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探討感染幽門螺旋菌治療失敗病例中細菌對抗生素抗藥性的形成

Investigation of antibiotic resistance in therapeutic failure of *Helicobacter pylori* infection

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目 錄

Abstract

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. *Phyllanthus urinaria* Linnea (Euphorbiaceae) is widely used in traditional Taiwanese medicine for the treatment of gastritis and ulcers. In this study, we examined the anti-inflammatory effects of *Phyllanthus urinaria* chloroform (PUC) and methanol (PUM) extracts, and its eight isolates on *Helicobacter pylori*-infected human gastric epithelial AGS cells. The results revealed that crude extracts PUC and PUM showed potent antimicrobial activity against *Helicobacter pylori* than pure isolates. On the other hand, *in vitro Helicobacter 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, *Helicobacter 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. These results open the possibility of considering *Phyllanthus urinaria* a chemopreventive agent for peptic ulcer or gastric cancer, but this bioactivity should be confirmed *in vivo* in the future.

Keywords: *Phyllanthus urinaria*, Isolates, Chloroform and methanol extracts, *Helicobacter pylori* infection, Human gastric epithelial cells, NF-κB, and IL-8.

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 *Helicobacter 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- *Helicobacter pylori* activity. In human gastric epithelial AGS cells *Helicobacter pylori*-induce 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 *Helicobacter 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-*Helicobacter pylori* activity has been widely demonstrated *in vitro* (Stamatis et al., 2003; Ustun et al., 2006; Ndip et al., 2007; Shih 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, *Phyllanthus urinaria* is traditionally used in Taiwan to reduce heat, 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 *Phyllanthus urinaria* have been reported, however, there is no report on its action against *Helicobacter pylori* induced inflammation. In our previous study, we analyzed *Phyllanthus 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., 2007). In the present study, we examined the antibacterial 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 *Helicobacter 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 *Helicobacter pylori* infection of AGS cells. With this approach, we hope to understand the protective mechanism of *Phyllanthus urinaria* on *Helicobacter pylori*-induced inflammation and increase its clinical potential in gastrointestinal disease.

Results and discussion

Growth inhibition of Helicobacter pylori

The anti-*Helicobacter pylori* activities of *Phyllanthus 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 *Helicobacter 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 (CLR), which is one of the most commonly used antibiotics for treatment of *Helicobacter 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 *Helicobacter pylori* inhibition of the extract. Our current results indicate that isolation of pure compounds from the crude *Phyllanthus urinaria* extract does not lead to an increase in the *Helicobacter pylori* inhibitory effect as expected. It is known that single agents are generally ineffective or poorly effective in eradicating *Helicobacter 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 MIC of pure isolates (**1**–**9** except **8**) and crude extracts (PUC and PUM) against *Helicobacter pylori*. The MIC ranged from 15.6 to 62.5 μM for pure isolates. The lowest MIC was 97.7 μg/mL recorded for the crude extracts PUC and PUM (Table 1). The MIC 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 MIC values were found to be lower than the antibacterial values tested suggesting that the isolates and extracts were bacteriostatic at lower concentrations and bactericidal at higher concentrations (Gerhard et al., 2002).

Treatment of PUC and PUM do not influence AGS cells viability

We then analyzed how *Phyllanthus 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. As shown in Fig. 2, at their maximum concentration (0.5 mg/ml) tested the extracts PUC and PUM have no significant effect upon AGS cell viability.

Inhibition of Helicobacter pylori adhesion to and invasion of AGS cells

Since the extracts PUC and PUM determined to inhibit *Helicobacter pylori* growth at an effective level. These two extracts were further assayed with regard to their ability to inhibit the adhesion of *Helicobacter pylori* to AGS cells. As shown in Fig. 3A, the extract PUC exhibited a marked anti-adhesion activity against *Helicobacter 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. 3B). Importantly, after treatment of PUC, the bacterial invasion activity was dramatically reduced more than 90% in a range of concentration of 0.125 to 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 *Helicobacter pylori* adhesion and invasion to AGS cells at lower concentrations. Thus, therefore, the extract PUC chosen for evaluation of anti-*Helicobacter pylori*-induced AGS cells inflammation.

