

CHINESE CHIVE SEED EXTRACT

Food and cosmetic ingredients with tonics, aphrodisiac, anti-fatigue, anti-ageing and immune boosting properties Improving lumbago, coldness, enuresis and frequent micturition

- CHINESE CHIVE SEED EXTRACT—P
 (Powder, Food Grade)
- CHINESE CHIVE SEED EXTRACT—WSP
 (Water—soluble Powder, Food Grade)
- CHINESE CHIVE SEED EXTRACT—PC (Powder, Cosmetic Grade)
- CHINESE CHIVE SEED EXTRACT—WSPC (Water—soluble Powder, Food Grade)
- CHINESE CHIVE SEED EXTRACT-LC (Liquid, Cosmetic Grade)
- CHINESE CHIVE SEED OIL
 (0il, Food Grade)



ORYZA OIL & FAT CHEMICAL CO., LTD.

ver . 13.0 YF



CHINESE CHIVE SEED EXTRACT

Health Ingredients with tonics, aphrodisiac, anti-fatigue, anti-aging and immune boosting properties

1. Introduction

[Chinese Chive]

Chinese chive (*Allium Tuberosum*) is an alliaceous perennial green vegetable of the liliaceae family. It is a bulb forming perennial plant with short rootstocks and branches into leaves (Fig. 1). Its leaves are long and flat with a common length of 20 to 30 cm and fascicular. Chinese chive usually blossoms from August through October with 20 to 40 small white flowers developing a hemispheroidal umbelliform. There are six stamens and three ovaries. The seeds are mature and produced in autumn (Fig. 2).

Chinese chive is believed to originate from the western region of China. It is resistant to both intense heat and cold weather and grows in a wide area in West Asia, India, South East Asia, East Asia, and Siberia.

Chinese chive is known as "Nira" in Japan, originated from the names "Kamira" (Kojiki, the Record of Ancient Matters, the oldest surviving book in Japan, 712), "Komira" (Honso-wamyo,



Fig.1 Whole plant of Chinese chive

an encyclopedia of Materia Medica in Japan, 918), and "Kukumira" (Manyou-syu, Collection of Ten Thousand Leaves, the oldest existing, and most highly revered, collection of Japanese poetry). Garlic was referred to as "Omira" while Chinese chive as "Komira" in Japanese. These names were further simplified to "Mira" over time and eventually known as "nira" which implies delicious taste. Nira had become the popular name for Chinese chive in the "Wakan-sansai-zue", a Japanese encyclopedia (1712).

[History of Chinese Chive as Food]

Chinese chive has been cultivated and eaten as vegetable in China for over 3,000 years. According to "Si Min Yue Ling", a book on agriculture and human's health written during the late *Han* dynasty, it is described that both leaves and flowers of Chinese chive are edible. It was then introduced to Japan during the *Yayoi* Period (300 BC to 300 AD). However, this has been unclear as some said that Chinese chive grew wild in Japan. A record indicated that Chinese chive had been cultivated in the 9th century (*Heian* Period). It was believed that Chinese chive was initially used as drug and mixed with rice gruel for consumption. In the *Edo* Period (1600-1867), according to





Fig.2 Ripe flowers and seeds

the "Compendium of Agriculture," a systematic agricultural technical book, the agronomist Antei Miyazaki described that Chinese chive was a well-known vegetable and commonly consumed by human. He also mentioned that Chinese chive improves general health and well-being due to its nutrients and warming effects on the body.

On the other hand, Chinese chive has not been cultivated or consumed in the western countries due to its distinctive garlic smell.

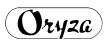
In Japan, Chinese chive has been commonly used for a long time in spite of the low consumption. In late 1960's, the consumption of Chinese chive increased dramatically upon change of human diet. Chinese chive is now produced mainly in Kochi, Chiba, and Tochigi prefectures of Japan.

Chinese chive is rich in carotene, vitamins (e.g. Bs, C, and E) and sulfuric compounds such as allyl sulfide contained in onion and garlic that contributes to its distinct smell. The allyl sulfide is antibacterial. It enhances absorption of vitamin B1 and promotes recovery from strenuous activity. Latest findings revealed that allyl sulfide is preventive against skin cancer, lung cancer, liver cancer, cancer of the large intestine and blood clots. With all its benefits, Chinese chive has been introduced into daily diet as healthy vegetable with stamina-enhancing properties.

[Chinese Chive and Chinese Herbal Medicine]

Chinese chive has been using as crude drug since 770 B.C.. Chinese chive seed was referred to as "Kyushi", "Kyusaishi" or "Kyusaijin" as a crude drug. Meanwhile, the functional effects of Chinese chive seed were documented in the vegetable section of the book "Ben Cao Gang Mu" written by Li Shi Zhen during the Ming dynasty, a famous encyclopedia of Traditional Chinese Materia Medica. According to the description, Chinese chive seed is "effective for nocturnal emission and hematuria," "warming the back and knees to cure lumbago, swelling and pain in knees, very efficacious for curing the symptom of sexual intercourse in dream", "replenishing liver and Mingmen to cure frequent micturition, enuresis and white vaginal discharge (leukorrhea) in woman".

Chinese chive seed has been used as traditional treatment from ancient time for lack of energy, lumbago, frequent urination and dribbling after urination. Considering the relationship between the symptoms described by traditional Chinese medicine and those described by the modern medicine, Chinese chive seed is expected to enhance general



health, aphrodisiac, relieve fatigue, prevent aging, and relieve symptoms of benign prostate hyperplasia (BPH). It is believed that Chinese chive seed will be synergistic with saw palmetto, sterol and pumpkin in the prevention of BPH.

Oryza Oil & Chemical Co., Ltd. prompted research and studies on Chinese Chive Seed Extract. Results revealed the revitalizing effect of Chinese Chive Seed Extract as potent aphrodisiac, energy enhancer, immune booster and anti-ageing. Research showed that Chinese Chive Seed Extract increases male blood testosterone suitable as an aphrodisiac while maintaining healthy level of neurotransmitters in response to stressful events. Oryza Oil & Chemical Co., Ltd. successfully commercialized the production of Chinese Chive Seed Extract as new generation health revitalizer for improving general health and well-being physically and emotionally.

2. Functional Components of Chinese Chive Seed Extract

S-allyl-L-cysteine and S-1-propenyl-L-cysteine are two important functional components identified in Chinese Chive Seed Extract. Other active components identified include saponins and ceramide(Fig. 3). These components have been reported to be neuroprotective^{1,2)}, aphrodisiac^{3,4)}, and cancer preventive agents^{5,6,7)}, repectively.

The seed of Chinese chive contains the highest amount of S-allyl-L-cysteine upon measurement and comparison with the leaf and flower of the plant (Fig. 4).

