ORYZA OIL & FAT CHEMICAL CO., LTD.

EVENING PRIMROSE EXTRACT

For the prevention of *Helicobacter pylori* infection

- EVENING PRIMROSE EXTRACT -PH
- EVENING PRIMROSE EXTRACT -WSPH

ORYZA OIL & FAT CHEMICAL CO., LTD.

ver. 1.2 NN

EVENING PRIMROSE EXTRACT

Functional food for the prevention of *Helicobacter pylori* infection

1. Introduction

Evening Primrose is a dicotyledonous plant of genus *Oenothera*. The seeds have been used for edible oil and also known to be a medicine in Europe. Evening Primrose oil has contains much γ-linolenic acid, which has well known to relieve obesity, diabetes mellitus, hypercholesterolemia, premenstrual syndrome (PMS), and so on.

Recently, polyphenols in plant seeds have been paid attention. Polyphenols prevent oxidation of lipids and scavenge active oxygen as a trigger of cause of various diseases. ORYZA OIL & FAT CHEMICAL CO., LTD. has been studying for the physiological function of polyphenols of the Evening Primrose seeds. We have recently developed an EVENING PRIMROSE EXTRACT (EPE), in which polyphenols are highly concentrated. ORYZA has discovered that EVENING PRIMROSE EXTRACT contains a considerably great amount of polyphenols and has marked antioxidative and antidiabetic actions. Recently, as new physiological activities of EVENING PRIMROSE EXTRACT, we also confirmed its marked antibacterial effects on *Helicobacter pylori* (*H. pylori*), and therefore, produced this extract on a commercial basis as a functional food with new appeal.

Evening Primrose

2. What is "EVENING PRIMROSE EXTRACT"?

2-1. EVENING PRIMROSE

EVENING PRIMROSE EXTRACT has been cultivated in North America or China to obtain oils from the seeds. Evening primrose is introduced to Japan as decorative plants which is also seen on the riverside or seashore. The following 4 species are known to be evening primrose: *Oenothera laciniata, Oenothera striata, Oenothera biennis* and *Oenothera erythrosepala*.

Evening primrose has been long cultivated mainly in North America and China. Indians in North America have a long history of the use of the whole plant of evening primrose for the treatment of various disorders such as the use of its root for the treatment of swelling and its seeds for the treatment of hemorrhoids and the promotion of health.

At present, the seeds of evening primrose are widely used as a source of γ-linoleic acid as a health supplement food. The whole plant or flower extract is drunk as tea. The whole plant and root are eaten as pickles, and the seeds are mixed in soup or the dough for muffins. Its seed extract began to be used as a health food recently. Thus, evening primrose is a dietary source that has been frequently consumed for a long time in various regions of the world.

2-2. Polyphenol content

We compared the content of polyphenol with various actions such as antidiabetic action between EVENING PRIMROSE EXTRACT and other plant extracts and found a considerably great content of polyphenol in EVENING PRIMROSE EXTRACT.

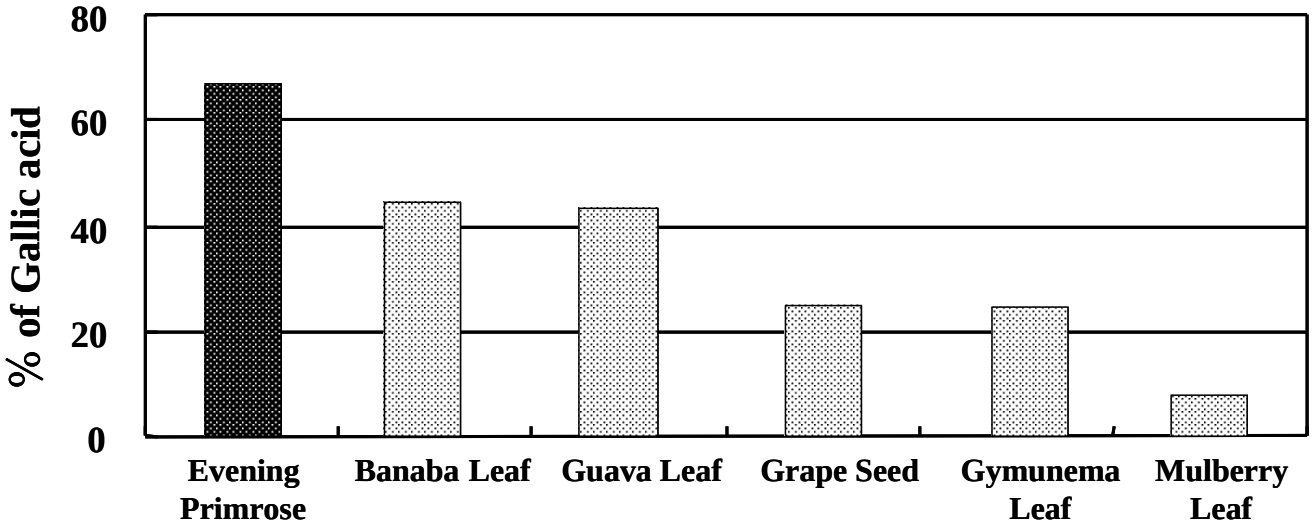


Fig. 1 Comparison of polyphenol contents among various plant extracts

2-3. Components of EVENING PRIMROSE EXTRACT

Gallic acid has astringent action in addition to antioxidative action, and ellagic acid has inhibitory action on melanin formation. Pentagalloyl glucose (PGG) has anti-inflammatory action, catechin has deodorant and antimicrobial actions, and proanthocyanidin has preventive action on atherosclerosis. As a new action of EVENING PRIMROSE EXTRACT abundantly containing these substances, we found its antimicrobial effects on *H. pylori*.

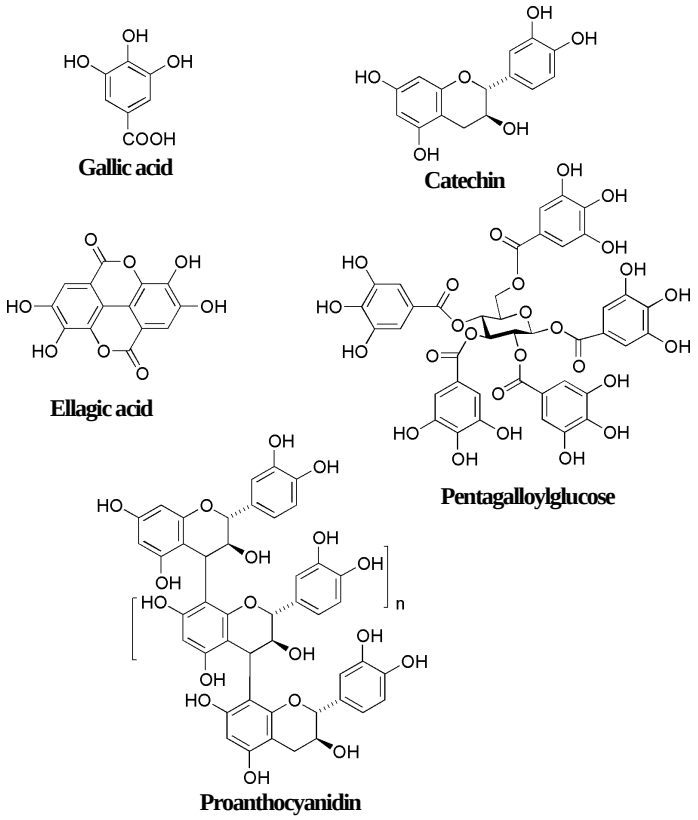


Fig. 2 Major Polyphenols of EVENING PRIMROSE EXTRACT

2-4. Antioxidative activity of EVENING PRIMROSE EXTRACT

The superoxide scavenging activity of EVENING PRIMROSE EXTRACT was measured by electron spin resonance (ESR). This extract had markedly high antioxidative activity (3.5×10^5 units/g). Consumption of foods with such high antioxidative activity is known to have preventive effects on various lifestyle-related diseases.

Assayed Items	Results	Assaying Method
Superoxide scavenging activity	**3.5×10^5 units/g※**	**Electron spin resonance (ESR)**

Test trustee: Japan Food Research Center Foundation

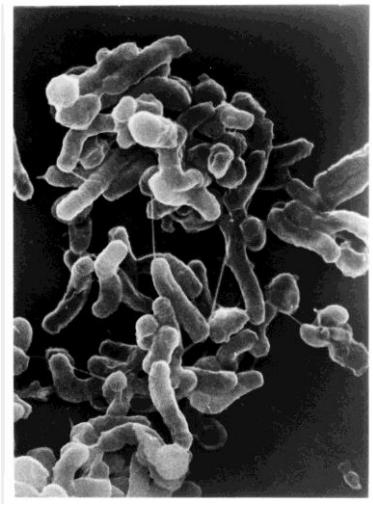
Research results issue number : 302010023-001

※The units was defined by J.M.McCord and I.Fridovich[*J.Biol. Chem.*,244,6049(1969)]

3. *H. pylori*

3-1. Discovery of *H. pylori*

In 1983, Warren and Marshall in Australia were the first to demonstrate the presence of *H. pylori* in the stomach. The term, *H. pylori* is derived from the helical shape of the bacterium, and the presence of a large amount of the bacterium in the pyloric region of the stomach. *H. pylori* moves by rotating its flagella like a propellor. The infection route is unclear, but oral infection ***via*** the mouth is considered to be the primary route.



