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Microanalysis of a Selective Potent Anti-*Helicobacter pylori* Compound in a Brazilian Medicinal Plant, *Myroxylon peruiferum* and the Activity of Analogues

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Abstract: A selective potent anti-*Helicobacter pylori* isoflavone was isolated from a Brazilian Medicinal Plant, *Myroxylon peruiferum*. The isolation bioassay-guided and the characterization of an active anti-*H. pylori* constituent was performed using the methanol extract of plant of minute amount. The active compound was identified as cabreuvin (**1**), an isoflavone derivative. The structure-activity relationships of several related compounds were also investigated. © 1999 Elsevier Science Ltd. All rights reserved.

Helicobacter pylori is a micro-aerophilic, Gram-negative, spiral-shaped flagellated bacterium which lives in the human gastric mucosa. *H. pylori* is the cause of gastric inflammation and peptic ulcers, and may be associated with gastric cancer.[1-2] Chemotherapy regimens for eradication of *H. pylori* involve the use of two antibiotics, such as amoxicillin and clarithromycin, in combination with a proton-pump inhibitor (PPI). However, since more than double the recommended doses of antibiotics are required for successful eradication of this organism, the present chemotherapeutic regimens cause side effects such as nausea and diarrhea, and promote the development of tolerant bacteria. Therefore, effective new drugs having selectivity for treating *H. pylori* infection without side effects are required.[3-9]

We have been searching for new lead anti-*H. pylori* compound from Brazilian medicinal plants. Methanol extracts of 80 species of Brazilian medicinal plants, purchased in São Paulo, were tested for their ability to inhibit the proliferation of *H. pylori*. The extracts of three species showed potent anti-*H. pylori* activity. This paper describes the search for the active compound in the extract of "Oleo vermerho" (trunk wood, *Myroxylon peruiferum*, Leguminosae) which is uesd for cystitis, bronchitis and diabetes, by using plant extract of minute amount and the evaluation of activity of analogues.

The methanol extract (MIC 62.5μg/mL)[10] of *M. peruiferum* was partitioned between ethyl acetate and water, and subsequently the aqueous layer was partitioned between n-butanol and water, as shown in Scheme 1. The three extracts thus obtained were subjected to the anti-*H. pylori* test. The activity was concentrated in the ethyl acetate extract (MIC 30μg/mL). This material (350.8μg) was fractionated by HPLC (ODS-column) using a 30-min. linear gradient mixture of 0-100% acetonitrile in water containing 0.1% trifluoroacetic acid.

The fractionation pattern of the HPLC is shown in Figure 1. The thirteen fractions obtained were freeze-dried after evaporating the solvent. Each fraction was then redissolved in the same volume of solvent, regardless of their actual weights. The assays were carried out using the appropriate concentration on reference to the MIC value of the ethyl acetate extract. The activity was concentrated in the eleventh fraction, H-11. The MIC value based on the pro-separated weight of the ethyl acetate extract was 35μg/mL. Other fractions were inactive. Accordingly, the compound contained in H-11 is thought to be the main anti-*H. pylori* compound in *M. peruiferum*. To characterize this bioactive compound, the ethyl acetate extract (19.47 mg) was separated by preparative TLC and the compound with an HPLC peak in the location identical to that of H-11 was isolated (2.88 mg). Its structure was analyzed by EI-MS and ^{1}H- and ^{13}C-NMR techniques including 2D-NMR (^{1}H-^{1}H COSY, ^{1}H-^{13}C COSY, HMBC).

This paper is dedicated to the memory of Prof. Yasuo Komoda.

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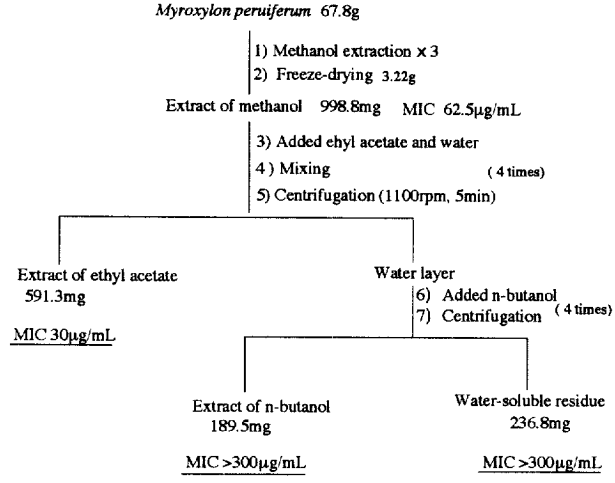
The bioactive compound was identified as an isoflavone, cabreuvin (**1**)[11] previously isolated from *M. peruiferum*[12a] and *M. balsamum*[12b]. This compound is obtained in quantity by the methylation of 3',4',7-trihydroxy-isoflavone **4**. Using **1** as an external standard, the amounts of **1** in the three extracts obtained by the solvent partition described above were determined by HPLC. The results indicate that the proportions of **1** in the ethyl acetate, n-butanol, and water extracts were 12.43%, 1.82%, and 0%, respectively. Cabreuvin (**1**) is thought to be responsible for most of the anti-*H. pylori* activity in the *M. peruiferum* extract.



