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Ethnopharmacological communication

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.

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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.

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Table 1
Growth inhibition of *Helicobacter pylori* by *Anisomeles indica* ethanol extracts and constituents.

Tested sample[a]	Inhibition zone (mm)[b]
H_2O Extraction	0
10% EtOH Ext	0
20% EtOH Ext	0
50% EtOH Ext	7
95% EtOH Ext	13
Acteoside	12
Compneoside	9
Ovatodiolide	23
AMX	14
CLR	21
MTZ	7
DMSO	0

[a] AMX, amoxicillin; CLR, clarithromycin; MTZ, metronidazole.

[b] The concentrations of water extract, ethanol extracts, and constituents from *Anisomeles indica* were 0.5 mg/ml. The standard antimicrobial agents including AMX (0.05 mg/ml), CLR (0.05 mg/ml), and MTZ (0.8 mg/ml) were used as positive control, whereas DMSO was used as a negative control.

3.2. Inhibitory effects against Helicobacter pylori-induced inflammation in human gastric epithelial cells

We then analyzed whether ethanol extracts of *Anisomeles indica* influenced NF-κB activation. As shown in Fig. 1A, the extraction from 50% ethanol inhibited the luciferase activity by only 14.2% at 400 μg/ml compared to the *Helicobacter pylori* infection alone. The extraction of 95% ethanol dose-dependently inhibited the luciferase activity from 9.6% to 48.1% at concentrations of 100–400 μg/ml compared to DMSO control. In addition, the positive control, OVT, effectively inhibited the luciferase activity by 64.6% at 0.06 μM compared to control. We then examined IL-8 production in AGS cells following infection with *Helicobacter pylori* in the presence of *Anisomeles indica* ethanol extracts or OVT. The extractions of 50% or 95% ethanol reduced the IL-8 secretion by 21.6–42.7%, and 31.1–69.4%, respectively, at the concentrations of 100–400 μg/ml compared to infection of *Helicobacter pylori* alone, whereas OVT reduced IL-8 secretion by 81.9% at 0.06 μM (Fig. 1B). These results indicate that *Anisomeles indica* extracts may decrease the IL-8-induced inflammatory responses through attenuation of NF-κB activity in *Helicobacter pylori*-infected gastric epithelial cells.