PUC Inhibit Helicobacter pylori-induced inflammation of AGS cells

Recent reports had demonstrated that *Helicobacter 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 *Helicobacter pylori* infection, NF-κB-luciferase constructs were used to determine luciferase expression following pretreatment of PUC and infection with *Helicobacter pylori*. When AGS cells were transiently transfected with NF-κB-luciferase construct, pretreatment of cells with PUC prior to *Helicobacter pylori* infection led to a dose-dependent reduction in the stimulation of luciferase activity. As shown in Fig. 4, 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 *Helicobacter pylori*-induced IL-8 production, AGS cells were either left untreated or pretreated with the PUC prior to *Helicobacter pylori* infection. The significant induction of the cytokine was inhibited by pretreatment of PUC (Fig. 4). 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. 4). The finding indicating that attenuation of IL-8 production by PUC pretreatment might contribute to prevent IL-8-induced inflammatory response. This may be due to inactivation of multiple intracellular signaling pathways induced by *Helicobacter pylori* infection and decrease of NF-κB translocation into

the nucleus (Li and Verma, 2002). Conventional therapies suppress not only *Helicobacter pylori* but also the intestinal bacterial flora which produces side-effects as abdominal pain and diarrhoea. In regards to this, since *Phyllanthus urinaria* seems to have a restricted antibacterial spectrum, which may helpful as an option in the management of the bacterial infection.

Conclusion

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

Experimental procedures

Isolation of compounds from Phyllanthus urinaria

The voucher specimen, and method for extraction and isolation were described previously (Fang et al., 2007). 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 ºC. 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., 2007).

Bacterial and cell culture

Helicobacter 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.

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 *Helicobacter 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 (0.2mM) 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.

Determination of minimum inhibitory concentration (MIC)

Broth microdilution MICs were determined in 96-well plates (Falcon) using twofold serial dilutions of isolates, extracts, or antibiotics. *Helicobacter 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 MIC 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.

Inhibition of Helicobacter pylori adhesion to and invasion into AGS cells

Helicobacter pylori adhesion to and invasion of cultured AGS cells was 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 *Helicobacter pylori* in log-phase. AGS cells were added with *Helicobacter 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 activity to inhibit *Helicobacter pylori* adhesion and invasion was determined as: % of inhibition = (CFUs of *Helicobacter pylori* in the presence of test sample/CFUs of *Helicobacter pylori* in the absence

of test sample) × 100.

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 *Helicobacter 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.

Interleukin-8 measurement

To detect IL-8 released by AGS cells during *Helicobacter pylori* infection, the levels of IL-8 was measured. AGS cells were added with various concentrations of PUC in cell culture medium before *Helicobacter pylori* infection. The treated cells then infected with *Helicobacter pylori* strain 26695 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).

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. $*P < 0.05$, $**P < 0.01$.

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Table 1. MIC of *Phyllanthus urinaria* isolates, and its chloroform (PUC) and methanol (PUM) extracts against *Helicobacter pylori*

Sample	MIC[a]
1	15.6
2	31.3
3	62.5
4	62.5
5	31.3
6	31.3
7	31.3
9	31.3
PUC	97.7
PUM	97.7
AMX	0.2
CLR	0.1
MTZ	100.0

[a] The concentration of isolates were in μM, and extracts and standard drugs were in μg/ml. Percentage of inhibition was estimated with respect to a control that was incubated only with the solvent (DMSO).

Fig. 1.

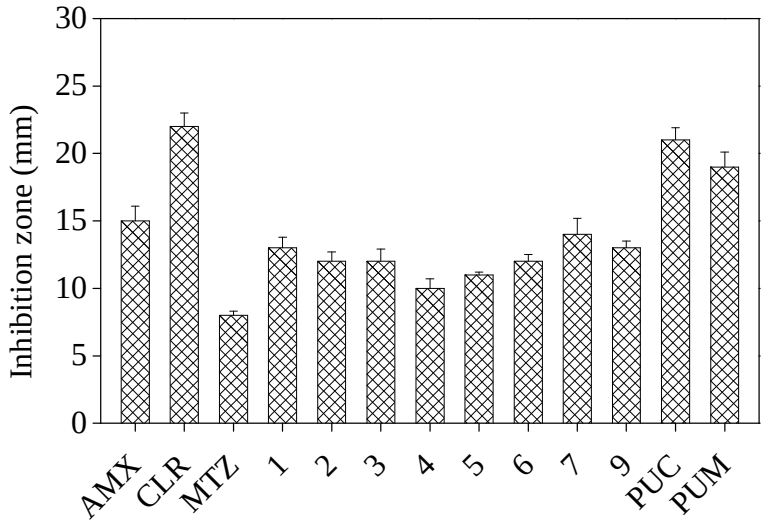

Inhibition zone (mm)
30
25
20
15
10
5
0
AMX
CLR
MTZ
1
2
3
4
5
6
7
9
PUC
PUM

Fig. 2.

