ORYZA OIL & FAT CHEMICAL CO., LTD.

CISTANCHE TUBULOSA EXTRACT

Food and cosmetic ingredients with tonics, memory improving, anti-aging, anti-fatigue, anti-sex dysfunction, immune boosting and fat metabolism accelerating properties

■ **CISTANCHE TUBULOSA EXTRACT-P25**

(Water-soluble Powder、Food Grade)

■ **CISTANCHE TUBULOSA EXTRACT-PC25**

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Ver.1.0 JT

Improve Memory・Anti-aging,-fatigue

CISTANCHE TUBULOSA EXTRACT

1. Introduction

Cistanche Tubulosa (Schenk) R. Wight is a plant that parasitizes the roots of Tamarix. Since it has no root or chloroplast, it cannot photosynthesize (Fig. 1). Therefore, it grows by absorbing the nutrition out of plants it parasitizes. In China, Cistanche Tubulosa is known as a rare Panax ginseng found in deserts and used as a pharmaceutical to cure Alzheimer's disease. In Japan, Cistanche Tubulosa has been categorized as a food after its food/pharmaceutical classification was revised by the Health, Labor and Welfare Ministry in 2005. It belongs to Cistanche, Orobanchaceae, same as the parasitic plant Cistanche salsa used in Chinese herbal medicine. According to the Chinese Comprehensive Pharmaceutical Dictionary, it supplements renal function, increases sexual power, and smoothes the intestines. The dictionary also states that it treats impotence, infertility, menstrual disorder, and psychroalgia of the back and knees. Cistanche salsa has been widely used clinically as a prescribed drug for supplementing renal function and nutritional fortification in China. However, collecting the valuable crude drug Cistanche salsa (certified as a class 2 national protected plant) has become difficult recently. Therefore, there is a growing awareness about Cistanche Tubulosa which belongs to the same family and has been reported to have similar effects and functions as Cistanche salsa.

Cistanche Tubulosa grows in the Takla Makan Desert in Hsinchiang Uighur Autonomous Region, China. It has a very strong vital energy to flower and fruit under severe desert conditions (Fig. 2). Its host tamarix grows to 6 meters tall and has small, dark pink flowers. This plant is used to protect against wind and sand (prevents the spread of yellow sand) in desert regions. Cistanche Tubulosa is now considered to be the key to greenification of deserts and prevention of global warming and the Chinese government recommends the incubation of Cistanche Tubulosa to stimulate local industries.

According to Mr. Keiichi Morishita’s report, the Hotan region, an oasis in the Takla Makan Desert, has one of the four longest life expectancies in the world. The percentage of elderly people that live to be over one-hundred years old in this region is the highest in China, over three times that of Okinawa Prefecture in Japan which is famous for its people’s longevity. Per 100,000 people, Okinawa has 51 people aged over 100 and Hotan has 183 such people. People of the Hotan region eat Cistanche Tubulosa daily and prepare it by slicing and then boiling with mutton in a pot or pickling it in tea or liquor in order to survive the harsh environmental conditions of the region. This habit is believed to be the key of people’s longevity in the region.

Oryza Oil & Chemical Co., Ltd. studied Cistanche Tubulosa extract jointly with Sinphar Pharmaceutical Co., Ltd. (pharmaceutical company in Taiwan) with the assistance of Peking University. Sinphar Pharmaceutical Co., Ltd. established a raw

ingredient production base and also a subsidiary 新疆天力砂生薬物有限公司 in Hotan. The company implements GAP plantation of Cistanche Tubulosa to stably provide high-quality, pesticide-free raw material. The company's products are safe and extremely stable in quality. Moreover, its products have been certified as organic foods by the government and raised in GMP facilities. Through joint research, we have discovered that Cistanche Tubulosa extract has activities to prevent aging of the brain and skin, increase sexual power, and accelerate fat metabolism in addition to its known activities. Sinphar Pharmaceutical Co., Ltd. and Peking University have also discovered that the extract has activities to improve brain functions, prevent aging or fatigue, and boost immune strength from test data accumulated in their long-term study.

Our Cistanche Tubulosa extract is the highest concentration ever (echinacoside 25% min, acteoside 9% min). We believe that the extract can be used in a wide variety of foods and cosmetics as a new ingredient to improve brain functions, vitalize the body, and enhance beauty.

Fig. 1 Cistanche Tubulosa

Fig. 2 Hsinchiang Uighur Autonomous Region (GAP plantation)

2. Functional Components of Cistanche Tubulosa

The main effective ingredients of Cistanche Tubulosa extract are phenylethanoid glycosides, especially echinacoside and acteoside (Fig. 3). Although echinacoside is known as the main component of the herb Echinacea, Cistanche Tubulosa contains a higher amount of echinacoside than any other plant. Acteoside (a type of polyphenols) has an extremely strong antioxidative property which is reported to be 15 times stronger than resveratrol (polyphenols contained in grapes) and 5 times stronger than vitamin C[1]. In recent study, new compounds (kankanoside and others) have been isolated[2] and vasorelaxing activity has been reported as a pharmacological action of the new compounds, echinacoside, and acteoside[3]. Active constituents in extracts of Cistanche salsa and Cistanche Tubulosa that belong to the same family, were compared and it was clarified that Cistanche Tubulosa has more active constituents (Fig. 4). Cistanche Tubulosa extract with high quantity of active constituents performs various bioactive functions as described below.

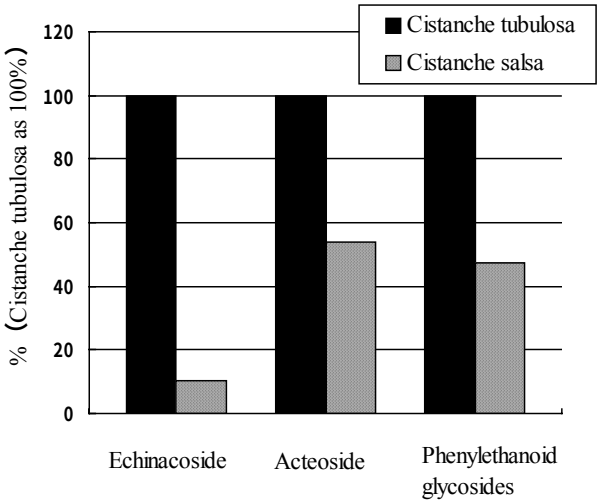
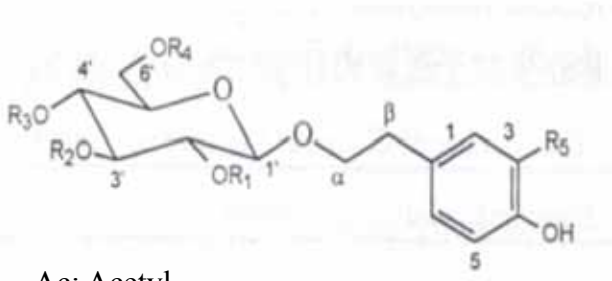


Fig. 4 Echinacoside, acteoside and phenylethanoid glycosides content of Cistanche salsa and Cistanche tubulosa

Compound Name	R1	R2	R3	R4	R5
2'-Acetylacteoside	Ac	Rha	Cf	H	OH
Acteoside	H	Rha	Cf	H	OH
Cistanoside A	H	Rha	Cf	Glc	OMe
Cistanoside C	H	Rha	Cf	H	OMe
Echinacoside	H	Rha	Cf	Glc	OH
Isoacteoside	H	Rha	H	Cf	OH
Tubuloside A	Ac	Rha	Cf	Glc	OH



Ac: Acetyl
Cf: trans-Caffeoyl
Glc: β-D-Glucopyranose
Rha: α-L-Rhamnopyranose

Fig. 3 Components of Cistanche Tubulosa Extract

References
1) Kanebo：news release, 2005.
2) Haihui Xie *et al*., *Chem. Pharm. Bull*., **54**(5), 669-675, 2006.
3) Yoshikawa M., *et al*., *Bioorg. Med. Chem*., **14**(22), 7468-7475, 2006.

Bioactivities of Cistanche Tubulosa Extract

(1) Improvement of brain function

(2) Anti-aging effect

(3) Skin beautifying effect

(4) Anti-fatigue

(5) Aphrodisiac effect

(6) Immune boosting effect

(7) Metabolism enhancing effect

(8) Antioxidant Activities

3. Physiological Function of Cistanche Tubulosa Extract

(1) Improvement of brain function

1) Improvement of learning and memory (*in vivo*)

There are three levels of mechanisms in consideration of learning and memory.
(1) Ability to acquire memory, referred to as learning ability
(2) Ability to store memory, referred to as consolidation
(3) Ability to elicit and recall information memorized

Cistanche Tubulosa extract was clarified to significantly influence all of these mechanisms. The test results are described below.

① Improve learning ability and retentive memory (Sinphar and Peking University data)

In order to evaluate Cistanche Tubulosa extract's activities to improve learning ability and consolidation, a step down test was carried out on mice[4]. In this method, a platform (safe area) is located on an electric wire with 36 V current and mice's learning ability and consolidation are evaluated by the time they spend on the platform and the number of electric shocks they receive (Fig. 5). Mice were trained by the device. Scopolamine (a drug to cause learning ability disorder by inhibiting acetylcholine receptor in the brain) was administered before the training started, and sodium nitrite (a drug to inhibit the synthesis of protein involved in the formation of memory by inducing oxygen deficit in the brain) was administered after the training in order to induce learning/memory disorder. As a result, the safe area time (latency) and the number of mistakes (times that mice got electric shocks) were significantly better in the Cistanche Tubulosa extract administration group as compared to the memory consolidation dysfunction model group. The levels were recovered to almost the same as normal mice that were trained (normal group) (Figs. 6 and 7). Cistanche Tubulosa extract was confirmed to perform a stronger activity than piracetam (positive control), a pharmaceutical to activate energy metabolism of brain cells. These results clarified that Cistanche Tubulosa extract significantly helps the brain recover from scopolamine-induced learning disorder and sodium nitrite-induced memory consolidation dysfunction and improve the brain functions of learning ability and formation of memory.

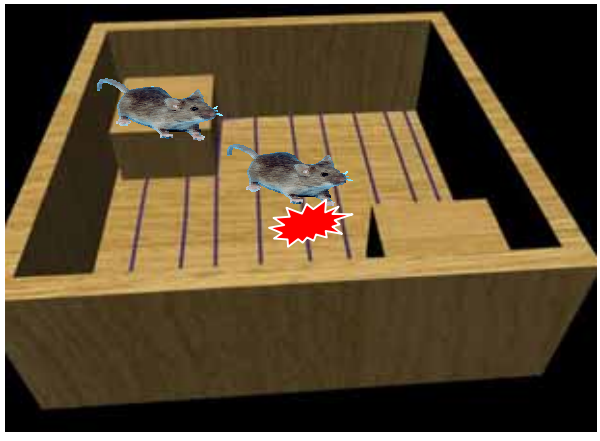


Fig. 5 Step down test

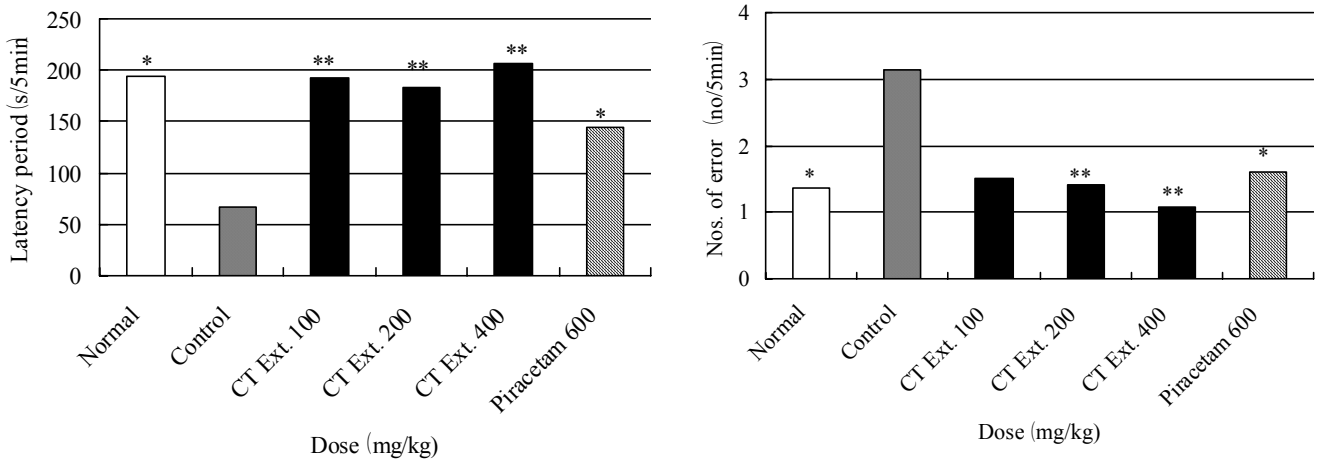


Fig. 6 Effect of Cistanche Tubulosa Extract (CT Ext.) on memory acquiring impaired mice model induced by scopolamine. *n*=12-15, *: $p<0.05$, **: $p<0.01$

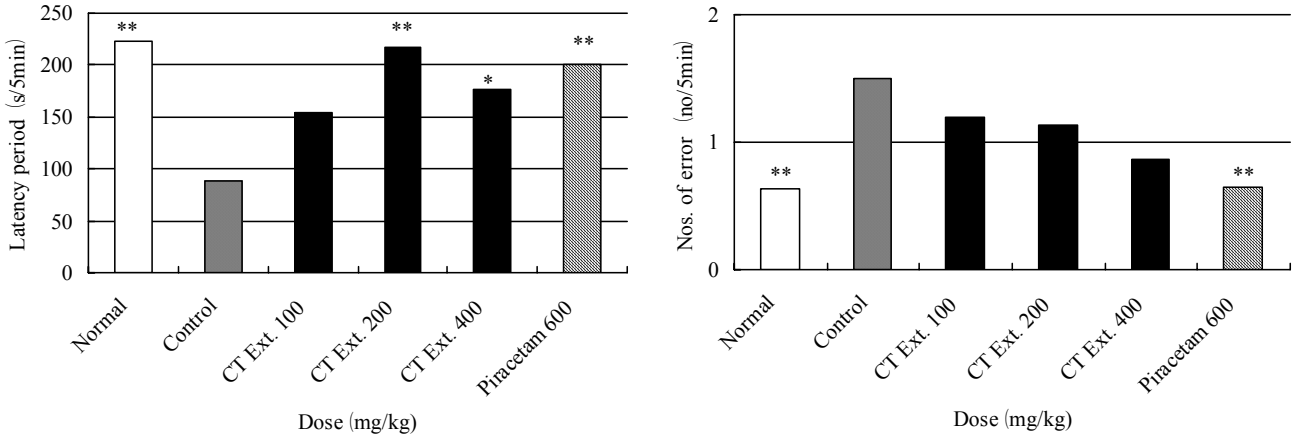


Fig. 7 Effect of Cistanche Tubulosa Extract (CT Ext.) on memory consolidation impaired mice model induced by sodium nitrite. *n*=14-15, *: $p<0.05$, **: $p<0.01$

4) Cong G., *et al.*, Effects of CTG on memory consolidation dysfunction of mice. *Traditional Chinese Drug Research and Clinical Pharmacology*., **16**(3), 162-164, 2005.