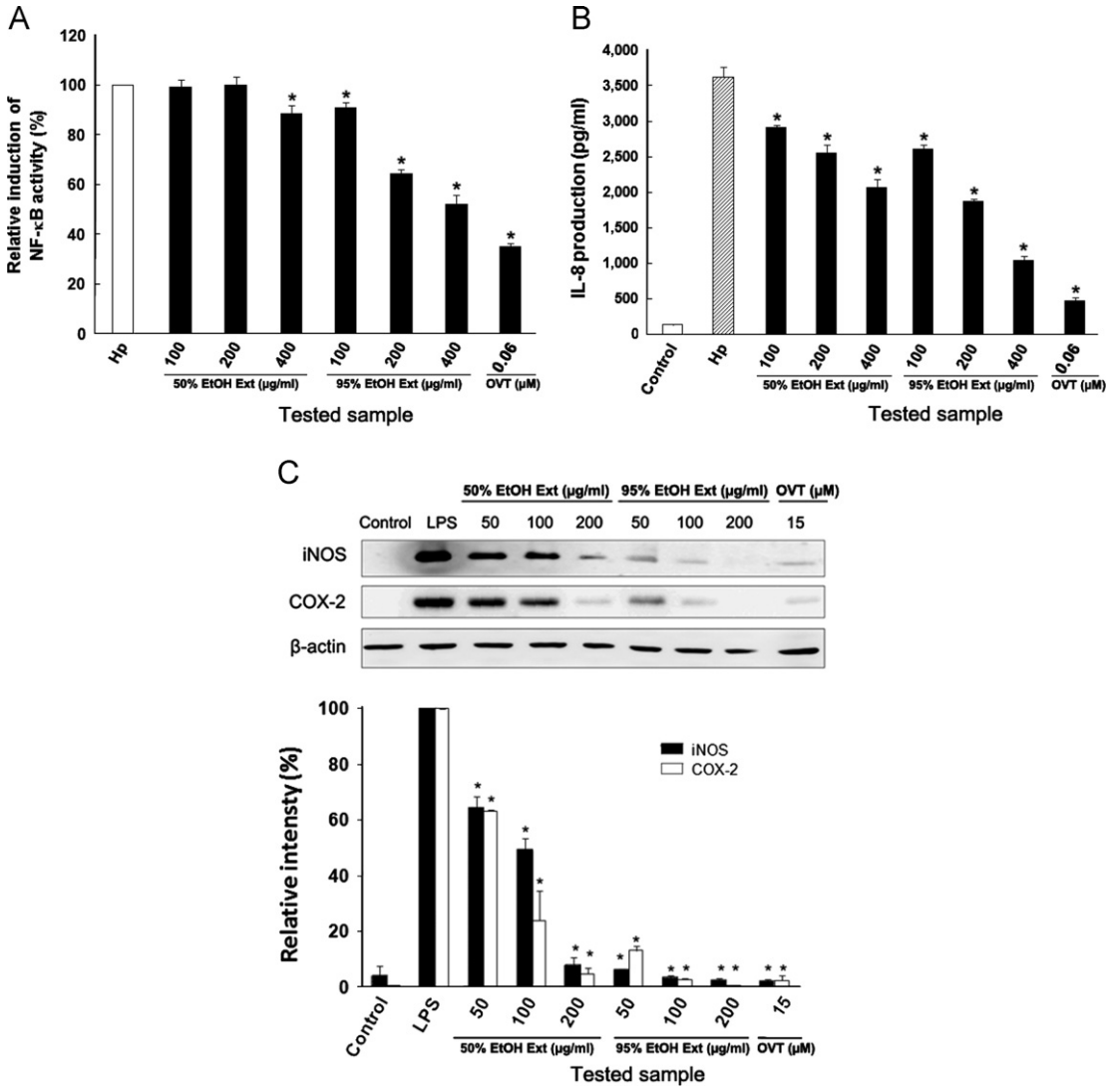

Fig. 1. *Anisomeles indica* ethanol extracts and OVT-mediated inhibition of inflammatory responses. Effects of the treatments on *Helicobacter pylori*-induced (A) NF-κB activity and (B) IL-8 secretion in AGS cells were determined. (C) The expressions of iNOS and COX-2 of LPS-stimulated macrophages were analyzed and protein expression levels of iNOS and COX-2 were quantified by densitometric analysis and normalized to β-actin. The data are presented as means ± standard deviations for three independent experiments. $^{*}P < 0.05$.

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3.3. *Anisomeles indica* extracts inhibited LPS-induced inflammation in macrophages

To further determine whether *Anisomeles indica* extracts inhibit *Helicobacter pylori*-induced inflammatory mediators, we determined the levels of NO production and expressions of iNOS in LPS-stimulated macrophage RAW264.7 cells. Our data revealed that *Anisomeles indica* 50% and 95% ethanol extractions dose-dependently inhibited NO production by 8.9–38.7%, and 39.6–82.2% at concentrations of 50–200 μg/ml, respectively (Supplemental Fig. S2A). The positive control OVT inhibited NO production by 83.8% at 15 μM. Additionally, in a dose-dependent manner, the extracts from 50% and 95% ethanol suppressed the TNF-α production of LPS-stimulated macrophage by 42.–74.6%, and 13.0–91.2%, at 50–200 μg/ml, respectively, whereas OVT suppressed TNF-α production by 55.8% at 15 μM (Supplemental Fig. S2B). Inflammation-related proteins iNOS and COX-2 were then examined by immunoblot analysis to investigate the effects of *Anisomeles indica* extracts inhibit *Helicobacter pylori*-mediated inflammatory responses in macrophages. As shown in Fig. 1C, the expression levels of iNOS and COX-2 were significantly decreased in LPS-induced macrophages treated with 95% and 50% ethanol extracts, as well as OVT. These results indicate that *Anisomeles indica* ethanol extracts that contain OVT may play a critical role in the attenuation of inflammation in macrophages.