An electron micrograph of *H.pylori*
Osaki *et.al.*,*J Med Microbiol*.2002

3-2. *H. pylori* infection

H. pylori is frequently detected in patients with gastritis or peptic ulcers, and considered to be a cause of the development, recurrence, and intractability of ulcer. *H. pylori* lives in the mucous layer and cell interstitium of the gastric mucosa particularly in the pyloric region and converts urea in the stomach into ammonia (NH_3) by urease produced by the bacterium itself. This ammonia neutralizes surrounding acid, expanding the habitat area of *H. pylori*, and may be involved in gastric mucosal injury. If *H. pylori* infection develops during early childhood, well-differentiated gastric cancer tends to develop after acute gastritis or atrophic gastritis. If *H. pylori* infection develops during adulthood, the gastric mucosa does not become atrophied, and duodenal ulcer tends to develop. At the age of 40 years or older, about 80% of the people are infected with *H. pylori*. Its high prevalence in the elderly is due to the poor sanitary environments such as waterworks and sewers. Thus, many people are infected with *H. pylori*, but only a low percentage develops ulcers. There are about 60,000,000 Japanese with *H. pylori* infection, but most of them are free of symptoms and live a healthy life. However, 90% of patients with gastric/duodenal ulcers have *H. pylori* infection.

3-3. Eradication of *H. pylori* and its disadvantages

For the eradication of *H. pylori*, 3-drug combination therapy (MAC*H*-1 study) consisting of a proton pump inhibitor and 2 antimicrobial drugs is effective. Its eradication rate is about 85%. However, studies have shown certain adverse effects of eradication therapy in 50.5%: soft feces (13.7%), diarrhea including watery feces (8.8%), dysgeusia including allotriogeusia and bitter taste (2.6%), and eruptions (1.4%). A follow-up study for 6 months or more after eradication has shown mild reflux esophagitis in 3.9% of patients in whom eradication was successful. This may be partly because the decreased gastric acid secretion improves (is normalized) after *H. pylori* eradication in gastric ulcers and gastritis accompanied by marked gastric mucosal atrophy.

3-4. Lifestyle of *H. pylori* and the antimicrobial action of EVENING PRIMROSE EXTRACT

Fig. 3 shows the pathogenic factors and lifestyle of *H. pylori*. The pathogenic factors include not only bacterium-associated factors such as structures of *H. pylori* and substances produced by *H. pylori* but also host-associated factors produced by gastric epithelial cells and immunocytes of the host.

The bacterium-associated pathogenic factors include: (1) urease (neutralization gastric acid by degrading urea and producing NH_3, allowing continuous bacterial infection in the stomach), (2) flagella (control of bacterial movements), (3) adhesin (involvement in bacterial adhesion to gastric epithelial cells), (4) catalase (anti-phagocytosis action), (5) SOD (anti-phagocytosis action), (6) VacA (vacuolization of gastric epithelial cells), (7) PAI (induction of cytokine production, Type IV secretion apparatus), (8) CagA (phosphorylation of tyrosine residues affecting the cytoskeleton), (9) LPS (induction of immunological cross reactions with gastric epithelial cells), (10) heat shock protein (action as an adhesion factor and induction of immunological cross reactions), and (11) NapA (leukocyte-activating factor). The host-associated pathogenic factors include: (1) cytokines such as IL-6 and IL-8 (induction of inflammation), (2) active oxygen (damage of gastric mucosal cells), (3) carbon monoxide (production of peroxinitrate as a superoxide that damages DNA).

EVENING PRIMROSE EXTRACT has bactericidal effects and exerts antimicrobial effects by acting on the bacterium-associated pathogenic factors (1) and (3) and the host-associated pathogenic factor (2). Detailed data are described in [4. Function of EVENING PRIMROSE EXTRACT].

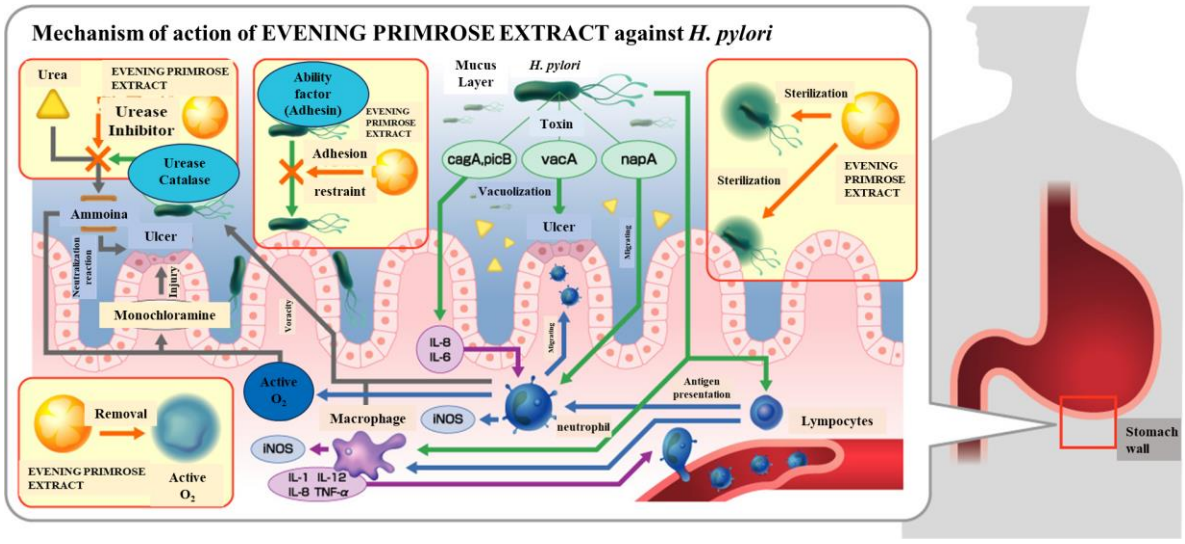


Fig. 3 Lifestyle of *H. pylori* and antimicrobial action of EVENING PRIMROSE EXTRACT

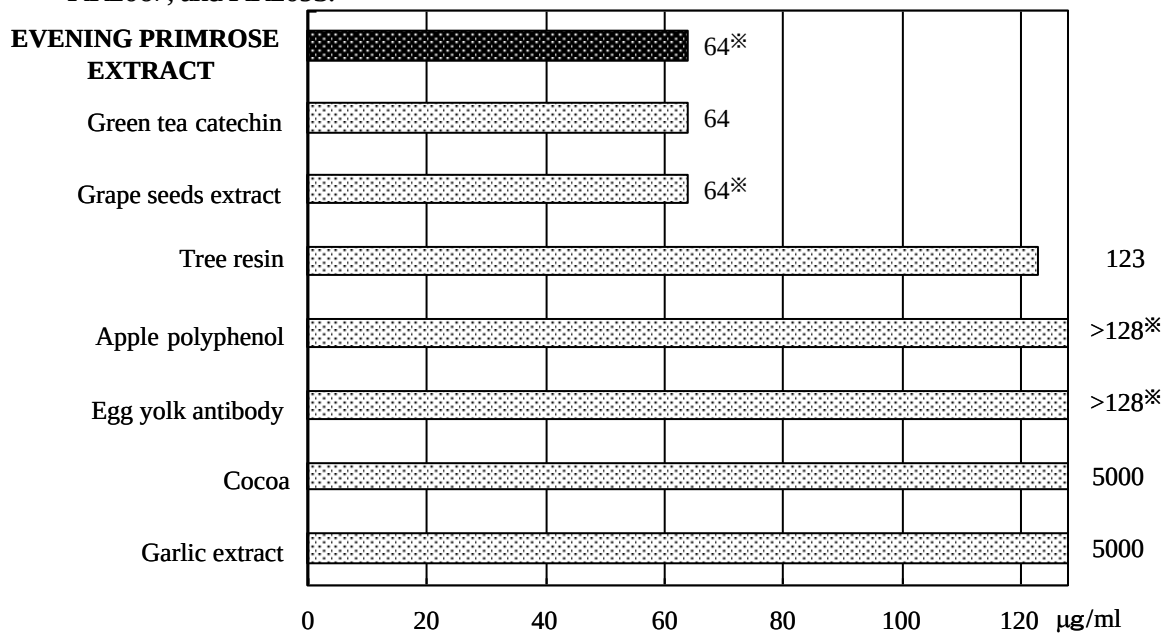
4. Function of EVENING PRIMROSE EXTRACT

4-1. Inhibition of the growth of *H. pylori*

We compared the minimum inhibitory concentration (MIC) among EVENING PRIMROSE EXTRACT and other various plant extracts and found that EVENING PRIMROSE EXTRACT has marked antimicrobial action with an MIC of 64 μg/ml (0.0064%).

