In Vitro Susceptibility of Helicobacter pylori to Isoquinoline Alkaloids from Sanguinaria canadensis and Hydrastis canadensis

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Methanol extracts of the rhizomes of Sanguinaria canadensis, and the roots and rhizomes of Hydrastis canadensis, two plants used traditionally for the treatment of gastrointestinal ailments, were screened for in vitro antibacterial activity against 15 strains of Helicobacter pylori. The rhizome extracts, as well as a methanol extract of S. canadensis suspension-cell cultures inhibited the growth of H. pylori in vitro, with a MIC90 range of 12.5–50.0 µg/ml. Three isoquinoline alkaloids were identified in the active fraction. Sanguinarine and chelerythrine, two benzophenanthridine alkaloids, inhibited the growth of the bacterium, with an MIC90 of 50.0 and 100.0 µg/ml, respectively. Protopine, a protopine alkaloid, also inhibited the growth of the bacterium, with a MIC90 of 100 µg/ml. The crude methanol extract of H. canadensis rhizomes was very active, with an MIC90 of 12.5 µg/ml. Two isoquinoline alkaloids, berberine and β-hydrastine, were identified as the active constituents, and having an MIC90 of 12.5 and 100.0 µg/ml, respectively. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: Helicobacter pylori; Sanguinaria canadensis; Hydrastis canadensis; berberine; sanguinarine.

INTRODUCTION

The discovery of the bacterium Helicobacter pylori (HP) as the main etiologic organism of chronic gastritis, peptic ulcer disease, and gastric cancer was the most significant discovery in the field of gastroenterology of the twentieth century (Graham, 1989, 1994). Helicobacter pylori-induced gastritis is now associated with duodenal ulcer disease, peptic ulcer disease, gastric carcinoma, primary gastric B-cell lymphoma, ischemic heart disease and hyperemesis gravidarum (Goodwin, 1997; Kuipers et al., 1997; Frigo et al., 1998; Markus and Mendall, 1998). Statistics from 1997 indicate that as much as one-half of the world’s population is infected with the bacterium (Breuer et al., 1997). Treatment of HP infections often consists of triple drug therapy, including a proton pump inhibitor or bismuth salts in combination with two antibiotics for 7–14 days (Sung et al., 1996; Salcedo and Al Kawas, 1998). While, triple and quadruple drug therapy is usually effective, antibiotic resistant strains of H. pylori have emerged, and are becoming problematic throughout the world (Graham et al., 1996; Fedorak et al., 1997; Dore et al., 1999). In view of the development of antibiotic resistance, HP is now viewed as a significant human pathogen in need of new chemotherapeutic agents for treatment and prevention (Doig and Trust, 1997).

The discovery and development of botanical extracts for the treatment of HP infections is one such strategy. Plant-based medicines have been used throughout history, in traditional systems of medicine, to treat a variety of gastrointestinal ailments, including stomachache, gastritis, diarrhea, and peptic ulcers. Two such plants, Sanguinaria canadensis L. (bloodroot, Papaveraceae) and Hydrastis canadensis L. (goldenseal, Ranunculaceae), are herbaceous perennials native to eastern Canada and the United States (Der Marderosian, 1977; Mahady, 1993). Traditionally, the Native American Indians used hot water extracts of both plants for the treatment of a variety of ailments including dyspepsia, gastritis and indigestion (Anderson, 1885; Veninga and Zaricor, 1976; Mahady et al., 1993).

The rhizomes of S. canadensis contain an acrid orange-red juice from which numerous isoquinoline alkaloids, including chelerythrine, protopine and sanguinarine (Fig. 1) have been isolated (Mahady et al., 1993). Experimental evidence has shown that aqueous and alcohol extracts of the rhizomes inhibit the growth of Staphylococcus aureus and Mycobacterium tuberculosis in vitro, and the constituents responsible for this activity were two of the benzophenanthridine alkaloids, sanguinarine and chelerythrine (D’Amico, 1950; Gottshall et al., 1949). Similarly, the roots and rhizomes of H. canadensis produce a yellow-orange juice containing isoquinoline alkaloids, including berberine and β-hydrastine (Fig. 1) (Der Marderosian, 1977) Crude extracts of the rhizome, as well as berberine have been shown to inhibit the growth of Staphylococcus aureus and E. coli in vitro (D’Amico, 1950; Hocking, 1977).
Because of their traditional use for the treatment of gastrointestinal disorders, and their purported antibacterial activities, methanol extracts of both plants were screened against 15 strains of 

*Helicobacter pylori*. This report describes the results of the in vitro susceptibility of 

*H. pylori* to the methanol extracts, as well as their constituent isoquinoline alkaloids.