4. Discussion

With increasing antimicrobial resistance, it is necessary to develop potent therapeutic agents of *Helicobacter pylori* infection. Previously, it has been reported that extractions of *Anisomeles indica* inhibited *Helicobacter pylori* growth and *Helicobacter pylori*-induced inflammation (Hsieh et al., 2008). Based on our recent study, OVT—one of the pure constituents isolated from *Anisomeles indica*—may be responsible for the antimicrobial activity and effects against *Helicobacter pylori*-infection (Rao et al., 2012). This study further suggested that it would be worth validating the molecular mechanisms by which *Anisomeles indica* acts against *Helicobacter pylori*-induced inflammation either in the gastric epithelium or macrophages, and to develop more efficient and feasible approaches for production of OVT-containing extracts from *Anisomeles indica*.

In the present study, 95% ethanol extract of *Anisomeles indica* contained a large amount of OVT (Supplemental Table S1), whose inhibition activity is much better than standard antibiotic MTZ (Table 1). Additionally, treatment of *Helicobacter pylori*-infected cells with 95% ethanol extracts of *Anisomeles indica* significantly attenuated the inflammatory response by decreasing NF-κB activation and IL-8 secretion (Fig. 1). These results indicate that 95% ethanol extract might be a potential approach to obtain vast amounts of OVT with bactericidal function as well as an inhibitory effect on *Helicobacter pylori*-induced inflammation.

Since high concentrations of reactive nitrogen species as free radicals could contribute to the oxidative stress and lead to an increase in DNA mutation rates, the production of NO by LPS-stimulated macrophages is one of the most important mechanisms responsible for the development of chronic gastritis into gastric carcinoma (Coussens and Werb, 2002). Our current study reveals that *Anisomeles indica* extracts (50% and 95% ethanol) can inhibit LPS-induced inflammation in macrophages, including the secretion of the pro-inflammatory cytokine TNF-α, and NO production (Supplemental Fig. S2), as well as the protein expressions of iNOS and COX-2 (Fig. 1C). These mediators are involved in inflammatory signaling pathways, suggesting that the anti-inflammatory activity was contained in the 50% and 95% ethanol *Anisomeles indica* extracts. Our present findings are consistent with a previous study which reported that the *Anisomeles indica* extracts may act like an anti-inflammatory agent by decreasing inflammatory mediator production (Hsieh et al., 2008).

This is the first report on the ethanol extracts from *Anisomeles indica* regarding their inhibitory effects against *Helicobacter pylori*-induced inflammatory mediators by gastric epithelial cells and macrophages. We demonstrate that the inhibitory activity against *Helicobacter pylori*-induced inflammatory responses was due to the *Anisomeles indica* ethanol extracts, which contained a great quantity of OVT. Since the *Anisomeles indica* ethanol extracts did not harm the host cells and a vast amount of OVT was contained in the 95% ethanol extract, which appears to be a suitable candidate to develop a new therapeutic agent against *Helicobacter pylori* infection and to prevent chronic superficial gastritis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2012.11.015.

References

Brandt, S., Kwok, T., Hartig, R., Konig, W., Backert, S., 2005. NF-kappaB activation and potentiation of proinflammatory responses by the *Helicobacter pylori* CagA protein. Proceedings of the National Academy of Sciences of the United States of America 102, 9300–9305.

Chang, C.S., Liu, J.F., Lin, H.J., Lin, C.D., Tang, C.H., Lu, D.Y., Sing, Y.T., Chen, L.Y., Kao, M.C., Kuo, S.C., Lai, C.H., 2012. Synthesis and bioevaluation of novel 3,4, 5-trimethoxybenzylbenzimidazole derivatives that inhibit *Helicobacter pylori*-induced pathogenesis in human gastric epithelial cells. European Journal of Medicinal Chemistry 48, 244–254.

Coussens, L.M., Werb, Z., 2002. Inflammation and cancer. Nature 420, 860–867.

Hsieh, S.C., Fang, S.H., Rao, Y.K., Tzeng, Y.M., 2008. Inhibition of pro-inflammatory mediators and tumor cell proliferation by *Anisomeles indica* extracts. Journal of Ethnopharmacology 118, 65–70.

Huang, T.C., Hsieh, C.F., Boufford, D.E., Kuoh, C.S., Ohashi, H., Peng, C.I., Tsai, J.L., Yang, K.C., Hsiao, A., Tsai, J.M. (Eds.), 2003. Flora of Taiwan,2nd Eds., National Science Council of the Republic of China, Taipei, pp. 437–448.

