オリザ

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なお、一部の項目については詳細を確認してください。
JAPANESE BUTTERBUR EXTRACT

Anti-allergic and pollenosis-preventive food material

1. Introduction

Pollenosis is a modern disease that affects 1 in 10 Japanese individuals. There are many who suffer from symptoms such as sniveling, sternutation, and itch of the eyes during the Japanese cedar and cypress pollen-dispersing period from February to May.

A number of anti-allergic food materials derived from plants, such as tiencha, perilla, tomatoes, and roses, have been developed, and data of their mechanisms and functions showing their effectiveness in humans are being collected. However, known anti-allergic materials originate from foreign countries to some extent. We found the effectiveness of Japanese butterbur by studying the literature on the anti-allergic activity of vegetables and fruit grown in Japan and performing screening tests.

As indicated by the name, Japanese butterbur (Petasites japonicus) is one of the few vegetables native to Japan. Japanese butterbur has a good flavor, and has long been popular among the Japanese as a food material in the early spring. There are more than 200 kinds of Japanese butterbur, including wild species grown in hills and cultivated vegetables, such as "Aichi wase fuki (P. japonicus Maxim.)", "Mizufuki", "Akita buki (P. japonicus Maxim. var. giganteus)", and "Nobisugidennen". Japanese butterbur is widely cultivated in Aichi, Gunma, and Osaka Prefectures. Among the cultivar species, P. japonicus Maxim. is representative due to its rapid growth and high yield. The production of Japanese butterbur is highest in Chita region in Aichi Prefecture, it was 6,800 t in the 2001 fiscal year, accounting for about 90% of that in Aichi Prefecture and about 40% of that in the whole country. Japanese butterbur used to be a synonym for the early spring, but it is now cultivated for a longer time and marketed from October to May.
We examined the anti-allergic activity of extracts of Japanese butterbur grown in Aichi Prefecture, where its production is highest, in cooperation with Japan Agricultural Co-operatives in Aichi Chita and Kyoto Pharmaceutical University, and found inhibitory effects on degranulation from mast cells, release of leukotrienes, and TNF-β production. Furthermore, we confirmed the in vivo effectiveness of JAPANESE BUTTERBUR EXTRACT, and evaluated the effectiveness in humans. Additionally, two sesquiterpenes, 6 polyphenols, and 2 triterpene glycosides were identified together with new sesquiterpene glycoside named potassium 2β-hydroxyfukinone 2-O-β-D-glucopyranoside-6'-sulfate (fukinoside A) as effective constituents. We consider that JAPANESE BUTTERBUR EXTRACT containing these active constituents can be used for products claiming anti-allergic activity for the alleviation of pollenosis and atopic dermatitis.

2. Functions and constituents of JAPANESE BUTTERBUR EXTRACT

(1) Traditional medicinal properties and recent studies

Japanese butterbur, which has another name, Hou-to-sai, has been used for detoxication, ejection of sluggish blood, and the treatment of bruises. Japanese butterbur contains many essential oil components, and there have been studies on sesquiterpenes in Japanese butterbur flower buds and rhizome\(^1\)\(^-\)\(^4\). The anti-allergic activity of some eremophilane type sesquiterpenes has been reported\(^5\). Researchers in Hokkaido and Osaka Prefecture, where the production of Japanese butterbur has recently been comparable to that in Aichi Prefecture, have studied the edible part of Japanese butterbur. The group of Dr. Tazaki at Obihiro University of Agriculture and Veterinary Medicine and Dr. Iwamoto at the Agricultural, Food and Environmental Sciences Research Center of Osaka Prefecture studied the biosynthetic pathway\(^6\) and quantification method\(^7\) of a polyphenol, fukinolic acid, which is characteristic of Japanese butterbur. With regard to the biological activity of Japanese butterbur, antioxidative activity\(^8\), collagenolytic activity\(^9\), neutrophil elastase inhibitory activity\(^10\), estrogentic activity\(^11\), vasodilative activity\(^12\), and amylase and carboxypeptidase inhibitory activities\(^13\) have

![Fig. 1. Known compounds of *P. Japonicus* and *P. hybridus*](image-url)
been reported. With regard to polyphenols, the DNA polymerase inhibitory activity of a phenylpropanoid, petaciphenol\textsuperscript{14,15}, has also been reported.

Recently, it has been reported that extracts of "European butterbur (Petasites hybridus)" roots improved symptoms in patients with allergic rhinitis\textsuperscript{16,17}. These studies indicated that the effects of European butterbur were similar to those of medical antihistamine preparations, and no side effects caused by suppression of the central nervous system were observed. The effective constituent is petasine, which has activities that not only suppress degranulation from mast cells, as observed in flavonoids, but also dilate capillary blood vessels and bronchial tubes and inhibit leukotriene synthesis\textsuperscript{18}. Therefore, unlike conventional anti-allergic materials, JAPANESE BUTTERBUR EXTRACT is expected to be effective at the occurrence of allergic symptoms.

References

(2) Anti-allergic constituents of JAPANESE BUTTERBUR EXTRACT

Japanese butterbur contains characteristic flavor constituents, monoterpenes and sesquiterpenes, and many polyphenols. However, as described above, it has been reported that sesquiterpenes are contained in rhizomes and flower buds, but there have been few studies on the edible aerial part. To investigate hydrophobic fraction of extracts of Japanese butterbur aerial parts, we have studied the anti-allergic active components using a criterion of the suppressive activity of degranulation of rat basophilic mast cells (RBL-2H3), and isolated below constituents. Additionally, we have isolated a novel sesquiterpene glycoside, potassium 2β-hydroxyfukinone 2-O-β-D-glucopyranoside-6'-sulfate.

Fig. 2. Constituents in aerial part of Japanese butterbur
Mechanism of the occurrence of allergy

IgE antibody binding to specific antigens, such as pollens, exists on the surface of mast cells in patients with allergic diseases. The binding of antigens that have invaded the body to IgE and its cross-linking by antigens induce phosphorylation of specific proteins, such as Lyn and Syk, and elevation of the concentration of intracellular calcium ion and diacylglycerol by inflow of calcium ions. Consequently, exocytosis of granules containing histamine (degranulation), production of leukotrienes by activation of 5-lipoxygenase, and production of TNF-α by activation of intranuclear cytokine transcription factors are induced. These substances produced by mast cells cause itch of eyes and nose and increased sniveling in pollenosis and bronchial constriction and nasal congestion in asthma. TNF-α is involved in inducing chronic allergic diseases and enhancing hypersensitivity (Fig. 3).

Fig. 3. Antigen-inducing mast cell activation and site of action of JAPANESE BUTTERBUR EXTRACT and PERILLA SEED EXTRACT
2. Synergic effect of JAPANESE BUTTERBUR EXTRACT and PERILLA SEED EXTRACT

We have placed "PERILLA SEED EXTRACT" on the market as an anti-allergic material. The extracts not only have effects of suppression of degranulation and inhibition of leukotriene synthesis but also show anti-inflammatory effects, and have been used in many anti-allergic food products.

