Anti-*Helicobacter pylori* Activity of Quinolone Alkaloids from Evodiae Fructus

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A biologically monitored fractionation of methanol extract of the fruit of *Evodia rutaecarpa* led to the isolation of six quinolone alkaloids, evocarpine (1), 1-methyl-2-[(4*Z*,7*Z*)-4,7-tridecadienyl]-4(1*H*)-quinolone (2), 1-methyl-2-[(6*Z*,9*Z*)-6,9-pentadecadienyl]-4(1*H*)-quinolone (3), 1-methyl-2-undecyl-4(1*H*)-quinolone (4), dihydroevocarpine (5), 1-methyl-2-pentadecyl-4(1*H*)-quinolone (6). They showed potent anti-*Helicobacter pylori* activity with the minimum inhibitory concentration (MIC) value of 10—20 μg/ml. However, they had no effect on *Helicobacter pylori* urease activity at the concentration of 300 μg/ml.

Key words *Helicobacter pylori*; *Evodia rutaecarpa*; quinolone alkaloid

Helicobacter pylori (HP) was isolated from the gastric antrum of chronic gastritis patients by Warren and Marshall in 1983.[1,2] HP promoted gastritis to gastric cancer. Pathogenic HP produces urease strongly, which hydrolyzes urea to CO_2 and ammonia. HP urease is considered to play a critical role in the pathogenesis of gastritis and peptic ulcer. Therefore, eradication of the bacteria and inhibition of the urease are important for the treatment of patients with gastroduodenal diseases.[3] Current clinical therapy relies on triple therapy schedules combining bithmuth salts, metronidazole or tinidazole, and tetracycline or amoxacillin. However, the attractiveness of such comprehensive regimens is clearly diminished by numerous side effects. Accordingly, there is a need for safe and effective treatment with a compound having excellent anti-HP activity.

During the screening program to discover such compounds from natural products, Evodiae fructus was found to show inhibitory activity against HP. In this paper, we report isolation of the active principle and inhibitory effect on HP growth and urease activity of quinolone alkaloids from Evodiae fructus.

MATERIALS AND METHODS

Materials Brucella agar and brucella broth were purchased from Difco Co. (U.S.A.). Horse serum was from Sigma Chem. Co. (U.S.A.). AnaeroPak Campylo was from Mitsubishi Gas Chemical Co., Inc. (Japan). ^{1}H- and ^{13}C-NMR spectra were measured on a Varian Unity 300 NMR (^{1}H: 300 MHz, ^{13}C: 75 MHz). MS were obtained on a Hewlett Packard 5989A mass spectrometer. The chemical shifts were expressed as δ values in ppm from tetramethylsilane (TMS) as internal standard. TLC was carried out on Si gel 60 F_{254} and RP-18 F_{254} plate (Merck, Darmstadt, Germany). Column chromatography was performed over Si gel 60 (Merck, particle size 70—230 mesh) and YMC*GEL ODS-A (YMC, particle size 70—230 mesh). The preparative HPLC apparatus consisted of a Shimadzu model LC-6AD pump and SPD-10A UV detector.

Plant Material The dried unripe fruits of *E. rutaecarpa* were purchased from a local herb market in Taejon, Korea in 1996. The authenticity of the plant was confirmd by D. S. Han, Emeritus Professor of Seoul National University.

Isolation of HP Growth-Inhibitory Components from Evodiae Fructus The dried, unripe fruits of *E. rutaecarpa* (5 kg) were milled and extracted 3 times with MeOH at room temperature. The MeOH extracts were concentrated to a residue under reduced pressure, and this was diluted with water. This aqueous solution was extracted with BuOH. The combined BuOH extracts, upon evaporation, yielded 222 g of solid material. The crude BuOH extracts were mixed with 200 g silica gel (70—230 mesh) and then subjected to column chromatography on silica gel, eluting with hexane and a gradient of hexane–EtOAc (20 : 1→1 : 1, v/v). The elution gave 16 fractions (each of 1000 ml) and each fraction was monitored by *in vitro* anti-HP activity. Fraction 7—9 with the highest inhibitory activity were combined and evaporated to give 18.4 g of solid material. The active fractions were applied to rechromatography over RP-18 (YMC*GEL, ODS-A, 60 Å, 70/230 mesh). Elution with a step gradient of MeOH/H_2O (50% MeOH→70% MeOH→90% MeOH→100% MeOH) was divided into three active fractions, EB-1 (13.6 g), EB-2 (0.7 g), EB-3 (0.5 g). Fraction EB-1 was further purified by semi-preparative HPLC (column, J'sphere ODS-H80, 4 μm, 80 Å, i.d. 20×150 mm; MeOH–H_2O (90 : 10); flow rate, 8 ml/min; UV, 254 nm) to give compounds **1**—**4**. Compounds **5** and **6** were isolated from EB-2 and EB-3 by using the same HPLC condition.

