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Bioevaluation of *Anisomeles indica* extracts and their inhibitory effects on *Helicobacter pylori*-mediated inflammation

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ABSTRACT

Ethnopharmacological relevance: *Helicobacter pylori* is associated with the majority of gastric disorders and the antibiotic resistant rates have increased annually worldwide. *Anisomeles indica* and its constituent, ovatodiolide (OVT), were shown to have bactericide activity against *Helicobacter pylori*. The aim of this study was to manufacture extracts containing the effective constituent, OVT, and evaluate their bactericidal function and the inhibition of inflammatory responses to *Helicobacter pylori* infection.

Materials and methods: Various concentrations of ethanol for extraction of *Anisomeles indica* were performed and the content of OVT was analyzed by high-performance liquid chromatography (HPLC). The anti-bacterial activity of *Anisomeles indica* ethanol extracts and the constituent OVT were determined. Additional experiments were performed to investigate the *Anisomeles indica* ethanol extracts and OVT to inhibit the *Helicobacter pylori*-induced inflammation of both gastric epithelial cells and macrophages.

Results: Amongst the extracts tested, 50% and 95% ethanol extracts contained large amount of OVT and showed potent anti-*Helicobacter pylori* activity. An in vitro *Helicobacter pylori*-infection model revealed that 95% ethanol extract attenuated *Helicobacter pylori*-induced nuclear factor kappa B (NF-κB) activity and interleukin (IL)-8 secretion of gastric epithelial cells. In addition, 95% ethanol extract significantly inhibited lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS), as well as production of nitric oxide (NO) and tumor necrosis factor α (TNF-α) by macrophages.

Conclusions: This study reveals that *Anisomeles indica* ethanol extracts containing OVT may be a potent and economic therapeutic agent for *Helicobacter pylori* infection and attenuation of *Helicobacter pylori*-mediated inflammation.

1. Introduction

Helicobacter pylori is a Gram-negative microaerophilic bacterium that commonly infects the human stomach and causes several types of gastrointestinal diseases (Marshall, 2002). Infection of *Helicobacter pylori* may persist in the human stomach for a life-time, resulting in release of interleukin-8 (IL-8), a crucial chemokine for neutrophil infiltration, leading to chronic inflammation (Yamaoka et al., 1996). Strains of *Helicobacter pylori* containing a functional cytotoxin-associated gene A (CagA) are linked to the mechanism of chronic gastritis due to *Helicobacter pylori* infection (Yamaoka et al., 1997). After injection by the type IV secretion system, CagA is phosphorylated, and subsequently induces an inflammatory response through activating nuclear factor-κB (NF-κB) translocation into the nucleus (Brandt et al., 2005). Additionally, during *Helicobacter pylori* infection, nitric oxide (NO)—an inflammatory mediator produced by inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in both macrophages and the gastric epithelium—is strongly associated with tissue inflammation and injury (Wilson et al., 1996). Therefore, inhibition of *Helicobacter pylori*-induced NF-κB activation, IL-8 secretion, and attenuation of NO production might be a useful therapeutic strategy for chronic gastritis.

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Treating *Helicobacter pylori*-infected patients clinically involves in combination with a proton pump inhibitor and various types of antibiotics, leading to more than 90% eradication of *Helicobacter pylori* and reduces the recurrence of peptic ulcers (Zullo et al., 2005). However, given the extensive treatment with antibiotics for decades, the failure rates due to antimicrobial resistance range from 20% to 40% (Megraud and Lamouliatte, 2003). Therefore, development of effective non-antibiotic therapeutic approaches with low manufacturing costs is urgently required.

Anisomeles indica is commonly used for numerous conditions, such as gastrointestinal disease, liver disease, as well as immune system deficiencies (Huang et al., 2003). It has been previously reported that extractions and isolated constituents of *Anisomeles indica* exhibit inhibition of inflammatory mediators and tumor cell proliferation (Hsieh et al., 2008; Rao et al., 2009). Additionally, ovatodiolide (OVT) as a pure constituent isolated from *Anisomeles indica* has been shown to have bactericide activity against *Helicobacter pylori*, resulting in reduction of *Helicobacter pylori* bacterial adhesion and invasion to human gastric epithelial (AGS) cells in our recent study (Rao et al., 2012). For economical purification of the effective constituent, we developed ethanol extracts containing OVT and examined their bactericidal function and attenuation of inflammatory responses in *Helicobacter pylori*-infected cells.

2. Materials and methods

2.1. Chemicals and reagents

Antibodies specific to iNOS and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Amoxicillin (AMX), clarithromycin (CLR), metronidazole (MTZ), and LPS (*Escherichia coli* O55: B5) were purchased from Sigma–Aldrich (St. Louis, MO). Whole plant of *Anisomeles indica* was obtained from Yusheng Co., Ltd. (Taichung, Taiwan).