In addition to suppressive effects of degranulation, JAPANESE BUTTERBUR EXTRACT have suppressive effects of leukotriene release and TNF-α production, which are not observed in PERILLA SEED EXTRACT, indicating that JAPANESE BUTTERBUR EXTRACT can alleviate various allergic symptoms.

The combination of JAPANESE BUTTERBUR EXTRACT and PERILLA SEED EXTRACT may have anti-allergic effects, as shown in Table 1. Fig. 4 shows the effective functions of these extracts in allergic reactions in which mast cells are involved.

Table 1. Synergic effect of JAPANESE BUTTERBUR EXTRACT and PERILLA SEED EXTRACT

<table>
<thead>
<tr>
<th></th>
<th>Enhancement of degranulation inhibitory activity</th>
<th>JAPANESE BUTTERBUR EXTRACT &amp; PERILLA SEED EXTRACT</th>
<th>Enhancement of relief on itch of eyes and nose, and nasal drip</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Inhibition of leukotriene synthesis and release</td>
<td>Inhibition of synthesis: PERILLA SEED EXTRACT</td>
<td>Enhancement of suppressive effect on rhinostenosis and bronchoconstriction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inhibition of release: JAPANESE BUTTERBUR EXTRACT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Suppression of TNF-α production</td>
<td>JAPANESE BUTTERBUR EXTRACT</td>
<td>Suppression of inducing chronic allergic diseases and enhancing hypersensitivity of eyes and nasal mucosa</td>
</tr>
</tbody>
</table>

Fig. 4 Outlines of the activities of our materials against allergic reactions in mast cells
3. Anti-type I allergic effect of JAPANESE BUTTERBUR EXTRACT

(1) Suppressive effect on degranulation (*in vitro*)

Suppressive effects of JAPANESE BUTTERBUR EXTRACT and conventional anti-allergic materials on the degranulation from mast cells were examined in rat basophilic mast cells (RBL-2H3) using the release of hexosaminidase as a parameter. It was found that JAPANESE BUTTERBUR EXTRACT had suppressive effects on degranulation at a level equivalent to that of tiencha extracts (Fig. 5).

![Graph showing suppressive effects](image)

Fig. 5. Suppressive effects of JAPANESE BUTTERBUR EXTRACT and conventional anti-allergic materials on antigen-inducing hexosaminidase release from RBL-2H3 cells. (n=4-6, mean ± S.E.)

<table>
<thead>
<tr>
<th>Material</th>
<th>IC50 (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JAPANESE BUTTERBUR EXTRACT</td>
<td>5.0</td>
</tr>
<tr>
<td>PERILLA SEED EXTRACT</td>
<td>15.2</td>
</tr>
<tr>
<td>Red perilla leaf extract</td>
<td>8.6</td>
</tr>
<tr>
<td>Tiencha extract</td>
<td>4.2</td>
</tr>
<tr>
<td>Rose petal extract</td>
<td>59.9</td>
</tr>
<tr>
<td>Butterbur root extract</td>
<td>6.8</td>
</tr>
</tbody>
</table>

The suppressive effects of JAPANESE BUTTERBUR EXTRACT components on degranulation from mast cells were examined in rat basophilic mast cells (RBL-2H3) using the release of hexosaminidase as a parameter. It was found all compounds except mussaendoside R had suppressive effects on degranulation (Fig. 6, Table 3).
Fig. 6. Suppressive effects of constituents in JAPANESE BUTTERBUR EXTRACT on antigen-inducing hexosaminidase release from RBL-2H3 cells (n=4, mean ± S.E.)

Table 3. IC50 value of constituents in JAPANESE BUTTERBUR EXTRACT on antigen-inducing hexosaminidase release from RBL-2H3 cells.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>IC50 (μg/ml)</th>
<th>Constituents</th>
<th>IC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-Fukinone</td>
<td>4.2</td>
<td>2β-Hydroxyfukinone</td>
<td>4.2</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>9.5</td>
<td>Fukinolic acid</td>
<td>2.1</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>8.6</td>
<td>3,5-Dicaffeoyl quinic acid</td>
<td>2.9</td>
</tr>
<tr>
<td>4,5-Dicaffeoyl quinic acid</td>
<td>3.3</td>
<td>4,5-Dicaffeoyl quinic acid methyl ester</td>
<td>4.0</td>
</tr>
<tr>
<td>Dotorioside II</td>
<td>4.0</td>
<td>Mussaendoside R</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Constituents</td>
<td></td>
<td>IC50 (μg/ml)</td>
<td></td>
</tr>
<tr>
<td>Potassium 2β-hydroxyfukinone-2-O-β-D-glucopyranoside-6'-sulfate</td>
<td>8.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

【Method】
RBL-2H3 cells were sub-cultured in Minimum Essential Medium (MEM medium, Sigma Inc.) containing fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37℃ with 5% CO2. RBL-2H3 cells were inoculated in flat-bottom 24-well microplates (2.0×10^5 cells in 400 μl/well) on the day before reaction. After incubation for 1 hour, rat anti-dinitrophenyl (DNP) monoclonal IgE antibody (Sigma Inc.) was added to the final concentration of 0.45 μg protein/ml, and the cells were incubated in the medium for 24 hours for sensitization. On the day of
reaction, the cells in each well were rinsed twice with 500 µl of Siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 25 mM PIPES), and preincubated in 160 µl Siraganian buffer containing 5.6 mM glucose, 1 mM CaCl₂, and 0.1% bovine serum albumin (BSA) at 37°C for 10 min. Subsequently, 20 µl of the test solution was added, and 10 min later, DNP-BSA (final concentration: 10 µg/ml) was added as an antigen. After incubation for 30 min, the reaction was terminated by chilling the plate with ice for 10 min, and 50 µl of the supernatant was transferred to a flat-bottom 96-well microplate. To each well, 50 µl of 1 mM p-nitrophenyl-N-acetyl-β-glucosaminide (PNAG) in 0.1 M citrate buffer was added, mixed, and incubated at 37°C for 1 hour.

After the reaction, 200 µl of reaction stopping solution (0.1 M NaHCO₃/Na₂CO₃, pH 10.0) was added, and the rate of hexosaminidase release was determined by measuring the absorbance at 410 nm using a microplate reader.

(2) Suppressive effect on leukotriene release (in vitro)

Leukotrienes are inflammation mediators that are synthesized from the cell membrane phospholipid of mast cells by antigen stimulation, and are involved in airway constriction in bronchial asthma. We examined JAPANESE BUTTERBUR EXTRACT and other known anti-allergic materials for their effects on the release of leukotrienes upon antigen stimulation. We found suppressive effects with JAPANESE BUTTERBUR EXTRACT but not with tiencha extract or PERILLA SEED EXTRACT (Fig. 7).