The polyphenol content was 64% in EVENING PRIMROSE EXTRACT, more than 80% in green tea catechin, 100% in grape seed extract, and 100% in apple polyphenol. This shows very marked antimicrobial action of polyphenol contained in EVENING PRIMROSE EXTRACT.

Experiments were performed by the agar dilution method using the following 24 bacterial strains.
Standard strains: ATCC43503, ATCC43504, ATCC43579, ATCC49503, and NCTC1163.
Clinically isolated strains: TK1003, TK1008, TK1021, TK1022, TK1023, TK1025, TK1029, TK1030, TK1036, TK1042, TK1402, KR2001, KR2002, KR2003, KR2005, KR2007, KR2009, KR2067, and KR2093.



* indicates values obtained by measurement at our company, and the others were cited from the literature and data.

Fig. 4 Comparison between EVENING PRIMROSE EXTRACT and other Anti-*H. pylori* compounds

Notes: Samples under the following conditions were used.
EVENING PRIMROSE EXTRACT without fillers
Green tea catechin containing 80% polyphenol or more
Grape seeds extract containing 100% polyphenol
Commercially available tree resin extract as tree resin extract
Apple polyphenol containing 100% polyphenol
Commercially available powdered cocoa as cocoa
Freeze-dried garlic press juice as garlic extract

Comparison of functional components in EVENING PRIMROSE EXTRACT showed marked antimicrobial action of pentagalloyl glucose (PGG) on *H. pylori*.

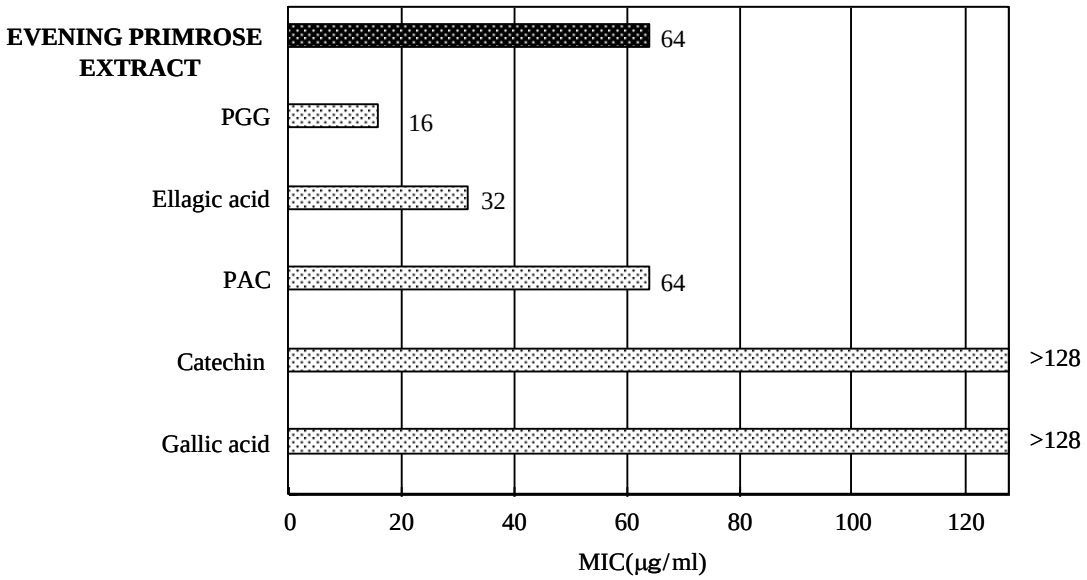


Fig. 5 Comparison of functional components in EVENING PRIMROSE EXTRACT

After culture of *H. pylori* with EVENING PRIMROSE EXTRACT without nutrition (such as in physiological saline), bactericidal effects of this extract were observed.

In Fig. 6, the Y-axis represents the count of *H. pylori*, and the X-axis represent hours (h). In the absence of EVENING PRIMROSE EXTRACT (0 μg), no changes in the bacterial count were observed. After addition of this extract at a concentration of 512 or 256 μg/ml, the bacterial count decreased below the detection limit. At an extract concentration of 128, 64, or 32 μg/ml, the bacterial count decreased below the detection limit after 24 hours. As shown in the figure, the count of *H. pylori* dose-dependently decreased.

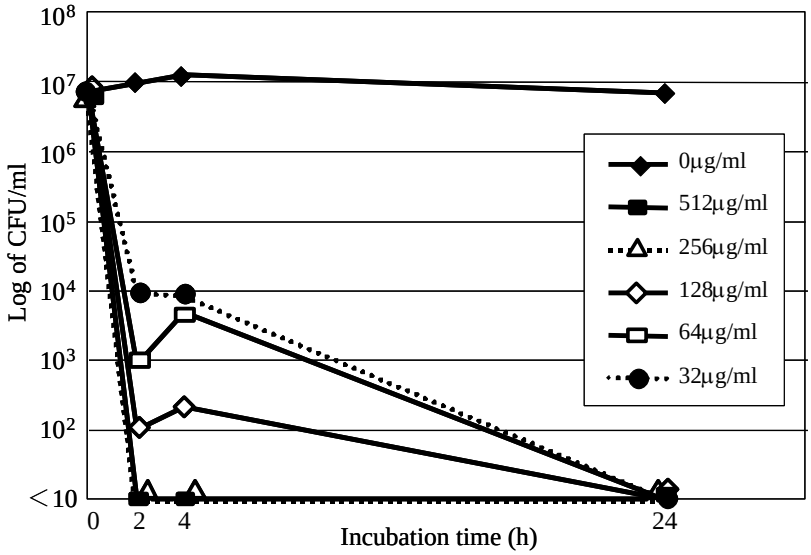


Fig. 6. Effects of EVENING PRIMROSE EXTRACT on *H. pylori* survival rates

TK1402 as a clinically isolated *H. pylori* strain was cultured in BHI agar supplemented with 7% equine defibrinated blood under a microaerobic condition and cultured with shaking in Hank's balanced salt solution in the presence of EVENING PRIMROSE EXTRACT at each concentration (512, 256, 128, 64, 32, and 0 mg/ml). After 0, 2, 4, and 24 hours, 0.1-ml samples were obtained, and the bacterial count was determined.

4-2. Inhibition of urease

H. pylori can live in the stomach with a very low pH because this bacterium neutralizes acid in the stomach by converting urea into ammonia using urease. Therefore, *H. pylori* cannot survive if the action of urease is inhibited. Since the produced ammonia changes the properties of the gastric mucosa and mucus, erosion of the gastric wall develops, resulting in inflammation. We examined the possible inhibitory action of EVENING PRIMROSE EXTRACT on urease and found marked inhibitory activity. Urease inhibitory effects were evaluated using sword bean-derived urease by the indophenol method.

The IC_{50} of EVENING PRIMROSE EXTRACT for urease was 8.54 μg/ml. Therefore, EVENING PRIMROSE EXTRACT is expected to inhibit the activity of urease produced by *H. pylori*, which prevents the formation of ammonia, inhibiting its injury of the gastric wall.

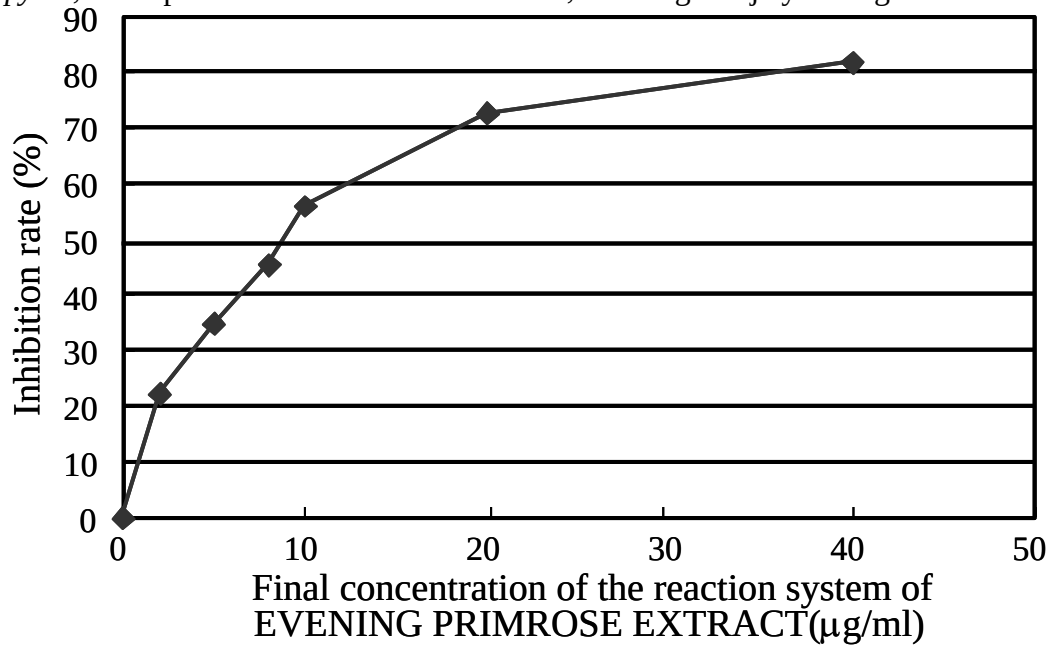


Fig. 7 Urease inhibitory action of EVENING PRIMROSE EXTRACT

Urease inhibition rate (%) = {(Ub – Us)/Ub} × 100
Urease activity (U) = Es - Eb
Es: Absorbance measured using enzyme solution
Eb: Absorbance measured using buffer instead of enzyme solution
Ub: Urease activity measured using water instead of extract
Us: Urease activity measured using extract

4-3. Inhibition of *H. pylori* adhesion

H. pylori with an adhesion factor can adhere to gastric epithelial cells and survive in the stomach. We examined the possible inhibitory effects of EVENING PRIMROSE EXTRACT on *H. pylori* adhesion to epithelial cells derived from gastric cancer and observed marked inhibition (82.45% at 16 μg/ml). Therefore, EVENING PRIMROSE EXTRACT is expected to inhibit *H. pylori* adhesion to gastric epithelial cells, preventing *H. pylori* infection.

