Comparative analysis of anti-*Helicobacter pylori* activities of FEMY-R7 composed of *Laminaria japonica* and *Oenothera biennis* extracts in mice and humans

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Helicobacter pylori-eliminating effects of FEMY-R7, composed of *Laminaria japonica* and *Oenothera biennis* extracts, were investigated in mice and humans. Male C57BL/6 mice were infected with the bacteria by intragastric inoculation (1×10^9 CFU/mouse) 3 times at 2-day intervals, and simultaneously, orally treated twice a day with total 20, 64 or 200 mg/kg/day FEMY-R7 for 2 weeks. In *Campylobcter*-like organism (CLO)-detection tests on gastric mucosa and feces, FEMY-R7 reduced the urease-positive reactivity in a dose-dependent manner; i.e., the positivity ratios were decreased to 70, 20, and 10% for gastric mocosa and to 80, 50, and 20% for feces. In a clinical sudy, human subjects, confirmed to be infected with *Helicobacter pylori*, were orally administered twice a day with capsules containing total 100, 320 or 1,000 mg/man/day FEMY-R7 (matching doses for 20, 64 or 200 mg/kg/day, respectively, in mice from a body surface area-based dose translation) for 8 weeks. FEMY-R7 decreased the positivity ratios in feces to 70, 40, and 30%, respectively. In bacterial culture, *H. pylori* was identified from the CLO-positive stools of mice and humans. The bacterial identification ratios exhibited a good correlation between the matching doses in mice and humans. It is suggested that FEMY-R7 could be a promising functional food without tolerance as an adjunct to reduce the dosage of antibiotics for the treatment of recurrent *H. pylori* infection.

Keywords: *Helicobacter pylori*, FEMY-R7, *Laminaria japonica*, *Oenothera biennis*, *Campylobcter*-like organism-detection test, bacterial identification

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Peptic ulcers are one of the common gastric illnesses in modern societies. Gastric erosions and ulcers are caused by various factors including over-secretion and retention of gastric acid, depletion of protective mucin layers, disturbances in focal blood flow, and local inflammation [1-4]. Ulcer-inducing agents cover non-steroidal anti-inflammatory drugs [5-9], alcohols [7,8, 10], stresses [4,7,8], gastric retention [7,8], gastric hypermotility and acetic acid accumulation [8,11-14], and *Helicobacter pylori* infection [1,2,15-17].

Since *H. pylori* infection is related to inflammations, the bacteria are detected in gastric ulcer (70%), gastritis (50-60%), and duodenal ulcer (90%) patients. It has been confirmed that *H. pylori* is a key factor for chronic active gastritis as well as development to gastric malignancies [18-20]. It is well known that *H. pylori* exacerbates erosions and ulcers by stimulating gastric secretion and retention [21,22]. Accordingly, it is believed that eradication of *H. pylori* is the most important for effective ulcer treatment in patients exhibiting chronic and repetitive

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incidence of gastritis [1,2,15,16,17,23].

In order to eliminate *H. pylori* and treat gastric ulcers, triple therapies composed of proton-pump inhibitors, such as pantoprazole and omeprazole, and antibiotics, such as clarithromycin, metronidazole, and amoxicillin, have been recommended [2]. However, it was recently confirmed that *H. pylori* exhibits a high tolerance to the antibiotics [24]. Therefore, in spite of relatively-weak potency compared with the antibiotics, natural products without tolerance during repeated treatments may contribute to the eradication of *H. pylori* from the stomachs.

Extracts of *Laminaria japonica* and *Cladosiphon okamuranus* has been widely used in Oriental medicine. Extensive researches demonstrated that the extracts of *L. japonica* and *C. okamuranus* contain fucoidan, a sulfate polysaccharide complex, as an active ingredient, that has anti-oxidative, anti-coagulative, and anti-inflammatory activities [25,26]. In addition, the beneficial effects of fucoidan on inflammatory diseases, ischemia, and immune dysfunction are reported [27,28]. Recently, investigators showed that fucoidan interfere with the attachment of *H. pylori* to gastric cells in animals and in humans [29,30]. On the other hand, tannins from *Oenothera biennis* seed also have anti-*H. pylori* activity [31]. Moreover, *L. japonica* extract (LJE) was confirmed to inhibit bacterial growth *in vitro* and to block adhesion and colonization of *H. pylori* in the gastric walls [32]. We also demonstrated that a combinational treatment with LJE and *O. biennis* seed extract (OBE) killed *H. pylori* and eliminated the bacteria from the mouse stomachs *in vitro* and *in vivo* [33]. Also, effectiveness of the combinational therapy in reducing *H. pylori* was confirmed by indirect analytical methods; i.e., urea breath test (UBT) and serum pepsinogen I/II levels, in human patients [34].