![Fig. 7. Inhibitory effects of JAPANESE BUTTERBUR EXTRACT and conventional anti-allergic materials on antigen-inducing leukotriene release from RBL-2H3 cells (n=6, mean ± S.E.)](image-url)
The same experimental procedures used in the degranulation tests were used in this test. The amount of leukotrienes released into the medium 30 min after antigen stimulation was determined by ELISA (Leukotriene C4/D4/E4 enzyme immunoassay system, Amersham-Pharmacia Inc.).

(3) Suppressive effect on TNF-α production (in vitro)

TNF-α is an inflammatory cytokine produced several hours after antigen stimulation (delayed phase of immediate type allergy), which is involved in the aggravation and chronicity of allergic diseases. JAPANESE BUTTERBUR EXTRACT showed dose-dependent suppressive effects on TNF-α production (Fig. 8). However, rose petal extract at low doses (1 and 10 µg/ml) and PERILLA SEED EXTRACT did not show suppressive effects.

![Graph showing suppressive effect on TNF-α production](image)

Fig. 8. Suppressive effect JAPANESE BUTTERBUR EXTRACT and conventional anti-allergic materials on antigen-inducing TNF-α production from RBL-2H3 cells (n=4, mean ± S.E.)

The same experimental procedures used in the degranulation tests were used in this test. The amount of TNF-α produced 4 hours after antigen stimulation was determined by ELISA (Tumor Necrosis Factor Alpha [(α)TNF α], Rat, Biotrak ELISA system, Amersham-Pharmacia Inc.).
(4) Passive cutaneous anaphylaxis (PCA) reaction in rats (in vivo)

The anti-allergic activity in vivo of JAPANESE BUTTERBUR EXTRACT was tested using the rat passive cutaneous anaphylaxis (PCA) reaction, which is a model of type I allergy. It was found that oral administration of JAPANESE BUTTERBUR EXTRACT 2 hours prior to the induction of reaction suppressed leakage of the dye into the skin due to inflammation (Fig. 9).

![Fig. 9. Effect of JAPANESE BUTTERBUR EXTRACT on PCA reaction (n=6, mean ± S.E.)](image)

【Method】

The back of male 7 weeks old Wistar rats was shaven, and anti-DNP-IgE diluted 12,500-fold in PBS (-) was subcutaneously administered (100 μl/site). After 2 days (after fasting for 20 hours), JAPANESE BUTTERBUR EXTRACT suspended in 5% acacia were orally administered, and 2 hours later, 0.5 ml of 1% Evans blue solution containing 1.5 mg/ml DNP-BSA was intravenously administered. The animals were sacrificed 30 min after administration, and the area of Evans blue spot on the skin was measured using a digital planimeter.

(5) Suppressive effect on leukotriene-inducing inflammation (in vivo)

The anti-inflammatory activity of JAPANESE BUTTERBUR EXTRACT was evaluated using inflammation models with subcutaneous administration of leukotriene D4. It was found that administration of 500 mg/kg of JAPANESE BUTTERBUR EXTRACT had anti-inflammatory effects.
Fig. 10. Effect of JAPANESE BUTTERBUR EXTRACT on dermal inflammation induced by leukotriene D₄ (n=4, mean ± S.E.)

【Method】
JAPANESE BUTTERBUR EXTRACT suspended in 5% acacia were orally administered to rats after fasting for 20 hours. After 2 hours, 0.5 ml of 1% Evans blue solution in PBS (-) was intravenously administered. The back of the rats was shaven, and 30 min after administration of Evans blue, 100 µl/site of 1 g/ml leukotriene D₄ solution or PBS (-) as a negative control was subcutaneously administered at 2 sites on either side of the median line. The animals were sacrificed 30 min after this administration, and the area of Evans blue spot on the skin was measured using a digital planimeter.

(6) Suppressive effect on vasoconstriction and bronchoconstriction( in vitro )
To suppress allergic symptoms that have already been onset, direct action to targeting tissue is required. For example, for congested nose in pollinosis, the blood vessel of nasal mucosa is contracted by histamine that was released from mast cell. On the other hand, airway contraction is occurred in bronchial asthma due to bronchial smooth muscle constriction induced by mast cell-derived leukotrienes. We examined the effects of JAPANESE BUTTERBUR EXTRACT and conventional anti-allergic materials on KCl- or norepinephrine-induced vasoconstriction and histamine- or leukotriene D₄-induced bronchoconstriction by using guinea pig trachea and rat thoracic aorta. As a result, JAPANESE BUTTERBUR EXTRACT and Tiencha extract relaxed vasoconstriction that was contracted by KCl (Fig. 11). Tiencha extract also suppressed vasoconstriction induced by norepinephrine, however JAPANESE BUTTERBUR EXTRACT was not suppressed. Hence, JAPANESE BUTTERBUR EXTRACT was found to possess non-specific suppressive activity against vasoconstriction. On the other hand, on bronchoconstriction, JAPANESE BUTTERBUR EXTRACT (1000 µg/mL) significantly suppressed histamine-induced bronchoconstriction. JAPANESE BUTTERBUR EXTRACT and PERILLA SEED EXTRACT significantly suppressed
bronchoconstriction induced by leukotriene D₄.

Rat thoracic aorta

Guine pig trachea

Fig. 11. Effects of JAPANESE BUTTERBUR EXTRACT and conventional anti-allergic materials on vasoconstriction and bronchoconstriction. (n=3-6, mean ± S. E., *: p<0.05, **: p<0.01, □: not tested)

These results suggest that JAPANESE BUTTERBUR EXTRACT has an inhibitory action against smooth muscle contraction of blood vessel and trachea, and it can expect healing effects against the allergic symptom (rhinostenosis and bronchocontraction) which has already occurred. Furthermore, as a result of examination of (+)-fukinone, a sesquiterpene in JAPANESE BUTTERBUR EXTRACT, on smooth muscle constriction, it suppressed KCl-induced vasoconstriction and histamine- and leukotriene D₄-induced bronchoconstriction (Tables 4-6).
Table 4. Effect of (+)-fukinone on vasoconstriction in rat thoracic aorta induced by KCl.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Relaxation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 (µg/ml)</td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>3.1 ± 2.3</td>
</tr>
<tr>
<td>(+)-fukinone</td>
<td>4</td>
<td>1.6 ± 0.3</td>
</tr>
</tbody>
</table>

mean ± S.E., **: p<0.01

Table 5. Effect of (+)-fukinone on bronchoconstriction in guinea pig trachea induced by histamine.

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>n</th>
<th>Control</th>
<th>(+)-fukinone</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>22.0 ± 4.7</td>
<td>15.7 ± 4.3</td>
</tr>
<tr>
<td>30</td>
<td>3</td>
<td>45.8 ± 4.5</td>
<td>34.6 ± 10.5</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>97.0 ± 8.5</td>
<td>92.9 ± 7.5</td>
</tr>
</tbody>
</table>

mean ± S.E., *: p<0.05

Table 6. Effect of (+)-fukinone on bronchoconstriction in guinea pig trachea induced by leukotriene D4 (LT)

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>n</th>
<th>Control</th>
<th>(+)-fukinone</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT 10 (nM)</td>
<td></td>
<td>-</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>104.5 ± 20.1</td>
<td>92.7 ± 9.5</td>
</tr>
<tr>
<td>100</td>
<td>3</td>
<td>51.6 ± 15.3</td>
<td></td>
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</tbody>
</table>

mean ± S.E
1) Constriction of rat thoracic aorta strips induced by KCl and norepinephrine.