In Fig. 8, the Y-axis indicates cell counts, and the X-axis indicates fluorescence intensity. Since *H. pylori* is labeled with fluorescein, fluorescence intensity increases with an increase in cells

with attached *H. pylori*, resulting in a shift of the peak to the right. ■ represents samples without EVENING PRIMROSE EXTRACT. The peak is observed on the right side, showing many cells with attached *H. pylori*. ■ represents samples without *H. pylori*. Since cells not labeled with fluorescence also emit only slight fluorescence, a peak is observed on the left side. With an increase in the concentration of EVENING PRIMROSE EXTRACT (16 ⇒ 32 ⇒ 64 μg/ml), the peak shifted from the right to left. This shows dose-dependent inhibition of *H. pylori* adhesion to cells by this extract.

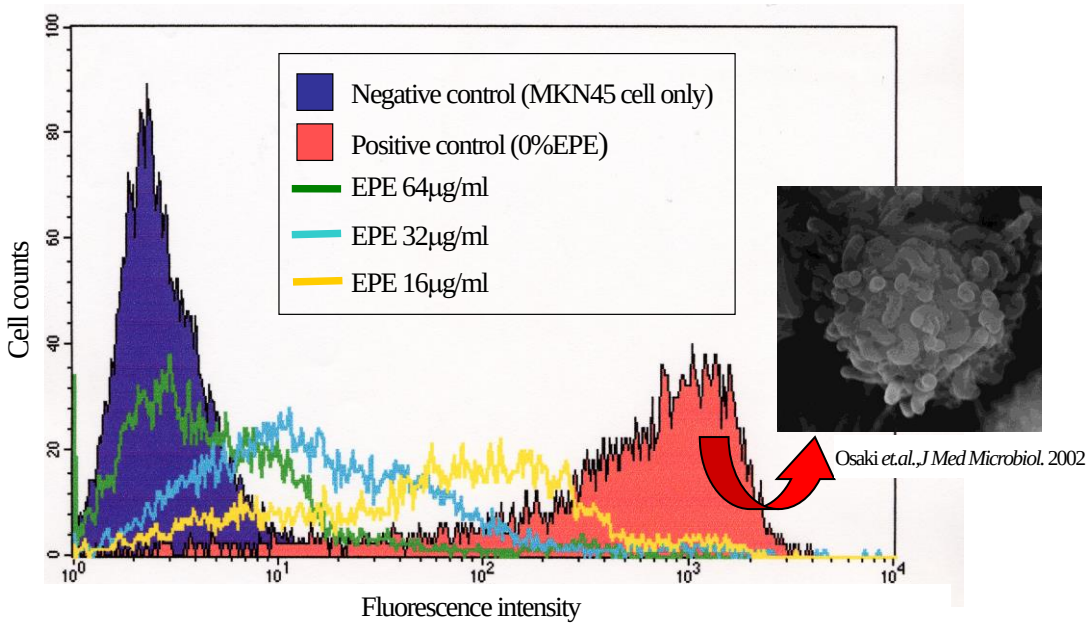


Fig. 8 Effects of EVENING PRIMROSE EXTRACT on *H. pylori* adhesion

TK1402 as a clinically isolated *H. pylori* strain (TK1402 strain) was cultured in BHI agar supplemented with 7% equine defibrinated blood under a microaerobic condition, adjusted to a concentration of 5×10^8 CFU/ml, and labeled with PKH-2 as a lipophilic fluorochrome. The TK1402 strain and an epithelial cell strain derived from human gastric cancer (MKN45 cells) were pretreated with EVENINGPRIMROSE EXTRACT(EPE) at each concentration (64, 32, and 16 μg/ml) at room temperature for 30 minutes. The TK1402 strain was mixed with MKN45 cells and reacted with *H. pylori* for 1 hour. Inhibition of *H. pylori* adhesion to MKN45 cells was analyzed by flow cytometry (FCM).

Table 1. Effects of EVENING PRIMROSE EXTRACT on *H. pylori* adhesion

Concentration of EVENING PRIMROSE EXTRACT （μg/ml）	Mean fluorescence intensity (adhesion inhibition rate, %)
64	15.12 ± 61.51 (98.10)
32	54.23 ± 230.86 (98.18)
16	139.56 ± 236.01 (82.45)
0	795.03 ± 594.44 (0)

Adhesion inhibition rates were calculated using the following equation

1 – (T/A – A') = adhesion inhibition rate (%)

A: fluorescence intensity of MKN45 cells not treated with EVENINGPRIMROSE EXTRACT

A': fluorescence intensity of MKN45 cells alone

T: fluorescence intensity of MKN45 cells treated with EVENINGPRIMROSE EXTRACT

4-4. Inhibition of *H. pylori* fixation

We examined the possible inhibitory effects of EVENING PRIMROSE EXTRACT on *H. pylori* fixation in the stomach by inhibiting its adhesion to gastric epithelial cells in animal experiments and found its inhibition. Thus, animal experiments also showed that EVENING PRIMROSE EXTRACT inhibits *H. pylori* adhesion to gastric epithelial cells, preventing *H. pylori* infection.

EVENING PRIMROSE EXTRACT was added to TK1402, a clinically isolated *H. pylori* strain (1-3 × 10^9 CFU/ml), to obtain a concentration of 1 mg/ml, which was orally inoculated to male MGS/Sea jirds aged 9 weeks at a concentration of 1 mg/jird once a day for 2 consecutive days. One week after oral inoculation, laparotomy was performed, and gastric mucosa was collected. Reverse transcription-polymerase chain reactions (RT-PCR) of the mucosal sample was performed using a specific primer for 16SrRNA of *H. pylori*, and the presence or absence of *H. pylori* fixation in the stomach was determined.

In Fig. 9, the band on the right side represents 16SrRNA (gene specific to *H. pylori*), showing the presence of *H. pylori*. In the control group (C), 4 of 5 jirds showed *H. pylori*. In the group treated with EVENING PRIMROSE EXTRACT (b), *H. pylori* was detected in 1 of 5 jirds, showing inhibition of *H. pylori* fixation in the stomach by this extract in 4 of the 5 jirds.

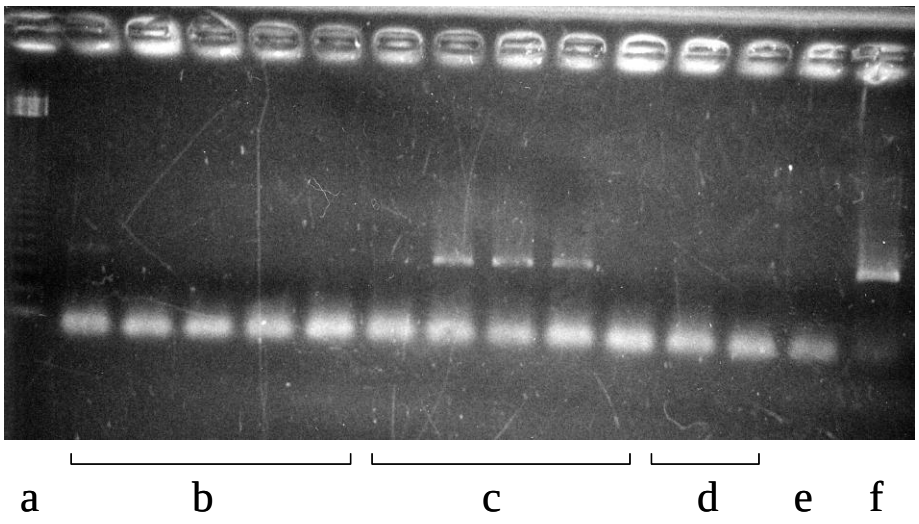


Fig. 9. Results of *H. pylori* detection in the gastric mucosa by RT-PCR method using 16SrRNA expression as a parameter in jirds 1 week after infection

a: DNA Ladder, b: oral inoculation of *H. pylori* + evening primrose extract, c: oral inoculation of *H. pylori*, d: oral inoculation of *H. pylori* + amoxicillin, e: (-) control, and f: (+) control. RT thermal cycle conditions: at 95°C for 15 minutes after reactions at 42°C for 45 minutes. PCR thermal cycle conditions: after degeneration at 95°C for 10 minutes, amplification of target DNA by 45 cycles (95°C, 45 seconds; 57°C,45 seconds; and 72°C, 1 minute). To confirm elimination of DNA, PCR was also performed without RT. Under the condition that the control (G3PDH) band (450 bp) is positive in all, the presence of the *H. pylori*-16S band (501 bp) was regarded as positive and its absence as negative.