**MATERIALS AND METHODS**

**Plant Materials and Extraction**

The cultivated rhizomes of *Sanguinaria canadensis* L. were collected, cleaned and air-dried before grinding. The ground rhizomes (100 g) were extracted three times with 500 ml of 95% methanol, and evaporated to dryness under reduced pressure. The dried crude methanol extracts were re-dissolved in a minimal amount of methanol and eluted through a Prep Sep® cyan column to produce extracts with a high concentration of isoquinoline alkaloids (Mahady *et al.*, 1993). The alkaloids, sanguinarine and chelerythrine, and protopine were isolated and characterized from the concentrated extracts using a previously described protocol (Mahady and Beecher, 1994). Suspension cell cultures of *S. canadensis* were maintained on a modified Gamborg B5 medium (2 g of tissue per 100 ml media), and subcultured every two weeks (Mahady *et al.*, 1993). The suspension cells (100 g) were harvested at fourteen days, dried and 9.86 g were extracted three times with 100 ml of 95% methanol. The extract was dried under reduced pressure and then re-dissolved in a minimal amount of methanol and eluted through a Prep Sep® cyan column as previously described above.

*Hydrastis canadensis* dried powdered roots and rhizomes were obtained from Frontier Natural Products, Norway, Iowa. The powdered plant materials (1718 g) were extracted with 95% methanol (MeOH), three times, 4000 ml for 48 h, 3500 ml for 24 h and 3300 ml for 24 h. The MeOH extract was evaporated under reduced pressure leaving a dark brown residue which was dissolved in 100% MeOH and filtered, resulting in a yellowish-brown crystalline substance. Repeated recrystallization from chloroform/MeOH yielded pure β-hydrastine (colorless crystals) and berberine (yellow crystals). The extracts and pure compounds were stored

![Figure 1.](image-url)
at -20 °C in sterile borosilicate glass vials. A 10 mg sample of each plant extract or pure compound was tested for antibacterial activity using the HP in vitro susceptibility assay.

**H. pylori assay**

Susceptibility testing was performed using the agar dilution procedure according to the guidelines described by the National Committee for Clinical Laboratory Standards (1997, 1999). The extracts were dissolved in methanol and sterile distilled water was used for further serial dilutions of the dissolved plant extracts. Final test sample of each plant extract or pure compound was adjusted to 19 mL of molten Mueller-Hinton agar (pH 7.3) supplemented with 10% sterile defibrinated horse blood. Growth control plates consisting of 20 mL of agar medium were included in each experiment. Petri plates incorporating minimal to maximum volumes of vehicle solvent were included as a growth control to ensure the viability of the organisms was not affected by the solvent used to dissolve the plant extracts. For quality control and comparative analyses, the antibiotic amoxicillin was also tested with each batch of plant extracts.

A total of 14 clinical isolates namely, assessment numbers: A2, A6, Ed, 002, 019A, 1022, 1050, 1058, 1060, 1080, 1153, 1175, 1452, 4126, and 1 American Type Culture Collection (ATCC) (Rockville, MD) strain (ATCC 43504) of *H. pylori* were used in the susceptibility testing. The clinical strains were coded to protect the identity of the patient from which they were obtained. Some of the isolates were obtained from the Microbiology Laboratory at the University of Illinois Medical Center (Chicago, IL), Abbott Laboratories (Abbott Park, IL), and Dr D. Y. Graham (Houston, TX). The isolates obtained from Abbott Laboratories include organisms obtained from patients in Richmond, VA; Charlottesville, VA; Nashville, TN; and Southampton, England. Clinical isolates were obtained from different geographic regions to ensure that the organisms were genetically distinct. Gram stain appearance, and a positive urease test confirmed the identification of each organism. The organisms were stored frozen at -70 °C in skimmed milk plus 17% glycerol.