A rat was anesthetized with ether and the thoracic aorta was removed. The aorta was cut helical like, and prepared 15 mm length specimen. The aorta preparation was attached to a Magnus apparatus and placed in an organ-bath filled with Krebs-Henseleit (118 mM NaCl, 2.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 2.5 mM CaCl$_2$, 25 mM NaHCO$_3$, 10 mM glucose, equilibrated with 95%N$_2$/5%O$_2$ gas) solution (6 ml). The organ bath was warmed at 37 °C and the solution was bubbled gently with 95%N$_2$/5%O$_2$ gas. The specimen was pulled gently with a tension of 1 g and the change in tension was recorded by MacLab 8e (AD instruments, Tokyo, Japan) via an isotonic transducer. After the tension was balanced, 3M KCl solution (100 µl) was added to the bath (final concentration: KCl 50 mM) and the change in tension was recorded by the extent of constriction plateaued. The specimen was washed 3 times with Krebs-Henseleit solution at 10-min intervals. This procedure was repeated again and the test sample diluted in DMSO (3 µl) was added accumulatively at 30-min intervals. The change in tension was recorded.

On the other hand, norepinephrine-induced aorta constriction was performed as follows. The aorta specimen was attached to the organ-bath filled with Krebs-Henseleit solution containing EDTA (37.2 mg/l) and tension was stabilized. A 1 mM nifedipine diluted with DMSO (6 µl) was added to organ-bath, and 1 mM norepinephrine (6 µl) was added 10 minutes later. After recording the contraction for 25 minutes, the specimen was washed 3 times at 10 min interval. Then, 1 mM nifedipine (6 µl) was added again, and a sample dissolved in DMSO (6 µl) was added 10 min later. After 10 min, norepinephrine was added accumulatively to become 1 nM～10 µM, and tension was recorded.

3) Constriction of guinea pig trachea strips induced by histamine and leukotriene D$_4$.

A guinea pig was anesthetized with ether and the trachea was removed. The trachea was cut into 3 to 4 mm length and its cartilage part was opened in Krebs-Henseleit solution containing 1 µM indomethacin. The tracheal preparation was attached to a Magnus apparatus and placed in an organ-bath filled with Krebs-Henseleit solution (6 mL). The organ bath was warmed at 37 °C and the solution was bubbled gently with 95%N$_2$/5%O$_2$ gas. The specimen was pulled gently with a tension of 1 g and the change in tension was recorded by MacLab 8e (AD instruments, Tokyo, Japan) via an isotonic transducer. After the tension was balanced, histamine or leukotriene D$_4$ solution (6 µL) was added to the bath (final concentration: histamine, 10 µM; leukotriene D$_4$, 10 nM) and the change in tension was recorded by the extent of constriction plateaued. The specimen was washed 3 times with Krebs-Henseleit solution at 10-min intervals. This procedure was repeated again and the test sample diluted in DMSO (6 µL) was added (final DMSO concentration, 0.1%). Ten minutes later, 1 to 10 µM histamine or 10 nM leukotriene D$_4$ was added accumulatively and the change in tension was recorded.
6. Effect of JAPANESE BUTTERBUR EXTRACT on patients with pollenosis

We asked 10 patients with Japanese cedar pollenosis, of whom 3 patients were evaluated using a questionnaire, to freely take JAPANESE BUTTERBUR EXTRACT (250 mg) for 4 weeks between the times before and during pollen dispersing, and examined allergy-related parameters in the blood immediately and 4 weeks (recovery period) after the completion of intake of JAPANESE BUTTERBUR EXTRACT. We also investigated the pollenosis symptoms during the intake period and over 4 weeks after the completion of intake (recovery period) using a questionnaire.

![Experimental protocol and change in amount of dispersed Japanese cedar pollen](image)

Although the amount of dispersed pollen was small in 2004, on March 11, when the intake of JAPANESE BUTTERBUR EXTRACT was completed, a large amount of pollen was observed in Nagoya City in Aichi Prefecture (36 pollen grains/cm²) and in Gifu City in Gifu Prefecture (12 pollen grains/cm²). The investigation using the questionnaire demonstrated that the symptoms, such as sternutation, olfaction, and itch of the eyes, were slightly milder during the period of intake of JAPANESE BUTTERBUR EXTRACT than during the recovery period (Fig. 13). The rate of basophils in the leukocytes was lower immediately after intake of JAPANESE BUTTERBUR EXTRACT than after the recovery period (Fig. 14). Since basophils exist in peripheral tissues as mast cells, suppression of the increase in the rate of basophils in the leukocytes may contribute to the prevention of type I allergy, in which mast cells are involved. The blood concentration of eosinophilic basicity protein (ECP) produced by eosinophils was lower immediately after intake of JAPANESE BUTTERBUR EXTRACT than after the recovery period (Fig. 15).
BUTTERBUR EXTRACT than after the recovery period, suggesting that the activity of eosinophils was suppressed during the intake period of JAPANESE BUTTERBUR EXTRACT.

Fig. 13. Changes in symptoms during ingestion period and recovery period
Fig. 14. Changes in blood parameter at right after ingestion and 4 weeks after ingestion
7. Stability of JAPANESE BUTTERBUR EXTRACT-P
   (in non-excipient form)

(1) Thermal resistance
   Evaluation of the heat stability of JAPANESE BUTTERBUR EXTRACT-P (in
   non-excipient form) showed no changes in the fukinone and total polyphenols contents
   even after heating at 120°C for 1 hour.

   ![Graph showing thermal resistance](image)

   Fig.15. Thermal resistance of JAPANESE BUTTERBUR EXTRACT-P
   (In non-excipient form, 100% as initial value)

(2) pH stability
   After storing of H2O solution of JAPANESE BUTTERBUR EXTRACT-P (in
   non-excipient form) without light shielding at room temperature for 1 week, fukinone
   and total polyphenols contents were measured. Both contents in JAPANESE BUTTERBUR
   EXTRACT-P (in non-excipient form) was found to be stable from acid to alkali range of pH.

   ![Graph showing pH stability](image)

   Fig.16. pH stability of JAPANESE BUTTERBUR EXTRACT-P
   (In non-excipient form, 100% as initial value)
(3) Solubility

JAPANESE BUTTERBUR EXTRACT-WSP (in non-excipient form) was dissolved in water (pH 3.5) and stored at room temperature, 40° and 4° for 12 weeks. The presence or absence of precipitation, turbid and versicolor was observed. JAPANESE BUTTERBUR EXTRACT-WSP (in non-excipient form) was highly soluble in water in the acid range.