4-5. Effect of *H. pylori* eradication

To determine whether EVENING PRIMROSE EXTRACT is effective against infections, we conducted an eradication test for *H. pylori* study using ddY mice.
The results showed that the number of bacteria decreased in a concentration-dependent manner, as shown in the figure below. Furthermore, 3 out of 7 animals were below the number of bacteria detection limit in the 240mg/kg administration group.

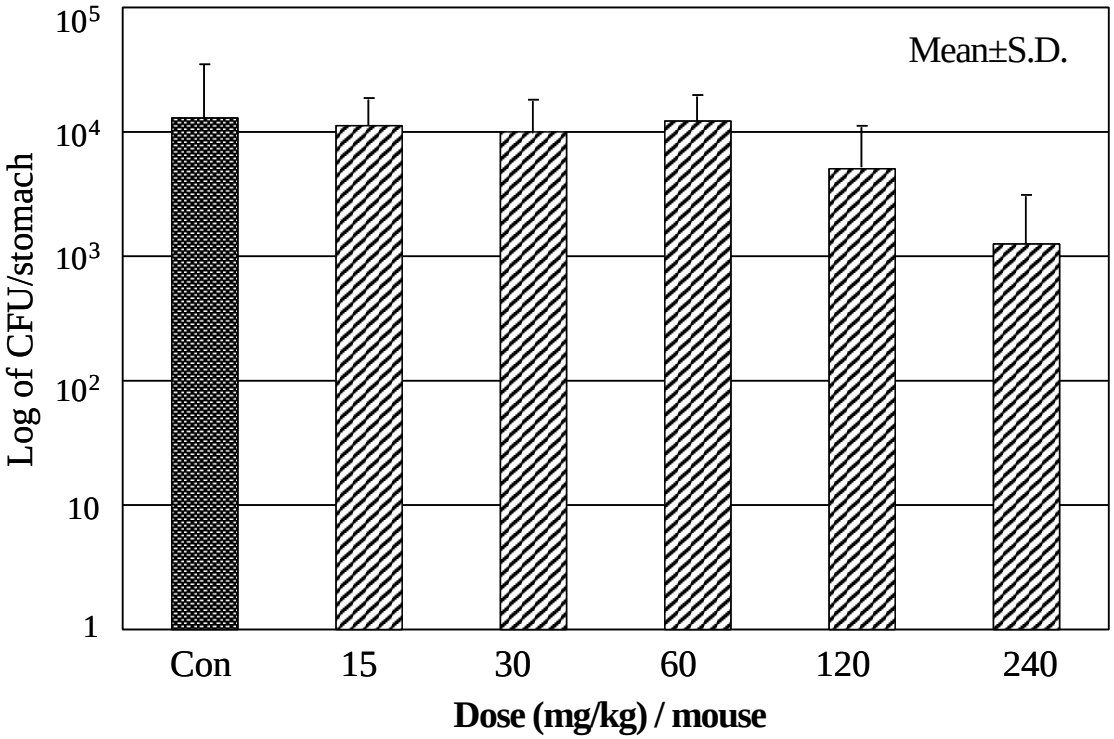


Fig. 10 *H. pylori* eradication effect using ddY mice

After fasting overnight, ddY mice (4 weeks old, male) were orally administered 0.5 ml of *H. pylori* at 10^8 CFU/ml per mouse, followed by using same manner two more times 12 and 24 h later. After 2 months of breeding, the mice were divided into groups of 7 per group and given EVENING PRIMROSE EXTRACT (15, 30, 60, 120, or 240 mg/kg) orally once daily for a week. From the evening of the final day of oral administration, subjects were fasted overnight. After laparotomy, the gastric mucosa was scraped off and coated on *H. pylori* detection agar medium, which was then cultured for 6 days to determine the number of bacteria.

4-6. Antiulcer Activity

Ammonia produced from urea by the urease of *H. pylori* is consider to be one of the causes of gastroduodenal ulcers. Therefore, we evaluated the antiulcer effects of EVENING PRIMROSE EXTRACT using an ammonia-induced ulcer model.

As a result (Fig. 11), the average ulcer coefficient in rats treated with 2% ammonia was 41.0 (photo; the reddish discoloration of the gastric mucosa is the area of ulcer development). The average ulcer coefficient in rats treated with 500 mg/kg of EVENING PRIMROSE EXTRACT was 1.6 (photo; no reddish discoloration of the gastric mucosa was observed). This indicates that EVENING PRIMROSE EXTRACT has antiulcer activity against ammonia ulcers when administered orally. Based on these results, it is considered that EVENING PRIMROSE EXTRACT acts directly on the gastrointestinal mucosa to prevent or cure ulcers.

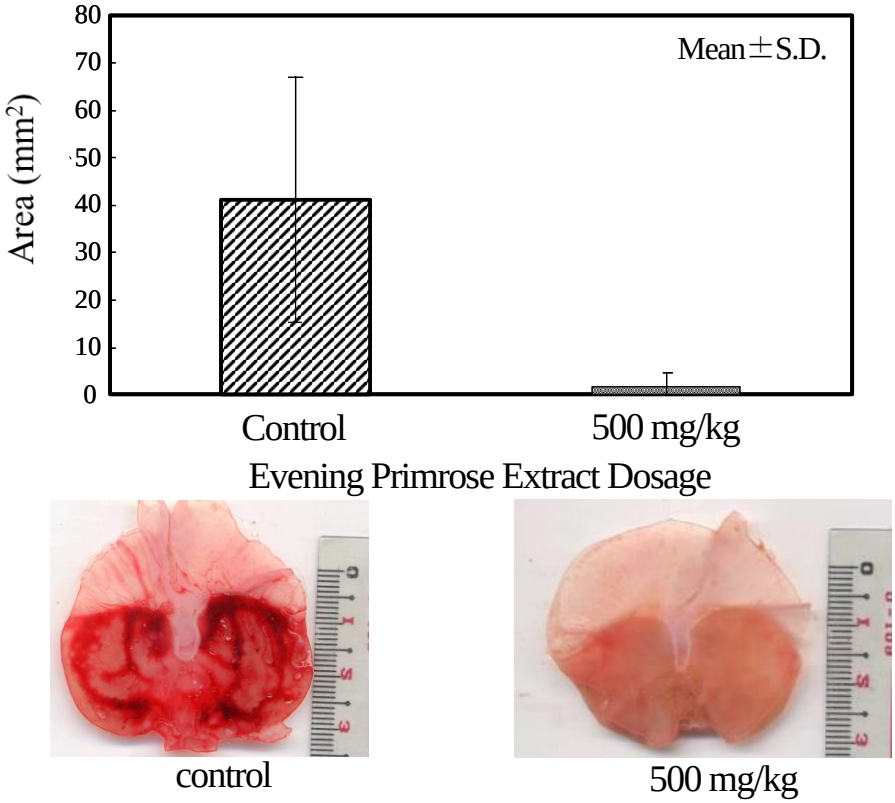


Fig. 11 Antiulcer action of EVENING PRIMROSE EXTRACT using ammonia ulcer model

Male Wistar rats (280-300g) were fasted for 24 hours and orally administered 0.5ml of 2% aqueous ammonia (10 mg of ammonia) per 100 g of rat and the rats were sacrificed by ether 30 minutes later. The stomach was removed and 8ml of 2% formalin was injected into the stomach and fixed for 10 min. The area of damage (mm^2) was measured using Scion Image and used as the ulcer index. EVENING PRIMROSE EXTRACT was suspended in 5% gum arabic powder and orally administered at 0.5ml per 100 g rats 30 min before administration of 2% aqueous ammonia. The control group was administered only 5% gum arabic powder solution.

4-7. Effect on lactic acid bacteria

Since EVENING PRIMROSE EXTRACT has an inhibitory effect on the growth of *H. pylori* which lives in the stomach, there is concern that it may have an adverse effect on intestinal lactic acid bacteria (so-called good bacteria).
Therefore, we examined EVENING PRIMROSE EXTRACT has an inhibitory effect on growth of lactic acid bacteria.
As a result, a comparison of the number of bacteria in the control (cont.: without EVENING PRIMROSE EXTRACT) and the group (EVENING PRIMROSE EXTRACT was added), almost no difference was observed between them (Fig. 12).

