using a standard gentamicin assay as previously described (Lai et al., 2006). The isolates **4**, **10** and **12**, and DMSO diluted in cell culture medium were added (to reach the indicated dilutions) directly to the cell culture medium for 10 min prior to inoculation of wells with *H. pylori* in log-phase. To determine the number of cell-adhesion bacteria, infected cells were washed three times to remove unbound bacteria and then lysed with distilled water for 10 min. Lysates were diluted in PBS, plated onto Brucella blood agar plates and cultured for 4–5 days, after which the CFUs were counted. To determine the number of viable intracellular bacteria, infected cells were washed three times in PBS and incubated with 100 μg/ml of the membrane-impermeable antibiotic gentamicin (Sigma–Aldrich) for 1.5 h at 37 °C to remove extracellular bacteria, followed by the same procedures as above to obtain CFUs. The adhesion or invasion activity was determined as the mean of at least six experiments performed in duplicate. The controls contained *H. pylori* infected AGS cells without test samples were used to establish 100% adhesion or invasion. The results were expressed as the percentage of relative inhibition of *H. pylori* adhesion or invasion, as compared with the controls.

2.6. Transient transfection of NF-κB reporter gene

NF-κB-Luc reporter plasmid was kindly provided by Dr. Chih-Hsin Tang, Department of Pharmacology, China Medical University

(Tang, Yang, Chen, & Fu, 2007). AGS cells were grown to 90% confluence in 12-well plates and was transfected using Lipofectamine 2000 (Invitrogen). After 24 h incubation, transfection was complete, and cells were incubated with various concentrations of isolates **4**, **10** and **12** and then infected with *H. pylori* for 6 h. To prepare cell lysates, 100 μl of reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. An equal volume of luciferase substrate (Promega) was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalised to transfection efficiency monitored by the cotransfected β-galactosidase expression vector obtained from Promega (Madison, MA, USA).

2.7. Interleukin-8 measurement

To detect IL-8 released by AGS cells during *H. pylori* infection, the levels of IL-8 was measured. AGS cells were added with various concentrations of isolates **4**, **10** and **12** in cell culture medium before *H. pylori* infection. The treated cells then infected with *H. pylori* at a MOI of 1:100. The supernatants were collected (after 24 h) and stored at −80 °C before analysis. The level of IL-8 in supernatants from AGS cell cultures was determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D systems), according to the manufacturer's instruction.

2.8. Statistical analysis

Data were analysed with Student's *t*-test and were expressed as mean values of at least three independent experiments ± SD. Differences in data values were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results and discussion

The $CHCl_3$ extract of the fresh fruiting bodies of *A. camphorata* was separated by extensive column chromatography over silica gel to yield the isolates **1**–**13** (Fig. 1). All the constituents were found (by TLC) to be present in the original extract of the *A. camphorata* materials. As a part of our ongoing study to discover the novel anti-*H. pylori* compounds from traditional medicines (Lai et al., 2008), the isolated constituents **1**–**13** were evaluated for their anti-*H. pylori* activity by the disk diffusion method (Lai et al., 2008). The subject isolates exhibited variable growth inhibition against *H. pylori* (Fig. 2). The ergostane-type triterpenes methyl antcinate B (**4**), antcins K (**10**) and A (**12**) exhibited significant potency with inhibition zones of 13, 12 and 10 mm, respectively, at a concentration of 0.2 mM. This effect was similar to the standard drug amoxicillin (AMX), which is one of the commonly used antibiotics for treatment of *H. pylori* infection (inhibition zone of 14 mm at 0.05 mg/ml). At 0.2 mM concentration, zhankuic acids A and C (**8** and **11**) were also shown to possess potent anti-*H. pylori* effect, with inhibition zones of 9 mm, greater than the antibiotic metronidazole (8 mm of inhibition zone at 0.8 mg/ml). Among the other isolates tested at 0.2 mM, all showed nearly similar inhibition zones in the range 5–7 mm to that of metronidazole. This is the first report on *A. camphorata* isolates for their anti-*H. pylori* activity.