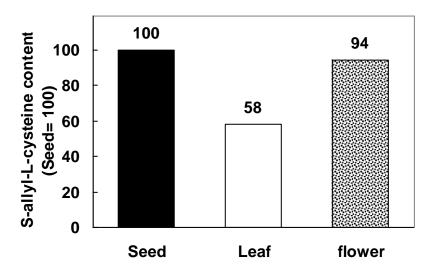


Fig. 4 The Contents of S-allyl-L-cysteine in different parts of Chinese chive plant.



Fig. 3 Chemical structures of functional components in Chinese Chive Seed Extract.

References:

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3. Physiological Function of Chinese Chive Seed Extract

a. Aphrodisiac

i. Increase Mating Frequency in Mice (in vivo)

The effect of Chinese Chive Seed Extract on mice's sexual performance was examined as illustrated in the picture on the right. Results revealed Chinese Chive Seed Extract increases the frequency of mating in mice (Fig. 5). In addition, time required to initiate mating is reduced in group treated with Chinese Chive Seed Extract (Fig. 6). Furthermore, blood samples of mice were collected for determination of blood testosterone concentration. As show in Fig. 7, blood testosterone



concentration was higher in group of mice treated with Chinese Chive Seed Extract.

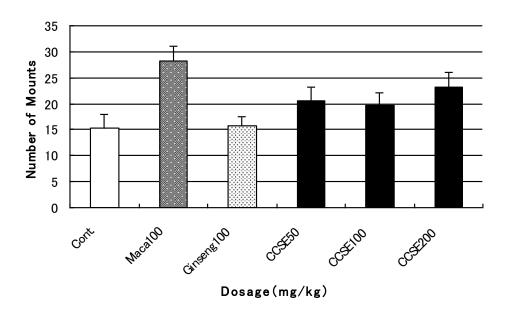


Fig. 5 The effect of Chinese Chive Seed Extract (CCSE) on mice mating frequency. (mean \pm S.E., n=5-12.)

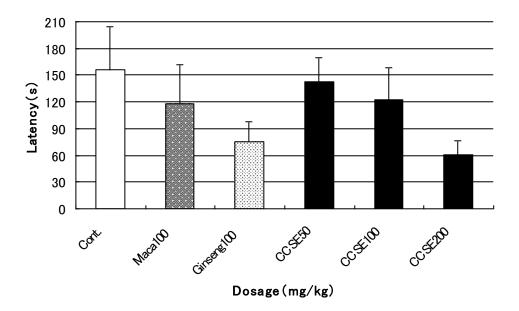


Fig. 6 The effect of Chinese Chive Seed Extract (CCSE) on latency time of mating in mice. (mean \pm S.E., n=5-12).

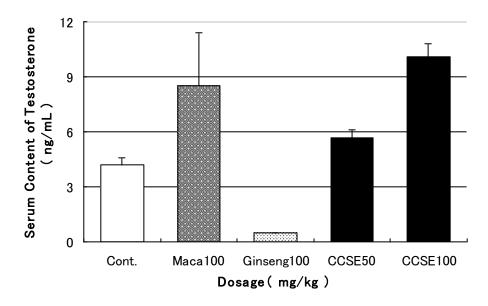


Fig. 7 The effect of Chinese Chive Seed Extract (CCSE) on blood testosterone concentration in mice. (mean \pm S.E., n=5-12).

The results indicate that Chinese Chive Seed Extract is aphrodisiac and enhances functions of reproductive system. In the meantime, the results also revealed that Chinese Chive Seed Extract is as effective as maca as an aphrodisiac while is more potent than that of the commonly known Panax Ginseng upon comparison.

[Test Method]



Mice (ddy, male & female, 6 months old) are separated into 6 groups, namely:

- i. Control (12 male & female mice, respectively)
- ii. Maca 100 (group treated with commercial Maca extract 100mg/kg, 6 male & female mice, respectively)
- iii. Ginseng 100 (group treated with commercial Ginseng extract 100mg/kg, 6 male & female mice, respectively)
- iv. CCSE 50 (group treated with Chinese Chive Seed Extract 50mg/kg, 12 male & female mice, respectively)
- v. CCSE 100 (group treated with Chinese Chive Seed Extract 100mg/kg, 12 male & female mice, respectively)
- vi. CCSE 200 (group treated with Chinese Chive Seed Extract 200mg/kg, 12 male & female mice, respectively)

Mice were housed in 12:12 of light:dark cycle (light phase: 08:00-20:00; dark phase: 20:00-08:00) environment with feed and water ad libitum. Test samples of the above extracts were given to mice orally on daily basis for 3 weeks. Experiments on mating of mice started 1-hour post administration of sample on day-21 (the last day of ingestion period). Male mice were then habituated in the test cage for 1 hour followed by mounting test for 30min with introduction of female mice. Tests were conditioned with red light on during dark cycle, starting 2 hours after light cycle. Time required to initiate mating (latency) and the number of mounts were recorded to evaluate the effects of test samples on mice sexual responses. Upon completion of mating tests, blood samples of mice were collected for determination of blood testosterone concentrations using Testosterone kit.

b. Anti-fatigue Activity (Mental, Physical)

<u>i. The Effect of Chinese Chive Seed Extract on Reserpine-induced Mental Fatigue Mice Model (in vivo)</u>

The effect of Chinese Chive Seed Extract on reserpine-induced mental fatigue was studied using mice model. Reserpine is an indole alkaloid antipsychotic antihypertensive drug that irreversibly binds to storage vesicles neurotransmitter such as dopamine (DA), norepinephrine (NE) and serotonin (5-HT). Reserpine-induced depletion neurotransmitters causes subsequent depression in humans. This experimental model was simulated by sub-cutaneous injection of reserpine in mice to induce mental stress and fatigue followed by a 6-minute swimming forced test illustrated on the right. Duration of immobile period was recorded and





compared.

As illustrated in Fig. 8, results showed that immobile period was shorter in groups of mice treated with Chinese Chive Seed Extract 100mg/kg and 200mg/kg, respectively. In addition, quantitative analysis of brain neurotransmitters and its metabolites showed that Chinese Chive Seed Extract promotes recovery of neurotransmitters and its metabolites in reserpine-induced stressed model (Table. 1). Similarly, the ratio of neurotransmitters / metabolites returned to normal levels in groups treated with Chinese Chive Seed Extract (Table. 2). Therefore, Chinese Chive Seed Extract promotes prompt recovery of mental stress and maintains healthy levels of neurotransmitters during stressful condition.

[Test Method]

30 mice (ddy, male, 5-week old) were separated into 5 groups, namely:

- i. Control group (without reserpine[Res -])
- ii. Reserpine group (treated with reserpine [Res+])
- iii. [Res+] and CCSE 50 (treated with [Res+] and Chinese Chive Seed Extract 50mg/kg) iv. [Res+] and CCSE 100 (treated with [Res+] and Chinese Chive Seed Extract 100mg/kg)
- v. [Res+] and CCSE 200 (treated with [Res+] and Chinese Chive Seed Extract 200mg/kg)

Mice were given the above test samples accordingly for 1 week. On day-6, one hour post administration of test samples, reserpine (1.5mg/kg) was given to mice (except those in control group) subcutaneously to induce stress and fatigue condition. 24-hour later, mice underwent a 6-minute forced swimming test. Duration of immobile period was recorded and compared. Cerebra of mice were removed for the study and analysis of brain neurotransmitters and its metabolites using HPLC-electrochemical detector.