② Ability to recall memorized information (Sinphar and Peking University data)

Training for a water maze test was conducted on mice four times a day for one week (Fig. 8). The training was carried out to make the mice remember the routes of the water maze. During the training period, the sample was orally administered every day. On the last day of the training, 30% ethanol was administered to induce disorder on mice's recall ability (ability to recall memorized information). Then, an evaluation was done in a water maze test. As a result, the mice that took the sample reached the goal in significantly shorter time as compared to the control group (Fig. 9). The error rate among the mice who made mistakes (Once when they get to the wrong good before getting to the correct one is recorded as one error.) was significantly lower in the group that took the sample (Table 1). Cistanche Tubulosa extract was also confirmed to have a stronger activity than piracetam (positive control). The results indicate that Cistanche Tubulosa extract has an activity to improve the ability to elicit or recall memorized information.

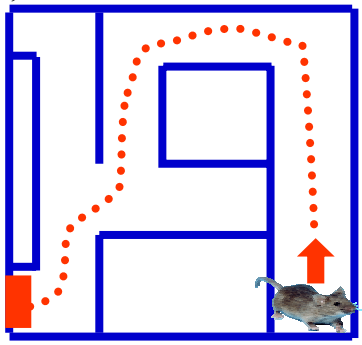


Fig. 8 Water maze test

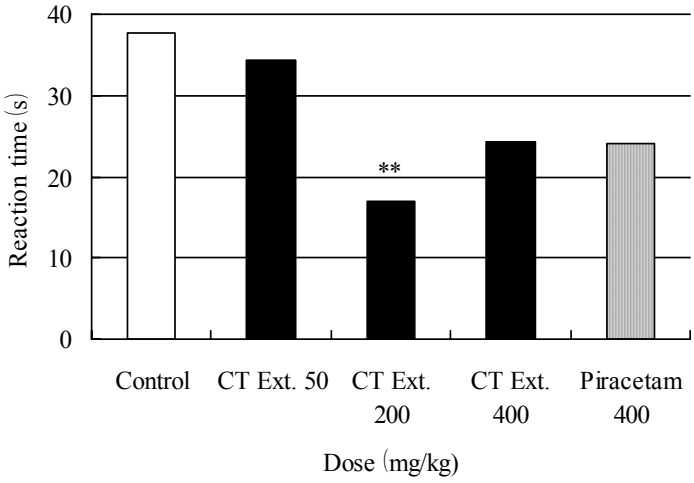


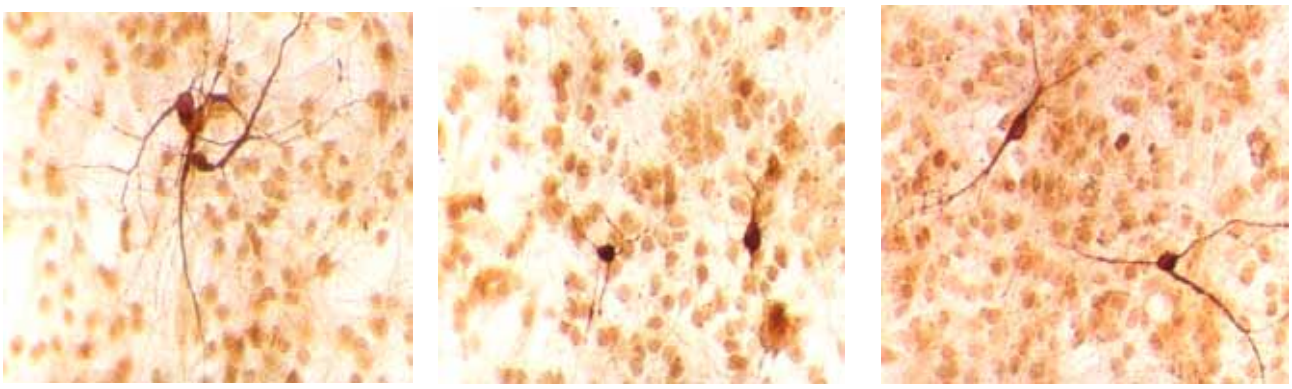
Fig. 9 Effect of Cistanche Tubulosa Extract (CT Ext.) on memory recall impairment. n=10-12, **: p<0.01

Table 1 Effect of Cistanche Tubulosa Extract (CT Ext.) on memory recall impaired model mice induced by alcohol. **: p<0.01

Group	Dose (mg/kg)	Before ethanol		After ethanol	
		Reaction time (s)	Error number／mice	Reaction time (s)	Error number／mice
Control	—	7.73±0.75	0/0	37.78±15.90	62/10
CT Ext.	50	7.46±0.13	0/0	34.40±21.71	47/8
CT Ext.	200	7.14±0.18	0/0	16.99±9.06**	8/3**
CT Ext.	400	7.91±0.19	0/0	24.38±27.84	46/7
Piracetam	400	8.00±0.46	0/0	24.08±32.52	54/6

2) Anti-apoptosis activity (*in vitro*, Sinphar and Peking University data)

Anti-apoptosis activity of Cistanche salsa, a congener of Cistanche Tubulosa, on brain neurons was evaluated[5]. Primary midbrain neurons taken from rat embryo (14 to 16 days old) were cultured to create midbrain neuron apoptosis models using a neurotoxin 1-methyl-4-phenylpyridium ion (MPP^+, 50 μmol/L). Then, Cistanche salsa extract (phenylethanoid glycoside content: 50 μg/mL) was given to the apoptosis models and their brain neurons were studied using a microscope over time. As a result, Cistanche salsa extract was clarified to significantly control brain neuron apoptosis. Moreover, in the group that Cistanche salsa extract was present, brain neuron source grew better and axons (protrusions extended from the cell body that output signals in neurons) were longer than that of the control group (MPP^+ administration group). It was confirmed that axons extended to almost the same as the normal group when Cistanche salsa extract was present (Fig. 10). These results clarified that Cistanche salsa extract improves brain functions by restoring damaged brain neurons. Cistanche Tubulosa extract is expected to perform the same activity because it belongs to the same family as Cistanche salsa and contains larger amounts of effective components.



Normal | Apoptosis induced by MPP^+ | MPP^++ Phenylethanoid glycosides

Fig. 10 The anti-apoptosis effects of phenylethanoid glycosides on mesencephalic neurons

5) Research on anti-apoptosis mechanism of phenylethanoid glycosides in *Cistanche salsa* in middle brain neurons. *Chinese Pharmacology Communication*., **19**(4), 50-51, 2002.

3) Rescue human fibroblasts (*in vitro*)

Echinacoside, a component of Cistanche Tubulosa, has been reported to rescue human fibroblasts (SHSY5Y) from TNFα-induced apoptosis[6]. After incubation of SHSY5Y cells ($1x10^4$ cell/well), echinacoside (1, 10 and 100 μg/mL) and TNFα (100 ng/mL) were added 36 hours before each valuation analysis. Cell viability was evaluated by the MTT assay method. The reactive oxygen species level in cells was evaluated by a staining method using a fluorescence dye 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA: After this reagent is metabolized in cells, fluorescence develops. The fluorescence intensity reflects the active oxygen level in the cell quantitatively.) Then activation of caspase (a group of cysteine protease constructing a signal communication channel to cause apoptosis on cells) was evaluated using a kit. As a result, Cell viability was higher in the echinacoside application group than the TNFα application group concentration-dependently (Fig. 11). Echinacoside was also confirmed to control the active oxygen level concentration-dependently (Fig. 12). Moreover, it was apparently confirmed to control the activation of an apoptosis effector caspase-3 (activated by apoptosis initiator caspase, it decomposes other proteins in cells and causes apoptosis) concentration-dependently (Fig. 13). These results clarified that echinacoside protects damaged fibroblasts by controlling the reactive oxygen species level in fibroblasts and also the activation of caspase-3. Cistanche Tubulosa extract is believed to perform a similar activity because it contains a larger amount of echinacoside.

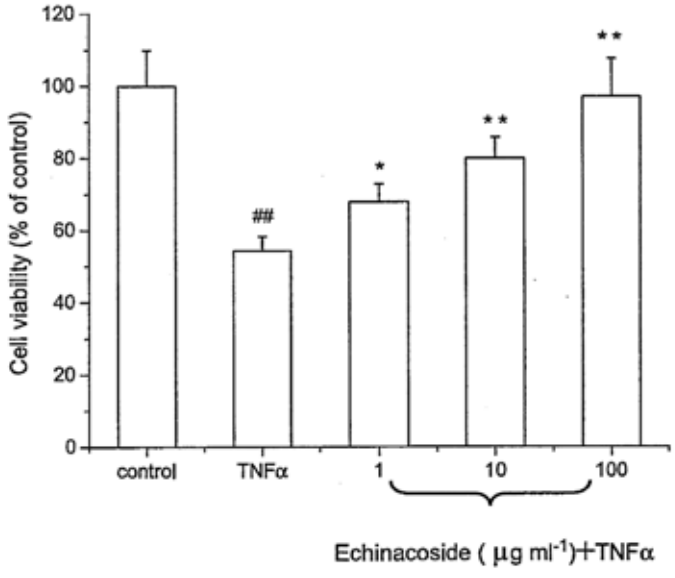
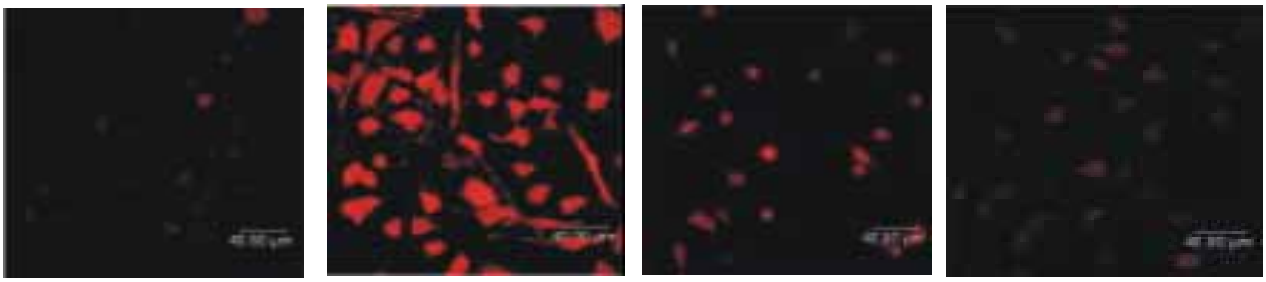


Fig. 11 Effect of echinacoside on TNFα-induced decrease in SHSY5Y cell viability. n=8, *: p<0.05, **: p<0.01 as compared to TNFα, ##: p<0.01 as compared to control cells



Control | Treatment with TNFα | Echinacoside 10 μg/mL | Echinacoside 100 μg/mL

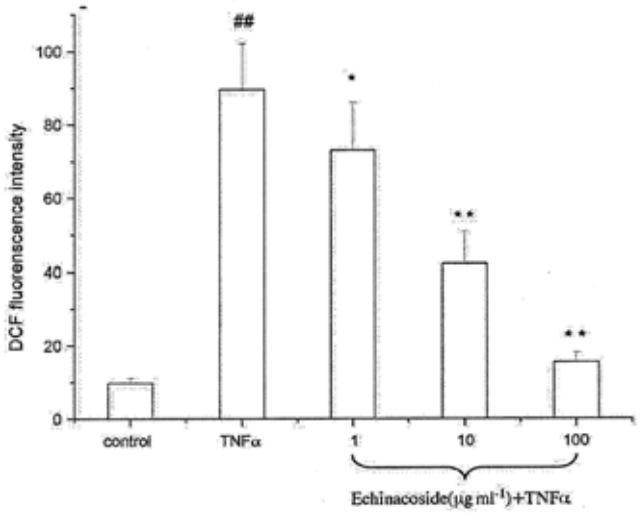


Fig. 12 Effect of echinacoside on level of intracellular reactive oxygen species in TNFα-induced SHSY5Y cells. *n*=8, *: $p<0.05$, **: $p<0.01$ as compared to TNFα, $^{\#\#}$: $p<0.01$ as compared to control cells

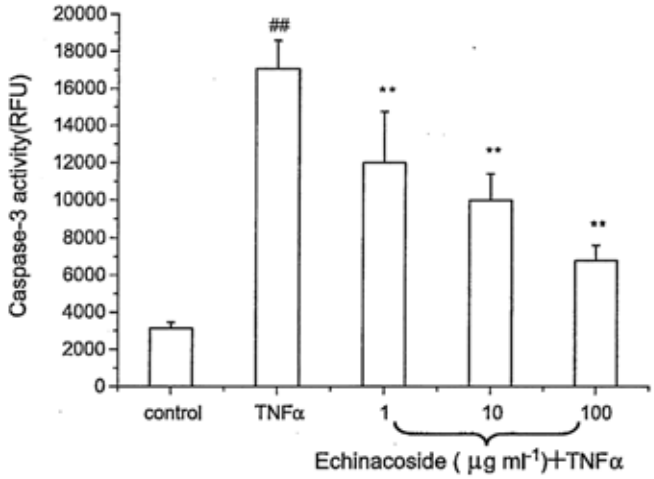


Fig. 13 Effect of echinacoside on TNFα-induced increase of caspase-3 activity. *n*=8, **: $p<0.01$ as compared to TNFα, $^{\#\#}$: $p<0.01$ as compared to control cells

6) Min D., *et al.*, Echinacoside rescues the SHSY5Y neuronal cells from TNFα-induced apoptosis. *European Journal of Pharmacology*., **505**, 11-18, 2004.

4) Prevent aging of the brain (*in vitro*, Oryza data)

In order to study Cistanche Tubulosa extract's activity to prevent aging of the brain, its influence on proliferation of human fibroblasts (SK-N-SH) was examined. In concrete, cell proliferation was evaluated by the MTT assay method. The extract's influence on neurite outgrowth was also observed using a microscope over time. As a result, Cistanche Tubulosa extract was confirmed to boost the proliferation of fibroblasts concentration-dependently (Fig. 14). Moreover, microscope photographs showed that neurite outgrowth and the formation of cell networks were accelerated when Cistanche Tubulosa extract was added as compared to the control (Fig. 15). These results indicate that Cistanche Tubulosa extract accelerates the proliferation of fibroblasts and may accelerate the change of fibroblasts into neurons by accelerating the neurite outgrowth even when fibroblasts are not damaged. Thus, the extract is believed to effectively prevent aging of the brain such as dementia (dementia is caused by denaturation and desquamation of cranial nerves) and improve brain functions. Echinacoside and acteoside, components of the extract, were also confirmed to perform the mitogenic activity. Especially, echinacoside performed a significant effect, even in low concentrations (Fig. 16). They performed the activity to accelerate the neurite outgrowth as well (Fig. 17). These results indicate that echinacoside and acteoside are partially involved in Cistanche Tubulosa extract's mitogenic activity and activity to accelerate the neurite outgrowth.