Bacterial Strains HP ATCC43504, NCTC11637 and NCTC11638 were purchased from ATCC and NCTC, respectively. Other HPs (HP82516, HP82548, HP4), clinical isolates selected from Korean gastroscopic sample, were also used. They were inoculated into brucella agar plates supplemented with 7% horse serum and cultured for 3 d at 37 °C in an anaerobic jar with AnaeroPak Campylo.

Growth Inhibition Assay of HP Growth inhibition assay of HP was performed as discussed.[4] One ml of isolated compounds was added to a petri dish containing unsolidified 7 ml brucella agar supplemented with 7% horse serum. Final concentrations of each isolated compound were 100, 80, 40, 20, 10, 5, 2.5, 1, 0.5, 0.25 and 0.1 μg/ml. Approximately 5×10^5 CFU of HP was then inoculated to the agar plates and

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Table 1. MICs of Quinolone Alkaloides (**1**—**6**) Isolated from Evodiae Fructus on the Growth of *Helicobacter pylori*

Compd.	MIC (μg/ml)						Inhibition % of urease activity[a]
	HP ATCC43504	HP NCTC11637	HP NCTC11638	HP 82516	HP 82548	HP 4	
1	10	10	20	10	20	10	0
2	10	10	10	10	10	10	0
3	10	10	20	10	10	10	0
4	10	10	20	10	10	10	0
5	10	10	20	10	10	10	0
6	10	10	20	10	10	10	0
Ampicillin	1	1	0.5	1	2	2	—
AHA[b]	—	—	—	—	—	—	93

a) HP urease activities were tested at the concentration of 300 μg/ml. *b*) AHA, acetohydroxamic acid.

cultured microaerobically for 3 d at 37 °C in an anaerobic jar (85% N_2, 10% CO_2, 6% O_2). The minimum inhibitory concentration (MIC) was determined after an incubation period of 72 h. Ampicillin was used as a positive control. All experiments were conducted in duplicate.

Preparation and Assay of HP Urease HP was inoculated from an agar plate into 30 ml of brucella broth supplemented with 10% fetal bovine serum in a 100 ml flask, which was placed in an anaerobic jar. The harvested cells were washed with 10 ml of 20 mM phosphate buffer, pH 7.0, sonicated and centrifuged at 5000×***g*** for 30 min. The resulting supernatant was used as the crude enzyme; its specific activity was 0.9 unit/mg protein. Urease activity was determined according to the method of Gutmann and Bergmeyer.[5]

RESULTS AND DISCUSSION

The BuOH extract of *E. rutaecarpa* (Rutaceae) was found to exhibit potent antibacterial activity against HP. Using an *in vitro* anti-HP activity test to guide isolation, the BuOH extract of *E. rutaecarpa* was fractionated by a series of normal and reverse phase column and semi-preparative HPLC chromatographic procedures to yield six quinolone compounds, evocarpine (**1**), 1-methyl-2-[(4*Z*,7*Z*)-4,7-tridecadienyl]-4(1*H*)-quinolone (**2**), 1-methyl-2-[(6*Z*,9*Z*)-6,9-pentadecadienyl]-4(1*H*)-quinolone (**3**), 1-methyl-2-undecyl-4(1*H*)-quinolone (**4**), dihydroevocarpine (**5**), and 1-methyl-2-pentadecyl-4(1*H*)-quinolone (**6**) (Fig. 1). These compounds gave a red-brown spot with Dragendorff's reagents on TLC and were identified as quinolone type alkaloid on the basis of their physicochemical properties and spectral data in comparison with those of published values.[6,7]

Table 1 shows the effect of six quinolone alkaloids (**1**—**6**) on the growth of HP and HP-urease activity. The MIC ranges for these compounds proved to be 10 to 20 μg/ml on the growth of HP, but had no effect on HP-urease activity at the concentration of 300 μg/ml. Ampicillin, which is clinically used for the eradication of HP, was used as postive control and exhibited an inhibitory effect (MIC value; 0.5—2 μg/ml) to HP growth.