In the present study, we investigated the comparative *H. pylori*-eliminating activities of FEMY-R7, a combinational preparation of LJE and OBE in mice and in humans by directly analyzing the presence of bacteria in feces through a *Campylobcter*-like organism (CLO)-detection test and bacterial identification.

Materials and Methods

Materials

FEMY-R7 containing LJE and OBE (1:1) was obtained from Misuba RTech Co. (Asan, Korea). After washing, drying and grinding, *L. japonica* was extracted with a hot water extraction method at 60ºC for 24 hours [33,34]. Subsequently, the extract was filtered through a 0.45 μm membrane, and concentrated in a vacuum evaporator. After sterilization, the extract was spray-dried. The *O. biennis* seeds were extracted with 60% ethanol, filtered, and concentrated. After sterilization, 67% maltodextran was added to the extract and spray-dried.

The 1:1 (v/v) mixture of LJE and OBE, named FEMY-R7 [33,34], was stored at 2ºC until use.

H. pylori culture and identification

H. pylori (ATCC49503) was obtained from American Type Culture Collection (Manassas, VA, USA), and cultured on brain heart infusion (BHI) broth in an anaerobic chamber with 10% CO_2, 5% O_2, and 85% N_2 at 37ºC with enough humidity [35].

Animals and treatment

Male C57BL/6 mice (body weights 25-27 g; Table 1) were procured from Daehan Biolink (Eumseong, Korea), and housed in a room with constant environmental conditions (23±2ºC; 55±10% relative humidity; 12-hour light-dark cycle; 150-300 lux brightness). Pellet feed and purified water were available *ad libitum*. All the animal experiments were conducted according to the Standard Operation Procedures (SOP), and approved by the Institutional Animal Care and Use Committee of Chungbuk National University, Korea (Approval No. CBNUR-284-11).

After 12-hour fasting, the mice (n=10/group) were orally inoculated with *H. pylori* (1×10^9 CFU/1 mL/mouse) 3 times at 2-day intervals [34], and simultaneously, orally treated twice a day with FEMY-R7 (total 20, 64 or 200 mg/kg/day) for 2 weeks.

Human subjects and treatment

Among total of 58 male patients (30-60 years old) who visited the Gastroenterology Department of the Catholic University Medical Center in Daegu, 40 patients were enrolled in the present study (Table 1). Enrollment criteria included infection of *H. pylori* which was confirmed by CLO test on the feces of patients without experience of *H. pylori* treatment with antibiotics within 4 weeks [34]. The study protocol was approved by the Institutional Review Board (IRB) of the Catholic University Medical Center in Daegu (Approval No. CR-10-074-RES-01-R), and written informed consent was obtained from all patients.

Table 1. Basic information on the mouse and human subjects

Species	Treatment (mg/kg/day)	Number of patients	Age	Sex
Mouse	Vehicle	10	6*	Male
	FEMY-R7 (20)	10	6*	Male
	FEMY-R7 (64)	10	6*	Male
	FEMY-R7 (200)	10	6*	Male
Human	Placebo	10	44.6±2.5[#]	Male
	FEMY-R7 (1.62)	10	45.2±3.3[#]	Male
	FEMY-R7 (5.2)	10	43.7±4.1[#]	Male
	FEMY-R7 (16.2)	10	46.1±3.8[#]	Male

*, weeks; [#], years

We designed a randomized, double-blind, placebo-controlled clinical trial for eradication therapy of *H. pylori*. The patients in treatment group were given twice a day FEMY-R7 capsules containing 1:1 mixture of LJE and OBE (total 1.62, 5.2 or 16.2 mg/kg/day: 100, 320, and 1,000 mg/man/day) before a meal for 8 weeks [34]. The patients in placebo control group were given only the capsules containing 1,000 mg microcrystalline cellulose. During the treatment period, the patients were requested to keep their own dietary lifestyle.

Bacterial identification

Three hours after the final administration, the mice were sacrificed, and their gastric mucosa (3×3 cm) from the pylorus was biopsied and minced. Additionally, 0.5 g feces of mice (from rectum and colon) and humans (after defecation) were collected, and suspended in the same volume of distilled water and filtered. The gastric mucosal and fecal samples were applied to CLO kits (Kimberly-Clark, Roswell, GA, USA), and incubated at 35°C for 24 hours to examine urease activity. The reaction (color change) was determined as negative for bright yellow, false (partially) positive for thick yellow, or positive for thick (dark) red [16,17,33-35].