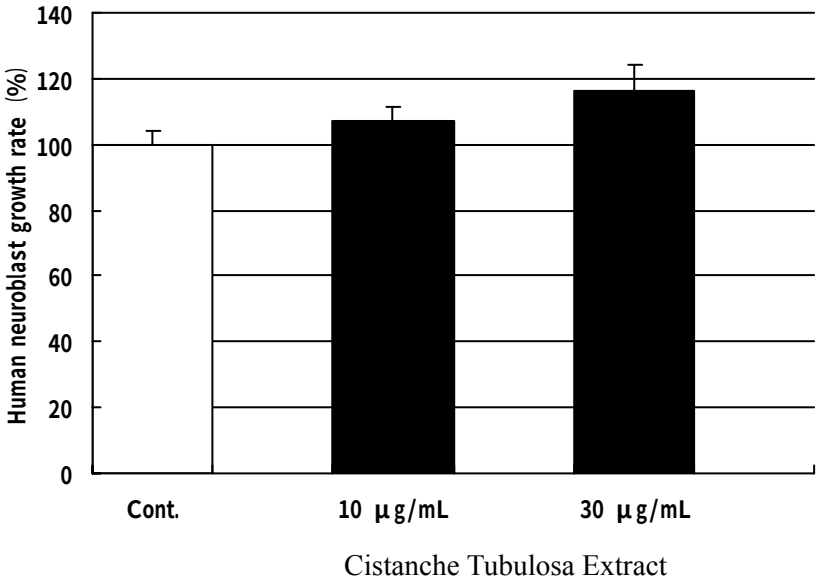


Fig. 14 Effect of Cistanche Tubulosa extract on boost the proliferation of fibroblasts（% of Control, Mean±S.D., *n*=5）

Control | Cistanche Tubulosa Extract 10 μg/mL | Cistanche Tubulosa Extract 30 μg/mL

Fig. 15 Effect of Cistanche Tubulosa extract on neurite outgrowth (microscope photographs, ×400)

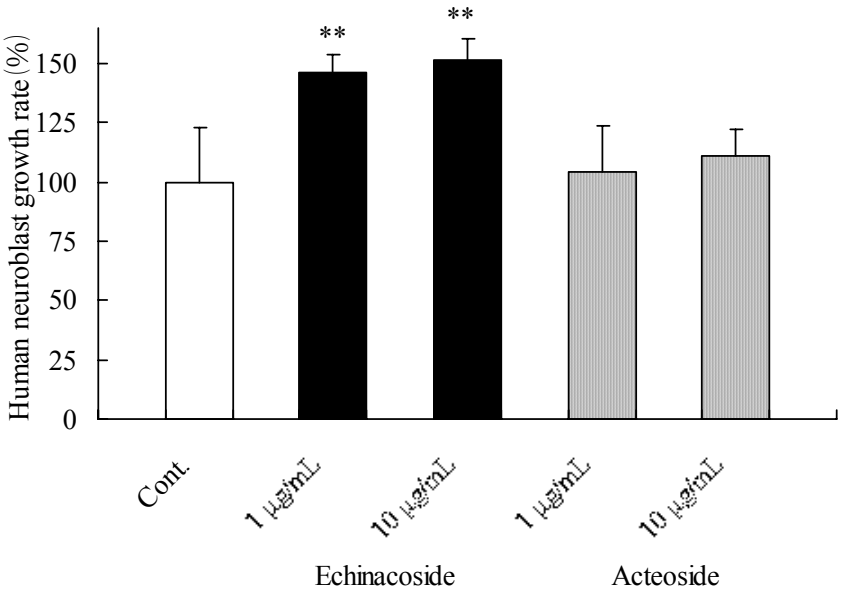


Fig. 16 Effect of echinacoside and acteoside on the proliferation of fibroblasts (% of Control, Mean±S.D., n=5 **: p<0.01)

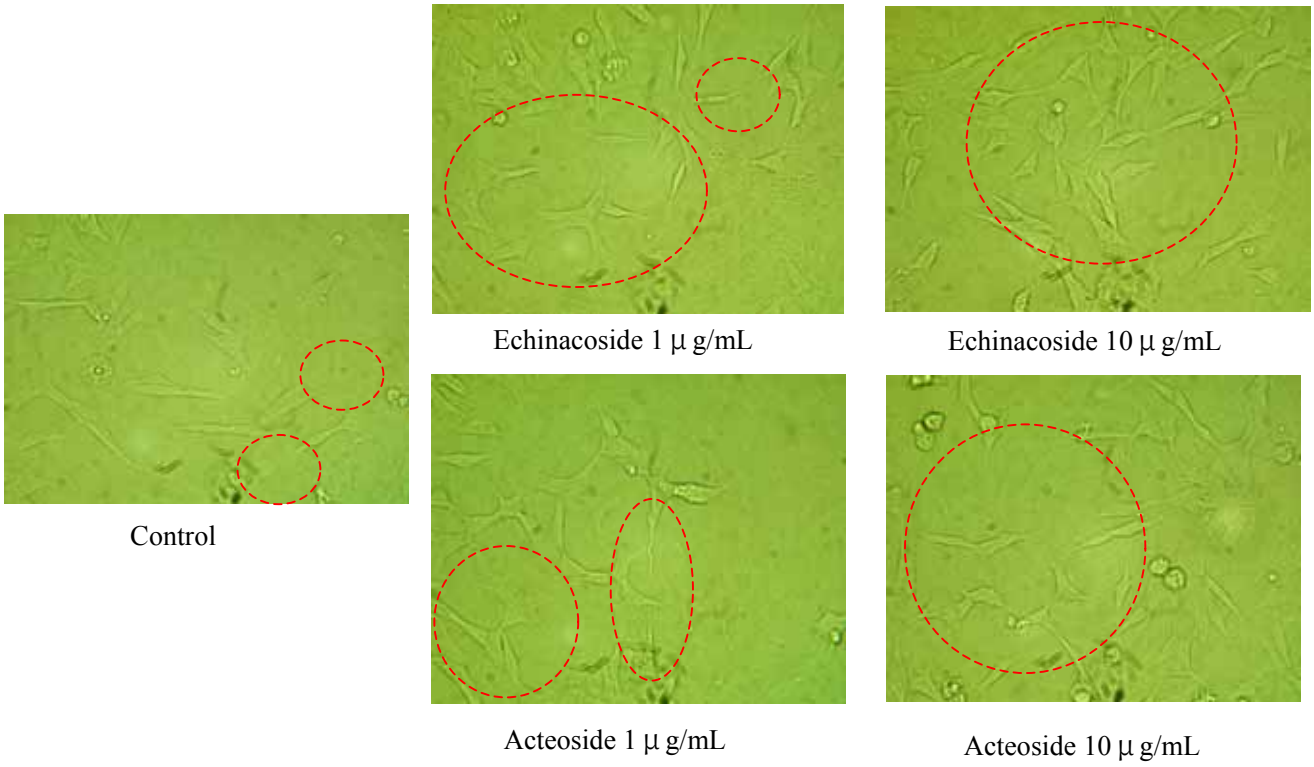


Fig. 17 Effect of echinacoside and acteoside on neurite outgrowth (microscope photographs, ×400)

【Method】

Human fibroblasts (SK-N-SH) were suspended ($1x10^4$ cells/mL) in a MEM medium (10% FCS, 100 units/mL penicillin, 100 μg/mL streptomycin contained) and 5 mL each was inseminated on a 60 mm petri dish. Cistanche Tubulosa extract prepared in various concentrations was added and changes on cell count were observed using a microscope over time. Proliferation of the cells was evaluated by the MTT assay method on the fifth and sixth day after the incubation started.

5) Influence on cerebral ischemia-reperfusion (*in vivo*)

Cistanche salsa, a congener of Cistanche Tubulosa, has been reported to influence cerebral ischemia-reperfusion and protect against apoptosis of CA_1 region of hippocampus (the part in the brain involved in memory and spatial learning ability. Extremely weak to ischemia and is the first pathological part involved in Alzheimer's disease) [7]. Mice were separated into five groups [sham surgery group, cerebral ischemia group, cerebral ischemia-reperfusion group, cerebral ischemia-reperfusion + positive control (gingko leave extract) group, and cerebral ischemia-reperfusion + Cistanche salsa extract group]. Each sample was orally administered to the mice for eight days. One hour after the final administration, the right carotid artery of the mice was pinched by a silken thread for three hours under anesthesia to induce cerebral ischemia models. Then reperfusion was carried out 24 and 48 hours later to create cerebral ischemia-reperfusion models. Cerebral ischemia area was measured by staining brain slices and the apoptosis rate in CA_1 region of hippocampus 24 hours after the cerebral ischemia-reperfusion was evaluated by the TUNEL method using a kit. As a result, cerebral ischemia was significantly improved and damage caused after reperfusion (24

and 48 hours later) was significantly reduced in the cerebral ischemia-reperfusion + Cistanche salsa extract group as compared to the control (cerebral ischemia-reperfusion) group (Table 2). Cistanche salsa extract was also confirmed to have almost equivalent activity as gingko leaf extract, positive control, to lower the apoptosis rate in CA_1 region in hippocampus 24 hours after the cerebral ischemia-reperfusion (Fig. 18). These results clarified that Cistanche salsa protects brain cells and prevents cerebral infarction and Alzheimer's disease by controlling apoptosis in the hippocampus. Cistanche Tubulosa extract is expected to perform a similar activity because it belongs to the same family as Cistanche salsa and contains larger amounts of the same effective components.

Table 2 Effect of Cistanche salsa on the percentage of infarction at ischemia-reperfusion 0, 24 and 48h in mice (Mean±S.D., **: p<0.01, n=13-18)

Group	Dose (mg/kg)	0h	24h	48h
		Area of cerebral ischemia (%)		
Sham surgery		0	0	0
Cerebral ischemia		57.47±5.37		
Cerebral ischemia-reperfusion		56.96±6.43	72.98±6.57	60.45±6.06
Gingko leave extract	100	20.32±3.45**	25.67±5.38**	23.83±3.78**
Cistanche salsa extract	62.5	27.23±5.66**	31.13±3.92**	22.27±5.32**
Cistanche salsa extract	125	21.45±4.47**	25.81±6.74**	20.06±4.69**
Cistanche salsa extract	250	22.03±6.22**	25.94±4.07**	22.14±4.75**

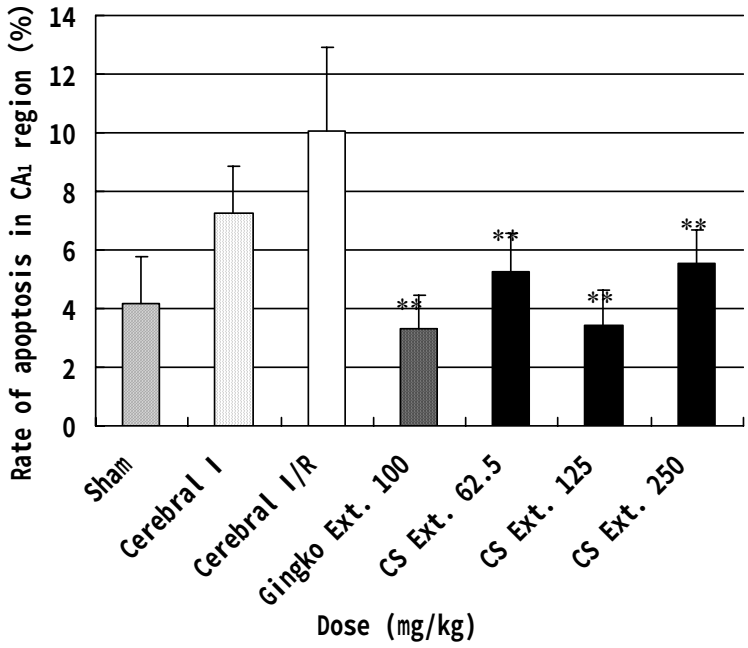


Fig. 18 Effect of Cistanche salsa extract (CS Ext.) on neuron apoptosis in CA_1 region at ischemia-reperfusion 24h (Mean±S.D., **: p<0.01, n=10)

7) Xiao-wen Wang, *et al.*, Protective effects of glycosides of *Cistanche* on cerebral ischemia-reperfusion damage of brain tissue in CA_1 region of hippocampus in awake mice. *Stroke and Nervous Diseases*., **10**(6), 325-328, 2003.

6) Increase the amount of brain neurotransmitters (*in vivo*)

Cistanche salsa, a congener of Cistanche Tubulosa, has been reported to increase the amount of brain neurotransmitters[8]. Each sample was administered to rats for 40 consecutive days. The rats' brains were taken out 12 hours after the final administration and the amount of brain neurotransmitters such as dopamine (DA), noradrenaline (NA), and serotonin (5-HT) was measured using the HPLC-ECD method. As a result, the amount of NA in the hypothalamus and 5-HIAA, a metabolite of serotonin, significantly increased (Table 3). Percentage of DA and its metabolite DOPAC significantly increased as well (Table 4). These results indicate that Cistanche salsa extract improves brain functions by increasing the amount of brain neurotransmitters. Cistanche Tubulosa extract is expected to perform a similar activity because it belongs to the same family as Cistanche salsa and contains larger amounts of effective components.

Table 3 The effect of Cistanche salsa extract (CS Ext.) on the level of monoamine transmitters in rat's hypothalamus. (μg/g tissue, Mean±S.D., *n*=6)

(mg/kg)	DA	DOPAC	NA	5-HT	5-HIAA
Cont.	0.42±0.05	0.08±0.02	1.58±1.09	1.84±0.15	0.85±0.15
CS Ext. 200	0.48±0.06	0.08±0.01	1.69±0.18	2.04±0.26	1.02±0.17
CS Ext. 400	0.46±0.04	0.06±0.01	1.98±0.19**	2.27±0.30	1.04±0.13*

*: $p<0.05$, **: $p<0.01$

Table 4 Recovery of the ratios of neurotransmitters/ metabolites in rat. (μg/g tissue, Mean±S.D., *n*=6)

(mg/kg)	DA/DOPAC	5-HT/5-HIAA
Cont.	5.64±1.41	2.16±0.23
CS Ext. 200	5.81±0.67	2.04±0.35
CS Ext. 400	7.62±1.20*	2.19±0.17

*: $p<0.05$

8) Influence of *Cistanche* on the amount of monoaminergic neurotransmitters in rat brain. *Chinese Herb*, **24**(8), 417-419, 1993.