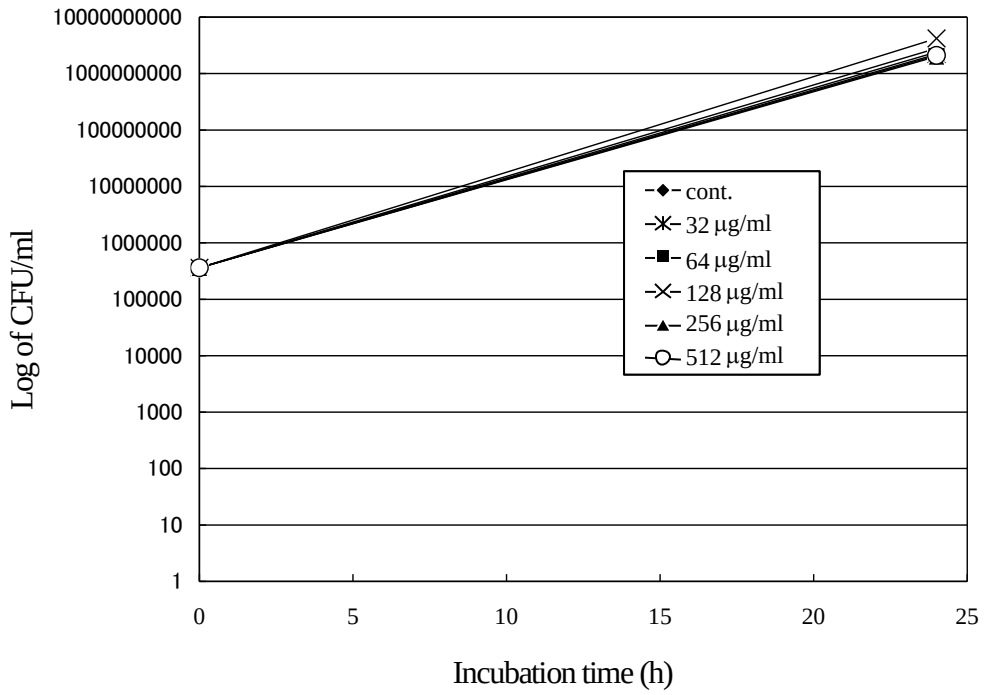


Fig. 12. Effect of EVENING PRIMROSE EXTRACT on lactic acid bacteria

4-8. Human Clinical Trials

A clinical trial was conducted to examine the effect of EVENING PRIMROSE EXTRACT on the number of *H. pylori* bacteria in the stomach and gastric mucosal inflammation in subjects with *H. pylori* infection. The subjects were divided into two groups: 5 subjects in the 120 mg group and 4 subjects in the 840 mg group, who took a capsule filled with EVENING PRIMROSE EXTRACT-PH. The capsules were taken two hours after dinner or on an empty stomach for eight consecutive weeks. The tests items were: ① urea breath test, ② fecal *H. pylori* antigen detection test (measuring *H. pylori* antigen in feces and determining the number of bacteria by absorbance), and ③ measurement of pepsinogen I/II ratio in blood (test to determine the degree of gastric mucosal inflammation).
The results showed that EVENING PRIMROSE EXTRACT was effective in suppressing inflammation of the gastric mucosa and reducing the number of *H. pylori* in the stomach.

① Urea breath test

The effect of EVENING PRIMROSE EXTRACT on *H. pylori* in the stomach was examined using a urea breath test. There are various test methods for determining *H. pylori* infection and the effectiveness of eradication, but the urea breath test is widely used as the simplest and most non-invasive method. When ^{13}C-labeled urea is orally administered, it is broken down into ammonia and carbon dioxide by the strong urease activity of the bacteria if *H. pylori* is present in the stomach. $^{13}CO_2$ is absorbed into the bloodstream from the gastrointestinal tract and released into the exhaled air through the lungs. The presence or absence of *H. pylori* infection is determined by measuring the amount of ^{13}C in the exhaled breath. There is a correlation between the exhaled breath $^{13}CO_2$ value (UBT value) and the number of *H. pylori* in the stomach, and a decrease in the UBT value indicates a decrease in the number of *H. pylori* in the stomach. The measurement results are shown in Fig. 13. It was found that the UBT value had decreased in both the low-dose and high-dose groups after two months of intake, compared to starting point. This result suggests that the number of *H. pylori* in the stomach has decreased.

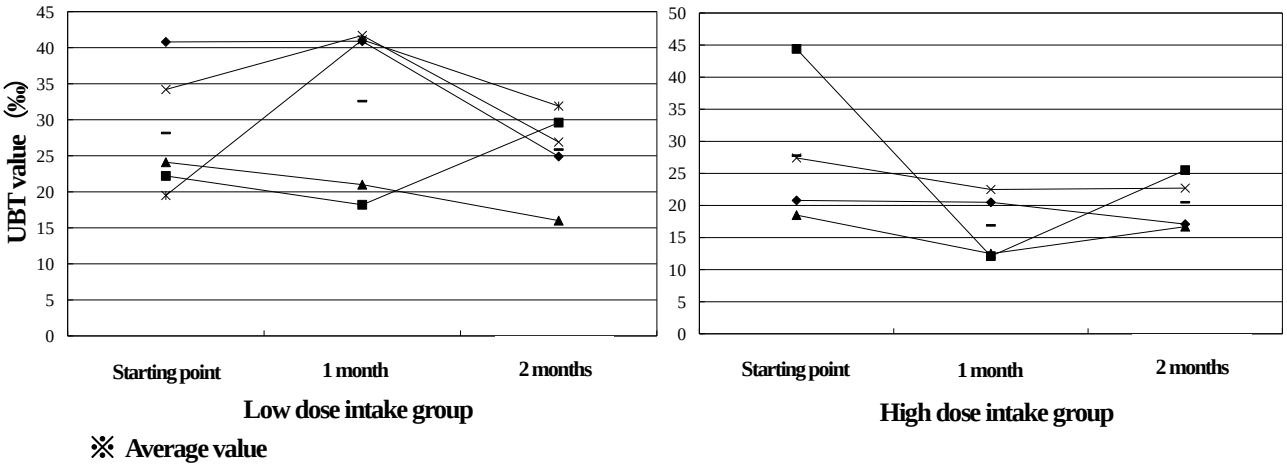


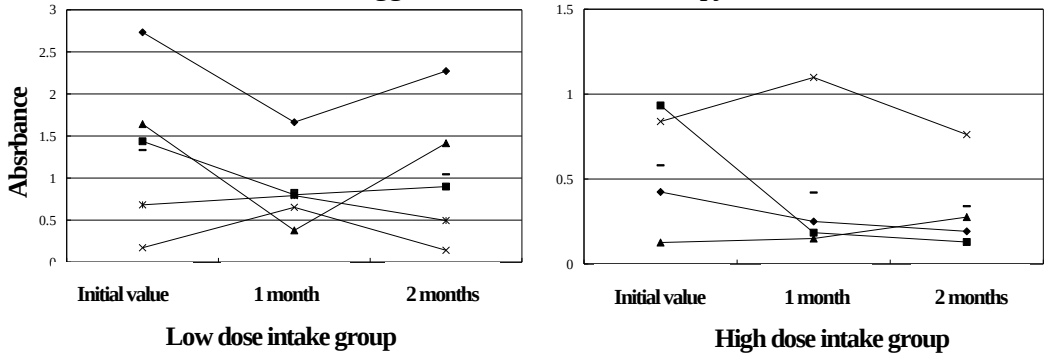
Fig. 13. Results of urea breath test

② Fecal *H. pylori* Antigen Test

The effect of EVENING PRIMROSE EXTRACT on *H. pylori* in the stomach was examined by ELSA detection method of *H. pylori* antigen in stool.

The fecal *H. pylori* antigen detection test is a new method that was developed in recent years. It detects *H. pylori* antigen in feces using ELISA, and is a more direct testing method than the urea breath test, etc. A decrease in the measured absorbance is considered to indicate a decrease in the number of *H. pylori* in the stomach.

The measurement results are shown in Fig. 14. Compared to the starting point, both low-dose and high-dose groups showed a decrease in fecal *H. pylori* antigen detection (absorbance) after 2 months of intake. This result suggests that the number of *H. pylori* in the stomach is decreased.



※ **Average value**

Fig. 14: Results of fecal *H. pylori* antigen test

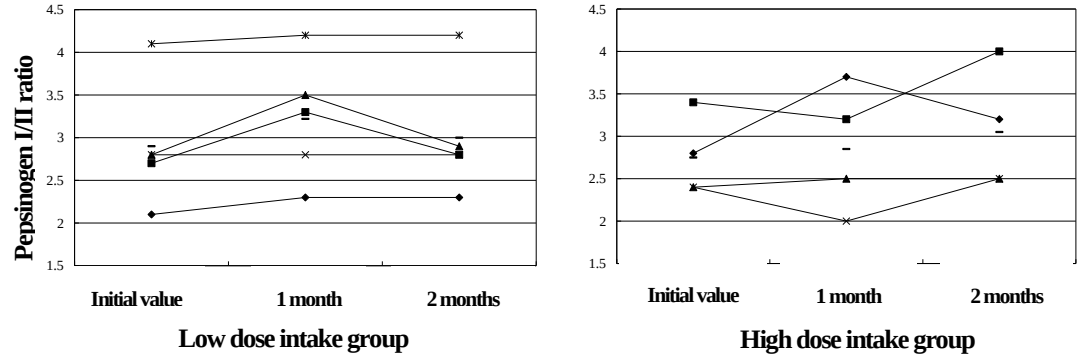
③ Measurement of blood pepsinogen I/II ratio

The effect of EVENING PRIMROSE EXTRACT on gastric mucosal inflammation was examined by measuring the blood pepsinogen I/II ratio.

