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 Δ-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

1
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 \( \gamma \)-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](image-url)
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](image)

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 ($3.5 \times 10^5$ units/g). Consumption of foods with such high antioxidative activity is known to have preventive effects on various lifestyle-related diseases.

<table>
<thead>
<tr>
<th>Assayed Items</th>
<th>Results</th>
<th>Assaying Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide scavenging activity</td>
<td>$3.5 \times 10^5$ units/g</td>
<td>Electron spin resonance (ESR)</td>
</tr>
</tbody>
</table>

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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.

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 (NH₃) 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 (MACH-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].
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.


<table>
<thead>
<tr>
<th>EVENING PRIMROSE EXTRACT</th>
<th>Green tea catechin</th>
<th>Grape seeds extract</th>
<th>Tree resin</th>
<th>Apple polyphenol</th>
<th>Egg yolk antibody</th>
<th>Cocoa</th>
<th>Garlic extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>64</td>
<td>64</td>
<td>123</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>5000</td>
<td>5000</td>
</tr>
</tbody>
</table>

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

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

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* can not 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.

\[
\text{Urease inhibition rate (\%)} = \frac{(\text{Ub} - \text{Us})}{\text{Ub}} \times 100
\]

\[
\text{Urease activity (U)} = \text{Es} - \text{Eb}
\]

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

**Fig. 7 Urease inhibitory action of EVENING PRIMROSE 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 \textit{H. pylori}, resulting in a shift of the peak to the right. \(\square\) represents samples without EVENING PRIMROSE EXTRACT. The peak is observed on the right side, showing many cells with attached \textit{H. pylori}. \(\square\) represents samples without \textit{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 \(\Rightarrow\) 32 \(\Rightarrow\) 64 \(\mu\)g/ml), the peak shifted from the right to left. This shows dose-dependent inhibition of \textit{H. pylori} adhesion to cells by this extract.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig8.png}
\caption{Fig. 8 Effects of EVENING PRIMROSE EXTRACT on \textit{H. pylori} adhesion}
\end{figure}

TK1402 as a clinically isolated \textit{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 \(5 \times 10^8\) 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 \(\mu\)g/ml) at room temperature for 30 minutes. The TK1402 strain was mixed with MKN45 cells and reacted with \textit{H. pylori} for 1 hour. Inhibition of \textit{H. pylori} adhesion to MKN45 cells was analyzed by flow cytometry (FCM).

\begin{table}
\centering
\caption{Table 1. Effects of EVENING PRIMROSE EXTRACT on \textit{H. pylori} adhesion}
\begin{tabular}{|c|c|c|}
\hline
Concentration of EVENING PRIMROSE EXTRACT (\(\mu\)g/ml) & Mean fluorescence intensity (adhesion inhibition rate, \%) \\
\hline
64 & 15.12 \(\Box\) 61.51 (98.10) & \\
32 & 54.23 \(\Box\) 230.86 (98.18) & \\
16 & 139.56 \(\Box\) 236.01 (82.45) & \\
0 & 795.03 \(\Box\) 594.44 (0) & \\
\hline
\end{tabular}
\end{table}

Adhesion inhibition rates were calculated using the following equation:

\[1 - \frac{T}{A - A'} = \text{adhesion inhibition rate} (\%)\]

\(A\): 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 × 10⁹ 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.

![16SrRNA](image)

**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.
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](image)

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](image)

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

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

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
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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

<table>
<thead>
<tr>
<th>Assayed Items</th>
<th>Results</th>
<th>Detected Limits</th>
<th>Assaying Method</th>
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<tbody>
<tr>
<td>BHC</td>
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<td>0.02ppm</td>
<td>Gas Chromatography</td>
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<tr>
<td>DDT</td>
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<td>0.02ppm</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>Aldrin</td>
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<td>0.01ppm</td>
<td>Gas Chromatography</td>
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<tr>
<td>Dieldrin</td>
<td>Not Detected</td>
<td>0.01ppm</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>Endrin</td>
<td>Not Detected</td>
<td>0.01ppm</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>Parathion</td>
<td>Not Detected</td>
<td>0.05ppm</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>Malathion</td>
<td>Not Detected</td>
<td>0.05ppm</td>
<td>Gas Chromatography</td>
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</table>

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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
Clastogenicity 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 orthocromic 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

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

### 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-\textit{H. pylori} compounds**
A total of 24 \textit{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\textsubscript{2}, 10% CO\textsubscript{2}, 85% N\textsubscript{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 \textit{H. pylori} cultured in the brucella broth was added and cultured under a microaerobic condition (37°C, 5% O\textsubscript{2}, 10% CO\textsubscript{2}, 85% N\textsubscript{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 \textit{H. pylori} survival rates**
The TK1402 strain, a clinically isolated \textit{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°C 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 (1-3 × 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.
EVENING PRIMROSE EXTRACT

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 °C, 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 × 10³ cfu/g            (Analysis for Hygienic Chemists)

**Moulds and Yeasts**

Max. 1 × 10² cfu/g            (Analysis for Hygienic Chemists)

**Coliforms**

Negative                     (Analysis for Hygienic Chemists)

**Composition**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evening primrose seed extract</td>
<td>33 %</td>
</tr>
<tr>
<td>Dextrin</td>
<td>67%</td>
</tr>
<tr>
<td></td>
<td>100%</td>
</tr>
</tbody>
</table>
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°C, 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**

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</table>

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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* The contents of this catalogue may be changed without prior notice.

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