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

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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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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 Helicobacter pylori-infection. In our previous study, we examined the anti-bacterial properties of this species chloroform (PUC) and methanol (PUM) extracts, and its eight isolates. 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 subjected to column chromatography (CC) and eluted with a gradient of hexane/EtOAc (the ratios of n-hexane/EtOAc were from 100:0 to 0:100) to afford compounds, methyl brevifolincarboxylate (1, 26 mg, 0.0033%), methylgallate (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 CH₂Cl₂/MeOH with a gradually increased ratio of methanol to afforded compounds, methyl brevifolinicarboxylate (6, 26 mg, 0.0033%), quercetin-3-α-L-rhamnopyranoside (quer cetin, 7, 8 mg, 0.001%), β-sitosterol-3-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 5% fetal bovine serum (Hyclone) and diluted to reach at 1 × 10⁸ CFUs/ml. The plates were incubated in a microaerophilic condition at 37 °C for 48 h. After 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.

2.4. 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 × 10⁸ 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 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 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.

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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 et al., 2007). AGS epithelium was 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 PUC 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 normalized 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 PUC in cell culture medium before H. pylori infection. The treated cells then infected with H. pylori strain 2,6695° at a MOI of 1:100. The supernatants were collected 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 (Rao et al., 2007).

2.8. Statistical analysis

The correlation of PUM and PUC anti-bacterial adhesion, invasion activity, and IL-8 secretion of treated AGS epithelial cells relative to DMSO treated control cells was determined by Student’s t-test. *P < 0.05, **P < 0.01.

3. Results and discussion

3.1. Growth inhibition of H. pylori

The anti-H. pylori activities of P. urinaria isolates 1–9 except 8, and extracts PUC and PUM were evaluated based on the disk agar diffusion method. The effect of β-sitosterol 3-O-β-D-glucopyranoside (8) was not tested because of its insolubility in culture media. DMSO used as negative control, showed no effect (0.2% total volume). As shown in Fig. 1, the extracts PUC and PUM were most active and inhibited H. pylori with inhibition zone of 21 and 19 mm at concentration of 100 mg/ml. This effect was nearly similar to the standard drug clarithromycin, which is one of the most commonly used antibiotics for treatment of H. pylori infection (inhibition zone of 22 mm at 0.05 mg/ml). Among the pure compounds tested at 0.2 mM concentration, all the isolates showed nearly similar inhibition zone range 10–14 mm to that of amoxicillin (inhibition zone of 15 mm at 0.05 mg/ml), another antibiotic used for the same purpose. Noticeably, the extracts PUC and PUM, and the tested isolates are more potent than another antibiotic metronidazole (8 mm of inhibition zone at 0.8 mg/ml). In this study, the tested isolates present in the crude extracts showed less inhibition at the concentration of 0.2 mM compared with the extracts PUC and PUM at 100 mg/ml. It seems these isolates contribute only in a minor extent to the H. pylori inhibition of the extract. Our current results indicate that isolation of pure compounds from the crude P. urinaria extract does not lead to an increase in the H. pylori inhibitory effect as expected. It is known that single agents are generally ineffective or poorly effective in eradicating H. pylori (Cavallaro et al., 2006). Hence, the activity of PUC and PUM extracts might either be caused by additional, not further identified plant compounds or might be mediated by the extract as a whole due to synergistic effects. This latter argument is consistent with the holistic approach of phytotherapy in that not single compounds but the extract as a multiple component mixture causes the desired effect (McChesney et al., 2007). Therefore, the crude extracts PUC and PUM are chosen for further experiments.

We next determined the MBC of pure isolates (1–9 except 8) and crude extracts (PUC and PUM) against H. pylori. The MBC ranged from 15.6 to 62.5 μM for pure isolates. The lowest MBC was 97.7 μg/ml recorded for the crude extracts PUC and PUM (Table 1). The MBC of standard drugs CLR and AMX which served as the positive control was 0.1 and 0.2 μg/ml, respectively, however, had a significant difference (P < 0.05) in activity to the isolates and extracts. The MBC values were found to be lower than the anti-bacterial values tested suggesting that the isolates and extracts were bacteriostatic at lower concentrations and bactericidal at higher concentrations (Gerhard et al., 2002).

### Table 1

<table>
<thead>
<tr>
<th>Sample</th>
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<tbody>
<tr>
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<td>CLR</td>
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*The concentration of pure isolates (1–9 except 8) were in μM whereas the crude extracts of chloroform (PUC) and methanol (PUM) and standard drugs (AMX, CLR, MTZ) were in μg/ml. Percentage of inhibition was estimated with respect to a control that was incubated only with the solvent (DMSO).*

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3.2. Treatment of PUC and PUM does not influence AGS cells viability

We then analyzed how *P. urinaria* extracts PUC and PUM treatment affects the viability of AGS cells. Assays were performed to assess the cell viability using trypan blue staining of untreated as well as test sample-treated AGS cells. At their maximum concentration (0.5 mg/ml) tested the extracts PUC and PUM have no significant effect upon AGS cell viability (data not shown).