In recent years, several other agents from natural products have been shown to exhibit anti-HP activity. Decursin and decursinol angelate with potent MIC value of 6—20 μg/ml were isolated from *Angelica gigas*. Protolichesterinic acid (Lichen *Cetraria islandica*), magnolol (*Magnolia officinalis*), capsaicin, thiosulfinate and berberine (*Coptis japonica*) have been reported as inhibitors of HP growth.[4,8—11] Recently, an inhibitor called CJ-13,564 with very potent anti-HP activity (MIC value of 0.1 ng/ml) was isolated from the actinomycetes *Peudonocardia* sp.[12] This compound has quinolone skeleton and structurally close relation to active compounds (**1**—**6**) isolated from *E. rutaecarpa*.

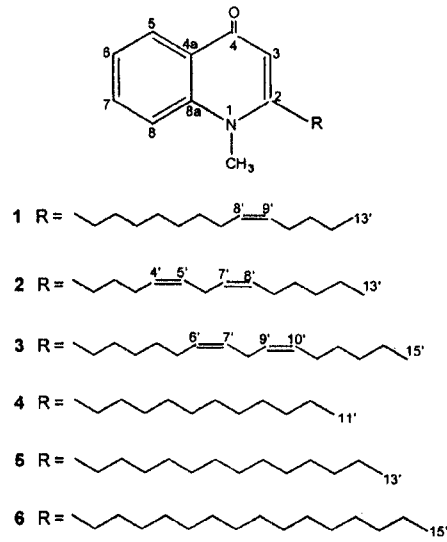

Fig. 1. The Chemical Structures of Quinolone Alkaloids (**1**—**6**) with Anti-*Helicobacter pylori* Activity from Fruit of *Evodia rutaecarpa*

Evodiae fructus is a Chinese herbal drug which has antiemetic, analgesic, uterotonic, antihypertensive and antibacterial activity. The quinolone alkaloids (**1**—**6**) discussed above have recently been isolated from fruits of *E. rutaecarpa* and are potent inhibitors of HP growth. It is suggested that the traditional uses of Evodiae fructus for abdominal pain, vomit and diarrhea would be due in part to inhibitory activity of this quinolone alkaloids.

REFERENCES

1) Warren J. R., Marshall B. J., *Lancet*, **1**, 1273—1275 (1983).
2) Marshall B. J., Barrett L. J., Prakash C., McCallum R. W., Guerrant R. L., *Gastroenterology*, **99**, 697—702 (1990).
3) Eaton K. A., Brooks C. L. Morgan D. R., Krakowka S., *Infect. Immun.*, **59**, 2470—2475 (1991).
4) Bae E.-A., Han M. J., Kim N.-J., Kim D.-H., *Biol. Pharm. Bull.*, **21**, 990—992 (1998).
5) Gutmann I., Bergmeyer H. U., "Methods of Enzymatic Analysis", Vol.

4, ed. by Bergmeyer H. U., Academic Press, Inc., New York, 1974, pp. 1791—1794.
6) Tang T.-Q., Fen X.-Z., Huang L., *Phytochemistry*, **43**, 719—722 (1996).
7) Sugimoto T., Miyase T., Kuroyanagi M., Ueno A., *Chem. Pharm. Bull.*, **36**, 4453—4461 (1988).
8) Hentschel E., Brandstatter G., Dragsics B., Hirschil A. M., Nemec H., Schutze K., Taufer M., Wurzer H., *N. Engl. J. Med.*, **28**, 308—312 (1993).
9) Fabry W., Okemo P., Ansorg R., *Chemotherapy*, **42**, 315—317 (1996).
10) Ingolfsdottir K., Hjalmarsdottir M. A., Sigurdsson A., Gudjonsdottir G. A., Brynjoflsdottir A., Steingrimsson O., *Antimicrob. Agents Chemother.*, **41**, 215—217 (1997).
11) Jones N. L., Shabib S., Sherman P. M., *FEMS Lett.*, **146**, 223—227 (1997).
12) Dekker K. A., Inagaki T., Gootz T. D., Huang L. H., Kojima Y., Kohlbrenner W. E., Matsunaga Y., McGuirk P. R., Nomura E., Sakakibara T., Sakemi S., Suvuki Y., Yamauchi Y., Kojima N., *J. Antibiot.*, **51**, 145—152 (1998)