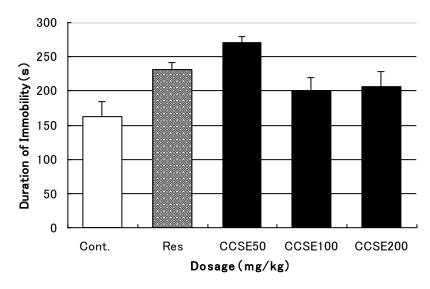


Fig. 8 The effect of Chinese Chive Seed Extract (CCSE) on reserpine-induced fatigue in mice (CCSE). Res: reserpine. (mean \pm S.E., n=6).

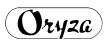


Table 1	The effect of Chinese Chives Seeds Extract (CCSE) on brain neurotransmitters
in reserp	ine-induced fatigue model in mice.

Croun	Contents of	Neurotransmitte	ers and Their M	letabolites (ng/	g Wet Brain Tis	sue Weight)
Group -	NE	DA	DOPAC	HVA	5-HT	5-HIAA
Cont.	224.2 ± 13.3	654.8 ± 25.6	89.4 ± 7.2	130.7 ± 4.3	317.8 ± 12.3	193.8 ± 7.9
Res	7.5 ± 2.5	53.7 ± 3.0	157.3 ± 12.0	260.3 ± 15.6	47.8 ± 3.4	336.8 ± 18.8
Res+ CCSE50	10.2±3.6	55.8±4.3	147.6 ± 12.1	249.5 ± 19.3	46.0 ± 1.8	296.9 ± 12.9
Res+ CCSE100	12.2 ± 2.2	57.2±7.8	142.5 ± 7.3	293.8 ± 14.4	52.7±6.7	298.7 ± 22.3
Res+ CCSE200	20.7±5.7	72.6±10.5	148.1 ± 7.0	291.5 ± 17.9	55.7±4.4	319.4 ± 13.2

Note: NE: norepinephrine, DA: dopamine, DOPAC and HVA: metabolites of dopamine, 5-HT: serotonin, 5-HIAA: metabolite of serotonin. (mean ± S.E., n=6).

Table 2 Recovery of the ratios of neurotransmitters/metabolites in reserpine-induced fatigue mice.

Group	DA/HVA	DA/DOPAC	5-HT/5-HIAA
Cont.	5.01	7.32	1.64
Res	0.21	0.34	0.14
Res+ CCSE50	0.22	0.38	0.15
Res+ CCSE100	0.19	0.40	0.18
Res+ CCSE200	0.25	0.49	0.17

ii. The Effect of Chinese Chive Seed Extract on Energy Production in L6 Muscle Cells (in vitro)

Fig. 9 illustrated the diagram of cell metabolism and energy production cascade. The following elements are involved in the energy production cascade:

1. Carnitine Palmitoyltransferase (CPT)

Metabolism of fatty acids takes place in cell mitochondria in a process known as beta-oxidation where energy is produced. Enzyme CPT catalyzes beta-oxidation by increasing the transport of fatty acids into inner mitochondria for energy production.

2. Acyl-CoA Oxidase (ACOX)

In subcellular level, beta-oxidation takes place in peroxisome where ACOX is responsible for the transport of fatty acids into peroxisome for energy production.

3. Cyclic AMP-dependent Protein Kinase (AMPK)

AMPK induces a cascade of events within cells in response to the ever changing energy charge of the cell. The role of AMPK in regulating cellular energy charge places



this enzyme at a central control point in maintaining energy homeostasis. Once activated, AMPK-mediated phosphorylation events switch cells to active ATP production (e.g. fatty acid and glucose oxidation).

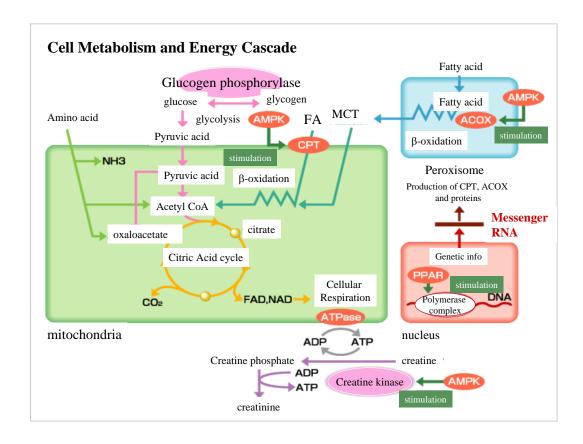


Fig.9 Energy production pathways in cells.

4. Glycogen Phosphorylase

Muscle cell glycogen appears to function as an immediate reserve source of available glucose for muscle cells. Glycogen is cleaved from the nonreducing ends of the chain by the enzyme glycogen phosphorylase for energy production. Glycogen Phosphorylase enhances energy supply of muscle cells.

5. Creatine Kinase

Creatine kinase (CK), also known as phosphocreatine kinase or creatine phosphokinase (CPK) is an enzyme that catalyzes the conversion of creatine to phosphocreatine. In tissues that consume ATP rapidly, especially skeletal muscle, but also brain and smooth muscle, phosphocreatine serves as an energy reservoir for the rapid regeneration of ATP, the major source of energy in biochemical reactions.

Polymerase chain reaction (PCR) is a biochemistry and molecular biology technique for enzymatically replicating DNA without using a living organism, such as $E.\ coli$ or



yeast. Researchers have used traditional PCR as a way to estimate changes in the amount of a gene's expression. When the products of the PCR process are run on agarose gel, a band corresponding to a gene will appear larger on the gel (note that the band remains in the same location relative to the ladder) it will just appear fatter or brighter.

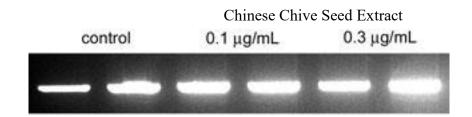


Fig.10 Typical electrophoresis image of AMPK gene expression on 2% agrose gel. Chinese Chive Seed Extract clearly enhanced the AMPK gene expression at concentrations of 0.1 μ g/mL and 0.3 μ g/mL in L6 cells when compared with control.

As confirmed by PCR (Fig. 11), Chinese Chive Seed Extract enhanced gene expression of CPT, ACOX-1, AMPK, Glycogen Phosphorylase & Creatine Kinase, which are important enzymes involved in the energy production cascade. Chinese Chives Seed Extract promotes energy production by activating the cell metabolism while preventing physical fatigue and tiredness.