Lai, C.H., Fang, S.H., Rao, Y.K., Geethangili, M., Tang, C.H., Lin, Y.J., Hung, C.H., Wang, W.C., Tzeng, Y.M., 2008. Inhibition of *Helicobacter pylori*-induced inflammation in human gastric epithelial AGS cells by *Phyllanthus urinaria* extracts. Journal of Ethnopharmacology 118, 522–526.

Lai, C.H., Rao, Y.K., Fang, S.H., Sing, Y.T., Tzeng, Y.M., 2010. Identification of 3′,4′, 5′-trimethoxychalcone analogues as potent inhibitors of *Helicobacter pylori*-induced inflammation in human gastric epithelial cells. Bioorganic & Medicinal Chemistry Letters 20, 5462–5465.

Lai, C.H., Wang, H.J., Chang, Y.C., Hsieh, W.C., Lin, H.J., Tang, C.H., Sheu, J.J., Lin, C.J., Yang, M.S., Tseng, S.F., Wang, W.C., 2011. *Helicobacter pylori* CagA-mediated IL-8 induction in gastric epithelial cells is cholesterol-dependent and requires the C-terminal tyrosine phosphorylation-containing domain. FEMS Microbiology Letters 323, 155–163.

Lu, D.Y., Chen, H.C., Yang, M.S., Hsu, Y.M., Lin, H.J., Tang, C.H., Lee, C.H., Lai, C.K., Lin, C.J., Shyu, W.C., Lin, F.Y., Lai, C.H., 2012a. Ceramide and Toll-like receptor 4 are mobilized into membrane rafts in response to *Helicobacter pylori* infection in gastric epithelial cells. Infection and Immunity 80, 1823–1833.

Lu, D.Y., Tang, C.H., Chang, C.H., Maa, M.C., Fang, S.H., Hsu, Y.M., Lin, Y.H., Lin, C.J., Lee, W.C., Lin, H.J., Lee, C.H., Lai, C.H., 2012b. *Helicobacter pylori* attenuates lipopolysaccharide-induced nitric oxide production by murine macrophages. Innate Immunity 18, 406–417.

Marshall, B., 2002. *Helicobacter pylori*: 20 years on. Clinical Medicine 2, 147–152.

Megraud, F., Lamouliatte, H., 2003. Review article: the treatment of refractory *Helicobacter pylori* infection. Alimentary Pharmacology & Therapeutics 17, 1333–1343.

Rao, Y.K., Fang, S.H., Hsieh, S.C., Yeh, T.H., Tzeng, Y.M., 2009. The constituents of *Anisomeles indica* and their anti-inflammatory activities. Journal of Ethnopharmacology 121, 292–296.

Rao, Y.K., Lien, H.M., Lin, Y.H., Hsu, Y.M., Yeh, C.T., Chen, C.C., Lai, C.H., Tzeng, Y.M., 2012. Antibacterial activities of *Anisomeles indica* constituents and their

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inhibition effect on *Helicobacter pylori*-induced inflammation in human gastric epithelial cells. Food Chemistry 132, 780–787.
Wilson, K.T., Ramanujam, K.S., Mobley, H.L., Musselman, R.F., James, S.P., Meltzer, S.J., 1996. *Helicobacter pylori* stimulates inducible nitric oxide synthase expression and activity in a murine macrophage cell line. Gastroenterology 111, 1524–1533.
Yamaoka, Y., Kita, M., Kodama, T., Sawai, N., Imanishi, J., 1996. *Helicobacter pylori cagA* gene and expression of cytokine messenger RNA in gastric mucosa. Gastroenterology 110, 1744–1752.
Yamaoka, Y., Kita, M., Kodama, T., Sawai, N., Kashima, K., Imanishi, J., 1997. Induction of various cytokines and development of severe mucosal inflammation by *cagA* gene positive *Helicobacter pylori* strains. Gut 41, 442–451.
Zullo, A., Gatta, L., De Francesco, V., Hassan, C., Ricci, C., Bernabucci, V., Cavina, M., Ierardi, E., Morini, S., Vaira, D., 2005. High rate of *Helicobacter pylori* eradication with sequential therapy in elderly patients with peptic ulcer: a prospective controlled study. Alimentary Pharmacology & Therapeutics 21, 1419–1424.