Pepsinogen I/II is used as an indicator of gastric mucosal atrophic gastritis, etc. Pepsinogen I/II is significantly lower as gastric mucosa atrophy progresses, and the more severe the atrophy, the lower the pepsinogen I/II values.

The measurement results are shown in Fig. 15. Compared to the starting point, pepsinogen I/II levels had increased in both low-dose and high-dose groups after two months of intake.

This result suggests that the inflammation of the gastric mucosa is reduced.



※ **Average value**

Fig. 15. Measurement results of blood pepsinogen I/II ratio

5. Stability of EVENING PRIMROSE EXTRACT

5-1. Thermal Resistance

The pyrolysis of EVENING PRIMROSE EXTRACT does not occur at a normal food processing temperature for 60 minutes.

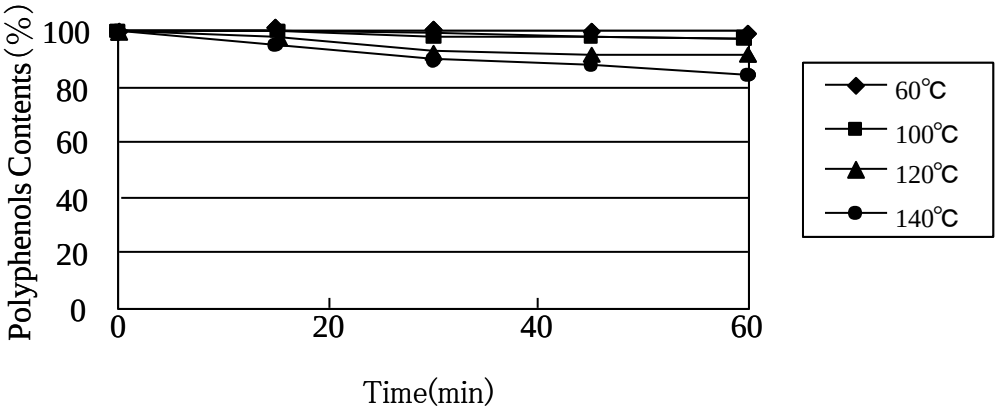


Fig. 10 Heat-Resistance of EVENING PRIMROSE EXTRACT

5-2. pH Stability

Polyphenols in EVENING PRIMROSE EXTRACT remains stable specially at neutral to acid field of pH.

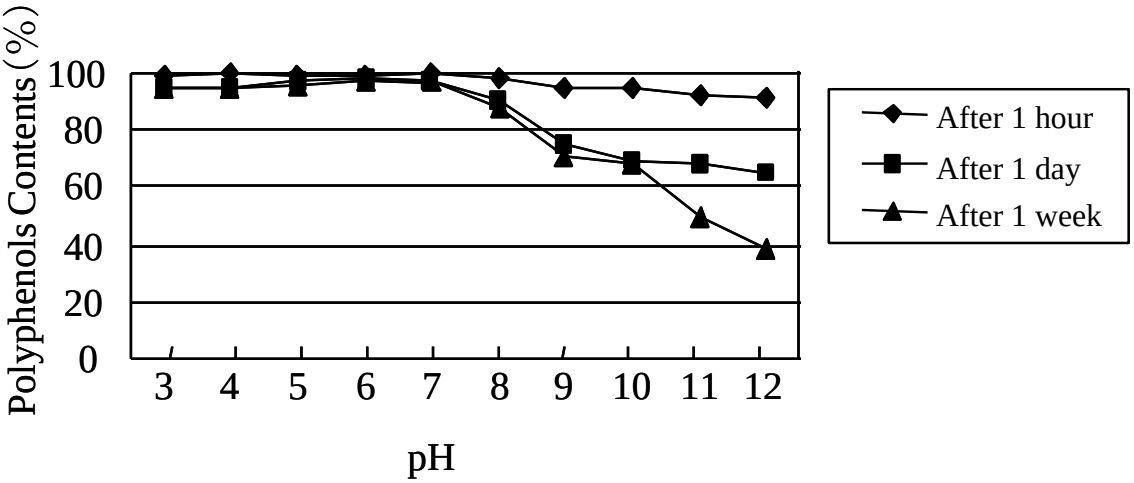


Fig. 11 Influence of pH on The Polyphenols Contents

% of polyphenols in 0.05% solution (80% aqueous methanol)

6. Daily Dosage of EVENING PRIMROSE EXTRACT

It is recommended to take more than 90~120mg of EVENING PRIMROSE EXTRACT -PH per day and more than 180~240mg of EVENING PRIMROSE EXTRACT-WSPH per day.

7. Nutrition facts of EVENING PRIMROSE EXTRACT

Items Analyzed	EVENING PRIMROSE EXTRACT -PH	EVENING PRIMROSE EXTRACT -WSPH
Water	1.5g／100g	2.0 g／100g
Protein	2.2 g／100g	2.6 g／100g
Fat	1.6 g／100g	0.1 g／100g
Ash	1.2 g／100g	6.4 g／100g
Saccharine	93.2 g／100g	86.1 g／100g
Dietary Fiber	0.3 g／100g	2.8 g／100g
Sodium	4.8mg ／100g	29.9mg ／100g
Energy	396kcal／100g	356 kcal／100g

The value of EVENING PRIMROSE EXTRACT-PH and EVENING PRIMROSE EXTRACT-WSPH was calculated with nutrition component analysis value of EVENING PRIMROSE EXTRACT-P and EVENING PRIMROSE EXTRACT-WSP.

EVENING PRIMROSE EXTRACT-P ： Test trustee: Japan Food Research Center Foundation
Research results issue number : 300080303-002

EVENING PRIMROSE EXTRACT-WSP : Test trustee: Japan Food Research Center Foundation
Research results issue number : 301060141-001

8. Acute Toxicity and Safety

8-1. Residual Agricultural Chemicals

Assayed Items	Results	Detected Limits	Assaying Method
BHC	Not Detected	0.02ppm	Gas Chromatography
DDT	Not Detected	0.02ppm	Gas Chromatography
Aldrin	Not Detected	0.01ppm	Gas Chromatography
Dieldrin	Not Detected	0.01ppm	Gas Chromatography
Endrin	Not Detected	0.01ppm	Gas Chromatography
Parathion	Not Detected	0.05ppm	Gas Chromatography
Malathon	Not Detected	0.05ppm	Gas Chromatography

Test trustee: Japan Food Research Center Foundation
Research results issue number : 300080303-001

8-2. Acute Toxicity

Five weeks old mice had been bred for two weeks after administering 5000mg/kg of the extract. No toxic effects were observed, thus the LD_{50}(rat) is more than 5000mg/kg.

8-3. Chromosome aberration test

Clastogenisity was evaluated in mammal culture cells (CHL/IU). In the short time treatment method, the doses of EVENING PRIMROSE EXTRACT were 75, 106.1, 150, 212.1, and 300 μg/ml without metabolic activation and 100, 141.1, 200, 282.8, and 400 μg/ml with metabolic activation. In the continuous treatment method, the doses were 39.5, 59.3, 88.9, 133.3, and 200 μg/ml in the 24-hour treatment test, and 19.8, 29.6, 44.4, 66.7, and 100 μg/ml in the 48-hour treatment test. As a result, EVENING PRIMROSE EXTRACT was not clastogenic for CHL/IU cells irrespective of the presence or absence of metabolic activation and irrespective of the treatment time.

8-4. Reverse mutation test (Ames test)

Ames tests were performed by the Ames plate method using a *Salmonella typhimurium* strain and *Escherichia coli* strain (WP2uvrA). As a result, EVENING PRIMROSE EXTRACT at any dose with or without metabolic activation did not significantly increase the incidence of revertant colonies. These results suggested that EVENING PRIMROSE EXTRACT is not mutagenic.

8-5. Micronucleus test in mice

This test was performed by intraperitoneal administration of EVENING PRIMROSE EXTRACT at 200 mg/kg as the maximum tolerable dose, and at 100 and 50 mg/kg in mice (7 mice/group). The mice were killed 24 or 48 hours after administration, and bone marrow was extracted, and its smear specimens were prepared and stained. Polychromatophilic and orthocyromic erythrocytes with micronuclei were measured. Peanut oil was intraperitoneally administered at a single dose as a solvent control, and cyclophosphamide was orally administered as a positive control. The incidence of polychromatophilic erythrocytes with micronuclei was not increased in the mice treated with EVENING PRIMROSE EXTRACT compared with the solvent control group. The positive control markedly increased the incidence of polychromatophilic erythrocytes with micronuclei. These results suggested that EVENING PRIMROSE EXTRACT is not genotoxic.

9. Practical Applications of EVENING PRIMROSE EXTRACT

EVENING PRIMROSE EXTRACT has 2 forms:

- EVENING PRIMROSE EXTRACT-PH(powder type)
- EVENING PRIMROSE EXTRACT-WSPH(water soluble type powder)

Applications	Examples
Drinks	Tea, blended tea, Protein shakes, and Nutritional drinks.
Dried foods	Soup, dried noodles, seasoning, pasta, cereal, oatmeal, and topping for pizza.
Confectionery	Candies, gum, cookies, pudding, Jelly, yogurt, chocolate
Snacks	Rice crackers, Cookies, and Wafers.
Fermentative foods	Bread and yogurt.