7) Prevent cerebral infarction and myocardial infarction (*in vivo*)

① Inhibition of platelet aggregation (Sinphar and Peking University data)

The influence of Cistanche Tubulosa extract on platelet aggregation was evaluated on rats. Each sample (positive control: aspirin) was orally administered to rats for seven days. Blood was taken from a main artery one hour after the final administration. Then, adenosine disodium diphosphate (ADP) solution was added to cause platelet aggregation and the aggregation rate was measured using a SPA-4 multi-functional aggregometer. As a result, platelet aggregation was significantly controlled in the Cistanche Tubulosa extract group as compared to the control group (Fig. 19). The activity was almost equivalent to that of the positive control aspirin.

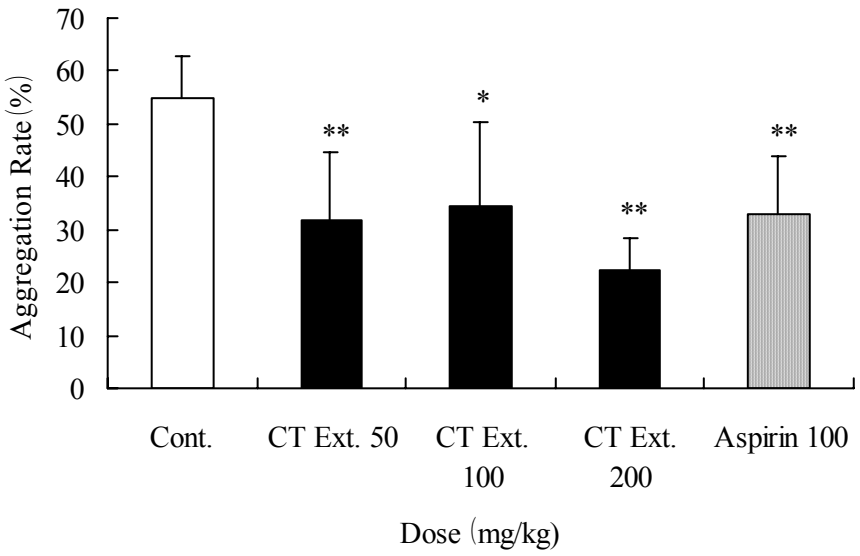


Fig. 19 Effect of Cistanche Tubulosa extract (CT Ext.) on platelet aggregation in rats (Mean± S.D., *: $p<0.05$, **: $p<0.01$, n=10-11)

② Inhibition of formation in vein bypass (Sinphar and Peking University data)

The influence of Cistanche Tubulosa extract on clot formation in vein bypass was evaluated on rats. Each sample (positive control: aspirin) was orally administered to rats for seven days. Vein bypass was isolated on the day of the final administration and clot weight was measured. As a result, clot formation in vein bypass was significantly inhibited in the Cistanche Tubulosa extract group as compared to the control group (Fig. 20). According to the test results, Cistanche Tubulosa extract is expected to prevent cerebral infarction and myocardial infarction.

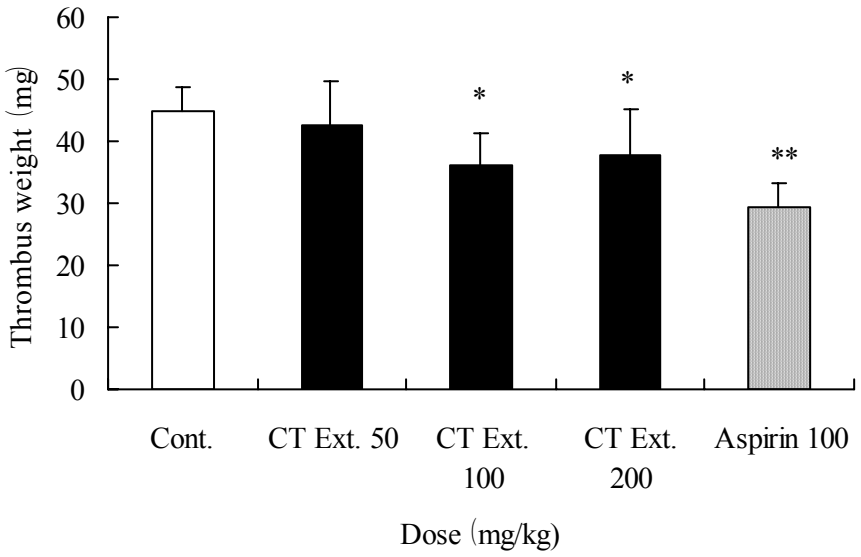


Fig. 20 Effect of Cistanche Tubulosa extract (CT Ext.) on thrombosis in artery-vein bypass in rat (Mean±S.D., *: $p<0.05$, **: $p<0.01$)

8) Clinical Trial (Phase I-III，Data from Sinphar group)

①Phase I

1.1 Toxicity Test on Single Dose Administration (Oral)

Experimental Group	1	2	3	4	5	6	7
Dosage (mg, single)	150	300	600	900	1500	1800	2400
Increasing times	1	2	4	6	10	12	16
Nos. of subject	2	4	6	6	4	4	4

Nos. of subject：30

Method：Oral (Echinacoside 25%, Acteoside 3%)

Result：

There was no abnormal change in body temperature, breathing frequency, heart rate, systolic arterial pressure, diastolic pressure, hepatic/renal functions, fasting blood glucose level, blood in general, urine in general, stool in general, or electrocardiogram on any subject people. There was no side effect on groups during the test. The result confirmed the safety of the extract in single administration on normal persons.

1.2 Toxicity Test on Continuous Administration (Oral)

Nos. of subject：12

Research centers：成都漢方医薬大学付属病院

Duration：10days （Start：2001.11）

Group	1	2
Dosage (mg/times)	600	900
Nos. of subject	6	6

Dosage：Oral Administration (three times a day) (Echinacoside 25%, Acteoside 3%)

Result：

There was no abnormal change in body temperature, breathing frequency, heart rate, systolic arterial pressure, diastolic pressure, hepatic/renal functions, fasting blood glucose level, blood in general, urine in general, stool in general, or electrocardiogram on any subject people. There was no side effect on groups during the test. The result confirmed the safety of the extract in continuous administration on normal persons.

1.3 Conclusion

There was no side effect or abnormal change in observation parameters during single and continuous administration safety tests conducted in phase I of Cistanche Tubulosa extract clinical test. This clarified that the extract would be safe to use in further clinical tests (Phase II, III). In phase II and III of the clinical tests, the extract was orally given to subject people three times a day. The dose was 600 mg per time (echinacoside 25% min, acteoside 3% min).

②Phase II

2.1 Method：According to the test method approved by the Ethics Committee, double blind tests were carried out at five research institutions. Subject patients were separated into two groups: a group to take Cistanche Tubulosa extract and another group to take the positive control (pharmaceutical product, Hydergine[*1]). Administration was carried out over three months. Through the observation of subjects' cognitive functions (mini mental state examination: MMSE), social ability (berg balance scale: BBS), and ability of daily living (ADL) and physical examination by a doctor before and after the administration, the influence of Cistanche Tubulosa extract on clinical treatment of vascular dementia was evaluated, comparing to the positive control group. Safety of the extract for clinical treatments was evaluated at the same time.

Research institutions：福建省漢方医薬研究院，戸州医学院付属漢方病院，陝西漢方医薬大学付属病院，西安市漢方病院，成都漢方医薬大学付属病院。
Duration：2002.3～2002.10

2.2 Nos. of subject：Cistanche Tubulosa extract：120
Positive control (pharmaceutical product, Hydergine)：120
Dosing period：Three months
Dosage：Oral Administration (600 mg, three times a day)

2.3 Result
2.3.1 Efficacy result

Efficacy ratio	MMSE	BBS	ADL	Symptoms
Cistanche Tubulosa Extract	75.66%	66.09%	50.43%	84.35%
Positive control	72.32%	54.46%	40.18%	70.54%

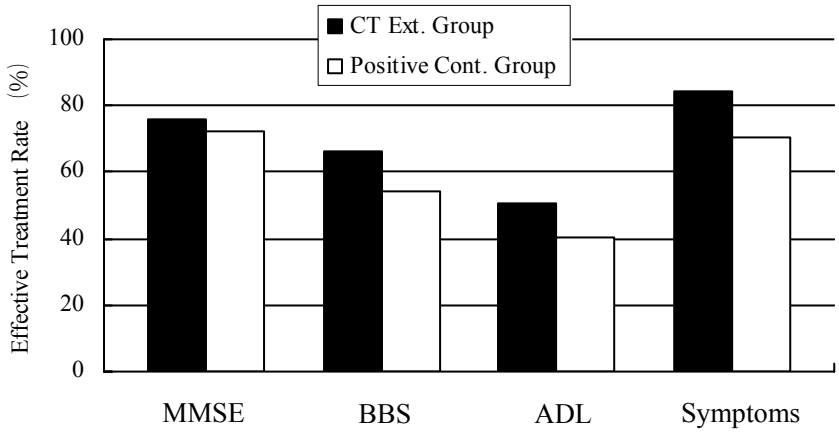


Fig. 21. The Treatment Effect of Cistanche Tubulosa Extract (CT Ext.) vs. Positive Control Group (Phase II)

2.3.2 Analysis on the Efficacy & Safety of Cistanche Tubulosa on Vascular Dementia

Cardiovascular Disease / Patient's Review	Treatment Rate	Improve	No changes	Worse
Mild (46 subjects)	63.04%	-	34.78%	2.17%
Moderate (61 subjects)	34.43%	44.26%	21.31%	0.00%
High (9 subjects)	22.22%	55.56%	22.22%	-

1. Cistanche Tubulosa Extract demonstrated effective treatment rate in patients with moderate symptoms of the diseases compared with positive control group.
2. Treatment rate of Cistanche Tubulosa Extract is similar to that in positive control group in patients with severe symptoms of the diseases.

2.3.3 Efficacy result of each facility

In the phase II of Cistanche Tubulosa extract clinical test on vascular dementia patients, there was no significant difference in results among the five research institutions ($p>0.05$). This indicates that the effectiveness of the extract was the same in each research institution.

2.4 Long-term efficacy

Three months after the completion of the phase II clinical test, a survey was carried out to learn about Cistanche Tubulosa extract's long-term effectiveness on vascular dementia. The subject patients' cognitive function was examined at their homes. As a result, cognitive function of the subjects who took Cistanche Tubulosa extract remained the same or even improved three months later. The efficacy rate was 90.32%. The efficacy rate of the positive control was 78.05%. At the survey visiting the patients who took Cistanche Tubulosa extract, the score to evaluate their cognitive function was 7.89±4.40 points higher than the score before the administration and 0.35±2.95 points higher than the score at the completion of the administration. The score to evaluate cognitive function of the patients who took the positive control was 5.73±3.23 points higher than the score before the administration. However, the score was 0.80±1.58 points lower than the score at the completion of the administration. The results show a significant difference between the two groups ($p<0.05$) and indicate that long-term effectiveness of Cistanche Tubulosa extract is higher than the positive control.

2.5 Safety evaluation

Before and after the administration, blood in general, urine in general, stool in general, hepatic function (ALT), renal function (BUN, Cr), and electrocardiogram of subjects of both groups were analyzed. As a result, Cistanche Tubulosa extract was confirmed to have no negative influence on safety indexes. Although there was a significant difference in the influence on electrocardiogram ($p<0.05$) as compared to the positive control (Hydergine), there was no significant difference in other safety indexes ($p>0.05$).

2.6 Conclusion

Results of the clinical tests described above indicate that Cistanche Tubulosa extract is effective for treatment of vascular dementia. Since no toxicity or side effect was seen during the clinical tests, Cistanche Tubulosa extract was confirmed to be safe for treatment of vascular dementia as well.

③Phase III

3.1 Method： According to the test method approved by the Ethics Committee, double blind tests were carried out at four research institutions. Subject patients were separated into two groups: a group to take Cistanche Tubulosa extract and another group to take the positive control (Hydergine). Administration was carried out over three months. Through the observation of subjects' cognitive functions (mini mental state examination: MMSE), social ability (berg balance scale: BBS), and ability of daily living (ADL) and physical examination by a doctor before and after the administration, the influence of Cistanche Tubulosa extract on clinical treatment of vascular dementia was evaluated, comparing to the positive control group. Safety of the extract for clinical treatments was evaluated at the same time.

Research institutions：成都漢方医薬大学付属病院，西安市漢方病院，戸州医学院付属漢方病院，福建省漢方医薬研究院。

Duration：2002.12～2003.8

3.2 Nos. of subject：Cistanche Tubulosa extract：333

Positive control (pharmaceutical product, Hydergine)：111

Dosing period：Three months

Dosage：Oral Administration (600 mg, three times a day)

3.3 Result

3.3.1 Efficacy result

Efficacy ratio	MMSE	BBS	ADL	Symptoms
Cistanche Tubulosa Extract	77.74%	72.10%	57.37%	91.19%
Positive control	64.15%	62.26%	38.68%	66.98%

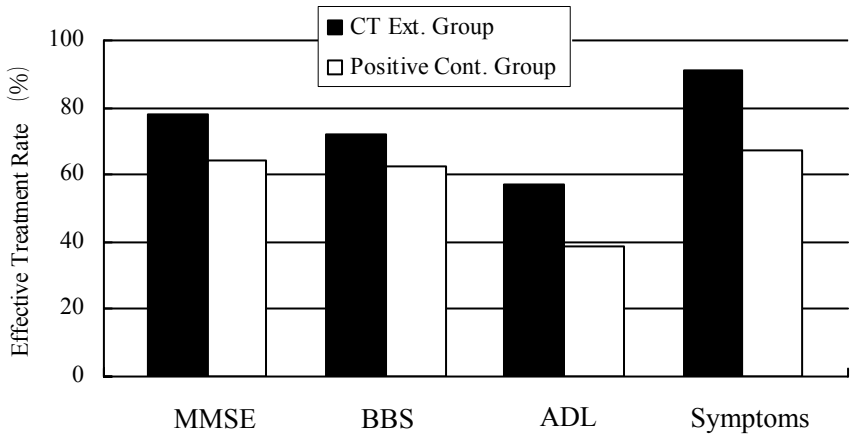


Fig. 22. The Treatment Effect of Cistanche Tubulosa Extract (CT Ext.) vs. Positive Control Group (Phase III)

3.3.2 Analysis on the Efficacy & Safety of Cistanche Tubulosa on Vascular Dementia

Cardiovascular Disease / Patient's Review	Treatment Rate	Improve	No changes	Worse
Mild (137 subjects)	83.94%	-	16.06%	0.00%
Moderate (182 subjects)	37.36%	49.45%	13.19%	0.00%
High (3 subjects)	66.67%	33.33%	0.00%	-

1. Cistanche Tubulosa Extract is more effective in patients with moderate symptoms of the diseases compared with positive control group.
2. No significant difference was observed on patients with severe symptoms when compared with positive control group.