<table>
<thead>
<tr>
<th></th>
<th>Room temperature (light shielding)</th>
<th>40° (without light shielding)</th>
<th>4° (without light shielding)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acid (pH3.5)</strong></td>
<td>Precipitation and Turbid</td>
<td>Neither precipitation nor turbid were observed for 12 weeks.</td>
<td>Neither precipitation nor turbid were observed for 12 weeks.</td>
</tr>
<tr>
<td><strong>Versicolor</strong></td>
<td>No versicolor was observed for 12 weeks.</td>
<td>No versicolor was observed for 12 weeks.</td>
<td>No versicolor was observed for 12 weeks.</td>
</tr>
</tbody>
</table>

8. Nutrition facts of JAPANESE BUTTERBUR EXTRACT-P (in non-excipient form)

<table>
<thead>
<tr>
<th></th>
<th>Results</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>0.8 g/100g</td>
<td>Heat drying method under reduced pressure</td>
</tr>
<tr>
<td>Protein*1</td>
<td>6.2 g/100g</td>
<td>Kieldahl method</td>
</tr>
<tr>
<td>Fat</td>
<td>3.6 g/100g</td>
<td>Acid fat dissolution method</td>
</tr>
<tr>
<td>Ash</td>
<td>20.9 g/100g</td>
<td>Direct ashing method</td>
</tr>
<tr>
<td>Carbohydrate*2</td>
<td>68.5 g/100g</td>
<td></td>
</tr>
<tr>
<td>Energy*3</td>
<td>331 kcal/100g</td>
<td></td>
</tr>
<tr>
<td>Dietary fiber</td>
<td>0.3 g/100g&gt;</td>
<td>Prosky method</td>
</tr>
<tr>
<td>Sodium</td>
<td>1200 mg/100g</td>
<td>Atomic absorption spectrophotometry</td>
</tr>
</tbody>
</table>

*1) N=6.25
*2) 100 – (moisture + protein + fat + ash)
*3) Factors for calculating the energy value: protein, 4; fat, 9; carbohydrate, 4; dietary fiber, 2

Test trustee: SRL, Inc.

Date of issue of the test result report: April 19, 2004
Research result issue number: No. 200404060017
9. Safety profile of JAPANESE BUTTERBUR EXTRACT-P
   (in non-excipient form, 100% as initial value)

(1) Residual agricultural chemicals
   The 33 agrochemicals, of which the standard levels of residues are established by
   the Food Sanitation Law (Ministry of Health, Labour and Welfare), were examined.

<table>
<thead>
<tr>
<th>No.</th>
<th>Chemical</th>
<th>Result</th>
<th>Detectable limit</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acephate</td>
<td>Not detected</td>
<td>1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>2</td>
<td>Iprodione</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>3</td>
<td>Etofenprox</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>4</td>
<td>Etrimfos</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>5</td>
<td>Chlorpyrifos</td>
<td>Not detected</td>
<td>0.05 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>6</td>
<td>Diethofencarb</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>7</td>
<td>Dichlorvos</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>8</td>
<td>Cyhalothrin</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>9</td>
<td>Cyfluthrin</td>
<td>Not detected</td>
<td>1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>10</td>
<td>Cypermethrin</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>11</td>
<td>Thiobencarb</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>12</td>
<td>Thiometon</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>13</td>
<td>Trichlamide</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>14</td>
<td>Trifluralin</td>
<td>Not detected</td>
<td>0.05 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>15</td>
<td>Tolecfos-Methyl</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>16</td>
<td>Parathion-Methyl</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>17</td>
<td>Bioresmethrin</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>18</td>
<td>Pyridaben</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>19</td>
<td>Pirimiphos-Methyl</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>20</td>
<td>Pyrethrins</td>
<td>Not detected</td>
<td>1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>21</td>
<td>Fenarimol</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>22</td>
<td>Fenitrothion</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>23</td>
<td>Fenobucarb</td>
<td>Not detected</td>
<td>0.5 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>24</td>
<td>Fenvalerate</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>25</td>
<td>Flucythrinate</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>26</td>
<td>Flutolanil</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>27</td>
<td>Procymidone</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>28</td>
<td>Permethrin</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>29</td>
<td>Pendimethalin</td>
<td>Not detected</td>
<td>0.05 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>30</td>
<td>Malathion</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>31</td>
<td>Myclobutanil</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>32</td>
<td>Methiocarb</td>
<td>Not detected</td>
<td>0.05 ppm</td>
<td>GC-MS</td>
</tr>
<tr>
<td>33</td>
<td>Lenacil</td>
<td>Not detected</td>
<td>0.1 ppm</td>
<td>GC-MS</td>
</tr>
</tbody>
</table>

Test trustee: Kyusai analysis institute Co., LTD.
Date of issue of the test result report: April 12, 2004
Research result issue number: No. 20040405-1
(2) Acute toxicity (LD₅₀)

JAPANESE BUTTERBUR EXTRACT (in non-excipient form, 2,000 mg/kg) was orally administered to male and female ICR mice aged 5 weeks under fasting, and the mice were maintained and observed for 14 days. Neither deaths nor abnormal in body weight compared with the controls were observed. Autopsy performed after the discontinuation of the experiment showed no macroscopic abnormalities in organs. Therefore, in male and female mice, the LD₅₀ of JAPANESE BUTTERBUR EXTRACT (in non-excipient form) by oral administration is 2,000 mg/kg.

10. Recommended daily dosage

It is recommended to take 250 mg/day of JAPANESE BUTTERBUR EXTRACT-P.

11. Applications

<table>
<thead>
<tr>
<th>Foods</th>
<th>Application</th>
<th>Health claim</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-allergic</td>
<td>1) Prevention of pollenosis</td>
<td>Drinks (beverage, juice, etc.), hard and soft gel capsule, tablet, candy, chewing, gum, cookies, chocolate, wafers, jelly, etc.</td>
</tr>
<tr>
<td></td>
<td>foods</td>
<td>2) Anti-atopic dermatitis, etc.</td>
<td></td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Anti-allergic</td>
<td></td>
<td>Lotion, cream, facial pack, body gel, etc.</td>
</tr>
<tr>
<td></td>
<td>cosmetics</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

JAPANESE BUTTERBUR EXTRACT-WSP, which is highly water-soluble, can be widely applied such as drinks among foods and lotions among cosmetics.

