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Antibacterial action of tryptanthrin and kaempferol, isolated from the indigo plant (*Polygonum tinctorium* Lour.), against *Helicobacter pylori*-infected Mongolian gerbils

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Abstract: We evaluated the effect of tryptanthrin and kaempferol, both isolated from *Polygonum tinctorium* Lour., against *Helicobacter pylori* colony formation in vitro and in *H. pylori*-infected Mongolian gerbils. *H. pylori* suspension was mixed with solution of tryptanthrin and/or kaempferol and placed onto agar plates. These plates were incubated at 37°C, under 10% CO_2 for 5 days, and the *H. pylori* colonies were counted. For the in vivo experiment, Mongolian gerbils were inoculated with *H. pylori* ATCC 43504 orally. After 4 weeks, the infected gerbils were given tryptanthrin and/or kaempferol, administered orally, twice a day for 10 days. The animals were killed and the number of live *H. pylori* in their stomachs was determined. In vitro both tryptanthrin and kaempferol significantly decreased the numbers of *H. pylori* colonies a dose-dependent manner. An additive effect on colony formation was observed with the combined use. In the in vivo experiment, oral administration of tryptanthrin and/or kaempferol significantly decreased the numbers of colonies in the gerbils' stomachs. We concluded that tryptanthrin and kaempferol were effective against *H. pylori* in vivo.

Key words: tryptanthrin, kaempferol, anti-*Helicobacter pylori* effect, Mongolian gerbil

Introduction

Since the isolation of *Helicobacter pylori* from human gastric biopsy specimens by Warren and Marshall in 1983,[1] many investigators have reported that *H. pylori* was associated with gastric disorders, e.g., chronic gastritis,[2,3] duodenal ulcer,[4] and gastric cancer.[5–7] As clinical strategies for the treatment of *H. pylori* infection, antibiotics are mainly used for the eradication of this bacterium, e.g., in dual or triple therapies with proton pump inhibitors (PPIs).[8,9]

Hirayama et al.[10] reported that Mongolian gerbils were a useful model for *H. pylori* infection. The symptoms in this model were demonstrated to be very similar to those in human gastritis, and this model has been shown to be suitable for the evaluation of antimicrobial drugs.[11,12]

It has been reported that many substances derived from plants (for example, flavonoids) have been used as drugs for the treatment of various diseases. Recently, we found that compounds isolated from *Polygonum tinctorium* Lour., a plant commonly known as indigo, had many biological activities in vitro; for example, anti-*H. pylori*,[13] antitumor,[14,15] antiviral,[16] and anti-inflammatory effects.[17] The substances showing anti-*H. pylori* activity were identified as tryptanthrin, kaempferol, 6-methoxykaempferol, and 3,5,4'-trihydroxy-6,7-methylenedioxy flavone.[13] In addition, other investigators have reported that flavonoids (kaempferol) isolated from plants had an antiulcer effect, based on the inhibition of platelet-activating factor (PAF) formation by gastric mucosa.[18]

In this study, we demonstrated the anti-*H. pylori* effect of tryptanthrin and/or kaempferol on an *H. pylori*-infected Mongolian gerbil model. These results suggest the possibility of a new therapeutic strategy, using plant-derived compounds.

Materials and methods

Bacteria

H. pylori ATCC43504 was used in this experiment. *H. pylori* was cultured in Brucella Broth (BBL, Cockeysville, MD, USA) supplemented with 10% heat-

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inactivated fetal calf serum (FCS; Dainippon Pharmaceutical, Tokyo, Japan) in a culture flask at 37°C, under 10% CO_2.

Animals

Mongolian gerbils (MON/Sea, male, 5 weeks old) were purchased from Seac Yoshitomi (Fukuoka, Japan). Twenty-five animals were divided into five groups. Five animals in each group were maintained on standard rodent chow and water available ad libitum.

Materials

An agar plate was prepared with Agar Noble (Difco Laboratories, Detroit, MI, USA) and horse blood (Japan Biological Material Center, Tokyo, Japan) by Skirrow's modified method.

Drugs

Tryptanthrin was purchased from Wako Pure Chemicals (Osaka, Japan) and kaempferol from Funakoshi (Tokyo, Japan). Omeprazole (OPZ) was obtained from Yoshitomi Pharmaceutical Industries (Osaka, Japan), clarithromycin (CAM) from Taisho Pharmaceuticals (Tokyo, Japan), and amoxicillin (AMPC) from Kyowa Hakko Industry (Tokyo, Japan). The drugs were dissolved in a solution of 0.5% hydroxypropyl methylcellulose (HPMC; Sigma St. Louis, MO, USA) before use. For single therapies, tryptanthrin and kaempferol were dissolved at a concentration of 10 mg/ml. For combined therapies, each drug was prepared at double concentration. For antibiotic plus OPZ therapy, AMPC (0.3 mg/ml), CAM (3 mg/ml), and OPZ (3 mg/ml) were prepared separately, and mixed just before use.