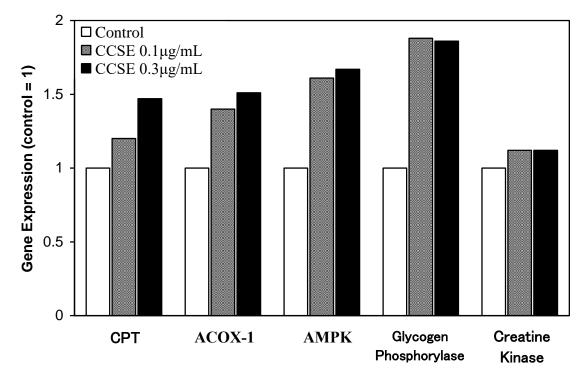


Fig.11 The effect of Chinese Chive Seed Extract (CCSE) on genetic expression of enzymes CPT, ACOX-1, AMPK, Glycogen Phosphorylase & Creatine Kinase in L6 cell line.



[Test Method]

Rat's L6 skeletal muscle myoblasts were cultured in α-MEM medium containing 2% FBS for 8 days. Chinese Chive Seed Extract solution of different concentrations in DMSO was added to the differentiated myoblast cells and continue culture for 24-hour. Myoblast cells were then broken down for RNA fraction using RNA extracting kit (RNeasy Micro Kit, QIAGEN). Complementary DNA (cDNA) was synthesized using reverse transcriptase (SuperScript III, Invitrogen) from RNA. Gene Expression was determined by PCR (polymerase chain reaction).

<u>iii. The Effect of Chinese Chive Seed Extract on Endurance Performance in Mice</u> (in vivo)

Further experiment was prompted to evaluate the effect of Chinese Chive Seed Extract on physical endurance using mice model. Mice were forced to perform swimming tests with an extra burden of 10% body weight. Total time required for mice's head to be completely immersed underneath water for the first 5 seconds was recorded.

Results revealed that 2-weeks' oral administration of Chinese Chive Seed Extract prolonged swimming time in mice. Swimming time increased with increasing concentration of Chinese Chive Seed Extract. Upon comparison with Panax Ginseng, swimming time in mice treated with Chinese Chive Seed Extract was longer. Therefore, Chinese Chive Seed Extract enhances endurance performance and prevents tiredness.

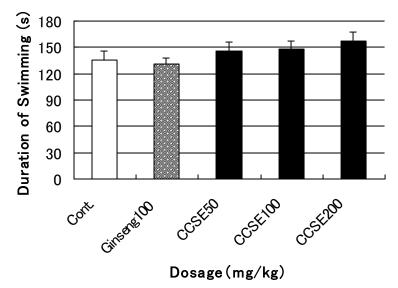


Fig.12 The Effect of Chinese Chive Seed Extract (CCSE) on endurance performance of mice in forced swimming test . (mean \pm S.E., n=7).

[Test Method]

- 35 Mice (ddY, male, 6-week old) were divided into 5 groups, namely:
- i. Control group
- ii. Ginseng 100 (group treated with commercial Ginseng Extract 100mg/kg)
- iii. CCSE 50 (group treated with Chinese Chive Seed Extract 50mg/kg)



iv. CCSE 100 (group treated with Chinese Chive Seed Extract 100mg/kg) v. CCSE 200 (group treated with Chinese Chive Seed Extract 200mg/kg)

Mice were given the above test samples accordingly daily for 2 weeks. On day-14, mice were loaded with extra burden of 10% body weight to perform forced swimming test. Total time required for mice's head to be completely immersed underneath water for the first 5 seconds was recorded as swimming time.

iv. The Effect of Chinese Chive Seed Extract on Spontaneous Movement in Mice (in vivo)

Further *in vivo* experiment was conducted to evaluate the effect of Chinese Chive Seed Extract on spontaneous movement in mice. As illustrated in Fig. 13 & 14, Chinese Chive Seed Extract demonstrated concentration-dependent increased in distance displaced and active time in mice in response to spontaneous locomotor movement. On the other hand, mice treated with caffeine shown restless behavior due to the stimulating effect of caffeine. Chinese Chive Seed Extract enhances spontaneous movement in mice without stimulating effect.

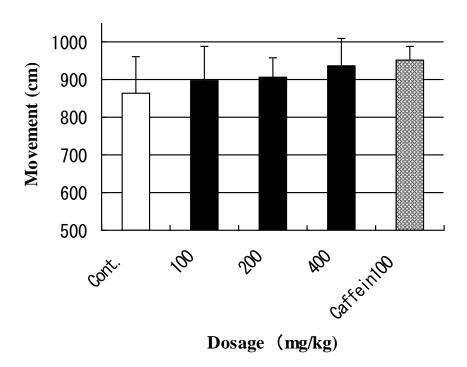


Fig.13 The effect of Chinese Chive Seed Extract on mice's movement distance in locomotor activity determination. (mean \pm S.E., n=5-7).



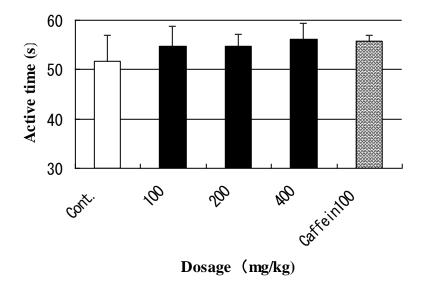


Fig.14 The effect of Chinese Chive Seed Extract on mice's active time in locomotor activity determination. (mean \pm S.E., n=5-7).

[Test Method]

Mice (ddY, male, 6-week old) were divided into 5 groups, namely:

- i. Control group
- ii. CCSE 100 (group treated with Chinese Chive Seed Extract 100mg/kg)
- iii. CCSE 200 (group treated with Chinese Chive Seed Extract 200mg/kg)
- iv. CCSE 400 (group treated with Chinese Chive Seed Extract 400mg/kg)
- v. Caffeine 100 (group treated with Caffeine 100mg/kg)

Mice were given the above test samples accordingly orally after overnight fasting. Mice were then relocated into a carton box (22cm x 26cm) 1-hour post administration of sample. Spontaneous activity of mice (distance displaced, active time) was video recorded and analyzed by a professional analysis software program for locomotor activity. Distance displaced (cm) and active time (sec) was calculated as spontaneous activity indexes.

c. Anti-ageing Effect

i. Prevention against Lipid-peroxidation in Liver of Old-aged Mice (in vivo)

The effect of Chinese Chive Seed Extract on lipid peroxidation in the liver of the ageing mice was studied. Liver MDA-value (malondialdehyde) was measured as an indication of the liver susceptibility to oxidative stress. As illustrated in Fig. 15, Liver MDA reduces with increasing concentration of Chinese Chive Seed Extract. Upon comparison, potency of Chinese Chive Seed Extract is stronger than that of Panax Ginseng. Chinese Chive Seed Extract is preventive against lipid-peroxidation, thus, anti-ageing.