Additional feces samples were centrifuged at 500×*g* for 3 min, and inoculated to a medium containing 20% glycerol for identification of *H. pylori* as follows [35]:

Oxidase: A colony was collected with a platinum-loop, and moved to a filter paper. After dropping *p*-phenylenediamine dihydrochloride solution, blue change following indolphenol blue production was determined as positive reaction.

Catalase: The colony grown in selective medium was placed on a slide, and bubbling following dropping 3% H_2O_2 was determined as positive reaction.

Nitrate reduction: Cultured colony was inoculated to nitrate broth, and incubated at 35°C for 48 hours. Reddish purple change by adding α-naphthylamine and sulfanilic acid was determined as positive reaction.

H_2S formation: The central portion of a colony was collected with a platinum-loop, and spread on the slant Triple sugar iron (TSI) medium. Black change following incubation at 37°C for 24 hours with the lead open was determined as positive reaction producing H_2S.

Results

Repeated intragastric inoculation (1×10^9 CFU/mouse, 3 times) of *H. pylori* to C57BL/6 mice revealed positive reaction (red color) in CLO test on the gastric mucosa (Table 2). The stomachs of mice orally treated with 20, 64 or 200 mg/kg/day FEMY-R7 for 2 weeks displayed

Table 2. Effect of FEMY-R7 on the reactivity in CLO test on the gastric mucosa and feces of mouse and human patients infected with *H. pylori*

Species	Treatment (mg/kg/day)	1	2	3	4	5	6	7	8	9	10	Positive ratio (%)
Mouse (stomach)	Vehicle control	●	●	●	●	●	●	●	●	●	●	100
	+FEMY-R7 (20)	●	●	●	●	●	●	◐	○	○	○	70
	+FEMY-R7 (64)	●	●	○	○	○	○	○	○	○	○	20
	+FEMY-R7 (200)	○	○	○	○	○	○	○	○	○	○	0
Mouse (feces)	Vehicle control	●	●	●	●	●	●	●	●	●	●	100
	+FEMY-R7 (20)	●	●	●	●	●	●	◐	◐	○	○	80
	+FEMY-R7 (64)	●	●	◐	◐	◐	○	○	○	○	○	50
	+FEMY-R7 (200)	◐	◐	○	○	○	○	○	○	○	○	20
Human (feces)	Placebo control	●	●	●	●	●	●	●	●	●	●	100
	+FEMY-R7 (1.52)	●	●	●	●	●	●	●	○	○	○	70
	+FEMY-R7 (5.2)	●	●	◐	◐	○	○	○	○	○	○	40
	+FEMY-R7 (16.2)	◐	◐	◐	○	○	○	○	○	○	○	30

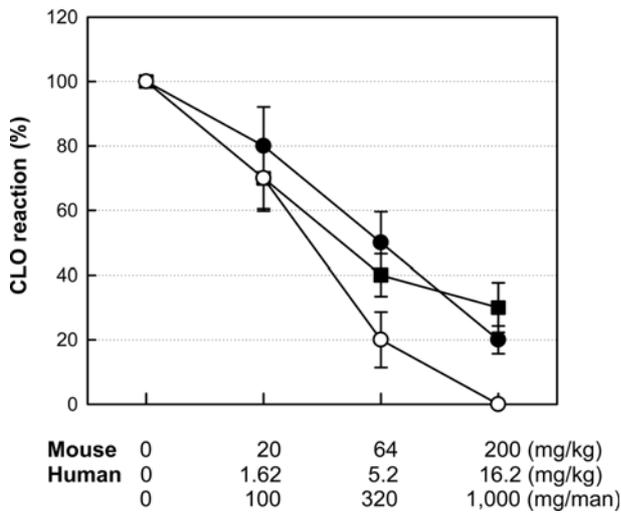○, negative; ◐, partially positive; ●, positive.

Figure 1. Dose-dependent *H. pylori*-eliminating activity of FEMY-R7 in mouse and human patients. *H. pylori* was detected with CLO kits from mouse stomach (○), mouse feces (●) or human feces (■).

positive reaction in 70% (including partial positivity), 20%, and 0% mice, respectively. By comparison, the CLO positive reactions from feces of the same mice were 80, 50, and 20% with 20, 64 and 200 mg/kg/day FEMY-R7, respectively, although there were several partially-positive mice. On the other hand, treatment of human patients with 1.62, 5.2 or 16.2 mg/kg/day (100, 320 or 1,000 mg/man/day) FEMY-R7 led to 70, 40, and 30% CLO-positive reaction in feces, respectively. Collectively, there was a good dose-response relationship in feces between mice and humans (Figure 1). However, the dose dependency in CLO reaction on mouse gastric mucosa was somewhat different from that of feces.