For susceptibility testing, the organisms were inoculated onto 5% sheep blood agar plates, and incubated at 37°C in a 10% CO2 atmosphere for 72 h. The organisms were then subcultured once to ensure reliable growth. An inoculum of each isolate was prepared by suspending the organism in 4.5 mL of sterile Mueller-Hinton broth and adjusting the turbidity to that of a 2.0 McFarland Standard using a spectrophotometer at 625 nm. This density produces a suspension of approximately 1 x 108 CFU/ml of *H. pylori*. The organisms were inoculated onto the agar plates containing consecutive dilutions of the plant extracts via a 32-prong inoculating device. The device delivers 8 µl per spot resulting in a final inoculum of approximately 1 x 106 CFU/spot. After the spots dried, the plates were incubated at 37°C in 10% CO2 and examined for growth after 3 days. All procedures were performed in duplicate. The minimum inhibitory concentration (MIC), defined as the lowest concentration of the compound at which there was no visible growth or only a faint haze, was determined for each plant extract and pure compound.

**RESULTS**

The minimum inhibitory concentrations (MIC) of crude methanol extracts of *Sanguinaria canadensis* and *Hydrastis canadensis*, and isolated chemical constituents are presented in Table 1. The 95% methanol extracts of both plants inhibited the growth of HP in vitro. The crude methanol extract of the roots and rhizomes of *H. canadensis* was slightly more active (MIC50 12.5 µg/ml; range 0.78–25 µg/ml) than the crude extract from the rhizomes or cell suspension cultures of *S. canadensis* (MIC50 12.5 µg/ml; range 12.5–100 µg/ml). Three isoquinoline alkaloids isolated from the rhizome extract of *S. canadensis* namely, sanguinarine, chelerythrine and protopine, inhibited the growth of the bacterium with an MIC50 of 50.0 to 100.0 µg/ml. Sanguinarine, was the most active of the three alkaloids, and inhibited the growth of all 15 HP strains with an MIC range of 6.25 to 50.0 µg/ml (Table 2). Two isoquinoline alkaloids, berberine and hydrastine, isolated from *H. canadensis*, inhibited the growth of *H. pylori* with an MIC50 of 12.5 and 100.0 µg/ml, respectively. Berberine was the most active alkaloid in the assay, and inhibited the growth of all 15 HP strains, with an MIC range of 0.78 to 25.0 µg/ml (Table 2). The MIC of amoxicillin was in the acceptable range established by NCCLS against the control strain, *H. pylori* ATCC 43504 (Table 1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample description</th>
<th>MIC50 µg/ml</th>
<th>MIC90 µg/ml</th>
<th>MIC range µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Sanguinaria canadensis</em> rhizome-MeOH</td>
<td>12.5</td>
<td>50</td>
<td>12.5–50</td>
</tr>
<tr>
<td>2.</td>
<td><em>S. canadensis</em> MeOH-Cyano fraction</td>
<td>25</td>
<td>50</td>
<td>12.5–100</td>
</tr>
<tr>
<td>3.</td>
<td><em>S. canadensis</em>-suspension cell cultures</td>
<td>50</td>
<td>50</td>
<td>25–50</td>
</tr>
<tr>
<td>4.</td>
<td>Sanguinarine</td>
<td>50</td>
<td>50</td>
<td>6.25–50</td>
</tr>
<tr>
<td>5.</td>
<td>Chelerythrine</td>
<td>100</td>
<td>100</td>
<td>25–100</td>
</tr>
<tr>
<td>6.</td>
<td>Protopine</td>
<td>100</td>
<td>&gt;100</td>
<td>25–100</td>
</tr>
<tr>
<td>7.</td>
<td><em>Hydrastis canadensis</em> rhizome-MeOH</td>
<td>12.5</td>
<td>50</td>
<td>0.78–50</td>
</tr>
<tr>
<td>8.</td>
<td>Berberine</td>
<td>12.5</td>
<td>25</td>
<td>0.78–25</td>
</tr>
<tr>
<td>9.</td>
<td>β-Hydrastine</td>
<td>100</td>
<td>100</td>
<td>25–100</td>
</tr>
<tr>
<td>10.</td>
<td>Amoxicillin</td>
<td>0.002</td>
<td>0.06</td>
<td>0.002–0.06</td>
</tr>
</tbody>
</table>

