Anti-\textit{Helicobacter pylori} activity of \textit{Plumbago zeylanica} L.

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Received 20 August 2004; received in revised form 15 October 2004; accepted 18 October 2004

First published online 25 November 2004

Abstract

It has been shown that the presence of infection by \textit{Helicobacter pylori} is strongly associated with gastric cancer and peptic ulceration. In western medicine, a 3-fold therapeutic regimen, emphasizing the use of antibiotics, is typically used to suppress \textit{H. pylori} activity. However, antibiotic drug resistance frequently develops as a consequence of such treatment. In our previous study, 50 Taiwanese folk medicinal plants were screened for their anti-\textit{H. pylori} activities. The results revealed that \textit{Plumbago zeylanica} L. had the highest inhibitory effects against \textit{H. pylori}. In this study, therefore, we have focused on establishing the anti-\textit{H. pylori} activities of \textit{P. zeylanica} L. Water and the organic solvents ethanol, ethyl acetate and acetone were used for \textit{P. zeylanica} L. extraction, obtaining yields of 1.66–6.84% (w/w). Excluding the water extract, higher anti-\textit{H. pylori} activity was demonstrated for all the extracts, both using the agar diffusion and dilution methods. The ethyl acetate extract exhibited the lowest minimum inhibitory concentrations against five \textit{H. pylori} strains, of which ranged from 0.32 to 1.28 mg ml$^{-1}$, followed, in ascending order, by the acetone, ethanol and water analogs. Bactericidal activity was determined for \textit{P. zeylanica} L. extracts, with the lowest minimum bactericidal concentrations (5.12–20.48 mg ml$^{-1}$) demonstrated for the ethyl acetate, followed, in ascending order, by the acetone and ethanol analogs. Bactericidal activity appeared to be in a dose-dependent manner. Through a broad pH range (2–7), bactericidal activity was not affected when extract concentrations were greater than or equal to the minimum bactericidal concentration. High stability was demonstrated for the ethyl acetate \textit{P. zeylanica} L. extract within pH range of 1–7, exhibiting all pH treatments bactericidal activity. © 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Anti-\textit{Helicobacter pylori} activity; \textit{Plumbago zeylanica} L; Bactericidal activity

1. Introduction

Infection with \textit{Helicobacter pylori} is strongly associated with duodenal and gastric ulcers [1]. Many of the epidemiological studies have indicated that high rates of \textit{H. pylori} infection may be related to the prevalence of gastric cancer and adenocarcinoma [2]. Various pharmacological regimens have been studied for the treatment of \textit{H. pylori} infection. Antibiotics [3,4], proton-pump inhibitors [5,6], H$_2$-blockers [7,8] and bismuth salts [9] are the suggested standard treatment modalities, typically combined in dual, triple and quadruple-therapy regimens to eradicate \textit{H. pylori} [10,3]. Problems encountered when administering these eradication regimens, include cost [10], the efficacy of antibiotics relative to pH, for instance, amoxicillin is most active at a neutral pH, and tetracycline has greater activity at a low pH [10], and resistance to the antibiotics [11]. Further, more than 15% of the patients undergoing such drug regimens experience therapeutic failure [10].

\textit{Plumbago zeylanica} L. is a semi-climbing subshrub that grows throughout Asia and Africa. This plant is distributed in thickets or grassland at low elevations in Taiwan [12]. The whole plant and its roots have been used as folk medicine in the treatment of rheumatic pain, dysmenorrhea, carbuncles, contusion of the extremities, ulcers and
elimination of intestinal parasites [13]. In traditional Indian medicine, *P. zeylanica* L. has been assigned medicinal properties and is used in formulations of a number of ayurvedic compounds [14]. In southwestern Nigeria, *P. zeylanica* L. is used in folk medicine to treat parasitic diseases, scabies and ulcers [15]. Pharmacological studies carried out by several workers have indicated that *P. zeylanica* L. extract has antiplasmodial [16], antimicrobial [13,17,18], antihyperglycemic [19], insecticidal [20] and antiallergic [21] properties. It also stimulates the central nervous system [22] and is cytotoxic for tumor cell activities [23].