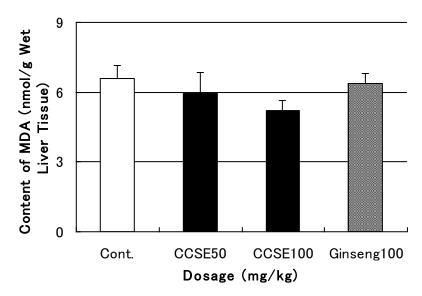


Fig.15 The effect of Chinese Chive Seed Extract on lipid peroxidation product (malondialdehyde: MDA) in the liver of ageing mice. (mean \pm S.E., n=6).

[Test Method]

Mice (ddY, male, 8-months old) were divided into 4 groups, namely:

- i. Control group
- ii. Ginseng 100 (group treated with Ginseng 100mg/kg)
- iii. CCSE 50 (group treated with Chinese Chive Seed Extract 50mg/kg)
- iv. CCSE 100 (group treated with Chinese Chive Seed Extract 100mg/kg)

Mice were given the above test samples accordingly daily for 4 weeks. Then, livers were removed for wet liver MDA measurement using thiobarbituric acid (TBA) method. In which, 10x of liver weight of 0.15M KCl was added for homogenization. To 0.5 mL of the Homogenate, 0.1M phosphoric acid (3mL) and 0.04M TBA (1mL) were added and heated at 100°C for 30 minutes. The reaction mixture was cooled with running tap water and then n-butanol (4mL) was added to extract the TBA-reactive substances. After centrifugation at 3,000rpm at 4°C for 10 minutes, supernatant layer was collected for absorbance measurement at 535nm.

d. Immune Boosting Effect

i. The Effect of Chinese Chive Seed Extract on Mice Phagocytic Activity (in vivo)

The effect of Chinese Chive Seed Extract on the immune system was evaluated. Phagocytic activity of mice was studied by injecting Indian ink (foreign substance) via the caudal vein of mice. The rate of removing the foreign substance from blood (K value, phagocytic index) was calculated and the weight of immune organs, spleen and thymus, was measured for the evaluation on mice's immune function.

As illustrated in Fig. 16, the phagocytic rate was greater in group of mice treated with Chinese Chive Seed Extract. In addition, weight of spleen and thymus of mice increased



after treatment with Chinese Chive Seed Extract (Table 3). The results indicate the immune boosting effect of the Chinese Chive Seed Extract.

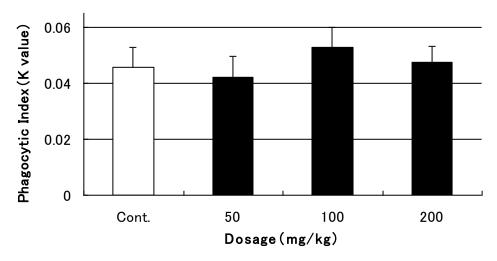


Fig.16 The Effect of Chinese Chive Seed Extract (CCSE) on phagocytosis in mice. (mean \pm S.E., n=10).

Table 3 The Effect of Chinese Chive Seed Extract (CCSE) on weight of immune organs, spleen and thymus. (mean \pm S.E., n=10).

Group (mg/kg	g) Body Weight (g) —	Weight (mg/10	ng/10g Body Weight)	
Group (mg/ kg	;) body Weight (g) —	Spleen	Thymus	
Cont.	29.9±1.7	43.8±1.9	24.9 ± 1.6	
CCSE50	28.5 ± 1.4	42.1 ± 2.3	30.8 ± 1.8	
CCSE100	29.2 ± 1.6	40.2 ± 1.7	29.2 ± 2.2	
CCSE200	29.5 ± 1.3	46.4 ± 2.2	29.7±2.5	

[Test Method]

40 mice (ddY, male & female, 4-week old) were separated into 4 groups, namely:

- i. Control group
- ii. CCSE 50 (group treated with Chinese Chive Seed Extract 50mg/kg)
- iii. CCSE 100 (group treated with Chinese Chive Seed Extract 100mg/kg)
- iv. CCSE 200 (group treated with Chinese Chive Seed Extract 200mg/kg)

Mice were given the above test samples accordingly daily for 1 week. On day-7, one hour post administration of sample, intravenous injection of Indian Ink (10mL/kg) was given to mice via caudal vein. At the thirtieth sec and the sixth min, $20~\mu\text{L}$ blood samples were taken from eye socket vein respectively and immediately put it into 2 mL of 0.1% Na₂CO₃ solution. The absorbance at 575 nm was measured. Phagocytic index was calculated as follows:

Phagocytic Index = (lgA1-lgA2) / (T2-T1)



lg: Logarithm, A1: Absorbance measured at 30 seconds post injection, A2: Absorbance measured 6 minutes post injection, T1: the first blood taken time post injection, i.e. 30 seconds, T2: the second blood taken time post injection, i.e. 6 minutes.

Spleen and thymus of mice were removed after blood collection mentioned above for weight measurement. Weights of spleen and thymus were indicated in mg/10g of body weight.

e. Skin Whitening Effect

B16 melanoma cells were used to study the effect of Chinese Chive Seed Extract on skin *in vitro*. As shown in Fig.17, Chinese Chive Seed Extract demonstrated a concentration-dependent inhibition on melanin production at concentration range of 0.1 μ g/mL to 100 μ g/mL. Consequently, Chinese Chive Seed Extract protects the skin against hyperpigmentation.

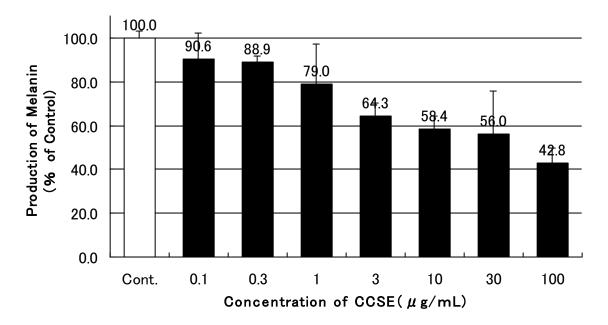


Fig.17 The Effect of Chinese Chive Seed Extract (CCSE) on melanin production in mouse B16 melanoma cells.

[Test Method]

B16 melanoma cells were cultured in α -MEM medium containing theophylline ($5x10^4$ cells/mL) with 200μ L each in a 48-well cell plate. Sample solutions of Chinese Chive Seed Extract were added to cells and continue culture for 3 days. Cells were crushed by ultrasound upon removal of culture medium. Absorbance was determined at wavelength 415nm.

f. Human Trial – The Effect of Chinese Chive Seed Extract on Blood Profile of Volunteer Subjects



Small-scale human trial was conducted on eight male volunteer subjects (aged 31-65) to examine the effect of Chinese Chive Seed Extract-P on blood profile. Blood samples were collected for blood profile analysis before and after ingestion of Chinese Chive Seed Extract-P (100 mg/day) for 28 days. As shown in Table 4, a slight decrease in lactic acid and ACTH (adrenocorticotropic hormone) level and a significant decrease in cortisol level, while a significant increase in noradrenalin level were observed (Fig. 18). Abnormal changes of the above parameters of the blood may cause the following symptoms as summarized in table 5.