Scheme 1.
Extraction and partition scheme of *M. peruiferum* and MIC data.

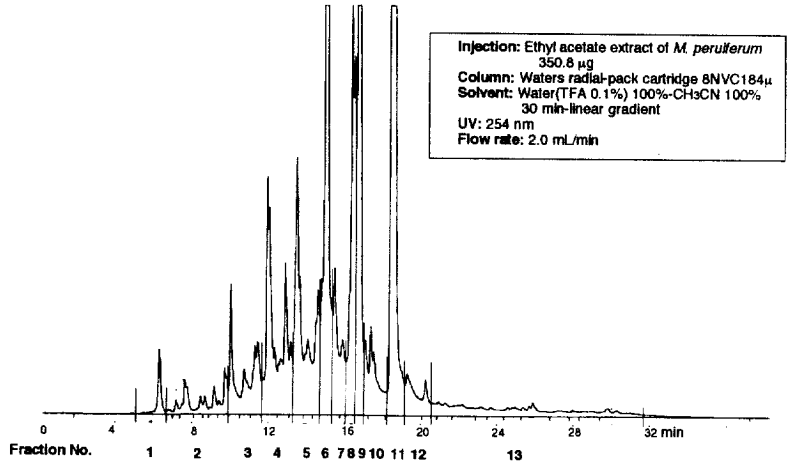


Figure 1.
HPLC chromatogram of ethyl acetate extract of *M. peruiferum*

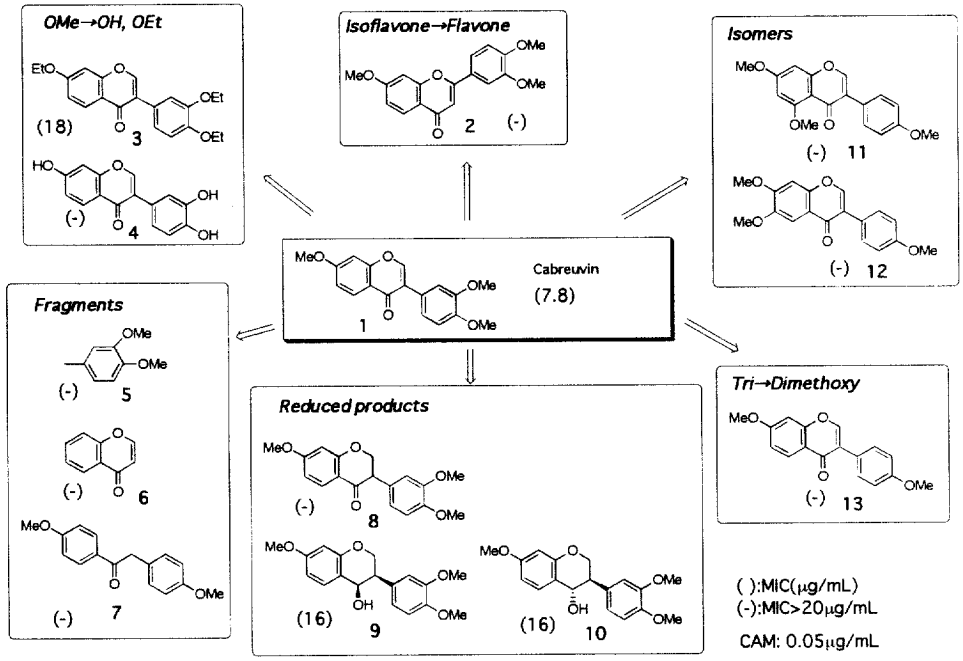
Structure-activity relationship studies of compounds related to cabreuvin (1).

The inhibition of the growth of *H. pylori* by the compounds related to **1** was evaluated, as shown in Scheme 2. These compounds were commercial products or compounds synthesized from them by known procedures[13]. The MIC values are listed underneath the structures of the respective compounds. While the triethoxy analogue **3**[14] preserved was half as bioactive as **1**, the demethylated compound **4** was inactive. Compounds **5**-**7**, which have partial structure of **1**, the isometric trimethoxyisoflavones **11**[17] and **12**, reduced product **8**[15], dimethoxyflavone **13**[18], and the corresponding flavone **2** did not have any bioactivity. Notably, both of the epimeric 4-hydroxyisoflavan derivatives **9** and **10**[16] had one half of the bioactivity of **1**. These results indicate that a delicate balance of the structural elements is necessary for the anti-*H. pylori* activity. Isoflavone or an isoflavan skeleton is a prerequisite, and the number and position of the phenolic hydroxy groups and their alkylation are critical. These results indicated 3'-alkoxy group is essential to their activity. Their structure-activity relationships are similar to those of the ichtyotoxicity of isoflavones.[19] They show that 7-methoxy and 3',4'-dimethoxy groups are responsible for an increase of the ichyotoxic power and any other substitution reduces the toxicity. The mechanism of ichyotoxicity is the inhibition of NADH oxidation in the respiratory chain. The similarity of both of the structure-activity relationships may imply that their mechanism of anti-*H. pylori* activity is also the inhibition of NADH oxidation.