3.3.3 Efficacy result of each facility

In the phase III of Cistanche Tubulosa extract clinical test on vascular dementia patients, there was no significant difference in results among the four research institutions ($p>0.05$). This indicates that the effectiveness of the extract was the same in each research institution.

3.4 Long-term efficacy

Three months after the completion of the phase III clinical test, a survey was carried out to learn about Cistanche Tubulosa extract's long-term effectiveness on vascular dementia. The subject patients' cognitive function was examined at their homes. As a result, cognitive function of the subjects who took Cistanche Tubulosa extract remained the same or even improved three months later. The efficacy rate was 64.71%. The efficacy rate of the positive control was 69.77%. At the survey visiting the patients who took Cistanche Tubulosa extract, the score to evaluate their cognitive function was 5.39±3.26 points higher than the score before the administration and 1.71±2.43 points higher than the score at the completion of the administration. The score to evaluate cognitive function of the patients who took the positive control was 4.47±2.70 points higher than the score before the administration. However, the score was 1.47±1.84 points lower than the score at the completion of the administration.

3.5 Safety evaluation

As a result, Cistanche Tubulosa extract was confirmed to have no negative influence on safety indexes.

3.6 Conclusion

Results of the clinical tests described above indicate that Cistanche Tubulosa extract is effective for treatment of vascular dementia. Since no toxicity or side effect was seen during the clinical tests, Cistanche Tubulosa extract was confirmed to be safe for treatment of vascular dementia as well.

*1: Hydergine (Nonproprietary name：Dihydroergotoxine mesylate)

A drug to improve brain metabolism and peripheral blood circulation. It improves blood flow by releasing vascular tone and dilating blood vessels. It also accelerates oxygen and blood supply to the brain and improves metabolism of brain cells.

(2) Anti-aging effect

1) Free Radical Scavenging Ability (*in vitro*)

Cistanche salsa extract (fraction in which phenylethanoid glycosides are the main components) has been reported to inhibit reactive oxygen species and protect DNAs[9]. In order to examine the extract's activity to inhibit reactive oxygen species (O_2-: Super oxide, OH: Hydroxyl radical, H_2O_2: hydrogen peroxide, $^1O_2$: singlet oxygen), IC_{50} (concentration of the sample to perform 50% inhibition) was measured by a chemiluminescence method. IC_{50} was also measured for the protective activity against DNA damage caused by hydroxyl radical using the same method. As a result, Cistanche salsa extract was confirmed to perform an extremely strong activity to inhibit reactive oxygen species and also protect DNA damaged by active oxygen (IC_{50}: 0.4211 μg/mL). IC_{50} values for each reactive oxygen species are listed in Table 5. These results indicate that Cistanche salsa extract has an anti-aging activity because it inhibits reactive oxygen species. Cistanche Tubulosa is expected to perform a similar activity because it belongs to the same family as Cistanche salsa and contains larger amounts of effective components.

Table 5. The Free Radical Scavenging Ability of Cistanche salsa extract

Free Radicals	IC_{50} (mg/mL)
O_2^- (superoxide)	0.0731
• OH (hydroxyl radical)	7.031
H_2O_2 (hydrogen peroxide)	0.098
$^1O_2$ (singlet oxygen)	0.1254

9) Xiaowen W., *et al.*, Free radical scavenging ability from *Cistanche* glycosides and its protection ability against DNA damage induce by OH. *Chinese Pharmaceutical Journal*, **36**(1), 29-32, 2001.

2) Enhances SOD activity and prevent lipid peroxidation (*in vivo*)

Cistanche salsa extract (fraction in which phenylethanoid glycosides are the main components) has been reported to perform activities to raise SOD activity and control lipid peroxidation[10]. Each sample was orally administered to mice for 18 days. Two hours after the final administration, blood was taken from the mice and SOD activity in red blood cells and serum MDA (malondialdehyde) content were measured. The extract's influence on DNA and RNA contents in organs (heart, brain, liver, and kidneys) was also evaluated. As a result, Cistanche salsa extract was confirmed to significantly increase SOD activity and control MDA content as compared to the control group (Fig. 23). Moreover, the extract was clarified to increase DNA and RNA contents

in the liver and kidneys (Tables 6, 7). The results apparently indicate that Cistanche salsa extract has an anti-aging activity because it controls lipid peroxidation and has an antioxidative activity. Cistanche Tubulosa is expected to perform a similar activity because it belongs to the same family as Cistanche salsa and contains larger amounts of effective components.

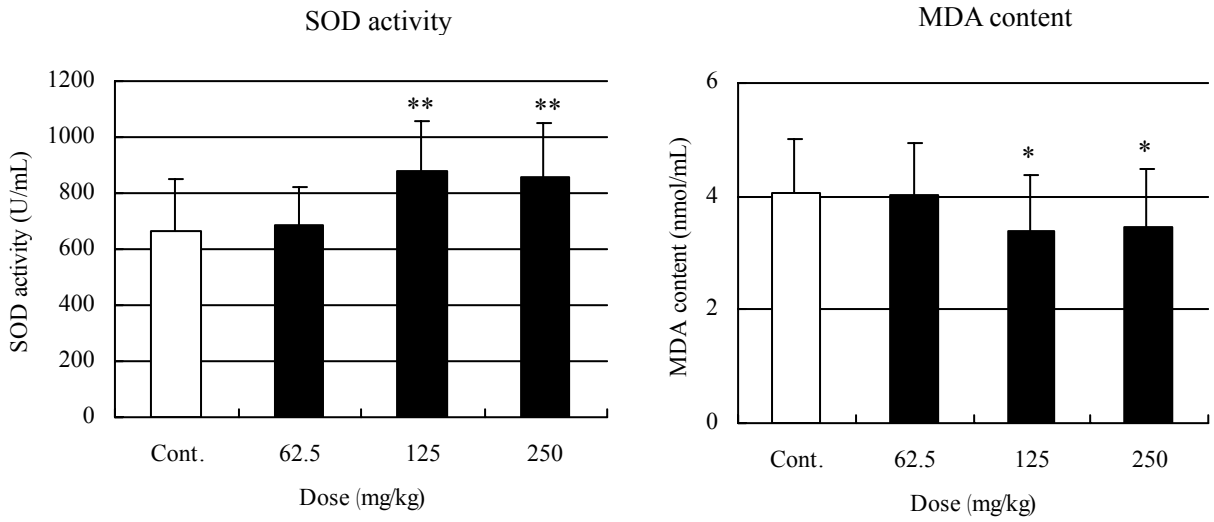


Fig. 23 Effect of Cistanche salsa extract on red blood cell SOD activity and serum MDA content in normal mice. (Mean±S.D., *: p<0.05, **: p<0.01, n=20-30)

Table 6. Effect of Cistanche salsa extract (CS Ext.) on organ DNA content in normal mice. (Mean±S.D., *: p<0.05, **: p<0.01, n=20-30)

Group	Dose(mg/kg)	DNA (μg/100mg)			
		heart	brain	liver	kidneys
Cont.		72.2±13.3	53.7±14.1	96.1±17.0	141.6±20.7
CS Ext.	62.5	73.7±14.2	58.7±20.0	96.4±10.2	146.2±20.9*
	125	72.2±14.6	58.1±16.6	105.3±10.6*	163.4±22.2**
	250	73.4±12.5	63.3±18.6	109.7±17.8**	164.3±19.2**

Table 7. Effect of Cistanche salsa extract (CS Ext.) on organ RNA content in normal mice. (Mean±S.D., **: p<0.01, n=20-30)

Group	Dose(mg/kg)	RNA (μg/100mg)			
		heart	brain	liver	kidneys
Cont.		76.1±17.9	81.2±17.3	253.5±56.7	133.4±17.9
CS Ext.	62.5	78.3±18.3	80.7±17.1	252.6±42.9	142.9±28.9
	125	76.4±18.4	81.4±15.3	299.5±52.9**	161.3±27.8**
	250	77.0±13.8	90.6±18.2	319.9±39.5**	167.3±25.6**

10) Linlin L., *et al.*, Effects on *Cistanche* glycosides anti-lipid peroxidation and anti-radiation. *China Journal of Chinese Material Medicine*, **22**(6), 364-367, 1997.

3) Cistanche species on peroxidation (*in vitro, in vivo*)

Cistanche Tubulosa (CT Ext.) and Cistanche salsa extracts (CS Ext.) were compared for their activity to control lipid peroxidation in the test described below[11].

1. Effect of Cistanche species extract on serum MDA content in rabbit (*in vitro*)
   Whole blood of rabbit was incubated with Cistanche Tubulosa extract or Cistanche salsa extract and the malondialdehyde (MDA) content, an index of serum lipid peroxidation) was measured by the TBA method. As a result, Cistanche Tubulosa and Cistanche salsa extracts significantly reduced the serum MDA content as compared to the control (Fig. 24). Cistanche Tubulosa demonstrated a stronger activity as compared to Cistanche salsa.

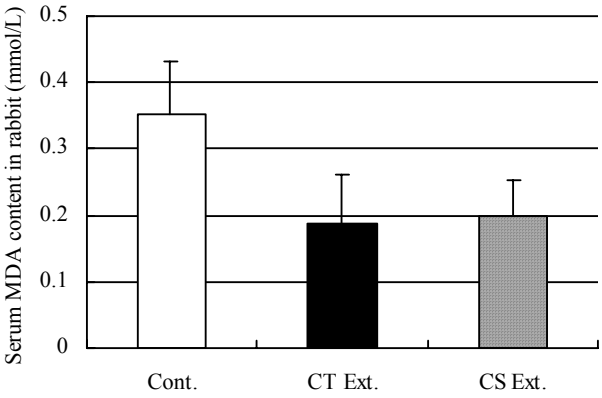


Fig. 24 Effect of Cistanche species extract on serum MDA content in rabbit (Mean±S.D., *: $p<0.05$, $n=8$)

2. Effect of Cistanche species extract on liver MDA content in mice (*in vitro*)
   Liver homogenate of mice was incubated with Cistanche Tubulosa extract or Cistanche salsa extract and the MDA content in the supernatant was measured by the TBA method. As a result, Cistanche Tubulosa and Cistanche salsa extracts significantly reduced the MDA content in the supernatant as compared to the control (Fig. 25). Cistanche Tubulosa demonstrated a stronger activity as compared to Cistanche salsa.

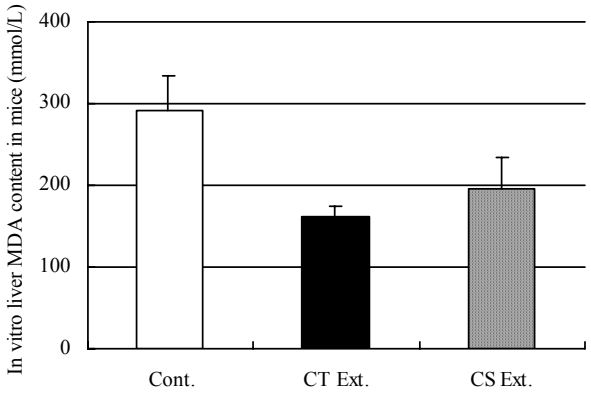


Fig. 25 Effect of Cistanche species extract on liver MDA content in mice (Mean± S.D., **: $p<0.01$, $n=8$)

3. Effect of Cistanche species extract on liver MDA content in mice with oral administration (*in vivo*)

Cistanche Tubulosa extract and Cistanche salsa extract were orally administered to mice once a day for ten consecutive days. On the eleventh day, the MDA content in the mice's liver homogenate was measured by the TBA method. As a result, Cistanche Tubulosa and Cistanche salsa extracts significantly reduced the MDA content in the liver as compared to the control (Fig. 26). The result clarified that Cistanche Tubulosa extract and Cistanche salsa extract perform the equivalent activity to control lipid peroxidation.

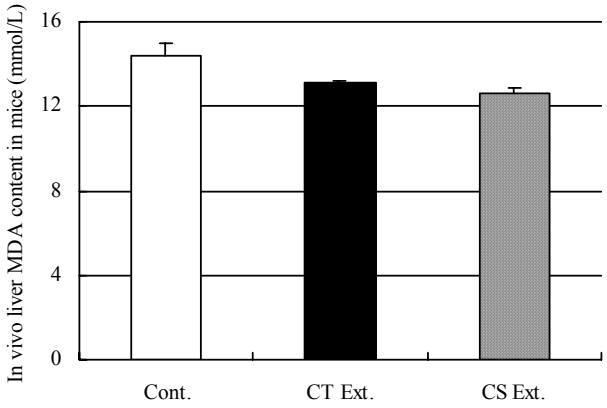


Fig. 26 Effect of Cistanche species extract on liver MDA content in mice (Mean± S.D., *: $p<0.05$, $n=6$)

11) Dawen S., *et al.*, The effects of traditional Chinese medicine *Cistanche* species on the immune function and lipid peroxidation. *Acta Academiae Medicinae Shanghai*, **22**(4), 306-308, 1995.

4) Anti-aging effect on aging mouse model (*in vivo*)

Echinacoside (ECH), an active constituent of Cistanche Tubulosa extract, has been reported to have an anti-aging activity on aged model mice[12]. D-galactose was subcutaneously administered to mice once a day for six consecutive weeks to create aged model mice. Each sample was orally administered for the same period of time and vitamin E (VE) was administered to the control group. On the following day of the final administration, blood was taken from the mice and their organs (heart, liver, kidney, and brain) were removed. The reactive oxygen species (ROS) level in tissue of the heart, liver, kidney, and brain was measured by the EPR (electron paramagnetic resonance) method. Blood GSH-Px activity was measured by the DTNB method [5,5'-dithiobis(2-nitrobenzoic acid) method] and serum SOD activity was measured by the EPR method to use as indexes of the body oxidation prevention system. The liver MDA content was also measured by the TBA method and brain MAO (monoamine oxydase) activity was measured using a MAO activity measurement kit. As a result, the reactive oxygen species level in tissue of the heart, liver, kidney, and brain was

significantly improved in the echinacoside group as compared to the aged model mice group (Fig. 27). Both echinacoside and vitamin E significantly increased blood GSH-Px activity and serum SOD activity and also significantly reduced the brain MAO activity and liver MDA content (Table 8). The results clarified that echinacoside, an active constituent of Cistanche Tubulosa extract, has an anti-aging activity. The anti-aging mechanism seems to be performed by echinacoside's activities to combat oxidation, control the liver MDA content, and reduce the brain MAO activity.