12. Packaging

- JAPANESE BUTTERBUR EXTRACT-P, WSP (Powder, food grade)
- JAPANESE BUTTERBUR POWDER (Powder, food grade)
- JAPANESE BUTTERBUR EXTRACT-PC, WSPC (Powder, cosmetic grade)
  - 5kg Interior packaging: aluminum-coated plastic bag
  - Exterior packaging: cardboard box
- JAPANESE BUTTERBUR EXTRACT –LC (Liquid, cosmetic grade)
  - 5kg Interior packaging: cubic polyethylene container
  - Exterior packaging: cardboard box

13. Storage

Store in cool, dry place. Avoid humidity.
14. Expression

<Food>
JAPANESE BUTTERBUR EXTRACT-P, JAPANESE BUTTERBUR EXTRACT-WSP
Expression: Japanese butterbur extract
JAPANESE BUTTERBUR POWDER
Expression: Japanese butterbur

<Cosmetics>
JAPANESE BUTTERBUR EXTRACT-PC
INCI name: Dextrin, Petasites Japonicus Leaf/Stem Extract
JAPANESE BUTTERBUR EXTRACT-WSPC
INCI name: Dextrin, Petasites Japonicus Leaf/Stem Extract
JAPANESE BUTTERBUR POWDER
INCI name: Petasites Japonicus Powder
JAPANESE BUTTERBUR EXTRACT-LC
INCI name: Butylene glycol, Water, Petasites Japonicus Leaf/Stem Extract
PRODUCT STANDARD

PRODUCT NAME

JAPANESE BUTTERBUR EXTRACT-P

FOOD

This product is extracted from Japanese butterbur, the stems and the leaves of *Petasites japonicus* (Compositae), with aqueous ethanol.

1. Appearance

Slightly yellow powder with slightly unique smell.

2. Certification Test

(1) Fukinolic acid

After a small amount of methanol (HPLC grade) is added to 250 mg of this product in a 10 ml volume flask, and the flask is treated with ultrasonic wave for 10 minutes. The solution is filtered through a 0.45 µm PTFE filter after addition of methanol (HPLC grade) to adequate volume. For preparation of standard solution, methanol (HPLC grade) is added to fukinolic acid, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions for 5 ml of test solution and standard solution. The peak of fukinolic acid is found in the HPLC chromatogram of test solution.

<HPLC condition>

Column : capcellpak C18 (4.6 mm ID x 250 mm)
Mobile phase: Solvent A = Citric acid solution (citric acidmonohydrate 2.1g in 1 L)
Solvent B = Methanol

<table>
<thead>
<tr>
<th>time(min)</th>
<th>0</th>
<th>19</th>
<th>25</th>
<th>35</th>
<th>50</th>
<th>60</th>
<th>61</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>100% A</td>
<td>100% A</td>
<td>80%A</td>
<td>80%A</td>
<td>60%A</td>
<td>60%A</td>
<td>100%B</td>
<td>100% B</td>
</tr>
<tr>
<td></td>
<td>20%B</td>
<td>20%B</td>
<td>40%B</td>
<td>40%B</td>
<td>60%A</td>
<td>100%B</td>
<td>100%B</td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Detector</td>
<td>UV 324 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column temperature</td>
<td>30 ºC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(2) Fukinone

This product (2.0g) in a centrifugation tube is suspended in purified water (10 ml), and treated with ultrasonic wave for 1 minute. Ethyl acetate (10 ml) is added to the suspension, and the tube is shaken for 3 minutes. Then the tube is centrifuged (room temperature, 3000 rpm, 10 minutes), and an ethyl acetate layer is collected (repeat this procedure three times). The collected ethyl acetate layer is evaporated and is dissolved in 20% acetonitrile (3 ml), and is filtered through a Sep-Pak C18 cartridge.

Then, the cartridge is washed with 70% acetonitrile (7 ml) and is eluted with absolute acetonitrile (5 ml). This solution is adjusted to 10 ml, and filtered through a 0.45 µm PTFE filter (test solution). For preparation of standard solution, acetonitrile is added to fukinone, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following
conditions for 5 ml of test solution and standard solution. The peak of fukinone is found in the HPLC chromatogram of test solution.

<HPLC condition>
Column : Shim-pack CLC-ODS (6.0 mm 150 mm)
Mobile phase : acetonitrile: water = 8:2
Flow rate : 1.0 ml/min
Detector : UV 254 nm
Column Temperature : 30 °C

3. Total polyphenols content
Max. 1.0 % (Folin-Denis method)

4. Total terpenoids content
Max. 300 µl / 100 g (Japanese Pharmacopoeia,
[In non-excipient form (JAPANESE BUTTERBUR EXTRACT)]
Essential oil content)
(Theoretical value in JAPANESE BUTTERBUR EXTRACT-P : Min. 60 ml / 100g)

5. Loss on Drying
Max. 10.0 % (Analysis for Hygienic Chemists, 1g, 105 °C, 2h)

6. Purity Test
(1) Heavy Metals
Max. 10 ppm (The Japanese Standards for Food Additives)

(2) Arsenic
Max. 1 ppm (Standard Method Analysis in Food Safety Regulation)

7. Standard Plate Counts
Max. 3 × 10³ cfu/g (Analysis for Hygienic Chemists)

8. Moulds and Yeasts
Max. 1 × 10³ cfu/g (Analysis for Hygienic Chemists)

9. Coliforms
Negative (Analysis for Hygienic Chemists)

10. Composition
Ingredients | Contents
--- | ---
Dextrin | 80 %
Japanese Butterbur Extract | 20 %
Total | 100 %
PRODUCT STANDARD

PRODUCT NAME

JAPANESE BUTTERBUR EXTRACT-WSP

FOOD

This product is extracted from Japanese butterbur, the stems and the leaves of *Petasites japonicus* (Compositae), with aqueous ethanol. This product is water-soluble.

1. Appearance

Slightly yellow powder with slightly unique smell.

2. Certification Test

Fukinolic acid

After a small amount of methanol (HPLC grade) is added to 250 mg of this product in a 10 ml volume flask, and the flask is treated with ultrasonic wave for 10 minutes. The solution is filtered through a 0.45 μm PTFE filter after addition of methanol (HPLC grade) to adequate volume. For preparation of standard solution, methanol (HPLC grade) is added to fukinolic acid, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions for 5 ml of test solution and standard solution. The peak of fukinolic acid is found in the HPLC chromatogram of test solution.

<HPLC condition>

Column : capcellpak C18 (4.6 mm × 250 mm)

Mobile phase: Solvent A = Citric acid solution (citric acidmonohydrate 2.1g / L)

Solvent B = Methanol

<table>
<thead>
<tr>
<th>time(min)</th>
<th>0</th>
<th>19</th>
<th>25</th>
<th>35</th>
<th>50</th>
<th>60</th>
<th>61</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>100% A</td>
<td>80% A</td>
<td>80% A</td>
<td>60% A</td>
<td>60% A</td>
<td>100% B</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100% A</td>
<td>20% B</td>
<td>20% B</td>
<td>40% B</td>
<td>40% B</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mobile phase: 100% A = 100% B

Flow rate : 1.0 ml/min
Detector : UV 324 nm
Column temperature : 30 ℃

3. Total polyphenols content

Max. 1.0 % (Folin-Denis method)

4. Loss on Drying

Max. 10.0 % (Analysis for Hygienic Chemists, 1g, 105 ℃, 2h)

5. Purity Test

(1) Heavy Metals

Max. 10 ppm (The Japanese Standards for Food Additives)

(2) Arsenic

Max. 1 ppm (Standard Method Analysis in Food Safety Regulation)

6. Standard Plate Counts

Max. $3 \times 10^3$ cfu/g (Analysis for Hygienic Chemists)
7. Moulds and Yeasts
Max. $1 \times 10^3$ cfu/g
(Analysis for Hygienic Chemists)

8. Coliforms
Negative
(Analysis for Hygienic Chemists)

9. Composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrin</td>
<td>80 %</td>
</tr>
<tr>
<td>Japanese Butterbur Extract</td>
<td>20 %</td>
</tr>
<tr>
<td>Total</td>
<td>100 %</td>
</tr>
</tbody>
</table>
This product is dried and grounded Japanese butterbur, the stems and the leaves of *Petasites japonicus* (Compositae).