Table 4 Comparison of blood parameters before and after treatment of Chinese Chive Seed Extract-P (100 mg/day) for 28 days in volunteer subjects.

	Before	After	Normal
Total cholesterols (mg/dL)	199.1 ± 33.7	193.3 ± 30.6	130~219
Triglycerides (mg/dL)	84.0 ± 34.7	95.3 ± 54.9	30~149
Phospholipids (mg/dL)	208.8 ± 30.4	205.8 ± 29.0	150~260
Free fat acids (mEq/L)	0.43 ± 0.16	0.44 ± 0.08	0.13~0.77
Creatinine (mg/dL)	0.78 ± 0.07	0.75 ± 0.04	0.61~1.04
Ketone (µmol/L)	28.1 ± 12.2	32.9 ± 25.7	Less than 76
Lactic acid (mg/dL)	9.9 ± 3.4	8.5 ± 3.0	3~17
Pyruvic acid (mg/dL)	0.89 ± 0.27	0.83 ± 0.25	0.30~0.95
ACTH (pg/mL)	36.4 ± 13.9	31.3 ± 14.0	7∼56
Adrenaline (ng/mL)	0.045 ± 0.019	0.046 ± 0.021	Less than 0.10
Noradrenaline (ng/mL)	0.146 ± 0.05	$0.188 \pm 0.041^{p < 0.05}$	0.07~0.31
Dopamine (ng/mL)	Less than 0.03	Less than 0.03	Less than 0.1
Cortisol (µg/dL)	12.6 ± 2.8	$10.8 \pm 2.8^{p < 0.01}$	6.2~19.4

Note: mean \pm SD, n=8.

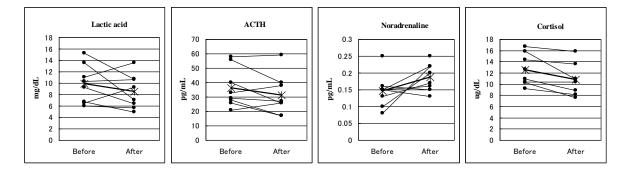


Fig.18 The Effect of Chinese Chive Seed Extract-P (100mg/day) on blood levels of lactic acid, ACTH, cortisol and noradrenaline in volunteer subjects after ingestion of Chinese Chive Seed Extract-P (100 mg/day) for 28 days. *: Average.



Table 5	Symptoms	related	to	abnormal	changes	on	blood	lactic	acid,	ACTH,
cortisol a	nd noradrena	aline.								

Parameters	Abnormal changes	Symptoms
Lactic acid	↑	Muscle fatigue
ACTH	^	Excessive secretion of cortisol
Cortisol	↑	Reduced resistance to infection and stress, feeling of fatigue, depression
Noradrenaline	•	Lack of energy, indifference, depression

Cortisol is the most potent glucocorticoid produced by the adrenal cortex. It is synthesized from cholesterol and its production is stimulated by pituitary adrenocorticotropic hormone (ACTH) which is regulated by corticotropin releasing hormone (CRH) (Fig. 19).

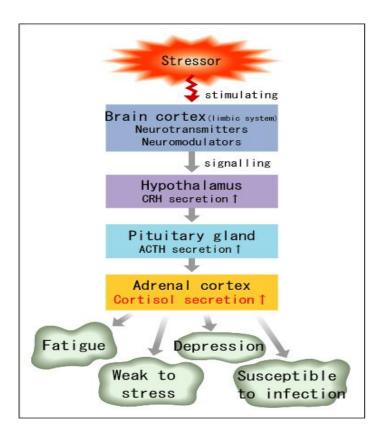
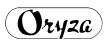


Fig. 19 Symptoms caused by stress-induced excessive secretion of cortisol.



Cortisol has been termed "the stress hormone" because it is secreted in higher levels during the body's 'fight or flight' response to stress, and is responsible for several stress-related changes in the body. Higher and more prolonged levels of cortisol in the bloodstream (like those associated with chronic stress) have been shown to cause many problems of the body, such as susceptible to infection and stress, lowered immunity, tiredness, depression and impaired cognitive performance. In recent years, there is an increasing demand for measures to promote recovery from exhaustion and stress via regulation of cortisol secretion as well as ACTH release. As revealed in the human trial, Chinese Chive Seed Extract can be expected to be suitable for this purpose.

Noradrenaline is released from the medulla of adrenal glands as a hormone into the blood and as neurotransmitter in the nervous system. Noradrenaline is also known as stress hormone or "the anger hormone" as it affects parts of the human brain where attention and responding actions are controlled, and along with epinephrine, it underlies the "fight or flight" response. It is usually released when a host of physiological changes are activated by stressful event. Noradrenalin is also essential for the transfer of information from short-term memory stored in the hippocampus to long-term memory stored in the cerebral neocortex.

Noradrenaline plays an important role in attention, focus and depression. The biological basis for depression is associated with deficiency in serotonin or noradrenaline, or both. Most anti-depressants act by increasing the amount of serotonin and noradrenaline available to postsynaptic cells in the brain. Similarly, the human trial with Chinese Chive Seed Extract revealed that noradrenaline level was raised towards a higher level within the normal range suitable for the maintenance of healthy mood and prevention against stress related depression.

In vitro and *in vivo* experiments of Chinese Chive Seed Extract demonstrated positive anti-fatigue and endurance enhancing activities. Subjective experiments conducted with questionnaire revealed that oral treatment of Chinese Chive Seed Extract reduces tiredness, improves morning erection and promotes physical health (Fig. 20).

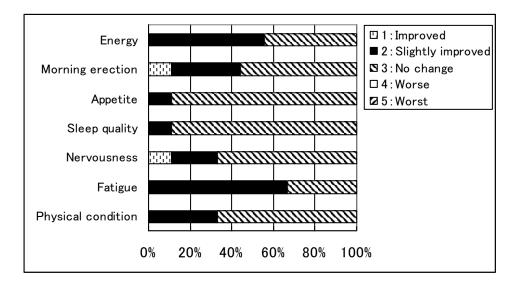


Fig.20 Subjective response on the effect of ingestion of Chinese Chive Seed Extract-P.



4. Stability of Chinese Chive Seed Extract

(I) Thermostability

Result of thermostability test on Chinese Chive Seed Extract (pure extract) revealed that its active component, S-allyl-L-cysteine remained stable at normal food processing temperature 100°C and 120°C for 1 hour (Fig. 21).