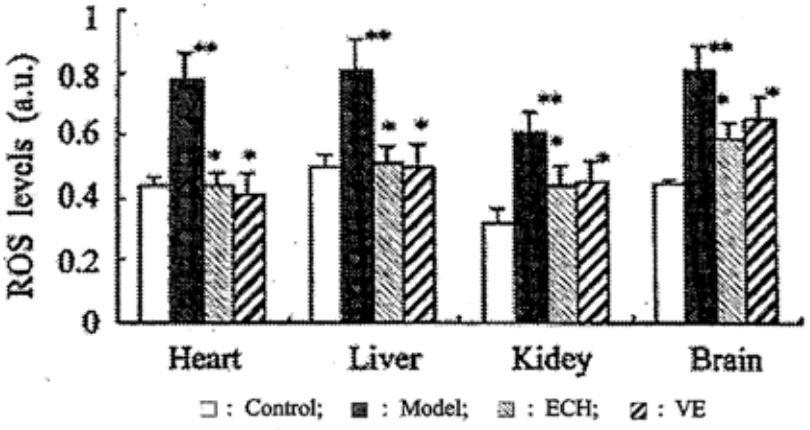


Fig. 27 The level of ROS in heart, liver, kidney and brain tissue among different groups (Mean ±S.D., **: p<0.01 vs control group *: p<0.01 vs model group, ECH: 50 mg/kg, VE: 50 mg/kg, n=10)

Table 8 The activities of whole blood GSH-Px, SOD in serum, MAO in brain and the content of MDA in liver among different groups of mice (Mean±S.D., n=10)

	GSH-Px (U/mgprot)	SOD (U_{ESR}/ml)	MDA (nmol/mgprot)	MAO (U/h/mgprot)
Control	60.63±7.80	350.5±11.3	1.52±0.20	24.76±1.19
Model	32.99±10.75**	300.0±14.2**	2.46±0.32**	28.09±3.76**
ECH (50 mg/kg)	54.27±7.97*	338.0±18.5*	1.95±0.21*	24.71±0.88*
VE (50 mg/kg)	45.87±7.42*	343.0±18.4*	1.95±0.37*	22.59±2.52*

**P<0.01 vs control group; *P<0.01 vs model group.

12) Gulinuer M., *et al.*, Anti-aging function study on echinacoside. *Acta Biochimica et Biophysica Sinica*, **20**(3), 183-187, 2004.

(3) Skin beautifying effect

1) Inhibition of hyaluronidase (*in vitro*, Data from Oryza)

Hyaluronidase is a hydrolase of hyaluronic acid and is distributed widely in tissues of animal body including the skin. Hyaluronic acid, the substrate of the enzyme, is a type of mucopolysaccharide existing in the skin, ligaments, joint fluids, and vitreous bodies in great quantity. In the skin, hyaluronic acid is involved in protecting cells, distributing nutrients, and maintaining cells' moisture content and elasticity. It also has a crucial role as joint fluid to maintain tissue structure/functions and lubricity. Hyaluronic acid content is known to decrease due to aging and diseases, which leads to dry skin, rough skin texture, lack of firmness or suppleness, dark spots, wrinkles, and arthritic pain caused by poorly lubricated joints. The activity of Cistanche Tubulosa extract, echinacoside, and acteoside to inhibit hyaluronidase (type I) expressing in the human body was evaluated in order to learn about their activity to prevent aging of the skin. As a result, Cistanche Tubulosa extract was confirmed to inhibit hyaluronidase (type I) (Fig. 28). The result indicates that Cistanche Tubulosa extract inhibits hyaluronidase in the human body. It also indicates that the extract has a potential activity to prevent aging of the skin. Echinacoside and acteoside were also confirmed to inhibit hyaluronidase (type I) (Fig. 29). This indicates that echinacoside and acteoside are involved in a part of Cistanche Tubulosa extract's hyaluronidase inhibitory activity in the human body.

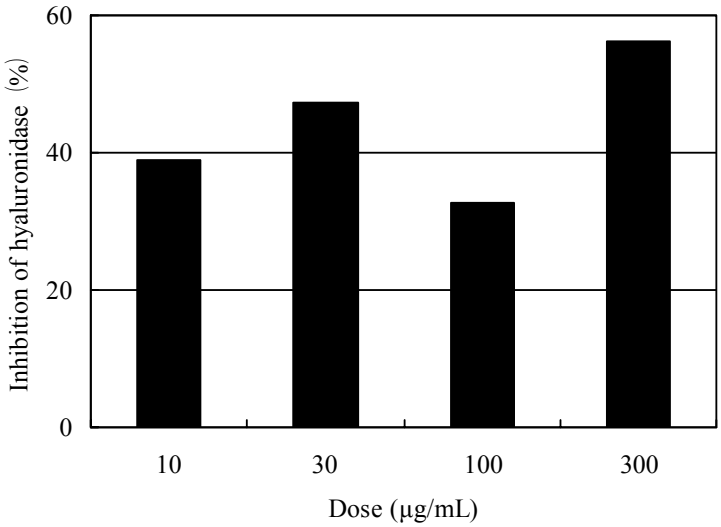


Fig. 28 Effect of Cistanche Tubulosa Extract on inhibit hyaluronidase (type I)

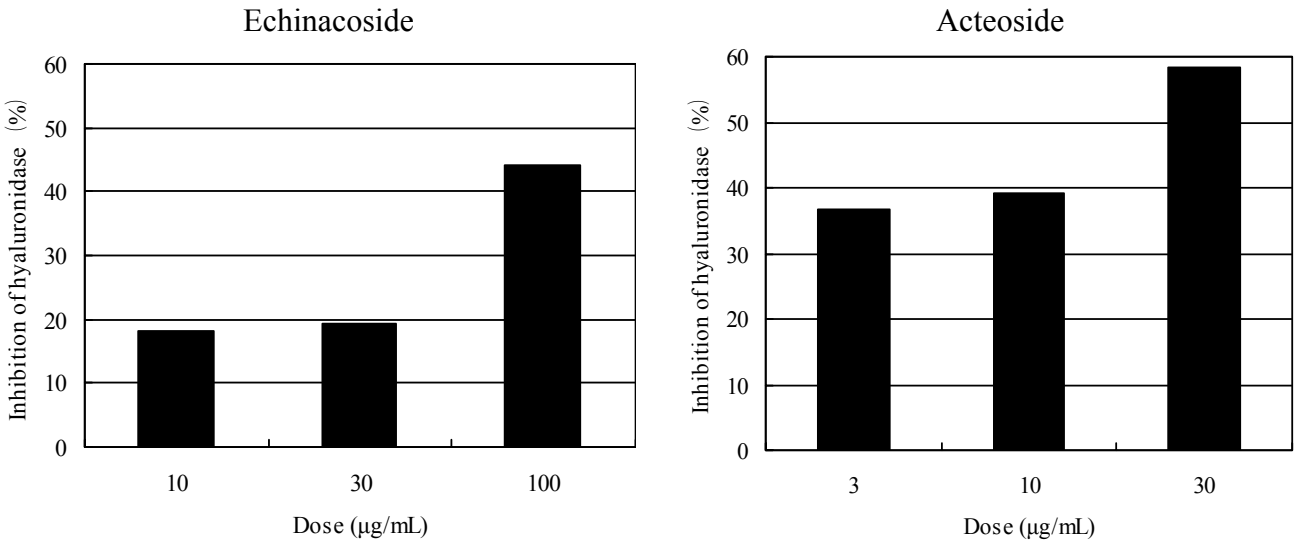


Fig. 29 Effect of echinacoside and acteoside on inhibit hyaluronidase (type I)

【Method】Each samples were dissolved in DMSO, and hyaluronic acid was hydrolized by hyaluronidase. After reaction with *p*-dimethylamino- benzaldehyde, absorbance was measured.

2) Prevention of Photo-ageing of Skin (*in vivo*, Data from Oryza)

One of the causes of skin's aging is active oxygen generated by UV ray. In order to learn about Cistanche Tubulosa extract's activity to prevent aging of the skin, photo-aged mice were created (by radiating UV ray to hairless mice) and the extract's influence on wrinkle formation and skin aging-related genes (hyaluronidase: Hyal1, collagenase: MMP-1) was evaluated. As a result, Cistanche Tubulosa extract was confirmed to control wrinkles formed by UV ray exposure at the concentration of 200 mg/kg (Fig. 30). As a result of a comparison in the expression of hyaluronidase and collagenase genes by RT-PCR, Cistanche Tubulosa extract (200 mg/kg) was confirmed to control the expression of hyaluronidase and collagenase genes caused by UV ray exposure (Fig. 31). The results were similar to the results of evaluation of the influence on wrinkle formation shown in Fig. 29. According to the results, Cistanche Tubulosa extract seems to control wrinkle formation by controlling the expression of skin aging genes. Thus, Cistanche Tubulosa extract was confirmed to control skin's aging caused by UV ray exposure.

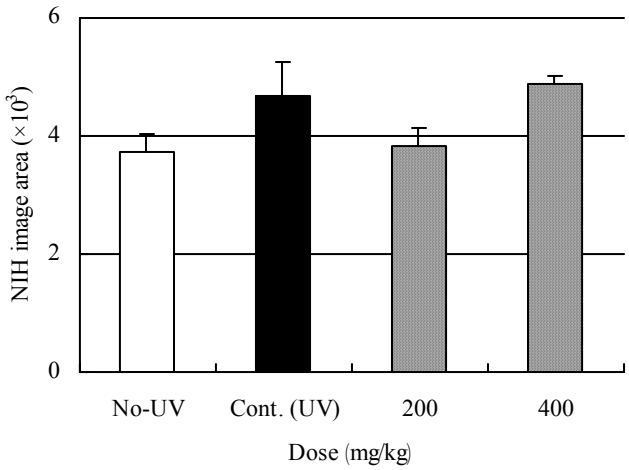


Fig. 30 Anti-wrinkle effect of Cistanche Tubulosa Extract (Mean±S.E., *n*=4)

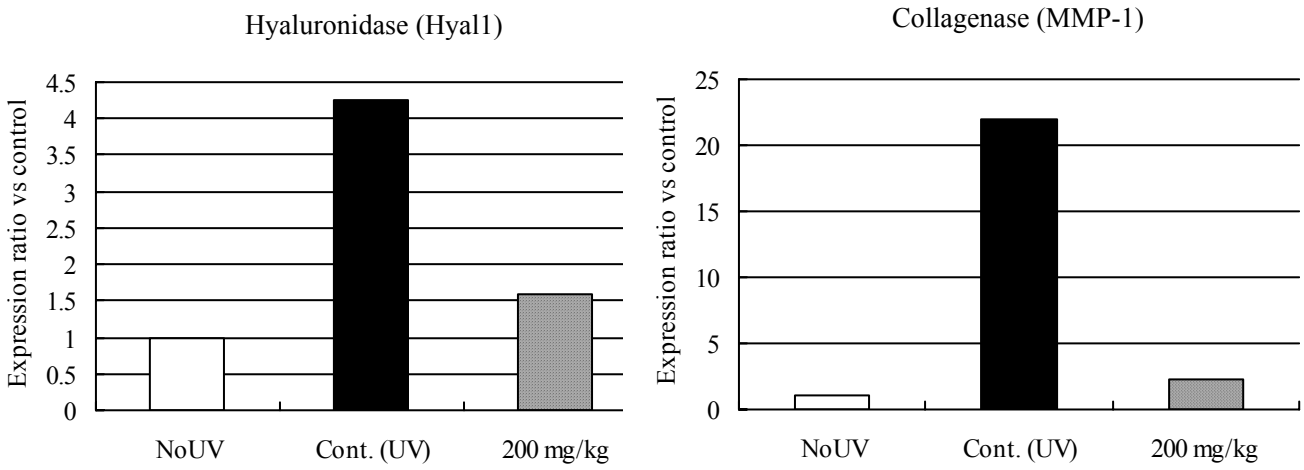


Fig. 31 The Effect of Cistanche Tubulosa Extract on Genetic Expression of Hyaluronidase and Collagenase (There is no RT-PCR analysis in group C.T. Ext 400mg/kg as RNA extraction was unsuccessful.)

【Method】Hairless mice were separated into four groups (No UV group, UV + solvent group, UV + 200 mg/kg of Cistanche Tubulosa extract group, and UV + 400 mg/kg of Cistanche Tubulosa). Each sample was orally administered for six consecutive weeks. The mice were exposed to UV ray three times a week for six weeks (1st week: 50 mJ/cm^2, 2nd week: 70 mJ/cm^2, 3rd and 4th weeks: 80 mJ/cm^2, 5th and 6th weeks: 200 mJ/cm^2). After the final administration, replicas of the back of the hairless mice were taken using Skin Cast. Then, image analysis was carried out on NIH images to evaluate the influence on wrinkle formation. The skin of the hairless mice was removed, RNA of the skin was extracted, and the expression of each gene was compared by RT-PCR after reverse transcriptase reaction.

(4) Anti-fatigue

Anti-fatigue of mice (*in vivo*)

Cistanche Tubulosa extract has been reported to have anti-fatigue activity with survival time under oxygen deficiency, survival time after the administration of sodium nitrite (toxic agent), and duration time in forced swimming as indexes[13].

1. Survival under anaerobic condition

Each sample was orally administered to mice for seven days. One hour after the last administration, mice were put in a 250 mL bottle (containing sodium carbonate) to measure the survival time under oxygen insufficiency. As a result, the survival time under oxygen insufficiency was significantly longer in the Cistanche Tubulosa extract group as compared to the control group (Fig. 32). The difference was concentration-dependent.

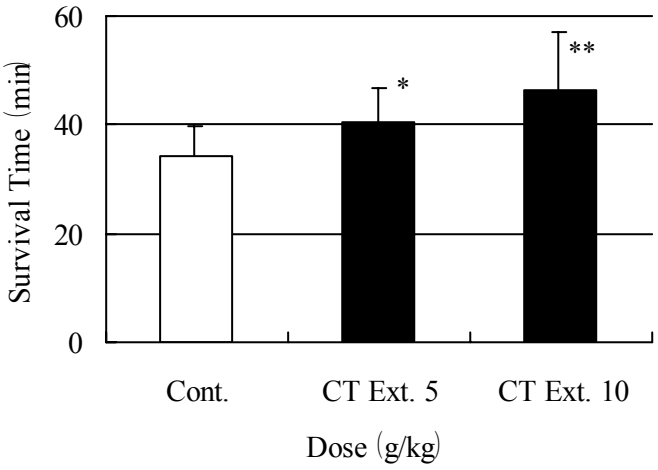


Fig. 32 Cistanche Tubulosa extract (CT Ext.) prolonged survival time under anaerobic condition (Mean±S.D., *: $p<0.05$, **: $p<0.01$, n=10-12)

2. Survival post administration of Sodium Nitrite (toxic agent)

Each sample was intraperitoneal administered to mice, sodium nitrite (250 mg/kg) was intraperitoneal administered 30 minutes later, and the survival time was measured. As a result, the survival time after the administration of sodium nitrite (toxic agent) was significantly longer in the Cistanche Tubulosa extract group as compared to the control group (Fig. 33). The effect was concentration-dependent.

