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Inhibition of *Helicobacter pylori*-induced inflammation in human gastric epithelial AGS cells by *Phyllanthus urinaria* extracts

Chih-Ho Lai[a], Shih-Hua Fang[b], Yerra Koteswara Rao[c], Madamanchi Geethangili[c], Chih-Hsin Tang[d], Ying-Ju Lin[e], Chien-Hui Hung[a], Wen-Ching Wang[f], Yew-Min Tzeng[c,*]

[a] Department of Microbiology, School of Medicine, China Medical University, Taichung, Taiwan, ROC
[b] Institute of Athletics, National Taiwan College of Physical Education, Taichung, Taiwan, ROC
[c] Institute of Biotechnology, Chaoyang University of Technology, Wufeng, Taiwan, ROC
[d] Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan, ROC
[e] Department of Medical Research, China Medical University Hospital, Taichung, Taiwan, ROC
[f] Institute of Molecular and Cellular Biology & Department of Life Sciences, National Tsing Hua University, Hsinchu, Taiwan, ROC

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ABSTRACT

Aim of the study: Helicobacter pylori is linked to a majority of peptic ulcers and to some types of gastric cancer, and its resistance to antibiotic treatment is now found worldwide. This study is aimed at evaluating the antimicrobial activity of *Phyllanthus urinaria* Linnea (Euphorbiaceae), chloroform (PUC) and methanol (PUM) extracts, and its eight isolates on *H. pylori*-infected human gastric epithelial AGS cells.
Materials and methods: The *in vitro* anti-bacterial activity of *P. urinaria* chloroform (PUC) and methanol (PUM) extracts, and its eight isolates were determined. Additional experiments were also performed to know the PUC and PUM ability to inhibit the *H. pylori* adhesion to and invasion of AGS cells, in addition to the effect of PUC on NF-κB activity as well as IL-8 synthesis during *H. pylori* infection of AGS cells.
Results: The results revealed that crude extracts PUC and PUM showed potent antimicrobial activity against *H. pylori* than pure isolates. On the other hand, *in vitro H. pylori*-infection model revealed that the inhibition of bacterial adhesion and invasion to AGS cells has dramatically reduced by treatment of extract PUC, while PUM has the same moderate effect. Furthermore, *H. pylori*-induced nuclear factor (NF)-κB activation, and the subsequent release of interleukin (IL)-8 in AGS cells were also inhibited by the extract PUC.
Conclusions: These results open the possibility of considering *P. urinaria* a chemopreventive agent for peptic ulcer or gastric cancer, but this bioactivity should be confirmed *in vivo* in the future.
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1. Introduction

The gram-negative bacterium *Helicobacter pylori* colonize the stomach where it can induce peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma (Gerhard et al., 2002; Peek and Blaser, 2002). The most accepted regime for the eradication of *H. pylori* infection currently includes a triple therapy, which combines the antibiotic clarithromycin (CLR) and amoxicillin (AMX) with a proton pump inhibitor such as omeprazole. This chemotherapy, however, sometimes produces side-effects and fails to eliminate infection in 10–30% of patients (Cavallaro et al., 2006). The occurrence of strains resistant to antibiotics would be expected to increase, and it is nowadays important to search for non-antibiotic substances with anti-*H. pylori* activity. In human gastric epithelial AGS cells, *H. pylori* induces inflammatory-associated gene expression, including activation of the nuclear factor kappa B (NF-κB), and production of interleukin (IL)-8 (Naumann, 2001). 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 and Verma, 2002). On the other hand, among the cytokines induced in the gastric mucosa colonized by *H. pylori*, IL-8 is one of the major proinflammatory cytokines, first isolated from monocytes as a neutrophil chemoattractant (Kido et al., 2001). IL-8 plays a crucial role in the initiation and maintenance of inflammatory response and recently has been identified to function as proangiogenic or carcinogenic factor based on the findings that gastric cancer cells in surgical specimens overexpressed IL-8 compared with corresponding normal mucosa (Kido et al., 2001).

Medicinal plants have been used as traditional remedies in treating and preventing gastrointestinal diseases for hundreds of years, and their anti-*H. pylori* activity has been widely demonstrated *in vitro* (Stamatis et al., 2003; Ustun et al., 2006; Ndip et al., 2007; Shih

* Corresponding author. Tel.: +886 4 23323000x3003; fax: +886 4 23304896.
E-mail address: ymtzeng@cyut.edu.tw (Y.-M. Tzeng).