The attachment of *H. pylori* to human epithelial cells is a key step in the initiation of infection. This process leads to the colonisation and efficient delivery of virulence factors such as cytotoxin-associated antigen A (CagA) into AGS cells, and finally the development of pathogenic diseases (Moss & Malfertheiner, 2007; Ofek & Sharon, 1990). A variety of food phytochemicals and extracts have been reported to inhibit *H. pylori* adhesion to gastric epithelium

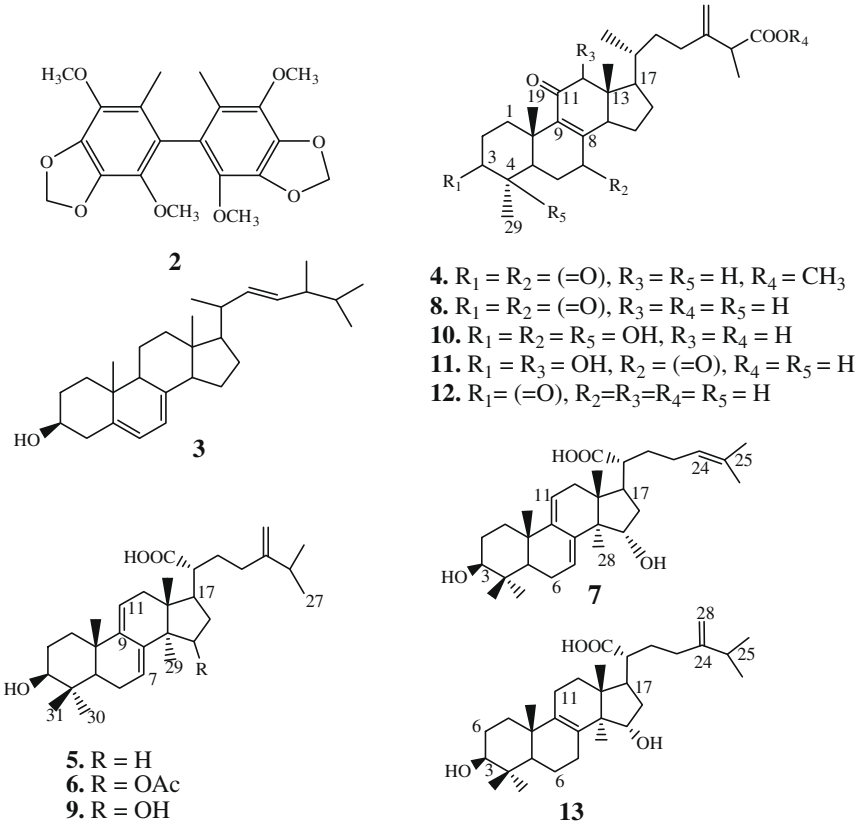

Fig. 1. Chemical structures of *Antrodia camphorata* isolated constituents.

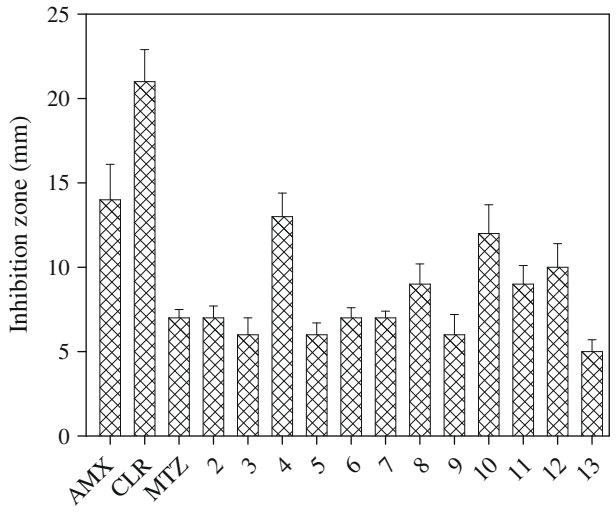

Fig. 2. Inhibitory effects of *Antrodia camphorata* isolates (0.2 mM) against *Helicobacter pylori*. DMSO was used as negative control, and the antibiotics amoxicillin (AMX, 0.05 mg/ml), clarithromycin (CLR, 0.05 mg/ml), and metronidazole (MTZ, 0.8 mg/ml) were used as positive control. Results were shown mean of different analysis of three independent experiments.

cells (Lee et al., 2008; Rohdewald & Beil, 2008). In the present study, the MTT assay was performed to assess the effect of *A. camphorata* isolates **1**–**13** on AGS cells viability. At their maximum concentration (0.2 mM) tested the isolates **1**–**13** have no significant ($p < 0.05$) cytotoxic effect upon AGS cell viability (data not shown). Then, since the isolates **4**, **10** and **12** were determined to inhibit *H. pylori* growth at an effective level (inhibition zone of >10 mm). These three isolates were further assayed with regard to their ability to inhibit the adhesion of *H. pylori* to AGS cells. AGS cells were washed 3 h after infection with *H. pylori* in the presence of isolates **4**, **10** and **12**. As shown in Fig. 3A, the isolate **4** exhibited a significant inhibition of *H. pylori* attachment was observed with a reduction of 63.3–72.9% in a range of concentrations between 0.005 and 0.02 mM compared with control DMSO. At these concentrations, isolate **10** has such effect with a reduction of 43.5–63.3%, while **12** has no activity (Fig. 3A). The results suggest that the mode of action of *A. camphorata* isolates **4** and **10** are related to antibacterial and, in addition, to anti-adhesion activities. On the other hand, a significant inhibition of bacterial invasion into AGS cells by isolate **4** and **10** was also found in the concentration range from 0.005 to 0.02 mM, while **12** has moderate effect only at the relatively higher concentration of 0.02 mM (Fig. 3B). Importantly, after treatment of isolate **4**, the bacterial invasion activity was dramatically reduced by more than 92.9% at a concentration of 0.01 mM. DMSO vehicle had no effect on adhesion and invasion assays. Results from this study demonstrate that isolates **4** and **10** have their ability to inhibit bacterial adhesion and invasion of AGS cells.