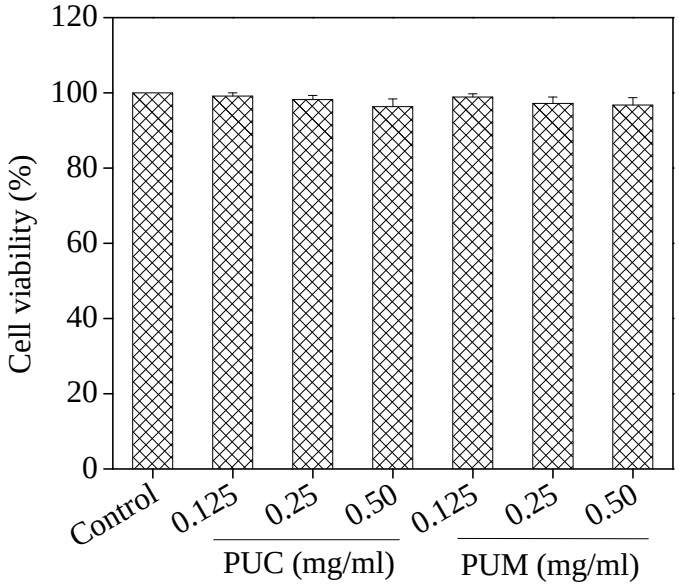
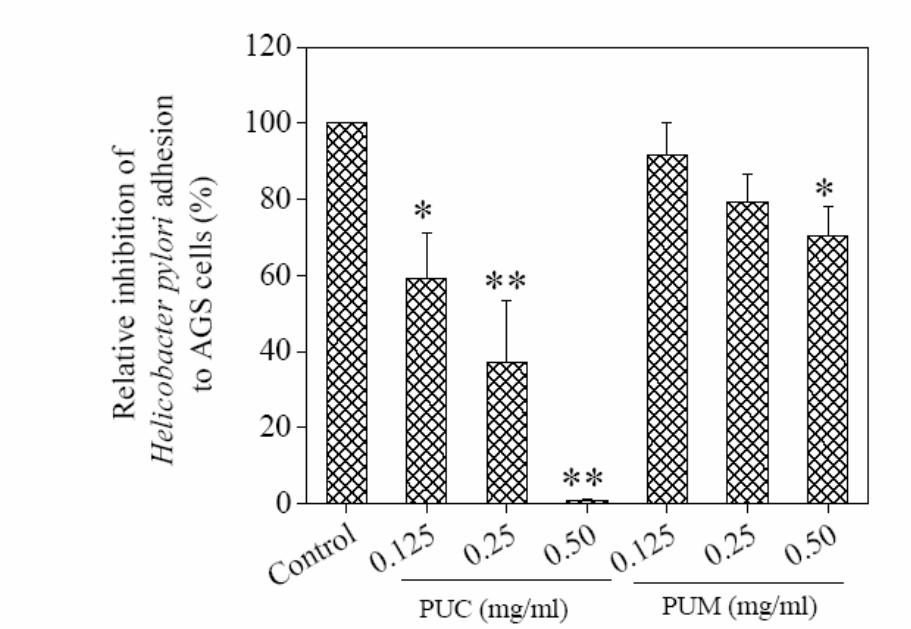
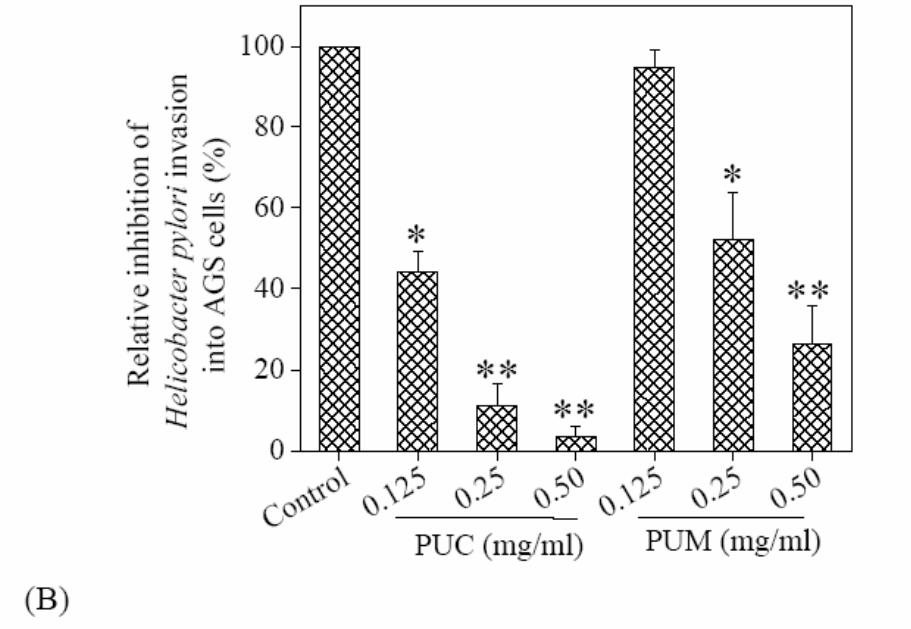

120
100
80
60
40
20
0
Cell viability (%)
Control
0.125
0.25
0.50
0.125
0.25
0.50
PUC (mg/ml)
PUM (mg/ml)

Fig. 3.