In our previous study, 50 Taiwanese folk medicinal plants were screened for their anti-*H. pylori* activities. The results revealed that *P. zeylanica* L. had the strongest inhibitory effect against *H. pylori*. In this study, therefore, we have focused on the anti-*H. pylori* activities of *P. zeylanica* L. Anti-*H. pylori* spectra were studied using the agar diffusion method and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) established using the agar dilution method; time-kill assay for the ethyl acetate extract on the bactericidal activity was determined and the effects of pH and pH stability on the bactericidal activities of *P. zeylanica* L. extract were also examined.

2. Materials and methods

2.1. Herbal plant and preparation of herbal extracts

Samples of *P. zeylanica* L. were collected and identified by Technician N. Y. Chiu from the Institute of Chinese Pharmaceutical Science China Medical College. Voucher specimens (No. 250481) were deposited at the Institute of Ecology and Evolutionary Biology, College of Life Sciences, National Taiwan University.

The dried, mixed, rhizome and radix of *P. zeylanica* L. were used for the preparation of herbal extracts in water, ethanol, acetone or ethyl acetate as extraction solvents. Two-hundred milliliters of extraction solvent were added to 30 g of ground (passed through a 60 mesh screen) herbal plant followed by stirring 1 h at room temperature. Subsequently, the mixture was centrifuged at 9000 rpm for 15 min at 4 °C and the residue was extracted twice with 2 × 200 ml of extraction solvent. The supernatants were mixed and concentrated to dryness in a rotary vacuum evaporator at less than 40 °C (below 50 °C in case of water extracts), and the concentrate was then weighed. Extraction yield (%, w/w) was calculated as a ratio of the weight of the concentrate to the weight of the *P. zeylanica* L. powder.

2.2. Bacterial strains and culturing

*Helicobacter pylori* BCRC 15415, BCRC 17021, BCRC 17023, BCRC 17026 and BCRC 17027 were obtained from the Bioresources Collection and Research Center (BCRC), Hsinchu, Taiwan, ROC.

The five strains of *H. pylori* were cultured in 5-ml tryptic soy broth (TSB, Difco, USA; each liter containing: a pancreatic digest of casein [17 g], an enzymatic digest of soybean meal [3 g], dextrose [2.5 g], sodium chloride [5 g], and dipotassium phosphate [2.5 g, pH 7.3]), with Columbia agar (bioMérieux, France; each liter containing: peptone [10 g], tryptose [10 g], *H. pylori* serum [3 g], corn starch [1 g], sodium chloride [5 g] and agar [13.5 g, pH 7.3]) slant containing 5% (v/v) defibrinated sheep blood formed at the bottom of the test tube. The broth was then incubated in a microaerophilic jar system (BBL), presenting a gas composition of 5% O2 and 10% CO2 in air (an OXOID BR 056A gas-generating kit was used for this purpose) at 37 °C for 72 h. The cell suspension was then diluted with 0.1% peptone to produce a cell concentration of 0.5–1.0 × 106 cfu ml−1 for antimicrobial testing.

2.3. Inhibitory-zone testing

The water, ethanol, acetone and ethyl acetate extracts of *P. zeylanica* L. were used for inhibitory-zone testing. The anti-*H. pylori* inhibitory-zone testing consisted of four extracts of *P. zeylanica* L. against five strains of *H. pylori*, according to the method of Johnson et al., (1995) [24]. A volume of 0.1-ml of each tested *H. pylori* suspension (0.5–1.0 × 106 cfu ml−1) was spread onto a Columbia agar plate containing 5% (v/v) defibrinated sheep blood. Wells of 7 mm in diameter were punched on the plates and 30 μl of the *P. zeylanica* L. extract [0.2 g ml−1; dimethyl sulfoxide (DMSO) as solvent] was individually incorporated into the wells. DMSO was used as control. The plates were diffused at 4 °C for 2 h and subsequently incubated in a microaerophilic jar system (5% O2 and 10% CO2) at 37 °C for 72 h. The clear zone around each well was observed and its diameter was examined.