In human gastric epithelial AGS cells *H. pylori*-infection causes infiltration of gastric mucosa with polymorphonuclear (PMN) leukocytes, which are responsible for the following gastric inflammation (Sempertegui, Diaz, & Mejia, 2007). Leukocyte adherence to the vascular endothelium is promoted by adhesion factors, triggered by activation of transcription factors as NF-κB. NF-κB is a ubiquitous transcription factor complex belonging to the Rel family of proteins, and is a crucial regulator of many cellular processes including the control of the immune response and inflammation (Li & Verma, 2002). The activated leukocytes are the source for massive free oxygen radical production in the mucosa (Kountouras, Chatzopoulos, & Zavos, 2001). These processes are initiated by the multifunctional protein, cag A, secreted by *H. pylori*, which triggers interleukin-8 expression by the NF-κB signalling pathway

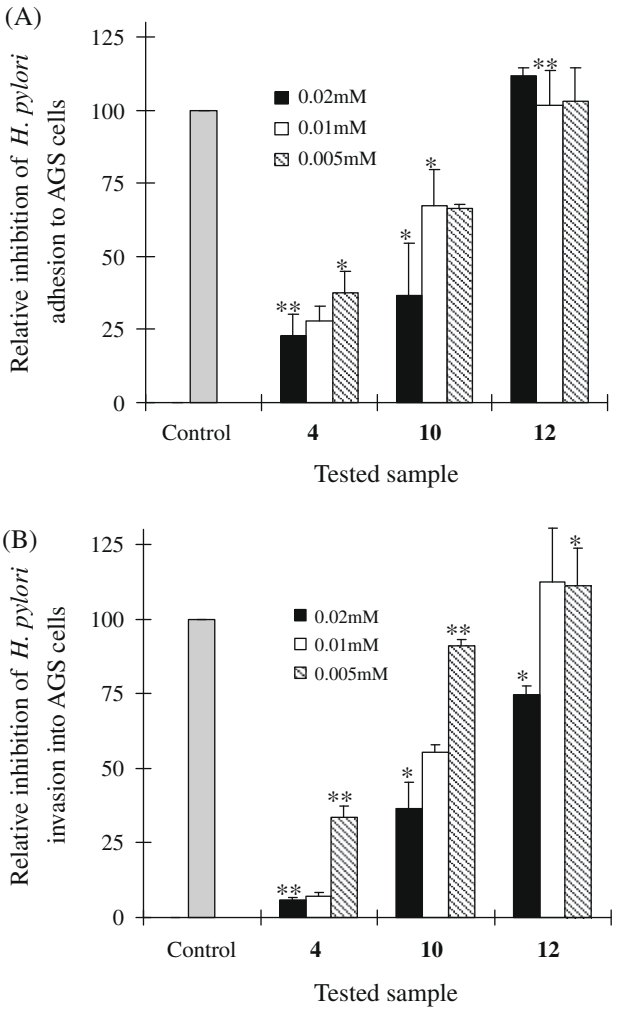

Fig. 3. Effect of *Antrodia camphorata* isolates on *Helicobacter pylori* anti-adhesion (A) and anti-invasion (B) of gastric epithelial cells. The bacteria to AGS cells that were untreated or treated with isolates **4**, **10** and **12** or DMSO control, followed by infection with *Helicobacter pylori* at a MOI of 100 for 6 h. Each experiment was shown represent mean values ± SD of at least six independent experiments. The significant difference was set at $^{*}p < 0.05$; $^{**}p < 0.01$.