1. Appearance  
Green powder with slightly unique smell.

2. Certification Test  
(1) Fukinolic acid  
This product (1.5 g) in a Erlenmeyer flask is suspended in 70% ethanol (15 ml), and refluxed for 2 hours at 70°C. After cooling, the suspension is filtered and the residue is washed with 70% ethanol (15 ml). The filtered solution is evaporated at 70°C, and the concentrate suspended in methanol (HPLC grade) is treated with ultrasonic wave for 10 min. The solution is filtered through a 0.45μm PTFE filter after addition of methanol (HPLC grade) to adequate volume. For preparation of standard solution, methanol (HPLC grade) is added to fukinolic acid, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions for 5 ml of test solution and standard solution. The peak of fukinolic acid is found in the HPLC chromatogram of test solution.

<HPLC condition>  
Column: capcellpak C18 (4.6 mm × 250 mm)  
Mobile phase: Solvent A = Citric acid solution (citric acid monohydrate 2.1g/L)  
Solvent B = Methanol  
Condition of gradient  

<table>
<thead>
<tr>
<th>time(min)</th>
<th>0</th>
<th>19</th>
<th>25</th>
<th>35</th>
<th>50</th>
<th>60</th>
<th>61</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>100% A</td>
<td>100% A</td>
<td>80%A</td>
<td>80%A</td>
<td>60%A</td>
<td>60%A</td>
<td>100%B</td>
<td>100%B</td>
</tr>
</tbody>
</table>

Flow rate: 1.0 ml/min  
Detector: UV 324 nm  
Column Temperature: 30°C

(2) Fukinone  
This product (5.0 g) in a Erlenmeyer flask is suspended in 70% ethanol (50 ml), and refluxed for 2 hours at 70°C. After cooling, the suspension is filtered and the residue is washed with 70% ethanol (30 ml). The filtered solution is evaporated at 70°C, and the concentrate is suspended in water (10 ml). The concentrate suspension poured into a centrifugation tube is treated with ultrasonic wave for 1 minute. Ethyl acetate (10 ml) is added to the suspension, and the tube is shaken for 3 minutes. Then the tube is centrifuged (room temperature, 3000 rpm, 10 minutes), and an ethyl acetate layer is collected (repeat this procedure three times).
collected ethyl acetate layer is evaporated and is dissolved in 20% acetonitrile (3 ml), and is filtered through a Sep-Pak C18 cartridge. Then, the cartridge is washed with 70% acetonitrile (7 ml) and is eluted with absolute acetonitrile (5 ml). This solution is adjusted to 10 ml, and filtered through a 0.45 μm PTFE filter (test solution). For preparation of standard solution, acetonitrile is added to fukinone, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions for 5 ml of test solution and standard solution. The peak of fukinone is found in the HPLC chromatogram of test solution.

<HPLC condition>
Column: Shim-pack CLC-ODS (6.0 mm × 150 mm)
Mobile phase: acetonitrile: water = 8:2
Flow rate: 1.0 ml/min
Detector: UV 254 nm
Column Temperature: 30 °C

3. Loss on Drying
Max. 10.0 % (Analysis for Hygienic Chemists, 1g, 105 °C, 2h)

4. Purity Test
(1) Heavy Metals
Max. 10 ppm (The Japanese Standards for Food Additives)

(2) Arsenic
Max. 1 ppm (Standard Method Analysis in Food Safety Regulation)

5. Standard Plate Counts
Max. 3 × 10^3 cfu/g (Analysis for Hygienic Chemists)

6. Moulds and Yeasts
Max. 1 × 10^3 cfu/g (Analysis for Hygienic Chemists)

7. Coliforms
Negative (Analysis for Hygienic Chemists)

8. Composition
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Japanese Butterbur</td>
<td>100 %</td>
</tr>
</tbody>
</table>
PRODUCT STANDARD

PRODUCT NAME

JAPANESE BUTTERBUR EXTRACT-PC
COSMETIC

This product is extracted from Japanese butterbur, the stems and the leaves of Petasites japonicus (Compositae), with aqueous ethanol.

1. Appearance
Slightly yellow powder with slightly unique smell.

2. Certification Test
(1) Fukinolic acid
After a small amount of methanol (HPLC grade) is added to 250 mg of this product in a 10 ml volume flask, and the flask is treated with ultrasonic wave for 10 minutes. The solution is filtered through a 0.45 µm PTFE filter after addition of methanol (HPLC grade) to adequate volume. For preparation of standard solution, methanol (HPLC grade) is added to fukinolic acid, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions for 5 ml of test solution and standard solution. The peak of fukinolic acid is found in the HPLC chromatogram of test solution.

<HPLC condition>
Column: capcellpak C18 (4.6 mm x 250 mm)
Mobile phase: Solvent A = Citric acid solution (citric acidmonohydrate 2.1g /L)
Solvent B = Methanol
Condition of gradient
<table>
<thead>
<tr>
<th>time(min)</th>
<th>0</th>
<th>19</th>
<th>25</th>
<th>35</th>
<th>50</th>
<th>60</th>
<th>61</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>100% A</td>
<td>100% A</td>
<td>80%A</td>
<td>80%A</td>
<td>60%A</td>
<td>60%A</td>
<td>100%B</td>
<td>100% B</td>
</tr>
<tr>
<td></td>
<td>20%B</td>
<td>20%B</td>
<td>20%B</td>
<td>40%B</td>
<td>40%B</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Flow rate: 1.0 ml/min
Detector: UV 324 nm
Column Temperature: 30 °

(2) Fukinone
This product (2.0g) in a centrifugation tube is suspended in purified water (10 ml), and treated with ultrasonic wave for 1 minute. Ethyl acetate (10 ml) is added to the suspension, and the tube is shaken for 3 minutes. Then the tube is centrifuged (room temperature, 3000 rpm, 10 minutes), and an ethyl acetate layer is collected (repeat this procedure three times). The collected ethyl acetate layer is evaporated and is dissolved in 20% acetonitrile (3 ml), and is filtered through a Sep-Pak C18 cartridge. Then, the cartridge is washed with 70% acetonitrile (7 ml) and is eluted with absolute acetonitrile (5 ml). This solution is adjusted to 10 ml, and filtered through a 0.45 µm PTFE filter (test solution). For preparation of standard solution, acetonitrile is added to fukinone, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following
conditions for 5 of test solution and standard solution. The peak of fukinone is found in the HPLC chromatogram of test solution.