Bacterial inoculation and administration of drugs

We established the *H. pylori*-infected gerbil model by Hirayama's method, with slight modification.[10] *H. pylori* ATCC 43504 was grown in a culture flask containing Brucella Broth supplemented with 10% FCS. *H. pylori* ($1–10 \times 10^9$ CFU / body) was orally inoculated to each gerbil after 24-h fasting. Four weeks after the inoculation, drugs were administered to the gerbils twice a day for 10 days.

Assessment of colony formation

The day after the final administration of drugs, the gerbils were killed and the stomachs were removed. The mucosa of each whole stomach was scraped with a spatula in 10 ml of phosphate-buffered saline (PBS) and suspended well. One hundred-microliter aliquots of these samples were spotted onto an agar plate, prepared by Skirrow's modified method. The plates were incubated at 37°C, under 10% CO_2, for 5 days, and the colonies of *H. pylori* were counted.

For the in vitro assay, tryptanthrin and kaempferol were dissolved in 100% dimethyl sulfoxide (DMSO), at concentrations of 5 mg/ml and 10 mg/ml, respectively. *H. pylori* suspension was mixed with these solutions and placed onto the agar plates. These plates were incubated under the same conditions as those mentioned above.

Statistical analysis

Data values were analyzed by one-way analysis of variance (ANOVA), except for the group with antibiotics plus PPI (positive control). When ANOVA indicated differences among the groups, pairwise comparisons of each experimental group versus the control group were performed by Dunnett's test. Differences were assessed by two-tailed test, with P values of 0.05 or 0.01 considered significant.

Results

Effects of tryptanthrin and/or kaempferol on colony formation of H. pylori *in vitro*

When *H. pylori* was incubated with tryptanthrin or kaempferol for 5 days, these compounds decreased the numbers of colonies in a dose-dependent manner (Fig. 1). At the dose of 10 μg/ml of tryptanthrin, colony formation was completely inhibited. In the presence of both compounds, an additive inhibitory effect against *H. pylori* colony formation was observed. In these experiments, the final concentration of DMSO was 1%. However, this concentration did not affect the colony formation (data not shown).

Effects of tryptanthrin and/or kaempferol against H. pylori*-infected Mongolian gerbils*

After tryptanthrin and/or kaempferol were orally administered to *H. pylori*-infected Mongolian gerbils twice a day for 10 days, the number of live *H. pylori* in their stomachs was determined (Fig. 2). Tryptanthrin (5.0 mg/body) significantly suppressed bacterial colonies in the gerbils' stomachs. Kaempferol, although less efficient than tryptanthrin, also significantly decreased the number of colonies. The combined administration of tryptanthrin and kaempferol resulted in a further decrease in the number of colonies compared with the single therapy, although there was no significant difference between the groups. The combination of AMPC, CAM and OPZ, when administered as a positive con-

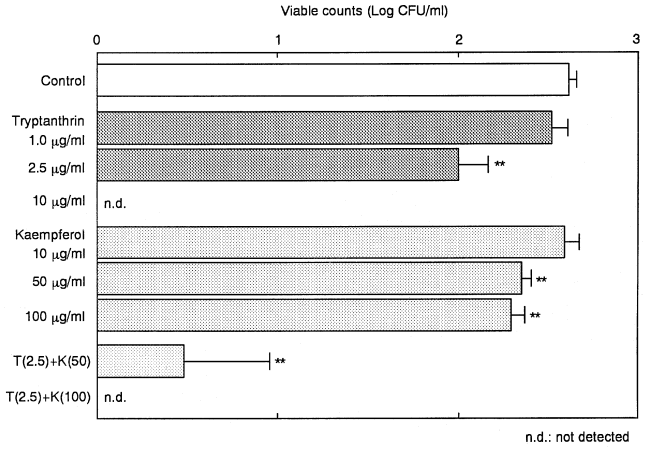

Fig. 1. Effects of tryptanthrin and kaempferol on *Helicobacter pylori* colony formation. *H. pylori* ATCC43504 was mixed with various concentrations of tryptanthrin, kaempferol, or both these agents. These suspensions were inoculated onto agar plates and the plates were incubated at 37°C, under 10% CO_2, for 5 days. After the incubation period, the number of *H. pylori* colonies was counted. Each value represents the mean ± SD of triplicate cultures. $^{**}P < 0.01$ compared with vehicle control. *n.d.*, not detected; *T*, tryptanthrin; *K*, kaempferol