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et al., 2007). *Phyllanthus urinaria* Linnea, one of the herbal plants belonging to the genus *Phyllanthus* (Euphorbiaceae), is widely distributed in tropical and subtropical countries including Taiwan. The species of *Phyllanthus* have long been used in folk medicine to treat kidney and urinary bladder disturbances, intestinal infections, diabetes, and hepatitis B in several parts of the world (Calixto et al., 1998). Particularly, *P. urinaria* is traditionally used in Taiwan to reduce heat, gastritis and ulcers, remove food stagnancy, improve eyesight, relieve inflammation, calm the liver, suppress *yang* hyperactivity of liver, detoxify poison from body and increase the flow of the urine (Chiu and Chang, 1998; Committee on Chinese Medicine and Pharmacy, 2003). Furthermore, the decoction from this species is commonly used as tea and juice in Taiwan for the treatment of inflammatory diseases (Kao, 1985). Although, various biological activities of *P. urinaria* have been reported, however, there is no report on its action against *H. pylori*-induced inflammation. In our previous study, we analyzed *P. urinaria* components, and their DPPH radical scavenging and inflammatory mediators, nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-6 growth inhibitory activities in LPS/IFN-γ-activated murine peritoneal macrophages (Fang et al., 2008). In the present study, we examined the anti-bacterial properties of this species chloroform (PUC) and methanol (PUM) extracts, and its eight isolates. Additional experiments were also performed to know the PUC and PUM ability to inhibit the *H. pylori* adhesion to and invasion of AGS cells, in addition to the effect of PUC on NF-κB activity as well as IL-8 synthesis during *H. pylori* infection of AGS cells. With this approach, we hope to understand the protective mechanism of *P. urinaria* on *H. pylori*-induced inflammation and increase its clinical potential in gastrointestinal disease.

2. Materials and methods

2.1. Isolation of compounds from P. urinaria

The voucher specimen, and method for extraction and isolation were described previously (Fang et al., 2008). Briefly, the dried plant material (800 g) was extracted with chloroform (PUC) and methanol (PUM), sequentially to yield the respective solvent extracts. After exhaustive extraction, the combined extracts were filtered, and the solvent was dried by rotary evaporation under reduced pressure at a temperature of maximally 35 °C. The solvent free extracts were used for the present study. The dried chloroform extract (PUC, 14 g, 1.75% w/w) was subjected to column chromatography (CC) and eluted with a gradient of *n*-hexane/EtOAc (the ratios of *n*-hexane/EtOAc were from 100:0 to 0:100) to afford phyllanthin (**1**, 55 mg, 0.0069%), phyltetralin (**2**, 12 mg, 0.0015%), trimethyl 3,4-dehydrochebulate (**3**, 10 mg, 0.0008%), methylgallate (**4**, 75 mg, 0.0094%), and rhamnocitrin (**5**, 14 mg, 0.0018%). The dried methanol extract (PUM, 11 g, 1.375%, w/w) was subjected to repeated column chromatography on a silica gel and eluted with CH_2Cl_2/MeOH with a gradually increased ratio of methanol to afforded compounds, methyl brevifolincarboxylate (**6**, 26 mg, 0.0033%), quercetin-3-α-L-rhamnopyranoside (quercitrin, **7**, 8 mg, 0.001%), β-sitosterol 3-*O*-β-D-glucopyranoside (**8**, 25 mg, 0.0031%), and rutin (**9**, 32 mg, 0.004%). All the derivatives were identified by comparing their physical and spectral data as reported in our previous communication (Fang et al., 2008).

2.2. Bacterial and cell culture

H. pylori, strain 26695 (ATCC 700392) was used as a reference strain. Bacterial strain was recovered from frozen stocks on Brucella agar plates (Difco) containing 10% sheep blood, 6 μg/ml vancomycin and 2 μg/ml amphotericin B under microaerophilic conditions for 48–72 h as described previously (Lai et al., 2002, 2006).

AGS cells (ATCC CRL 1739; human gastric adenocarcinoma epithelial cell line) were cultured in F12 (Hyclone) supplemented with 10% de-complement FBS (Hyclone). Penicillin and streptomycin (GIBCO BRL) were also added if needed. In the bacterial adhesion, invasion assay, and induced IL-8 secretion, the cell culture medium was not supplemented with antibiotics.

2.3. Determination of anti-bacterial activity

The *in vitro* anti-bacterial activities of the isolates and extracts [dissolved in 0.2% (v/v) of dimethylsulfoxide (DMSO, Sigma–Aldrich)] were determined by disk agar diffusion method (Castillo-Juarez et al., 2007). Briefly, a total volume of 100 μl of *H. pylori* suspension (1×10^8 colony forming units (CFUs)/ml) was spread onto Mueller Hinton agar plates (BBL) containing 10% sheep blood. Sterile paper disks (6 mm, BBL) were placed on the agar surface with 10 μl of isolates (0.2 mM) and extracts (100 mg/ml) individually. DMSO was used as negative control and 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. After 72 h for incubation at 37 °C under the microaerophilic condition with humidity, the inhibition zone was determined in diameter.

2.4. Determination of minimum bactericidal concentration (MBC)

Broth microdilution MBCs were determined in 96-well plates (Falcon) using two-fold serial dilutions of isolates, extracts, or antibiotics. *H. pylori* were suspended in Brucella broth (Difco) containing 5% fetal bovine serum (Hyclone) and diluted to reach at 1×10^6 CFUs/ml. The plates were incubated in a microaerophilic condition at 37 °C for 48 h. Following incubation, 100 μl aliquots of the broth were plated onto Brucella agar plates containing 10% sheep blood to determine the viable CFUs. The MBC was defined as the lowest concentration of the tested sample completely inhibited visible bacterial growth on Brucella agar plate. The final DMSO concentration in the assay never exceeded 0.52% (v/v) and did not have any effect in the growth at this concentration.

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

H. pylori adhesion to and invasion of cultured AGS cells were done using a standard gentamicin assay as previously described (Lai et al., 2006). The extracts PUC and PUM, 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. AGS cells were added with *H. pylori* at a MOI of 50 and incubated at 37 °C for 6 h. 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.

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