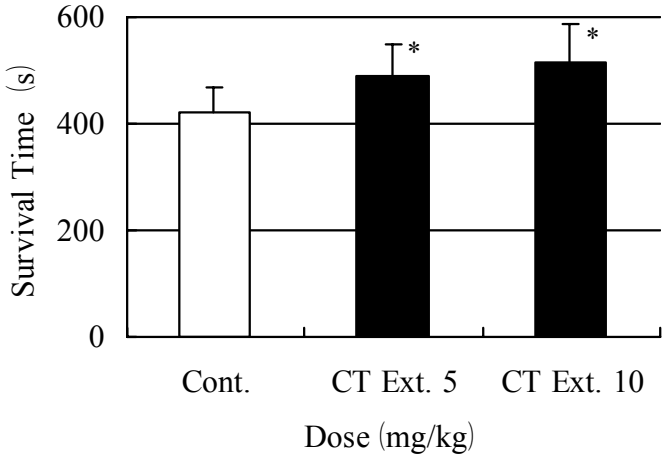


Fig. 33 Prolong Survival Time post administration of Sodium Nitrite (Mean±S.D., *: $p<0.05$, $n=10$)

3. Endurance under forced swimming test

Each sample was orally administered to mice for seven days. One hour after the last administration, a forced swimming test was conducted on the mice with a weight of 5% of their body weight. The time spent until the mice went under water and their breathing stopped was measured. As a result, the tolerance time in the forced swimming test was significantly longer in the Cistanche Tubulosa extract group as compared to the control group (Fig. 34). The effect was concentration-dependent. The result indicates that Cistanche Tubulosa extract has an anti-fatigue activity on mice.

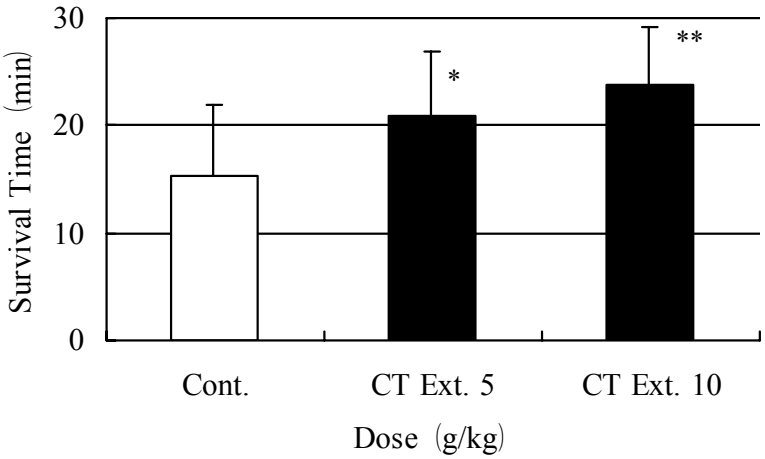


Fig. 34 Enhance Endurance under forced Swimming condition (Mean±S.D., *: $p<0.05$, **: $p<0.01$, $n=12$)

13) Zhiqiang W., *et al.*, Effects of CTG on oxygen insufficiency tolerance and fatigue tolerance. *Chinese Traditional Herbal Drugs*, **27**(supplementary issue), 137-138, 1996.

(5) Aphrodisiac effect

1) Effects of the constituents of Cistanchis herba on sex behavior in stressed mice (*in vivo*)

Cistanche salsa extract (fraction where phenylethanoid glycosides are the main components) and its components echinacoside and acteoside have been reported to have an activity to improve sexual ability[14]. The influence of Cistanche salsa extract and its components on sexual behavior reduced by stress was evaluated for 15 consecutive days. Ten female mice and one male mouse were put in one place for ten minutes and the number of mice that experienced mounting or intromission, the number of such behavior, and also the time spent until they started mounting were measured. Groups and the number of days that significant difference was seen as compared to the control group were described below. In the Cistanche salsa extract group, 3 and 5 days for the number of mounting and intromission respectively and 4 days for the time spent to start both mounting and intromission. In the echinacoside and acteoside groups, 4 and 5 days for the number of sexually active mice respectively, 3 to 6 days for the number of mounting and intromission, and 4 to 6 days for the time spent until starting mounting and intromission respectively. The results clarified that Cistanche salsa extract (fraction where phenylethanoid glycosides are the main components) contains active constituents to prevent the reduction of sexual behavior of stressed mice, which are echinacoside and acteoside. Cistanche Tubulosa is expected to perform a similar activity because it belongs to the same family as Cistanche salsa and contains larger amounts of effective components.

14) Sato T., *et al.*, Pharmacological studies on *Cistanchis Herba*. I. Effects of the constituents of *Cistanchis Herba* on sex and learning behavior in chronic stressed mice (1), *Yakugaku Zasshi*, **105**(12), 1131-1144, 1985.

2) Enhance male hormone production (*in vitro, in vivo*, Data from Oryza)

1. The effect on male hormone production gene expression in the liver

Influence of Cistanche Tubulosa extract (CT Ext.) on the expression of genes of enzymes involved in the synthesis of male hormone (testosterone) and its active form (dihydrotestosterone) in the liver was examined. Cistanche Tubulosa extract (400 mg/kg) was administered to mice once a day for two weeks and the total RNA in the liver was derived. DNA micro array analysis was conducted on one specimen each of the control group and Cistanche Tubulosa extract administration group. In the specimen from the Cistanche Tubulosa extract administration group, the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD), which is involved in testosterone, was enhanced by 1.5 times. The expression of the genes of steroid 5α-reductase 2 and aldo-keto reductase, which the synthesis of dihydrotestosterone, doubled (Fig. 35). Then, the expression of genes not included in the targets of micro array analysis, except for C17-20 lyase, was examined by RT-PCR. As a result, the gene expression of P450 SCC, 17α-hydroxylase, 17β-hydroxysteroid dehydrogenase, and steroid 5α-reductase 2 was enhanced (Fig. 36). Especially, gene expression of 5α-reductase 2 was 15 times higher as compared to the control group. These results clarified that Cistanche Tubulosa extract (400 mg/kg) enhances the expression of genes of enzymes involved in the production of male hormone in the liver.

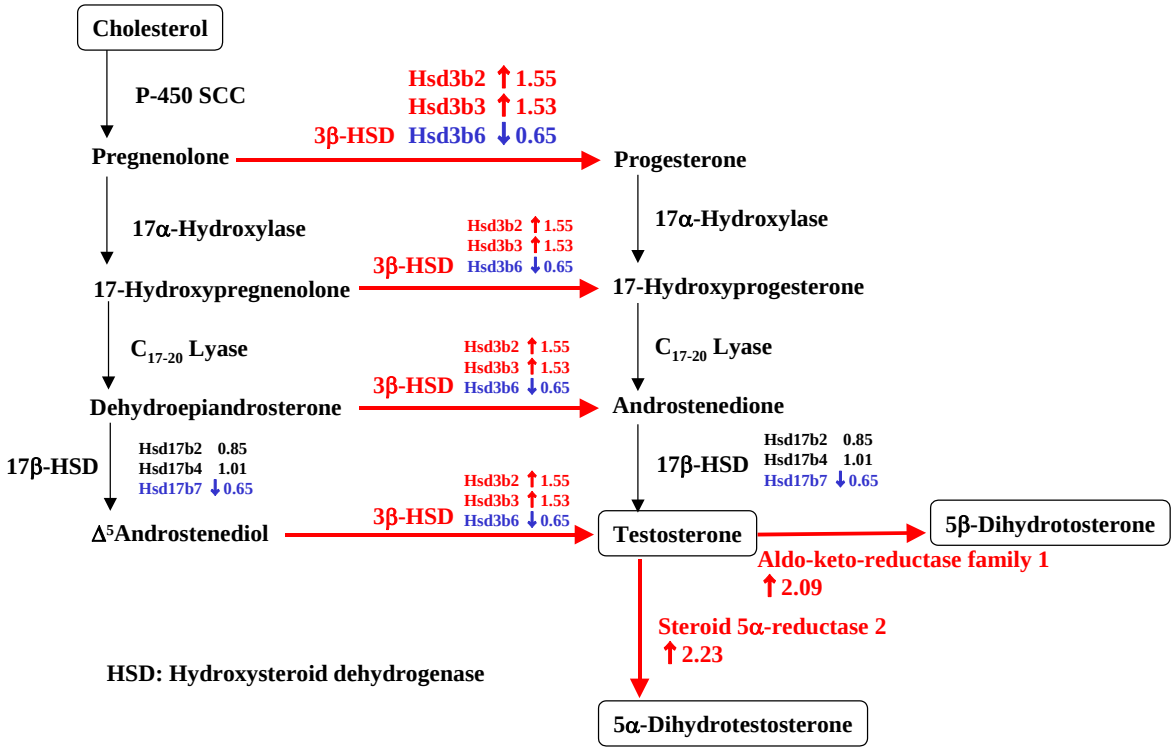


Fig. 35 DNA micro array analysis in liver [↑（Increase）, ↓（Decrease）and fold change rate is relative to control=1]

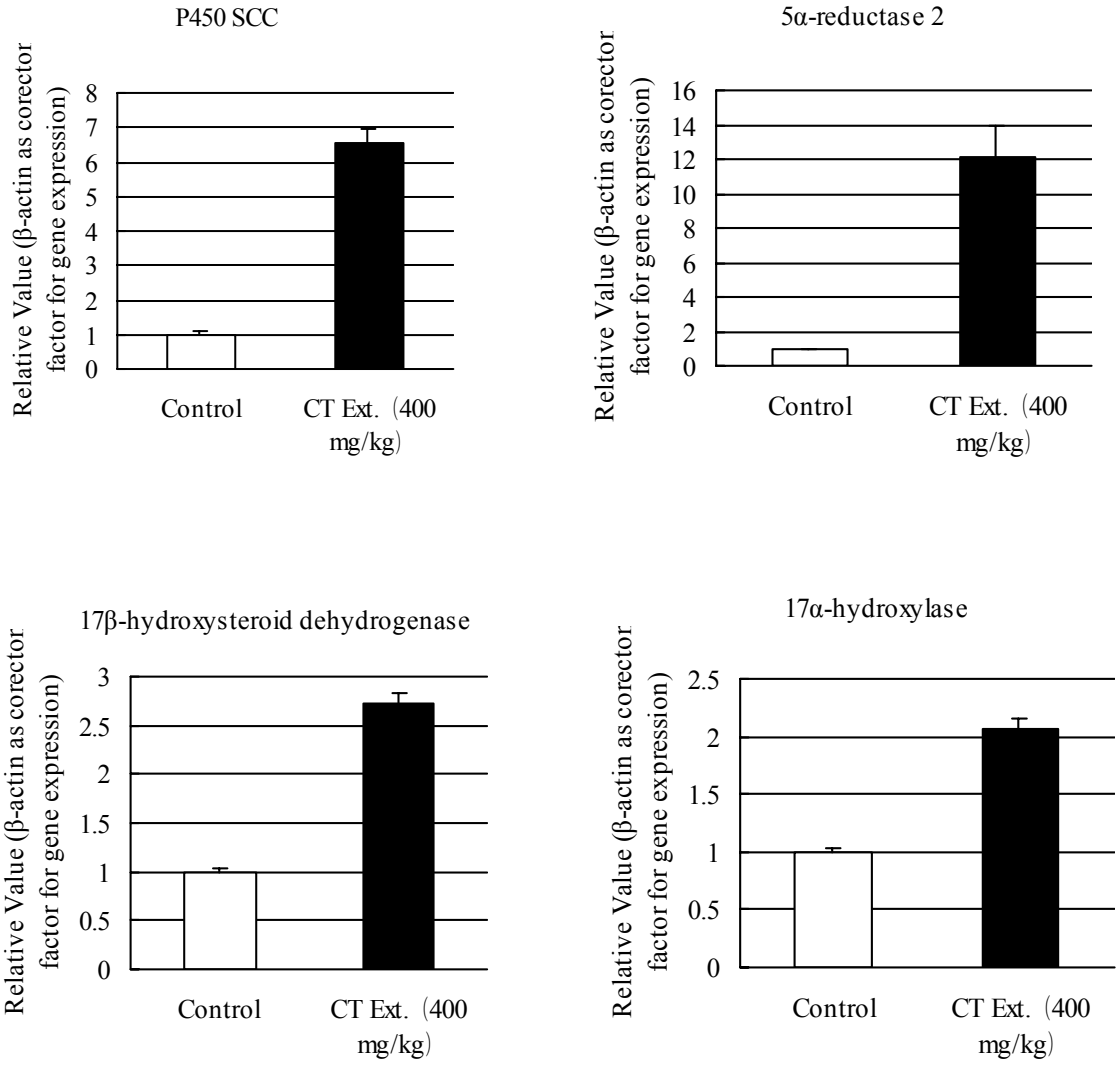


Fig. 36 RT-PCR analysis in liver (Mean±S.E., *n*=3-7)

2. The effect on male hormone production gene expression in the testis

After the examination on the liver, the extract's (CT Ext.) activity on the expression of genes of 5α-reductase 1 and 2 in the testis was evaluated. The result shows no enhancement in gene expression in the testis (Fig. 37).

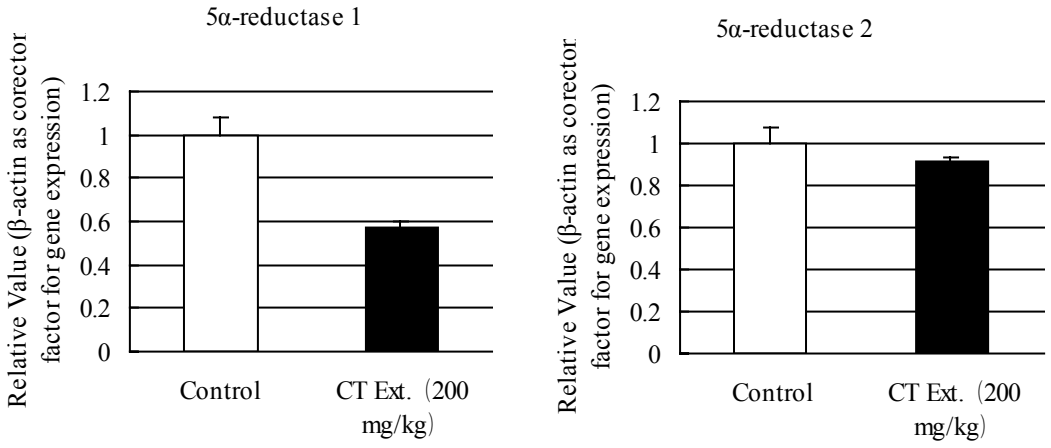


Fig. 37 RT-PCR analysis in testis (*n*=5-7)

【Method】Cistanche Tubulosa extract (200 mg/kg) was orally administered to five month old, male ddY mice for two weeks. After stabilizing their testis by "RNA later," RNA was derived using a kit manufactured by Quiagen. c-DNA was created by the reverse transcription reaction in the conventional manner and the gene expression was examined by RT-PCR.