2.4. Minimum inhibitory concentration and minimum bactericidal concentration testing

The MICs for the water, ethanol, acetone and ethyl acetate extracts of *P. zeylanica* L. were established against the five strains of *H. pylori*, using a broth-dilution method [25]. Each of the *P. zeylanica* L. extracts was dissolved in DMSO and 2-fold diluted in Columbia agar containing 5% (v/v) defibrinated sheep blood. DMSO was used as control. A volume of 0.1-ml of cell suspension (0.5–1.0 × 106 cfu ml−1) was spread onto Columbia agar plates containing 5% (v/v) defibrinated sheep blood. Colonies that had formed on the plates were enumerated following incubation in a microaerophilic jar system (5% O2 and 10% CO2 in air) at 37 °C for 72 h. The MICs for the *P. zeylanica* L. extracts
against H. pylori were determined. The MIC was defined as the lowest concentration of the test sample at which no colony of the test bacteria on the plate was formed.

The MBCs for the ethanol, acetone and ethyl acetate extracts of P. zeylanica L. were determined. For MIC testing, like as the MIC testing, a broth-dilution method [25] was used. Each of the P. zeylanica L. extracts was dissolved in DMSO and 2-fold diluted in Columbia agar containing 5% (v/v) defibrinated sheep blood or TSB, and was then individually added to test tubes, with Columbia agar slants formed at the bottom of the test tubes and the TSB layer to be covered on the slants, both the slant and broth contained the same concentrations of the extract. DMSO was used as control. H. pylori suspensions were added to the TSB layer to produce 0.5–1.0 $\times$ 10⁵ cfu ml⁻¹ of the initial bacterial count. Following incubation in a microaerophilic jar system (5% O₂ and 10% CO₂ in air) at 37 °C for 72 h. A volume of 0.1-ml of each suspension was spread onto Columbia agar plates containing 5% (v/v) defibrinated sheep blood, but lacking the ethyl acetate P. zeylanica L. extract. After incubation in a microaerophilic jar system (5% O₂ and 10% CO₂ in air) at 37 °C for 72 h, the colonies formed were subsequently enumerated. The MIC was defined as the lowest concentrations of the P. zeylanica L. extract giving complete inhibition of colony formation of the test bacteria at the latter cultivation.

2.6. Effect of the pH on bactericidal activity

The ethyl acetate extract of P. zeylanica L. was used to examine the effect of pH on bactericidal activity. Five milliliters of the Columbia agar containing 5% (v/v) defibrinated sheep blood (adjusted to pH 2–7 with 3 N HCl) at final concentrations of 0–20.48 mg ml⁻¹ of the ethyl acetate P. zeylanica L. extract were added to the test tubes to create slants at the bottom. Four milliliters of TSB (adjusted to pH 2–7 with 3 N HCl) containing the same concentration of the ethyl acetate P. zeylanica L. extract were poured onto the slants. Untreated slant broth was used as control. A volume of 0.1-ml of the H. pylori BCRC 17023 cell suspension was added to the TSB to produce 1.0 $\times$ 10⁸ cfu ml⁻¹ of the initial bacterial count. The broths were incubated in a microaerophilic jar system (5% O₂ and 10% CO₂ in air) at 37 °C for 24 h. Each of the broths was sampled and diluted by 10-fold dilutions in 0.1% peptone solution. A volume of 0.1-ml from each of the diluted samples was spread onto Columbia agar plates containing 5% (v/v) defibrinated sheep blood, but lacking the ethyl acetate P. zeylanica L. extract. After incubation in a microaerophilic jar system (5% O₂ and 10% CO₂ in air) at 37 °C for 72 h, the colonies formed on the Columbia agar plates were enumerated.