<HPLC condition>
Column: Shim-pack CLC-ODS (6.0 mm x 150 mm)
Mobile phase: acetonitrile:water = 8:2
Flow rate: 1.0 ml/min
Detector: UV 254 nm
Column Temperature: 30°

3. Total polyphenols content Max. 1.0 % (Folin-Denis method)

4. Total terpenoids content Max. 300 µl / 100 g (Japanese Pharmacopoeia, Essential oil content)
   [In non-excipient form (JAPANESE BUTTERBUR EXTRACT)]
   (Theoretical value in JAPANESE BUTTERBUR EXTRACT-PC: Min. 60 µl / 100 g)

5. Loss on Drying Max. 10.0 % (1g, 105°, 2h)

6. Purity Test
   (1) Heavy Metals Max. 10 ppm (The Second Method)
   (2) Arsenic Max. 1 ppm (The Third Method)

7. Standard Plate Counts Max. 1 x 10^2 cfu/g (Analysis for Hygienic Chemists)

8. Moulds and Yeasts Max. 1 x 10^2 cfu/g (Analysis for Hygienic Chemists)

9. Coliforms Negative (Analysis for Hygienic Chemists)

10. Composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrin</td>
<td>80 %</td>
</tr>
<tr>
<td>Petasites Japonicus Leaf/Stem Extract</td>
<td>20 %</td>
</tr>
<tr>
<td>Total</td>
<td>100 %</td>
</tr>
</tbody>
</table>

PRODUCT STANDARD

PRODUCT NAME

JAPANESE BUTTERBUR EXTRACT-WSPC

COSMETIC

This product is extracted with aqueous ethanol from Japanese butterbur, the stems and the leaves of *Petasites japonicus* (Compositae). This product is water soluble.

1. Appearance
   Slightly yellow powder with slightly unique smell.

2. Certification Test
   (1) Fukinolic acid
   After a small amount of methanol (HPLC grade) is added to 250 mg of this product in a 10 ml volume flask, and the flask is treated with ultrasonic wave for 10 minutes. The solution is filtered through a 0.45 µm PTFE filter after addition of methanol (HPLC grade) to adequate volume. For preparation of standard solution, methanol (HPLC grade) is added to fukinolic acid, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions for 5 l of test solution and standard solution. The peak of fukinolic acid is found in the HPLC chromatogram of test solution.

   <HPLC condition>
   Column : capcellpak C18 (4.6 mm × 250 mm)
   Mobile phase: Solvent A = Citric acid solution (citric acidmonohydrate 2.1 g L⁻¹)
   Solvent B = Methanol
   Condition of gradient
   
<table>
<thead>
<tr>
<th>time(min)</th>
<th>0</th>
<th>19</th>
<th>25</th>
<th>35</th>
<th>50</th>
<th>60</th>
<th>61</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>100% A</td>
<td>100% A</td>
<td>80%A</td>
<td>80%A</td>
<td>60%A</td>
<td>60%A</td>
<td>100%B</td>
<td>100% B</td>
</tr>
<tr>
<td>Flow rate :</td>
<td>1.0 ml/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Detector :</td>
<td>UV 324 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Column Temperature :</td>
<td>30 °</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

   (2) Fukinone
   This product (2.0g) in a centrifugation tube is suspended in purified water (10 ml), and treated with ultrasonic wave for 1 minute. Ethyl acetate (10 ml) is added to the suspension, and the tube is shaken for 3 minutes. Then the tube is centrifuged (room temperature, 3000 rpm, 10 minutes), and an ethyl acetate layer is collected (repeat this procedure three times). The collected ethyl acetate layer is evaporated and is dissolved in 20% acetonitrile (3 ml), and is filtered through a Sep-Pak C18 cartridge. Then, the cartridge is washed with 70% acetonitrile (7 ml) and is eluted with absolute acetonitrile (5 ml). This solution is adjusted to 10 ml, and filtered through a 0.45 µm PTFE filter (test solution). For preparation of standard solution, acetonitrile is added to fukinone, and the concentration is prepared 0.1 mg/ml (standard solution). HPLC analysis is performed according to the following conditions.
conditions for 5 1 of test solution and standard solution. The peak of fukinone is found in the HPLC chromatogram of test solution.

HPLC condition:
Column: Shim-pack CLC-ODS (6.0 mm x 150 mm)
Mobile phase: acetonitrile:water = 8:2
Flow rate: 1.0 ml/min
Detector: UV 254 nm
Column Temperature: 30°C

3. Total polyphenols content
Max. 1.0 % (Folin-Denis method)

4. Loss on Drying
Max. 10.0 % (1g, 105°C, 2h)

5. Purity Test
(1) Heavy Metals
Max. 10 ppm (The Second Method)

(2) Arsenic
Max. 1 ppm (The Third Method)

7. Standard Plate Counts
Max. 1 x 10^2 cfu/g (Analysis for Hygienic Chemists)

8. Moulds and Yeasts
Max. 1 x 10^2 cfu/g (Analysis for Hygienic Chemists)

9. Coliforms
Negative (Analysis for Hygienic Chemists)

10. Composition

<table>
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<tr>
<td>Dextrin</td>
<td>80 %</td>
</tr>
<tr>
<td>Petasites Japonicus Leaf/Stem Extract</td>
<td>20 %</td>
</tr>
<tr>
<td>Total</td>
<td>100 %</td>
</tr>
</tbody>
</table>

PRODUCT STANDARD
PRODUCT NAME

JAPANESE BUTTERBUR EXTRACT-LC
COSMETIC

This product is extracted from Japanese butterbur, the stems and the leaves of Petasites japonicus (Compositae), with aqueous 1,3-butyleneglycol.

1. Appearance
Brown liquid with scentless or slightly unique smell.

2. Certification Test
Mix this product (30 ml) with water (3.5 ml), and Folin-Denis reagent (0.2 ml) and saturated Na$_2$CO$_3$ solution (0.4 ml) are added. The solution reveals blue color. (Polyphenols)

3. Purity Test
(1) Heavy Metals
Max. 10 ppm (The Second Method)

(2) Arsenic
Max. 1 ppm (The Third Method)

4. Standard Plate Counts
Max. $1 \times 10^2$ cfu/g (Analysis for Hygienic Chemists)

5. Moulds and Yeasts
Max. $1 \times 10^2$ cfu/g (Analysis for Hygienic Chemists)

6. Coliforms
Negative (Analysis for Hygienic Chemists)

7. Composition

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Contents</th>
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<tbody>
<tr>
<td>Butylene Glycol</td>
<td>69 %</td>
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<tr>
<td>Water</td>
<td>30 %</td>
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<tr>
<td>Petasites Japonicus Leaf/Stem Extract</td>
<td>1 %</td>
</tr>
<tr>
<td>Total</td>
<td>100 %</td>
</tr>
</tbody>
</table>

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From product planning to OEM – For any additional information or assistance, please contact:

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