The antimicrobial activity of cabreuvin (**1**) was also tested against other microorganisms: Gram-positive bacteria (*Bacillus subtilis*, *Micrococcus lutea*, and *Bacteroides fragilis*), Gram-negative bacteria (*E. coli* and *Pseudomonas aeruginosa*) and yeasts (*Saccharomyces cerevisiae* and *Candida albicans*). Compound **1** was completely inactive against all of these organisms, even at a concentration of 625μg/mL. It is noteworthy that the bioactivity of **1**, although moderate, is highly selective against *H. pylori*.[20]

The active constituent was identified as an isoflavone derivative, cabreuvin (**1**), which has a moderate MIC value, but a highly-selective activity only against *H. pylori*. Further modifications of this isoflavone with a potent anti-*H. pylori* activity are investigated at present.

Scheme 2.
Anti-*H. pylori* activity of cabreuvin and some related compounds



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10. **Culture Medium for *H. pylori*.** *H. pyroli* 31A strain derived from patient of stomach ulcer was kindly provided by Dr. Takeshi. Itoh (Tokyo Metropolitan Reserch Laboratory of Public Health). *H. pyroli* was grown in test tubes in a liquid medium (5 mL) containing Brain-Heart Infusion Broth (Difco, Detroit, MI, USA) with 10% fetal bovine serum (Upstate Biotechnology Inc., Lake Placid, NY, USA) in an atmosphere of 10% CO_2, 5% O_2 and 85% N_2 at 37 °C for 48h.
Determination of minimum inhibitory concentrations (MICs) against *H. pylori*. Serial doubling dilutions of each compound and clarithromycin were made in 10% aqueous dimethylsulfoxide. The culture medium containing 5% of the culture of *H. pylori* and 10% of the solution of each compound was incubated at 37°C for 48 h. The MIC was defined as the lowest consentration of compounds, at which visible growth was inhibited.
11. **Cabreuvin (1):** ^{1}H NMR ($CDCl_3$) δ 3.91 (s, 3H,4'-OMe),3.92 (s, 3H, 7-OMe), 3.93(s, 3H, 3'-OMe), 6.86 (d, *J*=2.4 Hz, 1H,H-8), 6.93(d, *J*=7.9 Hz, 1H, H-5'), 7.00(dd, *J*=9.2, 2.4 Hz, 1H, H-6), 7.05(dd, *J*=7.9,1.8 Hz, H-6'), 7.21 (d, *J*=1.8 Hz, 1H, H-2'), 7.88(s, 1H, H-2), 8.22(d, *J*=9.2, 1H, H-5) ^{13}C NMR ($CDCl_3$) δ 55.82 (C-7,O*Me*), 55.99(C-3',O*Me*), 55.99(C-4',O*Me*), 100.15 (C-8), 111.24(C-5'), 112.62(C-2') 114.60(C-6), 118.44(C-4a), 121.06(C-6'), 124.78(C-1'), 124.97(C-3), 127.79(C-5), 148.83(C-3'), 149.16(C-4'), 152.22(C-2), 157.94(C-8a), 164.04(C-7), 175.94(C-4).
Synthesis: Cabreuvin (1). To a solution of 3',4',7-trihydroxyisoflavone (**4**) (48 mg, 0.18 mmol) and diisopropylethylamine (0.27 mL, 1.6 mmol) in a mixture of MeOH (5 mL) and dioxane (5 mL) at room temperature was added a solution of 10% trimethylsilyldiazomethane in *n*-hexane (2.7 mL, 1.6 mmol) and the reaction mixture was stirred at room temperature for 16 h. The solution was then evaporated and the residue was purified by preparative HPLC using a 80-min linear gradient of 40-80% acetonitrile in water to give pure **1** (50 mg, 90%).
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13. **Derivatives.** Unless otherwise noted, chemicals were commercially available and used without further purification. 3',4',7-Trimethoxyflavone (**2**), 3',4',7-trihydroxyisoflavone (**4**) and 4',6,7-trimethoxyisoflavone (**12**) were purchased from Indofine (Somerville, NJ, USA), 3,4-dimethoxytoluene (**5**) from TCI (Tokyo, Japan), chromone (**6**) and desoxyanisoin (**7**) from Aldrich (Milwaukee, WI, USA), genistein from Sigma (St. Luis, Mo, USA) and formononetin from Funakoshi (Tokyo, Japan). All of the compounds synthesized here have been reported previously. Physical data were identical with reported values for all the compounds.
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20. **Determination of MICs against other microorganisms.** MICs were determined by a conventional well agar method. The plates were incubated at 37°C for 24 h.