3.3. Inhibition of *H. pylori* adhesion to and invasion of AGS cells

Since the extracts PUC and PUM determined to inhibit *H. pylori* growth at an effective level. These two extracts were further assayed with regard to their ability to inhibit the adhesion of *H. pylori* to AGS cells. As shown in Fig. 2A, the extract PUC exhibited a marked anti-adhesion activity against *H. pylori* with a concentration range from 0.125 to 0.5 mg/ml (*P* < 0.05 and *P* < 0.01 compared to 0.5% (v/v) of DMSO), while PUM has significant effect only at 0.5 mg/ml (*P* < 0.05 compare to 0.5% of DMSO). On the other hand, a significant inhibition of bacterial invasion into AGS cells by PUM was also found with a reduction of more than 60% in a range of concentrations from 0.25 to 0.5 mg/ml (Fig. 2B). Importantly, after treatment of PUC, the bacterial invasion activity was dramatically reduced more than 90% in a range of concentration of 0.125–0.5 mg/ml. Results from this study demonstrate that PUC and PUM have their ability to inhibit bacterial adhesion and invasion of AGS cells. Noticeably, PUC was more potent than PUM in the inhibition of *H. pylori* adhesion and invasion to AGS cells at lower concentrations. Thus, the extract PUC chosen for evaluation of anti-*H. pylori*-induced AGS cells inflammation.

3.4. PUC inhibits *H. pylori*-induced inflammation of AGS cells

Recent reports had demonstrated that *H. pylori*-induced IL-8 release from AGS cells is mediated activation of NF-κB (Brandt et al., 2005). To examine whether the PUC has its ability to inhibit inflammatory mediators in response to *H. pylori* infection, NF-κB-luciferase constructs were used to determine luciferase expression following pretreatment of PUC and infection with *H. pylori*. When AGS cells were transiently transfected with NF-κB-luciferase construct, pretreatment of cells with PUC prior to *H. pylori* infection led to a dose-dependent reduction in the stimulation of luciferase activity. As shown in Fig. 3, the extract PUC inhibited the luciferase activity by 35.96, 71.34 and 93.35% at concentration of 0.125, 0.25 and 0.50 mg/ml, respectively, compared to DMSO control. The exact mechanism whereby PUC inhibits NF-κB activation needs to study further.

A functional consequence of increased NF-κB activity is a parallel increase in IL-8 expression (Kim et al., 2006). To analyze whether PUC could prevent the *H. pylori*-induced IL-8 production, AGS cells were either left untreated or pretreated with the PUC prior to *H. pylori* infection. The significant induction of the cytokine was inhibited by pretreatment of PUC (Fig. 3). Even administration of 0.125 mg/ml inhibited IL-8 production comparable with that of DMSO-treated control cells, and the inhibition reached to 29.40% at concentration of 0.5 mg/ml (Fig. 3). The finding indicating that attenuation of IL-8 production by PUC pretreatment might contribute to prevent *H. pylori*-induced inflammatory response. This may be due to inactivation of multiple intracellular signaling pathways induced by *H. pylori* infection and decrease of NF-κB translocation into the nucleus (Li and Verma, 2002). Conventional therapies suppress not only *H. pylori* but also the intestinal bacterial flora which produces side-effects as abdominal pain and diarrhea (Di Mario et al., 2006). In regards to this, since *P. urinaria* seems to have a restricted anti-bacterial spectrum, which may helpful as an option in the management of the bacterial infection.
4. Conclusion

In summary, our results showed that the PUC of *P. urinaria* has its ability to inhibit *H. pylori* infection of epithelial cells and diminish the secretion of bacterial-induced inflammatory cytokine. Thus, indicates that PUC may be developed to a new potent drug for anti-*H. pylori* infection of cells. Future studies are needed to clarify the molecular mechanisms by which PUC inhibits *H. pylori*-mediated activation of NF-κB expression in gastric epithelial cells and to identify additional targets in gene regulation.

Acknowledgements

This study was supported by National Science Council of Taiwan (NSC96–2811-M-324–001), and by China Medical University (CMU-95–193), and in part by Tomorrow Medical Foundation (TMF2007–02). We thank Chiou-Yan Lin, and Yu-Fen Tseng for expert technical assistance.

References