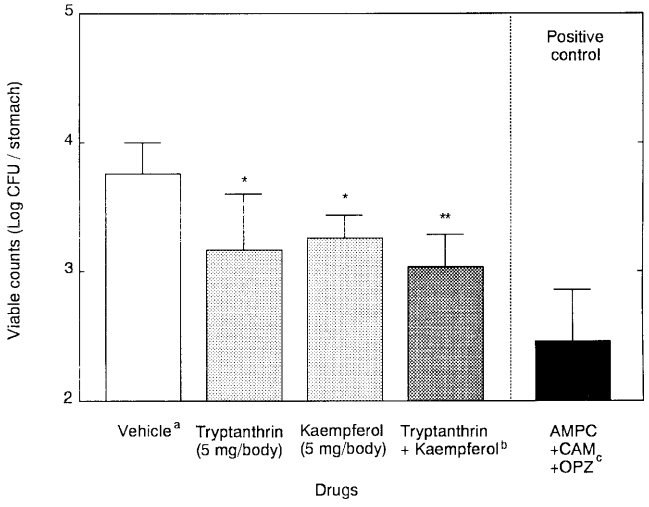

Fig. 2. Viable cell counts of *H. pylori* in stomachs of *H. pylori*-infected Mongolian gerbils after treatments with tryptanthrin and/or kaempferol. Drugs were administered orally to gerbils twice a day for 10 days, 4 weeks after *H. pylori* inoculation. The day after the final drug administration, the animals were killed. Each value represents the mean ± SD for five animals. Statistical analyses were performed between results for the control group and the tryptanthrin and/or kaempferol groups, but not the positive control. [a]0.5% hydroxypropyl methylcellulose (HPMC); [b]drug suspension consisting of tryptanthrin (10 mg/ml) and kaempferol (10 mg/ml) in 0.5 ml of vehicle; [c]drug suspension consisting of amoxicillin (*AMPC*; 0.1 mg/ml), clarithromycin (*CAM*; 1 mg/ml), and omeprazole (*OPZ*; 1 mg/ml) in 0.5 ml vehicle. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with vehicle control

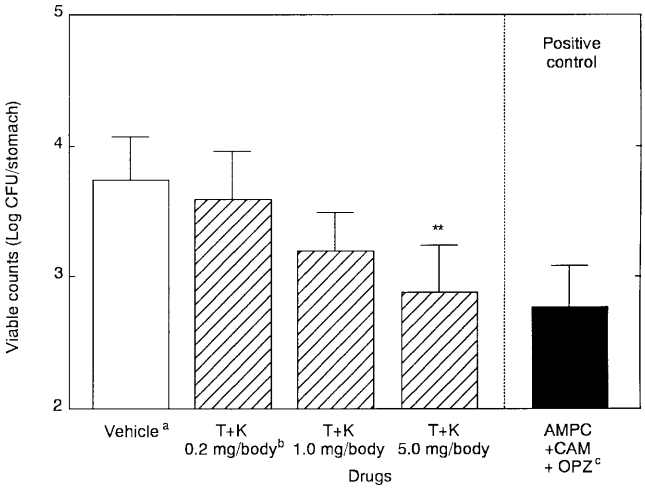

Fig. 3. Combined effects of tryptanthrin (*T*) and kaempferol (*K*) against viable counts of *H. pylori* in stomachs of *H. pylori*-infected gerbils. Four weeks after *H. pylori* inoculation, the combined administration of tryptanthrin and kaempferol was performed twice a day for 10 days. Each value represents the mean ± SD for five animals. Statistical analyses were performed between the results for the control group and the tryptanthrin and kaempferol groups, but not the positive control. [a]0.5% HPMC; [b]drug suspension consisting of tryptanthrin (0.4 mg/ml) and kaempferol (0.4 mg/ml) in 0.5 ml of vehicle; [c]drug suspension consisting of amoxicillin (*AMPC*; 0.1 mg/ml), clarithromycin (*CAM*; 1 mg/ml), and omeprazole (*OPZ*; 1 mg/ml) in 0.5 ml of vehicle. $^{**}P < 0.01$ compared with vehicle control

trol, also significantly reduced the number of colonies in the stomach.