Relative inhibition of *Helicobacter pylori* adhesion to AGS cells (%)
120
100
80
60
40
20
0
*
**
**
*
Control
0.125
0.25
0.50
0.125
0.25
0.50
PUC (mg/ml)
PUM (mg/ml)

(A)


Relative inhibition of *Helicobacter pylori* invasion into AGS cells (%)
100
80
60
40
20
0
*
**
**
*
**
Control
0.125
0.25
0.50
0.125
0.25
0.50
PUC (mg/ml)
PUM (mg/ml)

(B)

Fig. 4.

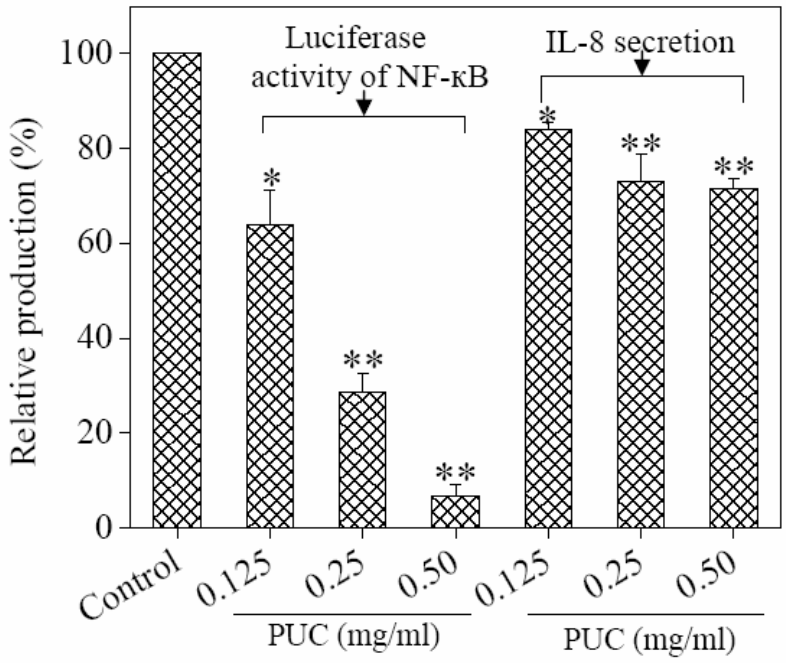

Luciferase activity of NF-κB
IL-8 secretion
Relative production (%)
100
80
60
40
20
0
*
**
**
*
**
**
Control
0.125
0.25
0.50
0.125
0.25
0.50
PUC (mg/ml)
PUC (mg/ml)

Figure legends

Fig. 1. Inhibitory effects of *Phyllanthus urinaria* isolates, and its extracts PUC and PUM against *Helicobacter pylori*. The concentration of isolates and extracts was 0.2 mM and 100 mg/ml, respectively. 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.

Fig. 2. Effect of *Phyllanthus urinaria* extracts PUC and PUM on AGS epithelial cell viability. Incubation was for 48 h at 37ºC. Data are displayed as percent of survival. The results are representative of at least three independent experiments.

Fig. 3. Effect of *Phyllanthus urinaria* extracts PUC and PUM on *Helicobacter pylori* anti-adhesion (A) and anti-invasion (B) of AGS cells. The bacteria to AGS cells that were untreated or treated with PUC or PUM, followed by infection with *Helicobacter pylori* at a MOI of 50 for 6 h. Each experiment was shown represent mean values ± SD of at least six independent experiments. The significant difference was set at * $P< 0.05$; ** $P<0.01$.

Fig. 4. Inhibitory effects of *Phyllanthus urinaria* extract PUC on *Helicobacter pylori*-induced NF-κB activation and IL-8 production by AGS cells. The luciferase activity and IL-8 levels in supernatants 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 * $P < 0.05$; ** $P<0.01$.

計畫成果自評部份

1. 研究內容與原計畫相當符合。

2. 目前已完成本計畫內容。

3. 由於此研究計畫接受**「明日醫學基金會」**研究經費的補助及**清大生科院王雯靜教授**與**明日醫學基金會執行長林懷正教授**的指導之下，成果已於 2007 年 12 月投稿期刊 Journal of Ethnopharmacology (JEP-D-07-01925)，該期刊於 INTEGRATIVE & COMPLEMENTARY MEDICINE 領域中排名第一名，初審已獲得通過並等待接受中(2008/2/13)，**我們非常感激明日醫學基金會及基金會同仁們對我們各方面的協助，希望將來能持續獲得基金會的補助與指導，並且期待能為台灣的醫學研究提供一些些貢獻**。