10. Packaging

EVENING PRIMROSE EXTRACT-PH
EVENING PRIMROSE EXTRACT-WSPH

5kg interior packaging: a double layered aluminum bag
exterior packaging: cardboard box

11. Storing Method

Store in cool, dry place. Avoid humidity.

12. Expression of EVENING PRIMROSE EXTRACT

Evening Primrose Seed Extract

※Please refer to your nation's standard.

Test methods

Fig. 1 Comparison of polyphenol contents among various plant extracts

Polyphenol was measured in samples dissolved in methanol solution by the Folin-Denis method described in the Methods of Food Function Research. Gallic acid was used as a standard.

Fig. 4 Comparison between EVENING PRIMROSE EXTRACT and other Anti-*H. pylori* compounds

A total of 24 *H. pylori* strains (5 standard strains and 19 clinically isolated strains) were used. The strains were subcultured in brucella agar supplemented with 7% equine defibrinated blood under a microaerobic condition (5% O_2, 10% CO_2, 85% N_2), and cultured in brucella broth supplemented with 10% fetal calf serum (FCS) under a microaerobic condition for 24 hours. Samples were added at each concentration to 10% FCS-supplemented brucella agar, where the *H. pylori* cultured in the brucella broth was added and cultured under a microaerobic condition (37°C, 5% O_2, 10% CO_2, 85% N_2) for 4 days, and bacterial proliferation was evaluated.

Fig. 5 Comparison of functional components in EVENING PRIMROSE EXTRACT

Performed by the methods used in Fig. 2.

Fig. 6 Effects of EVENING PRIMROSE EXTRACT on *H. pylori* survival rates

The TK1402 strain, a clinically isolated *H. pylori* strain, was cultured in brain heart infusion (BHI) supplemented with 7% equine defibrinated blood under a microaerobic condition, cultured with shaking in Hank's balanced salt solution in the presence of EVENING PRIMROSE EXTRACT at each concentration (512, 256, 128, 64, 32, 0 μg/ml), and 0.1ml samples were obtained after 0, 2, 4, and 24 hours, and bacterial cells were counted.

Fig. 7 Urease inhibitory action of EVENING PRIMROSE EXTRACT

Sword bean-derived urease was used. Enzyme solution (50 μl) adjusted to a final concentration of 0.02 units/ml was thoroughly mixed with EVENING PRIMROSE EXTRACT in test tubes, left at 37°C for 15 minutes. After addition of 300 μl of 100 mM phosphate buffer containing 400 mM urea, the mixture was immediately shaken and left at 37°C for 15 minutes. The reaction was terminated by adding 100 μl of 1 N sulfuric acid. The obtained reaction solution was mixed with 2.5 ml Solution A (5.0 g phenol and 25 mg sodium nitroprusside dissolved in 500 ml water) and 2.5 ml Solution B (2.2 g disodium hydrogenphosphate and 2.5 g sodium hydroxide that were dissolved in about 300 ml water, mixed with 3.0 ml sodium hypochlorite containing 10% or more effective chlorine, and adjusted with water to a volume of 500 ml) and left at 65°C for 20 minutes. The above 1 unit of urease is defined as activity that forms 1 micromol ammonia at 25° for 1 minute.

Urease activity in the obtained mixture solution was measured from absorbance at a wavelength of 630 nm, and the urease inhibition rate was calculated.

Fig. 8 Effects of EVENING PRIMROSE EXTRACT on *H. pylori* adhesion

The TK1402 strain (5×10^8 CFU/ml) as a clinically isolated *H. pylori* strain was labeled with PKH-2 as a lipophilic fluorochrome, and adhesion to an epithelial cell strain derived from human gastric cancer (MKN45 cells) was analyzed by flow cytometry (FCM).

Fig. 9 Results of *H. pylori* detection in the gastric mucosa by RT-PCR method using 16SrRNA expression as a parameter in jirds 1 week after infection

The effects of EVENING PRIMROSE EXTRACT on *H. pylori* fixation were evaluated in male MGS/Sea jirds aged 9 weeks. EVENING PRIMROSE EXTRACT was added to the TK1402 strain ($1\text{-}3 \times 10^9$ CFU/ml), a clinically isolated *H. pylori* strain, to obtain a concentration of 1 mg/ml and orally inoculated (1 ml/jird) once a day for 2 consecutive days. One week after oral inoculation, laparotomy was performed, and gastric mucosa was collected. Reverse transcription-polymerase chain reactions (RT-PCR) of the obtained gastric mucosa were performed using a primer specific to 16SrRNA of *H. pylori*, and the presence or absence of *H. pylori* fixation in the stomach was determined.

Fig. 10 H. pylori eradication effect using ddY mice

After fasting overnight, ddY mice (4 weeks old, male) were orally administered 0.5 ml of *H. pylori* at 10^8 CFU/ml per mouse, followed by using same manner two more times 12 and 24 hours later. After 2 months of breeding, the mice were divided into groups of 7 per group and given EVENING PRIMROSE EXTRACT (15, 30, 60, 120, or 240 mg/kg) orally once daily for a week. From the evening of the final day of oral administration, subjects were fasted overnight. After laparotomy, the gastric mucosa was scraped off and coated on *H. pylori* detection agar medium, which was then cultured for 6 days to determine the number of bacteria.

Fig.11 Antiulcer action of EVENING PRIMROSE EXTRACT using ammonia ulcer model

Male Wistar rats (280-300g) were fasted for 24 hours and orally administered 0.5ml of 2% aqueous ammonia (10 mg of ammonia) per 100 g of rat and the rats were sacrificed by ether 30 minutes later. The stomach was removed and 8ml of 2% formalin was injected into the stomach and fixed for 10 min. The area of damage (mm^2) was measured using Scion Image and used as the ulcer index. EVENING PRIMROSE EXTRACT was suspended in 5% gum arabic powder and orally administered at 0.5ml per 100 g rats 30 min before administration of 2% aqueous ammonia. The control group was administered only 5% gum arabic powder solution.

Fig. 12 Effect of evening primrose extract on lactic acid bacteria

Lactic acid bacteria (NBRC No. *Lactobacillus brevis*) and EVENING PRIMROSE EXTRACT were added to MRS-broth medium and cultured for 24 h. Then, 0.1 mL of the medium was coated on BCP-added plate count agar. After 48 h incubation, the number of bacteria was measured.

Fig. 13 Results of urea breath test

Fig. 14 Results of fecal H. pylori antigen test

Fig.15 Measurement results of blood pepsinogen I/II ratio

A clinical trial was conducted to examine the effect of evening primrose extract on the number of *H. pylori* bacteria in the stomach and gastric mucosal inflammation in subjects with *H. pylori* infection. The subjects were divided into two groups: 5 subjects in the 120 mg group and 4 subjects in the 840 mg group, who took a capsule filled with EVENING PRIMROSE EXTRACT-PH. The capsules were taken two hours after dinner or on an empty stomach for eight consecutive weeks. The tests items were: ① urea breath test, ② fecal *H. pylori* antigen detection test (measuring *H. pylori* antigen in feces and determining the number of bacteria by absorbance), and ③ measurement of pepsinogen I/II ratio in blood (test to determine the degree of gastric mucosal inflammation).

PRODUCT STANDARD

PRODUCT NAME

EVENING PRIMROSE EXTRACT-PH

(FOOD)

The product including polyphenols are extracted from evening primrose (*Oenothera biennis*) seeds with ethanol.

Appearance	It is light red-brown color powder which has slightly unique smell.	
Polyphenols Content	Min. 18.0 %	(Folin-Denis method)
Loss on Drying	Max. 5.0 %	(Analysis for Hygienic Chemists, 1g, 105℃, 2h)
Purity Test		
(1) Heavy Metals	Max. 10 ppm	(The Japanese Standards for Food Additives)
(2) Arsenic	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation)
Standard Plate Counts	Max. 1×10^3 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	contents
Evening primrose seed extract	33 %
Dextrin	67%
	100%

PRODUCT STANDARD

PRODUCT NAME

EVENING PRIMROSE EXTRACT-WSPH

(FOOD)

The product including polyphenols are extracted from evening primrose (*Oenothera biennis*) seeds with water.

Appearance	It is light red-brown color powder which has slightly unique smell.	
Polyphenols Content	Min. 15.0 %	(Folin-Denis method)
Loss on Drying	Max. 5.0 %	(Analysis for Hygienic Chemists, 1g, 105℃, 2h)
Purity Test		
(1) Heavy Metals	Max. 10 ppm	(The Japanese Standards for Food Additives)
(2) Arsenic	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation)
Standard Plate Counts	Max. 1×10^3 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	contents
Evening primrose seed extract	33 %
Dextrin	67%
	100%

ORYZA OIL & FAT CHEMICAL CO., LTD. striving for the development of the new functional food materials to promote your health.

■ **From product planning to OEM** - For any additional information or assistance, please contact :

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