Because of the difference in the CLO test results between stomach and fecal samples of mice, we cultured and identified *H. pylori* from feces of mice and humans. *H. pylori* was identified from the stools of all (100%) the

mouse and human patients treated with vehicle or placebo preparation (Table 3). However, FEMY-R7 treatment at 20, 64, and 200 mg/kg/day decreased the bacterial detection from the mouse feces to 60, 20, and 0%, respectively. Similarly, 1.62, 5.2, and 16.2 mg/kg/day FEMY-R7 in humans reduced the *H. pylori*-identification ratio to 70, 20, and 0%, respectively.

Discussion

It is well known that *H. pylori* secretes urease to survive in acidic environment and to invade gastric mucosa, inducing gastritis, ulcers, and sometimes stimulation of gastric cancers. In a previous investigations [33,34], we demonstrated that FEMY-R7 has an anti-bacterial activity against *H. pylori*. In CLO test, treatment with FEMY-R7 for 2 weeks eliminated *H. pylori* from the stomach of mice in a dose-dependent manner, displaying a near-full efficacy at 200 mg/kg/day. In the present study, such a bacteria-eradicating efficacy of FEMY-R7 was confirmed again as analyzed with CLO kits on the mouse gastric mucosa. However, the CLO reaction from mouse feces did not match that of gastric mucosa; i.e., there were several partially-positive reactions in feces of mice that displayed negative reaction in the stomach.

We administered FEMY-R7 to naturally-infected human patients at doses of 1.62, 5.2 or 16.2 mg/kg/day (100, 320 and 1,000 mg/man/day for 60 kg men) to match the doses in mice, according to the body surface area-based dose translation (extrapolation) [36]. Notably, the dose dependency in the CLO reaction from human feces was in parallel with that from mouse feces. More interestingly, the bacterial identification ratios exhibited a good correlation between the matching doses in mice and humans. It was also observed that bacteria was not identified from the feces showing partially-positive reaction in CLO test.

Table 3. Identification of *H. pylori* through culture of feces from mouse and human subjects infected with *H. pylori* followed by treatment with FEMY-R7

Species	Treatment (mg/kg/day)	1	2	3	4	5	6	7	8	9	10	Positive ratio (%)
Mouse (feces)	Vehicle control	+	+	+	+	+	+	+	+	+	+	100
	+FEMY-R7 (20)	+	+	+	+	+	+	-	-	-	-	60
	+FEMY-R7 (64)	+	+	-	-	-	-	-	-	-	-	20
	+FEMY-R7 (200)	-	-	-	-	-	-	-	-	-	-	0
Human (feces)	Placebo control	+	+	+	+	+	+	+	+	+	+	100
	+FEMY-R7 (1.62)	+	+	+	+	+	+	+	-	-	-	70
	+FEMY-R7 (5.2)	+	+	-	-	-	-	-	-	-	-	20
	+FEMY-R7 (16.2)	-	-	-	-	-	-	-	-	-	-	0

-, negative; +, positive.

Therefore, it is inferred that the partially-positive reaction may be due to the urease enzyme released from dead *H. pylori* remaining in the stools.

In our recent study, the effectiveness of FEMY-R7 was also confirmed in humans; i.e., daily intake of 300 mg/man/day FEMY-R7 for 8 weeks significantly lowered Delta over baseline-value (BOD) in the expiratory breath in UBT [34]. Although treatment with 300-320 mg/man FEMY-R7 for 8 weeks was not enough to fully eliminate the bacteria in our previous and present studies as analyzed by UBT and feces CLO test, it was confirmed that longer periods (>8 weeks) or higher doses (>320 mg/man) should fulfill the requirement for full clearance of *H. pylori* from the stomach.

Although triple therapies (for example, omeprazole+clarithromycin+amoxicillin or omeprazole+clarithromycin+metronidazole) consisting of two antibiotics had been recommended as a standard treatment for *H. pylori* [2], it has been recently demonstrated that the widespread use of antibiotics led to a rapidly-increasing bacterial resistance [24,37]. Thus, there is a strong rationale for the development of novel anti-bacterial agents without resistance and adverse-effects. In the present study, we showed the efficacy of FEMY-R7 in eliminating *H. pylori* from mice and humans by analyzing the correlations between gastric mucosal and fecal samples from mice as well as between fecal samples from mice and humans. In conclusion, it is suggested that FEMY-R7, a combinational regimen composed of LJE and OBE, could be a promising candidate overcoming tolerance of antibiotics or an adjunct to reduce the dosage of antibiotics for the treatment of recurrent *H. pylori* infection.

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Conflict of interests The authors declare that there is no financial conflict of interests to publish these results.

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