(Brandt, Kwok, Hartig, Konig, & Backert, 2005). IL-8 plays a crucial role in the initiation and maintenance of inflammatory response and is identified to function as a proangiogenic or carcinogenic factor (Kido et al., 2001). Therefore, the isolates **4**, **10** and **12** were additionally assessed with regard to their inhibitory effects against *H. pylori*-induced NF-κB activation and IL-8 secretion from AGS cells. When AGS cells were transiently transfected with NF-κB-luciferase construct, pretreatment of cells with isolates **4**, **10** and **12** prior to *H. pylori* infection led to a dose-dependent reduction in the stimulation of luciferase activity. As shown in Fig. 4A, the isolate **4** exhibited a significant inhibition of luciferase activity with a reduction of 0.26–0.46% in a range of concentrations between 0.005 and 0.02 mM compared with the control (DMSO) value of 1. At these concentrations, isolate **10** has such effect with a reduction of 0.05–0.27%, while **12** has a moderate effect (inhibition 0.14%) only at a relatively higher concentration of 0.02 mM (Fig. 4A). The exact mechanism whereby these isolates inhibited NF-κB activation needs further study. We further examined the effect of isolates **4**, **10** and **12** on *H. pylori*-induced IL-8 production by AGS cells. IL-8 production in AGS cells infected with *H. pylori* with or without pre-incubation of isolates **4**, **10** and **12** raging from 0.005 to 0.02 mM was measured. Pretreatment with 0.005, 0.01,

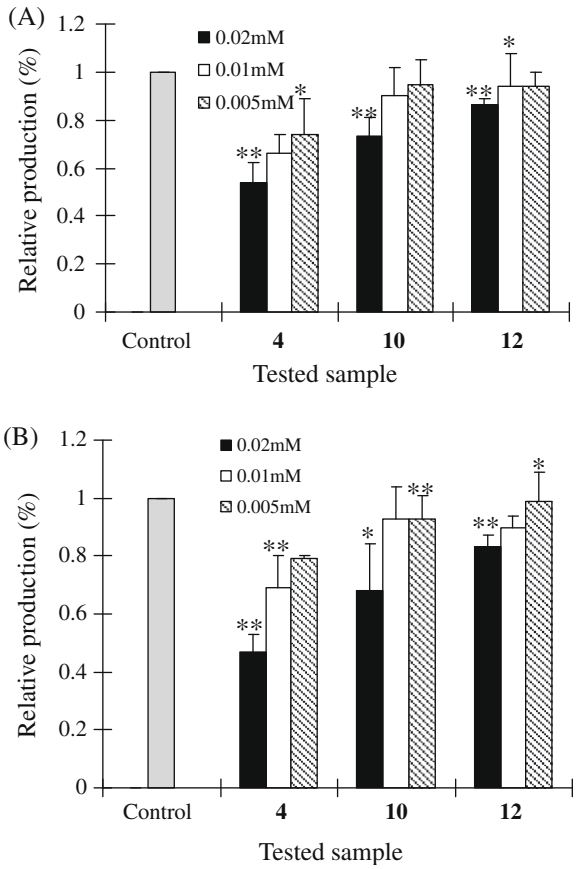

Fig. 4. Inhibitory effect of *Antrodia camphorata* isolates on *Helicobacter pylori*-induced NF-κB activation (A) and IL-8-luciferase activity (B), in gastric epithelial cells. The luciferase activity was determined as described in materials and methods. Results are shown by mean values ± SD from at least three independent experiments. The significant difference was set at $^{*}p < 0.05$; $^{**}p < 0.01$.

and 0.02 mM of isolates **4** and **10** in *H. pylori*-infected AGS cells for 24 h significantly decreased the IL-8 production by 26%, 34%, 46%; and 5%, 10% and 27%, respectively, compared to *H.* pylori infected cells with DMSO control (Fig. 4B). The isolate **12** has lower IL-8 inhibitory effect (14%) at higher concentration of 0.02 mM (Fig. 4B). These results indicate that isolates **4** and **10** might have a role in the attenuation of the IL-8 signal produced by gastric epithelial cells in response to *H. pylori* infection. This may be due to inactivation of multiple intracellular signalling pathways induced by *H. pylori* infection and decrease of NF-κB translocation into the nucleus (Li & Verma, 2002). To the best of our knowledge, we demonstrate here for the first time that *A. camphorata* isolates inhibited *H. pylori*-associated inflammation in AGS cells.

4. Conclusion

In summary, this study demonstrated that *A. camphorata* isolates displayed potential anti-*H. pylori* activity. The isolates methyl antcinate B and antcin K inhibit the *H. pylori* adhesion and invasion to AGS cells. In addition, these isolates also inhibit the IL-8 protein secretion and NF-κB signal activation in *H. pylori*-infected gastric epithelial cells. This inhibition of NF-κB activation and IL-8 secretion by methyl antcinate B and antcin K might contribute to the potential role of *A. camphorata* as an anti-inflammatory action in *H. pylori*-induced gastric epithelial cells damage. Future studies are needed to clarify the molecular mechanisms by which *A. camphorata* inhibits *H. pylori*-mediated activation of NF-κB expression in AGS cells and to identify additional targets in gene regulation.

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