2.2. Plant materials, extraction, and purification

The air-dried stems (500 g) of *Anisomeles indica* were extracted with 4.5 L of distilled water or ethanol (10%, 20%, 50%, and 95%) under reflux for 2 h. After filtering individually, the concentrated extracts were freeze-dried, resulting in dark brown solid masses (45.1, 41.2, 33.3 and 11.0 g/kg, respectively). OVT from *Anisomeles indica* was prepared as described previously (Rao et al., 2012). The active compound, OVT contained in the various extracts was confirmed by high-performance liquid chromatography (HPLC) [column: RP C18e 4.6 × 250 mm, 5 μm (Merck, Rahway, NJ)] (Rao et al., 2012). The mobile phase consisted of acetonitrile and 0.1% trifluoroacetic acid (TFA) in water, 64:36 (UV detection at 265 nm). Representative HPLC chromatograms are shown in Supplemental Fig. S1.

2.3. Cell and bacterial culture

AGS cells (ATCC CRL 1739) and RAW264.7 cells (ATCC TIB-71) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured as described previously (Lu et al., 2012a). *Helicobacter pylori* 26695 (ATCC 700392) were routinely cultured on Brucella blood agar plates (Becton Dickinson, Franklin Lakes, NJ) containing 10% sheep blood under 5% CO_2 and 10% O_2 conditions at 37 °C for 48 h (Lu et al., 2012b).

2.4. Determination of anti-Helicobacter pylori activity

Anti-*Helicobacter pylori* activities of chemical constituents and ethanol extracts from *Anisomeles indica* were determined by the disc agar diffusion method as described previously (Lai et al., 2010), while three standard antimicrobial agents (AMX, CLR, and MTZ) were used as positive controls (Lai et al., 2008).

2.5. NF-κB reporter luciferase assay

Cells were cultured in a 12-well plate (Nunc, Roskilde, Denmark) and then transfected with NF-κB-luc reporter plasmid using Lipofectamine 2000 (Invitrogen) as described previously (Lai et al., 2011). Cells were treated with various concentrations of ethanol extracts of *Anisomeles indica* followed by infection with *Helicobacter pylori* for 6 h. The transfected cells were lysed, and luciferase assays were performed with the Dual-Luciferase Reporter Assay System and normalized by co-transfection with a β-galactosidase expression vector (Promega, Madison, MA).

2.6. Measurement of cytokines

After cells were treated with various concentrations of ethanol extracts in cell culture medium, the cells were infected with *Helicobacter pylori* at an MOI of 1:100. The supernatants from cell cultures were collected, the levels of IL-8 and tumor necrosis factor-alpha (TNF-α) were determined by using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN).

2.7. Determination of nitric oxide production and cell viability assay

Nitric oxide (NO) production was determined using the Griess reagent (Sigma–Aldrich) (Lu et al., 2012b). The MTT assay was used to measure the cytotoxicity of tested agents in AGS or RAW264.7 cells as described previously (Chang et al., 2012).

2.8. Statistical analysis

The data are presented as mean ± standard deviation of triplicate experiments. The Student's *t*-test was used to calculate the statistical significance of experimental results and the symbol '*' indicates $P < 0.05$ compared with untreated controls.

3. Results

3.1. Growth inhibition of Helicobacter pylori by Anisomeles indica extracts

Our recent study showed that the pure constituent OVT isolated from *Anisomeles indica* was able to inhibit *Helicobacter pylori*-induced inflammation in human gastric epithelial cells (Rao et al., 2012). To further manufacture large amounts of OVT-containing extracts, various concentrations of ethanol for extraction of *Anisomeles indica* were performed and the content of OVT was analyzed by HPLC (Supplemental Fig. S1). The concentrations of OVT were 0.04, 0.14, and 0.16 mg/g in 20%, 50%, and 95% ethanol extracts, respectively (Supplemental Table S1). However, there was no OVT in water or 10% ethanol extracts. We then evaluated their inhibitory activity against *Helicobacter pylori* growth. By using the agar disk diffusion approach, the *Anisomeles indica* extracts showed a wide range of inhibitory effects against *Helicobacter pylori* growth with inhibition zones ranging from 0 to 13 mm (Table 1). *Anisomeles indica* 50% and 95% ethanol extracts showed inhibition zone values of 7 and 13 mm, respectively. Our data indicates that the extraction by 95% ethanol provided greater activity than water and other ethanol extractions. In the three purified constituents, the OVT exhibited a much better activity against *Helicobacter pylori* than aceoside and compneoside.