We identified the colonies as the *H. pylori* ATCC 43504 strain inoculated to gerbils by performing analysis of the DNA sequence of the 16S rRNA gene, which was amplified by the polymerase chain reaction (PCR), based on the method of Ho et al.[19] (data not shown).

Dose-dependency of combined administration of tryptanthrin and kaempferol in H. pylori*-infected gerbils*

Next, we evaluated the combined effects of tryptanthrin and kaempferol, at three different doses, in *H. pylori*-infected gerbils. Combined administration of tryptanthrin and kaempferol decreased the viable counts of *H. pylori* in gerbils' stomachs dose-dependently, with the dose of 5.0 mg/body of tryptanthrin and kaempferol decreasing viable *H. pylori* significantly (Fig. 3).

Discussion

In this study, we showed that tryptanthrin and kaempferol had an anti-*H. pylori* effect both in vivo and

in vitro. In another study tryptanthrin and kaempferol were isolated from an ethyl acetate extract of *P. tinctorium* Lour. and evaluated in terms of anti-*H. pylori* activity.[13]

In the present study, first, we evaluated the effects of tryptanthrin and kaempferol on *H. pylori* colony formation in vitro. The agents inhibited the colony formation in a dose-dependent manner, and an additive effect was observed with their combined administration. According to this result, we prepared *H. pylori*-infected Mongolian gerbils to determine the antibacterial effect of the agents in vivo. After the inoculation of *H. pylori*, tryptanthrin and kaempferol were administered singly or combined. The administration of tryptanthrin at 5.0 mg/body, and of kaempferol at 5.0 mg/body and the combined therapy of tryptanthrin and kaempferol significantly decreased the number of *H. pylori* colonies in the stomachs of the gerbils. Neither acute toxicity nor marked loss of body weight was observed in the gerbils during the experimental period (data not shown). Furthermore, macroscopic observation did not show any marked changes in internal organs resulting from the administration of tryptanthrin (data not shown). Other investigators have reported the intraperitoneal administration of kaempferol (15 mg/body) to rats[20] and its anti-inflammatory effect; in that experiment, no side effect of kaempferol was observed. Thus, we considered that kaempferol would be safe in vivo. In our study, *H. pylori* eradication was not complete. However, it is expected that complete bacterial clearance could be possible if the period of drug administration were to be extended.

Kaempferol is a well known flavonoid in the plant kingdom and is reported to have various biological activities, such as anti-oxidative[20–22] and anti-inflammatory effects.[23–25] Its anti-inflammatory effect was attributed to the inhibition of nitric oxide (NO) production, and the transcription of cyclooxygenase.[26] In *H. pylori*-positive gastric ulcer patients, it has been shown that mucosal NO and peroxynitrite formation were increased, suggesting that *H. pylori* promoted NO gastric stress.[27] Furthermore, eradication of *H. pylori* decreased inducible NO synthase (iNOS) and macrophage immunoreactivity in the gastric mucosa.[27] Thus, it is likely that kaempferol can suppress the inflammation induced by *H. pylori*.

Tryptanthrin has widespread distribution in plants,[28–30] and has antifungal[31] and antibacterial effects.[31–33] Recently, we have reported both an anti-*H. pylori* effect of tryptanthrin[13] and its cytotoxicity to malignant tumor cells.[14] In the present study, tryptanthrin inhibited *H. pylori* colony formation at a lower concentration than that of kaempferol in vitro. In infected gerbils, 1.25 mg/body (twice a day, for 10 days) of tryptanthrin reduced viable cell counts of *H. pylori* in the stomachs (data not shown). Furthermore, our preliminary data showed that the combination of an antibiotic (CAM) and tryptanthrin had a synergistic effect on colony formation in vitro, indicating the possibility of decreasing the dose of CAM. For the eradication of *H. pylori*, triple therapies that include two antibiotics and a PPI have been the main choice and have achieved high eradication rates.[34] In the metronidazole amoxicillin clarithromycin *H. pylori* (MACH) studies,[35,36] however, several side effects, such as gastrointestinal symptoms in the group with high doses of AMPC and CAM, and neural disorders in the group with metronidazole and CAM, have been observed. In combinations with metronidazole, an antiprotozoal drug, the eradication rate rose, and the dose of the antibiotics could be reduced. However, the risk of carcinogenicity still remains with the use of metronidazole.[34] Taking these results into consideration, we believe that the findings of our preliminary study suggest that, with a combined therapy of tryptanthrin plus antibiotics, the dose of antibiotics can be decreased and side effects can therefore be alleviated.

In conclusion, there is a possibility that plant-derived compounds, such as tryptanthrin and kaempferol, would be applicable for anti-*H. pylori* therapy.

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