2.7. Ethyl acetate P. zeylanica L. extracts – pH stability

The pH stability of the ethyl acetate P. zeylanica L. extract on bactericidal activity was tested. The pH-treated extracts were prepared using the Clark and Lubs (pH 1–2) and McIlvaine buffers (pH 3–7) [8% (w/v)] [26]; being all the extracts stored at room temperature for 2 h.

Five milliliters of Columbia agar, containing 5% (v/v) of defibrinated sheep blood at 10.24 mg ml⁻¹ of the pH-treated ethyl acetate P. zeylanica L. extracts were added to each test tube to create slants at the bottom. Five milliliters of TSB containing the same concentrations of the pH-treated ethyl acetate extract as the slant were poured onto the slants. Untreated slant broth was used as control. A volume of 0.1-ml of H. pylori BCRC 17023 cell suspension was added to the TSB to produce 1.0 $\times$ 10⁸ cfu ml⁻¹ of the initial bacterial count. Each broth was sampled and diluted by 10-fold dilutions in 0.1% peptone solution. A volume of 0.1-ml of each diluted sample was then spread onto Columbia agar plates containing 5% (v/v) defibrinated sheep blood, but lacking the ethyl acetate P. zeylanica L. extract. After incubation at 37 °C for 72 h in a microaerophilic jar system (5% O₂ and 10% CO₂ in air), the colonies grown on the Columbia agar plates were enumerated.
3. Results and discussion

3.1. Extraction yields

P. zeylanica L. extraction was accomplished using solvents of different polarities (water, ethanol, acetone and ethyl acetate; polarity indexes \((P)\) of 10.2, 5.2, 5.1 and 4.4, respectively). The greatest yields were obtained using water and ethanol (6.84% and 6.28% (w/w), respectively), followed, in descending order, by acetone and ethyl acetate (2.03% and 1.66%, respectively).

3.2. Anti-H. pylori spectra of P. zeylanica L. extract

The agar diffusion method [24] was used to study the anti- \(H.\) \(pylori\) spectra of the four \(P.\) \(zeylanica\) L. extracts. The results are presented in Table 1. Among the four extracts, ethanol, acetone and ethyl acetate extracts exhibited relatively high anti- \(H.\) \(pylori\) activities, forming large-diameter clear zones on the plate and for all the test strains. Less anti- \(H.\) \(pylori\) activity was observed in case of the water extract, with inhibitory zones very unclear. Ethanol, acetone and ethyl acetate are classified as moderate polarity solvents. Thus, we assumed that the \(P.\) \(zeylanica\) L. extract presenting a greater anti- \(H.\) \(pylori\) activity would also have a greater content of compounds with moderate polarity. A variety of compounds have been isolated from \(P.\) \(zeylanica\) L. including: naphthoquinones, such as plumbagin \([13,27]\), droserone, isoshinanolone and the new variant, 1,2(3)-tetrahydro-3,3’-biplumbagin \([27]\); meteroterpens, such as bakuchiol and 12-hydroxyisobakuchiol \([28]\); C-glucosylflavonoids; and saponaretin \([28]\). Given that all of these have moderate polarity, it seems reasonable to suggest that contributors to the anti- \(H.\) \(pylori\) activities of \(P.\) \(zeylanica\) L. are composed by one or more of the above compounds.