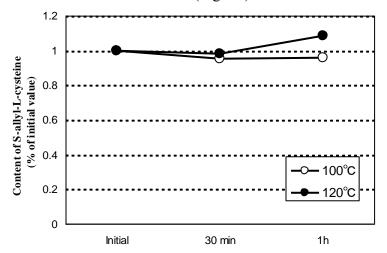


Fig.21 Thermostability of Chinese Chive Seed Extract (initial value=1).

(II) pH stability

Chinese Chive Seed Extract-WSP was used for pH stability test. Solution of Chinese Chive Seed Extract-WSP was prepared and adjusted to the required pH level for storage at room temperature for 1 day and 1 week, respectively. Result showed that content of S-allyl-L-cysteine, active component of Chinese Chive Seed Extract remained stable at normal pH and acidic condition. However, content of S-allyl-L-cysteine was degraded by approximately 10% under alkaline condition after 1 week (Fig. 23).

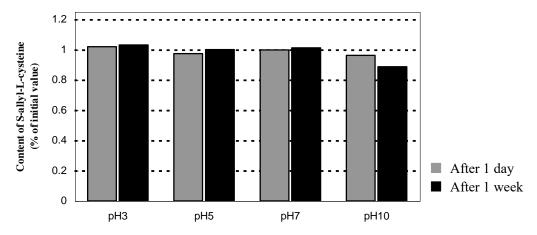


Fig.22 pH stability of Chinese Chive Seed Extract.



(III) Stability in Aqueous Solution

0.4% solution of Chinese Chive Seed Extract-WSP (water soluble poweder) at pH3.5 was prepared. The solution was stored at room temperature (light & dark conditions), 40°C (dark condition) and 5°C (dark condition), respectively for 2 weeks. Visual observation was conducted (table below) and there was no precipitation, turbidity and color change observed. Chinese Chive Seed Extract-WSP is highly stable in aqueous solution.

	Liquid stability (0.4% solution, pH 3.5)					
	Room temperature (light condition)	25℃ (dark condition)	40°C (dark condition)	5°C (dark condition)		
Precipita tion, turbidity	Negative	Negative	Negative	Negative		
Color changes	Negative	Negative	Negative	Negative		

5. Nutritional Information

Description	Amount/100 g	Note	Analytical Method
	Chinese Chive		
	Seed Extract-P		
Energy	354.7 (kcal)	1	Atwater Method (Revised)
Protein	5.7 (g)	2	Kjeldahl Method
Fat	1.5 (g)		Acid degradation
Carbohydrate	79.6 (g)	3	
Water	2.7 (g)		Heat-drying at reduced
			atmospheric pressure
Ash	10.5 (g)		Direct Incineration
Dietary fiber	0.0 (g)		Prosky Method
Sodium	24 (mg)		Atomic absorption
			spectrophotometory
Table Salt	0.1 (g)		Sodium Equiv. value
Zinc	0.6 (mg)		Inductively coupled
			plasma-atomic emission
			spectrometry

1. Energy expression standard (Ministry of Health and Welfare's announcement No. 176)



Conversion factor: Protein 4, fat 9, sugar 4, dietary fiber 2

2. Nitrogen, protein conversion factor: 6.25

3. Carbohydrate expression standard (Ministry of Health and Welfare's

announcement No. 176)

Calculation: 100 - (water + protein + fat + ash)

Test trustee: SRL, Inc

Date of analysis: September 13, 2006

Test No.: 200608310029

6. Safety Profile (pure extract)

(I) Residual Agricultural Chemicals

The Chinese chive seeds are in compliance with the standards stipulated in the Food Sanitation Law by The Ministry of Health, Labour & Welfare. The 229 agricultural chemicals were measured and they were all below the criteria.

Assay Method: Determined by GC-ECD/NPD, confirmed by GC-MS.

Test Trustee: R J Hill Laboratories Ltd., New Zealand.

Date of analysis: August 4, 2006

Reference No.: 426366

The Chinese Chive Seed Extract (with no diluents added) was again examined for 525 residual agricultural chemical compounds following the provisions of the Food Hygiene Law and pesticide legislation. As a result, contents of all compounds were confirmed to be below the standard values (measurable limits).

Test trustee: Food Safety Evaluation and Analysis Center, Masis Co., Ltd.

Date of test report issued: May 27, 2010

Report No. 38675

(II) Acute Toxicity (LD₅₀)

Acute Toxicity of Chinese Chive Seed Extract was conducted according to the Pharmaceuticals Guidelines on Single-Dose Toxicity Test. 2000 mg/kg of Chinese Chive Seed Extract (maximum dosage without burden on animals) was given to ddY mice (male & female) of 5-week old followed by close observation for 14 days. No abnormalities and fatal event observed. No abnormalities detected upon autopsy. Thus LD₅₀ of Chinese Chives Seeds Extract is deduced to be >2,000 mg/kg for both male and female mice.

(III) Mutagenicity Test (Ames test)

Following the OECD Guideline No. 471, and Commission Directive 2004/73/EC, Ames test was performed. The test was performed by using *Salmonella typhimurium* TA98 and TA100, under the conditions with or without S9mix.

The result showed Chinese Chive Seed Extract possessed no mutagenicity at the concentrations of 19.5 to $5000 \mu g/plate$.



7. Recommended Dosage

Recommended daily dose: 100mg/day for Chinese Chive Seed Extract –P & WSP.

8. Commercial Application

	Applications	Claims	Examples
Foods	General Tonic	1. aphrodisiac 2. anti-fatigue (mental & physical) 3. anti-aging	Beverages, hard & soft capsules, tablets, candies, chewing gums, chocolates, wafers, jellies etc
Cosmet ics	Beauty cosmetic	4. immune boosting	Body lotions, body gel etc.

9. Packaging

CHINESE CHIVE SEED EXTRACT-P (Powder, food grade)

CHINESE CHIVE SEED EXTRACT-WSP (Water-soluble, food grade)

CHINESE CHIVE SEED EXTRACT-PC (Powder, cosmetic grade)

CHINESE CHIVE SEED EXTRACT-WSPC (Water-soluble, cosmetic grade)

5kg Interior packaging : Aluminium bag Exterior packaging : Cardboard

CHINESE CHIVE SEED EXTRACT-LC (Liquid, cosmetic grade)

5kg Interior packaging: Cubic polyethylene container

Exterior packaging: Cardboard

CHINESE CHIVE SEED OIL (Oil, food grade)

16kg Interior packaging: Tin can
Exterior packaging: Cardboard

10. Storage

Store in a cool, dry and dark place.