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Previously published investigations have demonstrated that medicinal plants, used traditionally to treat peptic ulcer disease and other gastrointestinal ailments, also inhibit the growth of HP in vitro. For example, *Terminalia spinosa*, an East African plant, inhibited the growth of HP, with a MIC range of 62.5–500 µg/ml (Fabry et al., 1996). Extracts of *Thymus vulgaris* and *Cinnamomum zeylanicum* also inhibited the growth of the bacterium, (Tabak et al., 1996) and extracts of garlic (*Allium sativum*) were effective, with an MIC range of 20–40.0 µg/ml (Sivam et al., 1997; Jonkers, 1999). Furthermore, extracts of *Pistacia lentiscus* and *Cetraria islandica*, plants used traditionally for the treatment of dyspepsia, also inhibit the growth of *H. pylori* in vitro (Ingdorfer et al., 1997; Huwez, 1998). Thus, there is a growing body of evidence suggesting that medicinal plants are good candidates for developing new agents for the treatment and prevention of HP infections.

Traditionally, extracts of *S. canadensis* and *H. canadensis* were employed as homeopathic and folk remedies for the treatment of various illnesses, including dyspepsia, gastritis, and ulcers (Anderson, 1885; Veninga and Zaricor, 1976). In fact in 1830, tinctures and extracts of *S. canadensis* were listed as digestants, in both the *United States Pharmacopoeia* and *National Formulary* (Harkrader, 1990). Furthermore, descriptions of the clinical use of *S. canadensis* for the treatment of gastritis and stomach ulcers were published as far back as 1882 (Winterburn, 1885). More recently, both plants have been popularized as ingredients of dietary supplements in the United States with a variety of structure-function claims (Mahady and Chadwick, 2001).

While crude extracts of *S. canadensis*, and *H. canadensis* have been shown to inhibit the growth of various microorganisms in vitro (Harkrader, 1990; Scazzocchio et al., 1998; Veninga and Zaricor, 1976; Walker, 1990), their antibacterial activity against *H. pylori* had not been previously reported. In this investigation, 95% methanol extracts of the roots and/or rhizomes of *S. canadensis* and *H. canadensis* inhibited the growth of 14 clinical isolates and one ATCC strain of *H. pylori*. All of the anti-helicobacter activity appeared in the alkaloid fractions of the extracts, with the active constituents consisting primarily of known isoquinoline alkaloids. Berberine and sanguinarine were the most active isoquinoline alkaloids tested, and have previously been reported to have antimicrobial activities (Walker, 1990; Gentry et al., 1998). Berberine has been reported to inhibit the growth of HP in vitro (Bae et al., 1998), and its efficacy against HP has been compared with that of other antibiotics in a clinical trial (Hu, 1993). Few side effects were observed in patients treated orally with 400 mg of berberine per day. Although there is little in the way of in vivo toxicological data for goldenseal, there is some information is available for berberine. The oral median lethal dose is 329 mg/kg body weight in mice and 100 mg/kg body weight in dogs, doses well above that needed to treat HP infections (Lampe, 1992). As for the safety of sanguinarine, no adverse effects have been observed in animals treated with up to 100 mg/kg, and the oral median lethal dose in rats was 1658 mg/kg, thus again demonstrating low in vivo toxicity (Becci et al., 1987; Keller and Meyer, 1989).

The mechanism by which these extracts and alkaloids exert their antibacterial effect on HP is not known. However, it has been shown in vitro that berberine blocks the adhesion of uropathogenic *Escherichia coli* to erythrocytes and epithelial cells through a mechanism that involves a reduction in the synthesis of fimbrial subunits and the expression of assembled fimbriae (Sun et al., 1992). Since, in *in vitro* and *in vivo* studies have both demonstrated that the attachment of *H. pylori* to the gastric epithelial cells is mediated by the fucosylated Lewis b histo-blood group antigen-binding adhesin (Iver et al., 1998; Gerhard et al., 1999) this interesting mechanism of action will be explored in future investigations with both berberine and sanguinarine.

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IN VITRO SUSCEPTIBILITY OF HELICOBACTER PYLORI

REFERENCES