3.3. Minimum inhibitory concentration and minimum bactericidal concentration testing

Inhibitory-zone testing is the primary method for evaluation of the susceptibility of the test samples against specific microorganisms. Given the relatively low sensitivity of this method, MIC testing is mainly used as complementary method to evaluate the antimicrobial activity of the test sample. The MIC results for the water, ethanol, acetone and ethyl acetate of \(P.\) \(zeylanica\) L. extracts are shown in Table 2. Among the four extracts, the ethyl acetate variant had the lowest MICs against the five \(H.\) \(pylori\) strains (0.32–1.28 mg ml\(^{-1}\)), which was followed, in ascending order, by the acetone and ethanol extracts (0.32–10.24 and 0.64–10.24 mg ml\(^{-1}\), respectively). In fact, there were minimal differences between the acetone and ethanol extracts. The water extract showed more than 10.24 mg ml\(^{-1}\) of MICs against four \(H.\) \(pylori\) strains, having the higher MIC in comparison to the values registered in the other three extracts. Both agar diffusion and dilution methods used in the evaluation of the \(P.\) \(zeylanica\) L. extract for \(H.\) \(pylori\) susceptibility, gave similar results as demonstrated in Tables 1 and 2. This indicated that, among the four tested solvents, the ethyl acetate was the most efficient solvent for the \(P.\) \(zeylanica\) L. compounds in terms of anti- \(H.\) \(pylori\) activity.

Cellini et al., (1996) reported that the phosphate extract of garlic possesses anti- \(H.\) \(pylori\) activity, with MICs for the extract against 19 strains of \(H.\) \(pylori\) ranging from 2 to 5 mg ml\(^{-1}\) [29], supporting our findings in this study. In contrast, the anti- \(H.\) \(pylori\) activity of the methanol extract of \(Miyroxylon\) \(peruiferum\), a medicinal plant of Brazil, with 62.5 mg ml\(^{-1}\) of MIC was lower than the equivalent in our study [30]. In our previous study [31], antagonistic activity against ten \(H.\) \(pylori\) strains was demonstrated for the ethyl acetate extract of \(Ephelantopus\) \(scaber\) Linn. (MIC range 2.56–10.24 mg ml\(^{-1}\)), a Taiwanese folk medicinal plant.

To determine whether a test compound had bactericidal activity, microorganisms were initially cultivated in broth and subsequently, sampled, inoculated and re-cultivated in the presence of the test compound contained in the broth and subsequently. If no colony formation was observed at that point, the test compound had bactericidal activity [32]. Thus, minimum bactericidal concentration was defined as the lowest concentration at which the test compound could give complete inhibition of growth. MBCs for the \(P.\) \(zeylanica\) L.

### Table 1

<table>
<thead>
<tr>
<th>(H.) (pylori) strain</th>
<th>Inhibitory zone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>BCRC 15415</td>
<td>+++(H)</td>
</tr>
<tr>
<td>BCRC 17021</td>
<td>++(b) (H)(^c)</td>
</tr>
<tr>
<td>BCRC 17023</td>
<td>+</td>
</tr>
<tr>
<td>BCRC 17026</td>
<td>+ (H)</td>
</tr>
<tr>
<td>BCRC 17027</td>
<td>++++(H)</td>
</tr>
</tbody>
</table>

\(^a\) Four extracts with concentrations of 0.2 g ml\(^{-1}\) and 30 μl were incorporated into each well.

\(^b\) ++++, >20 mm (dia); ++++, 16–20 mm; ++, 11–15 mm; +, 8–10 mm; –, ≤7 mm.

\(^c\) hazy zone.

### Table 2

<table>
<thead>
<tr>
<th>(H.) (pylori) strain</th>
<th>MIC (mg ml(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>BCRC 15415</td>
<td>&gt;10.24</td>
</tr>
<tr>
<td>BCRC 17021</td>
<td>1.28</td>
</tr>
<tr>
<td>BCRC 17023</td>
<td>&gt;10.24</td>
</tr>
<tr>
<td>BCRC 17026</td>
<td>&gt;10.24</td>
</tr>
<tr>
<td>BCRC 17027</td>
<td>&gt;10.24</td>
</tr>
</tbody>
</table>
extracts are presented in Table 3, exhibiting ethanol, acetone and ethyl acetate extracts bactericidal activity. The lowest MBCs against the five strains of *H. pylori* (5.12–20.48 mg ml\(^{-1}\)) was shown for the ethyl acetate extract and followed by the acetone and ethanol analogs (MBC 5.12–81.92 and 10.24–81.92 mg ml\(^{-1}\), respectively). Hence, MBC and MIC results appeared to be consistent (Table 2).