11. Expression

<Food>

CHINESE CHIVE SEED EXTRACT-P, CHINESE CHIVE SEED EXTRACT-WSP

Expression: Chinese Chive Seed Extract



CHINESE CHIVE SEED OIL

Expression: Chinese Chive Seed Oil

<Cosmetic>

CHINESE CHIVE SEED EXTRACT-PC, CHINESE CHIVE SEED EXTRACT-WSPC

Expression: Dextrin, Allium Tuberosum Seed Extract INCI Name: Dextrin (and) Allium Tuberosum Seed Extract

CHINESE CHIVE SEED EXTRACT-LC

Expression: Butylene Glycol, Water, Allium Tuberosum Seed Extract

INCI Name: Butylene Glycol (and) Water (and) Allium Tuberosum Seed Extract



PRODUCT NAME : **CHINESE CHIVE SEED EXTRACT-P** (FOOD)

This product is extracted from chinese chive ($Allium\ tuberosum\ Rottl.$) seeds with aqueous ethanol. It guarantees minimum of 0.1 % S-allyl-L-cysteine.

<u>Appearance</u>	Yellowish powder with light unique smell.			
S-allyl-L-cysteine	Min. 0.1 %	(HPLC)		
Loss on Drying	Max. 10.0 %	(Analysis for HygienicChemists, 1g, 105 °C, 2 hr)		
Purity Test (1)Heavy Metals (as Pb)	Max. 10 ppm	(Sodium Sulfide Colorimetric Method)		
(2) Arsenic (as As ₂ O ₃)	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)		
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)		
<u>Coliforms</u>	Negative	(Analysis for Hygienic Chemists)		
<u>Composition</u>	Ingredient Maltodextrin Chinese Chive Seed Total	Content 80 % Extract 20 % 100 %		



PRODUCT NAME : **CHINESE CHIVE SEED EXTRACT-WSP** (FOOD)

This product is extracted from chinese chive (*Allium tuberosum* Rottl.) seeds with aqueous ethanol. It guarantees minimum of 0.1 % S-allyl-L-cysteine. This product is water-soluble.

<u>Appearance</u>	Yellowish powder with light unique smell.				
S-allyl-L-cysteine	Min. 0.1 %	(HPLC)			
Loss on Drying	Max. 10.0 %	(Analysis for HygienicChemists, 1g, 105 °C, 2 hr)			
Purity Test (1)Heavy Metals (as Pb)	Max. 10 ppm	(Sodium Sulfide Colorimetric Method)			
(2) Arsenic (as As ₂ O ₃)	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation, The Third Method, Apparatus B)			
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)			
<u>Coliforms</u>	Negative	(Analysis for Hygienic Chemists)			
<u>Composition</u>	Ingredient Maltodextrin Chinese Chive Seed Total	Content 80 % Extract 20 % 100 %			



PRODUCT NAME : **CHINESE CHIVE SEED EXTRACT-PC** (COSMETIC)

This product is extracted from chinese chive (*Allium tuberosum* Rottl.) seeds with aqueous ethanol. It guarantees minimum of 0.1 % S-allyl-L-cysteine.

<u>Appearance</u>	Yellowish powder with light unique smell.	
S-allyl-L-cysteine	Min. 0.1 %	(HPLC)
Loss on Drying	Max. 10.0 %	(Analysis for HygienicChemists, 1g, 105 °C, 2 hr)
Purity Test (1)Heavy Metals (as Pb)	Max. 10 ppm	(The Second Method of The Japanese Standards of Quasi-Drug Ingredients)
(2)Arsenic (as As ₂ O ₃)	Max. 1 ppm	(The Third Method of The Japanese Standards of Quasi-Drug Ingredients)
Standard Plate Counts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10 ² cfu/g	(Analysis for Hygienic Chemists)
<u>Coliforms</u>	Negative	(Analysis for Hygienic Chemists)
<u>Composition</u>	Ingredient Dextrin Chinese Chive Seed Extra Total	Content 80 % ract 20 % 100 %



PRODUCT NAME : CHINESE CHIVE SEED EXTRACT-WSPC (COSMETIC)

This product is extracted from chinese chive (*Allium tuberosum* Rottl.) seeds with aqueous ethanol. It guarantees minimum of 0.1 % S-allyl-L-cysteine. This product is water-soluble.

Appearance	Yellowish powder with light unique smell.	
S-allyl-L-cysteine	Min. 0.1 %	(HPLC)
Loss on Drying	Max. 10.0 %	(Analysis for HygienicChemists, 1g, 105 °C, 2 hr)
Purity Test (1)Heavy Metals (as Pb)	Max. 10 ppm	(The Second Method of The Japanese Standards of Quasi-Drug Ingredients)
(2)Arsenic (as As ₂ O ₃)	Max. 1 ppm	(The Third Method of The Japanese Standards of Quasi-Drug Ingredients)
Standard Plate Counts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10 ² cfu/g	(Analysis for Hygienic Chemists)
<u>Coliforms</u>	Negative	(Analysis for Hygienic Chemists)
Composition	Ingredient Dextrin Chinese Chive Seed Extra Total	Content 80 % ract 20 % 100 %



PRODUCT NAME : <u>CHINESE CHIVE SEED EXTRACT-LC</u> (COSMETIC)

This product is extracted from chinese chive (*Allium tuberosum* Rottl.) seeds with aqueous ethanol and then the extract is redissolved in aqueous 1,3-butylene glycol.

<u>Appearance</u>	Yellowish liquid. Light unique smell.
-------------------	---------------------------------------

<u>Certification Test</u> Add 0.5 mL ninhydrin reagent to 0.5 mL of the product. While

Amino acids heating, the solution will turn into bluish purple color.

Purity Test

(1) Heavy Metals (as Pb) Max. 10 ppm (The Second Method of The

Japanese Standards of Quasi-Drug

Ingredients)

(2)Arsenic (as As₂O₃) Max. 1 ppm (The Third Method of The

Japanese Standards of Quasi-Drug

Ingredients)

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

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

<u>Coliforms</u> Negative (Analysis for Hygienic Chemists)

<u>Composition</u> <u>Ingredient</u> <u>Content</u>

Butylene Glycol 50 %
Water 49 %
Chinese Chive Seed Extract 1 %
Total 100 %

31



PRODUCT NAME: CHINESE CHIVE SEED OIL (FOOD)

This oil is extracted and refined from chinese chive (Allium tuberosum Rottl.) seeds.

Appearance Red-yellow liquid oil with light unique smell.

Acid Value Max. 0.50

Saponified Value 175~195

<u>Iodine Value</u> 115∼140

Peroxide Value Max. 2.0 meq/kg

Purity Test

(1)Heavy Metals (as Pb) Max. 10 ppm (Sodium Sulfide Colorimetric Method)

(2) Arsenic (as As₂O₃) Max. 1 ppm (Standard Methods of Analysis in Food

Safety Regulation, The Third Method,

Apparatus B)

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

Moulds and Yeasts Negative (Analysis for Hygienic Chemists)

<u>Coliforms</u> Negative (Analysis for Hygienic Chemists)

<u>Composition</u> <u>Ingredient</u> <u>Content</u>

Chinese Chive Seed Oil 100 %



ORYZA OIL & FAT CHEMICAL CO., LTD. striving for the development of the new functional food materials to promote health and general well-being.

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