### 3.4. Time-kill assay

Ethyl acetate extracts of *P. zeylanica* L. were added to the *H. pylori* BCRC 17023 broth to produce final concentrations of 0–20.48 mg ml\(^{-1}\). At different time intervals during cultivation, the broth was sampled, diluted and the total colony counts on the plates were enumerated in order to determine the relationship between the concentration of ethyl acetate extract, treatment time and bactericidal activity (Fig. 1). Only anti- *H. pylori* activity and not bactericidal activity was determined, where concentration of the ethyl acetate extract was lower than the MBC (10.24 mg ml\(^{-1}\) against *H. pylori* BCRC 17023), it was solely four logs of bacterial counts decreased. Bactericidal activity was observed where concentration of the ethyl acetate extract was equal to the MBC value, being necessary 8 h to eradicate the *H. pylori* BCRC 17023. When concentration of the ethyl acetate extract (20.48 mg ml\(^{-1}\)) in the broth was greater than the MBC, the time needed to kill the *H. pylori* BCRC 17023 was reduced to 4 h. In summary, our results indicated that the bactericidal action of the ethyl acetate extract of *P. zeylanica* L. appeared to be dose dependent (Fig. 1).

### 3.5. Effect of pH on bactericidal activity

For the treatment of *H. pylori* infections, antibiotics are typically used in therapy regimen. However, the efficacy of the antibiotics appears to be pH dependent. For example, amoxicillin is most active at neutral pH and tetracycline presents greater activity at low pH [10]. In this study, therefore, the effect of the pH on the bactericidal activity given by the ethyl acetate *P. zeylanica* L. extract was investigated. The results shown in Fig. 2, where concentration of the ethyl acetate extracts in the broth was above the MBC value, bactericidal activity was demonstrated and appeared to be independent of the pH (unaltered at pH 2–7). However, when concentration of the ethyl acetate extract was lower than the MBC, it was observed a dose-dependent anti-*H. pylori* action, where the total bacterial counts in the broth increased when the pH was also raised. Similar results were also obtained in the anti-*H. pylori* activity of *Elephantopus scaber* Linn. [31] when the concentration of the extract was greater than its MBC value, i.e., presenting a bactericidal action not influenced at pH 2–7. Fig. 2 showed that in the control samples, *H. pylori* did not survive at pH 2 and 3 and it only started to grow when the pH was above 3.
3.6. Ethyl acetate extract of *P. zeylanica* L. – pH stability

When administrated orally, the drugs commonly will remain in the stomach for 1–2 h, where the gastric-juice pH is typically 1–2, thus the activity of drugs being influenced by the acidic pH. In this study, the stability of the ethyl acetate *P. zeylanica* L. extract was examined relative to its pH; i.e., the extract was resuspended in different buffer solutions (pH 1–7 for 2 h) to determine the effect on the bactericidal activity. The results showed that the ethyl acetate *P. zeylanica* L. extracts had a high stability in a wide range of pH (1–7), exhibiting all pH treatments bactericidal activity at 10.24 mg ml\(^{-1}\) with the bactericidal activity. The results showed that the ethyl acetate *P. zeylanica* L. extracts had a high stability in a wide range of pH (1–7), exhibiting all pH treatments bactericidal activity at 10.24 mg ml\(^{-1}\) and, therefore, no colony formation detected on the plate (data not shown).

In summary, the bactericidal activity of the ethyl acetate *P. zeylanica* L. extract appeared either to be independent of pH fluctuations or to be very stable (Fig. 2), which offers significant advantages, i.e., greater convenience and reduced cost for the treatment of *H. pylori* infections in comparison to proton-pump inhibitors or H\(_2\)-blockers treatments.

References