3. The effect on mouse testosterone level

Based on the results of 1 and 2, the influence of Cistanche Tubulosa extract (CT Ext.) on blood male hormone (testosterone) concentration was evaluated. As a result, there was no significant difference though there was an increasing tendency in testosterone concentration (Table 9).

Table 9. The effect of Cistanche Tubulosa Extract on mouse testosterone level

	Dose (mg/kg)	*n*	Testosterone (ng/mL)
Mice (5 month)	-	6	29.53±10.4
CT Ext.	200	7	40.41±29.64
	400	7	79.11±44.66

Mean±S.E.

【Method】Cistanche Tubulosa extract was orally administered to five month old, male ddY mice for two weeks and whole blood was collected. After separating the serum, testosterone content was measured using the Testosterone EIA kit (Cayman Chemical Corporate).

4. Effect on testosterone production in Leydig cells

Leydig cell exists in the testis and produces testosterone. Using Leydig cell line (R2C), the extract's influence on testosterone production was evaluated. As a result, Cistanche Tubulosa extract and its main component echinacoside showed the activity to increase testosterone (Fig. 38).

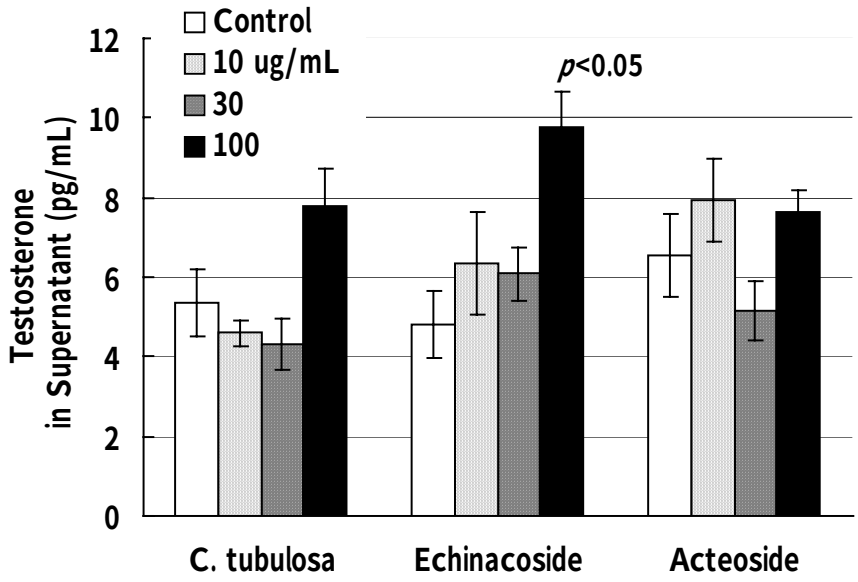


Fig. 38 Effect of Cistanche Tubulosa extract, echinacoside and acteoside on testosterone production in Leydig cells (n=4, Mean±S.E)

【Method】Leydig cells (R2C) derived from testicular cancer of rat were cultured in a 24-hole plate (2.5×10^5 cells/500 uL) and incubated for 24 hours. The sample was added, the cells were incubated for four hours, and the supernatant was separated to measure the testosterone content.

(6) Immune boosting effect

The effect of Cistanche Tubulosa extract on mouse lymphatic cells (*in vivo*)

Cistanche salsa extract (fraction in which phenylethanoid glycosides are the main components) has been reported to enhance immune strength[15]. ^{60}Co (cobalt 60) was radiated on mice in order to lower their immune strength. Each sample was then orally administered to the mice for 15 consecutive days. On the last day, blood was taken from the mice and specimen slides were prepared by staining. The diameter of lymphocytes (cells that attack bacteria and viruses), which served as the index of immune strength, was measured under a microscope to evaluate the extract's activity. As a result, the size of lymphocytes in the Cistanche salsa extract group was significantly enlarged as compared to the control group (Fig. 39). Cistanche Tubulosa extract is expected to perform a similar activity because it belongs to the same family as Cistanche salsa and contains larger amounts of effective components.

Cistanche Tubulosa extract also has been reported to activate lymphoid cells and increase the killing rate for cancer cells[16]. These reports convinced that Cistanche Tubulosa extract enhances immune strength.

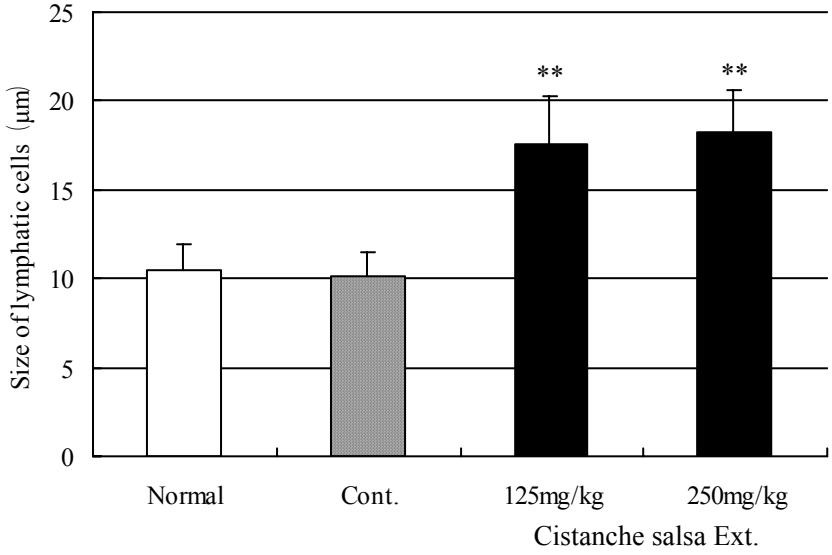


Fig. 39 Compare of Lymphocyte size (Mean±S.D., **: p<0.01, n=15)

15) Xiaowen W., *et al.*, Morphological changes of peripheral blood corpuscles of radiated mice feeded with *Cistanche*. *ACTA Academiae Medicinae Xinjiang*, **18**(2), 83-86, 1995.

16) Dawen S., *et al.*, The effects of traditional Chinese medicine *Cistanche* species on the immune function and lipid peroxidation. *Acta Academiae Medicinae Shanghai*, **22**(4), 306-308, 1995.

(7) Metabolism enhancing effect

1) The effect on cholesterol metabolism (*in vivo*, Data from Oryza)

The influence of Cistanche Tubulosa extract (CT Ext.) on the expression of genes involved in lipid metabolism in the liver was examined. Cistanche Tubulosa extract (400 mg/kg) was administered to mice for two weeks and the total RNA in their liver was derived.

DNA micro array analysis was conducted on one specimen each of the control group and Cistanche Tubulosa extract administration group. The result showed that Cistanche Tubulosa extract controlled the expression of enzymes involved in the synthesis of cholesterol as shown in Fig. 40. HMG CoA reductase participates in the synthesis mevalonic acid from 3-hydroxy-methyl-3-methylglutaryl CoA (HMG CoA) and is a rate-determining enzyme in cholesterol synthesis. It is known that the inhibition of this enzyme is effective to treat hypercholesterolaemia. Controlling the expression of genes of cholesterol biosynthetic enzymes is believed to be effective to control cholesterol synthesis.

Furthermore, the expression of genes of apolipoprotein B, VLDL receptor, and lipoprotein lipase was enhanced in the mice that took Cistanche Tubulosa extract (Table 10). The expression of genes of apolipoprotein B and VLDL receptor was examined on other mice by RT-PCR and the expression of both was enhanced (Fig. 41). Since they both are involved in transfer and cellular uptake of cholesterol in blood, the test results indicate that Cistanche Tubulosa extract may lower blood cholesterol level.

Cholesterol level in the serum and liver of the mice that took Cistanche Tubulosa extract was measured. LDL-cholesterol/pre β+β lipoprotein ratio (fraction containing VLDL and LDL-cholesterol) slightly reduced and HDL-cholesterol/α-lipoprotein ratio (fraction containing HDL-cholesterol) slightly increased (Table 11). The change was slight because normal mice were used in the test. A clearer result is expected in a future test on hypercholesterolaemia (pathological condition) model animals.

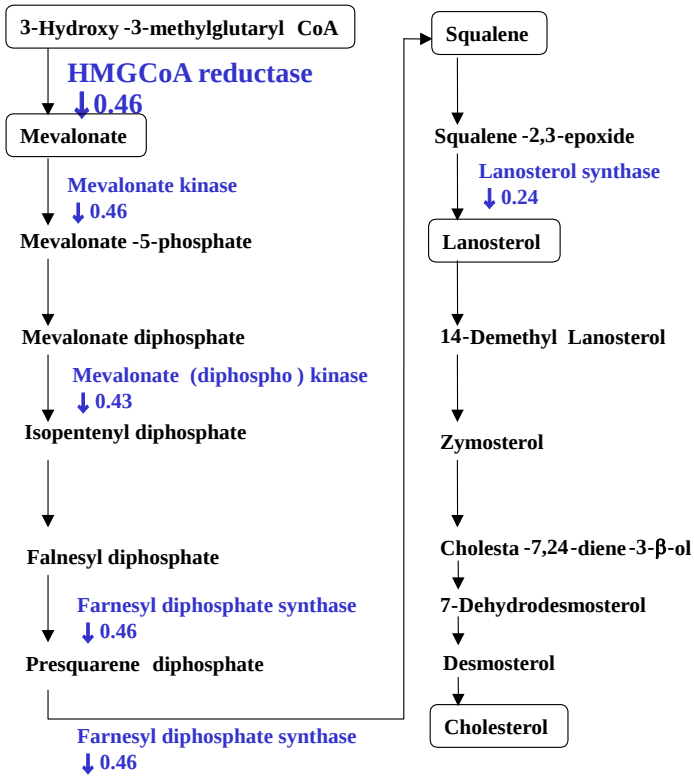


Fig. 40 DNA micro array analysis in Liver [↓ (Decrease) and fold change rate is relative to control=1]

Table 10 Up-regulation of cholesterol transporter gene DNA Micro Array analysis

	Fold Change	Function
Apolipoprotein B	↑2.87	Delivery VLDL cholesterol
VLDL receptor	↑9.00	Uptake of VLDL into cells
Lipoprotein lipase	↑2.08	Degradation and uptake of VLDL into cells

Fold change rate is relative to control=1

Table 11 Blood Lipid Profile of Liver Homogenate of mouse serum

	Total Cho. (mg/dL)	LDL-Cho. (mg/dL)	Ratio of Preβ+βlipo protein (%)	HDL-Cho. (mg/dL)	Ratio of α-lipoprotein (%)	Liver Cho. (mg/g)
Control	129.8±12.8	10.0±1.4	17.0±1.5	119.8±11.3	76.1±1.9	3.1±0.1
CT Ext.400mg/kg	132.0±8.7	9.5±0.5	16.4±0.9	123.8±7.7	78.3±1.2	3.2±0.2

Mean±S.E., *n*=5-7.

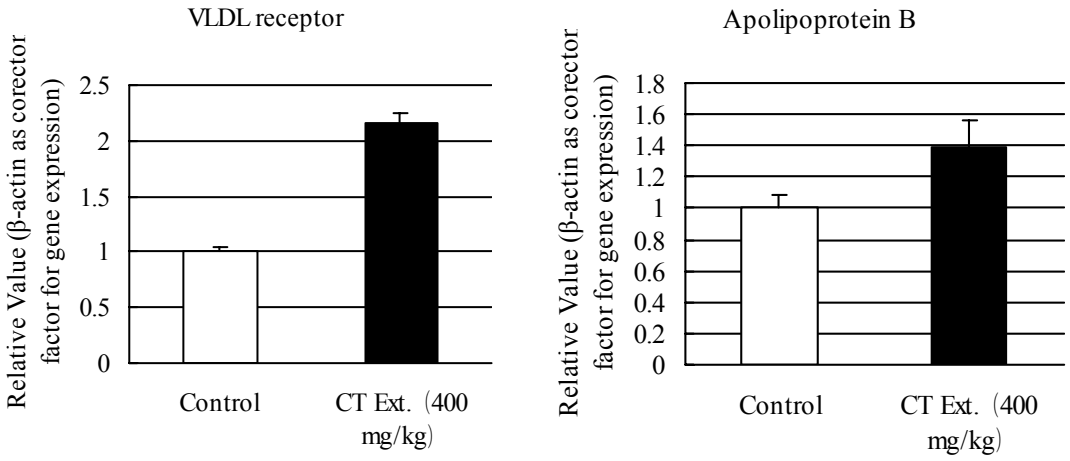


Fig. 41 RT-PCR analysis in Liver (enhancement of apolipoprotein B and VLDL receptor, Mean±S.E.)

2) The Effect of Cistanche Tubulosa Extract on Fatty Acid Metabolism (*in vivo,* Data from Oryza)

Genes involved in lipid metabolism in the liver were analyzed. As a result, the expression of the genes listed in Table 12 was enhanced. Lipin 1 and PPARα control lipid metabolism. Lipin 1 was recently confirmed to have functions to accelerate lipid metabolism. Acetyl-CoA acyl transferase and CPT are enzymes involved in beta-oxidation. The former is involved in actual beta-oxidation and the latter is involved in uptake of fatty acid into mitochondria. Enhancement of the gene expression of these proteins indicates that Cistanche Tubulosa extract (CT Ext.) accelerates the metabolism of fatty acid in the liver. For lipin 1 and CPT1, gene expression in other mice was examined by RT-PCR. As a result, genes of both were clearly enhanced as shown in Fig. 42. According to the results, Cistanche Tubulosa extract is expected to accelerate lipid metabolism in the liver.

Table 12 Genetic Expression of enzymes of Fatty Acid Metabolism

	Fold Change	Function
Lipin 1	↑5.11	Regulate lipid metabolism
PPARα	↑2.14	Regulate lipid metabolism (β-oxidation)
Acetyl-CoA acyl transferase 1A	↑2.78	Regulation of β-oxidation
Acetyl-CoA acyl transferase 1B	↑2.07	Regulation of β-oxidation
Carnitine palmitoyl transferase (CPT) 1	↑2.67	Uptake of fatty acids into Mitochondria (Rate limiting enzyme of β-oxidation)

Fold change rate is relative to control=1

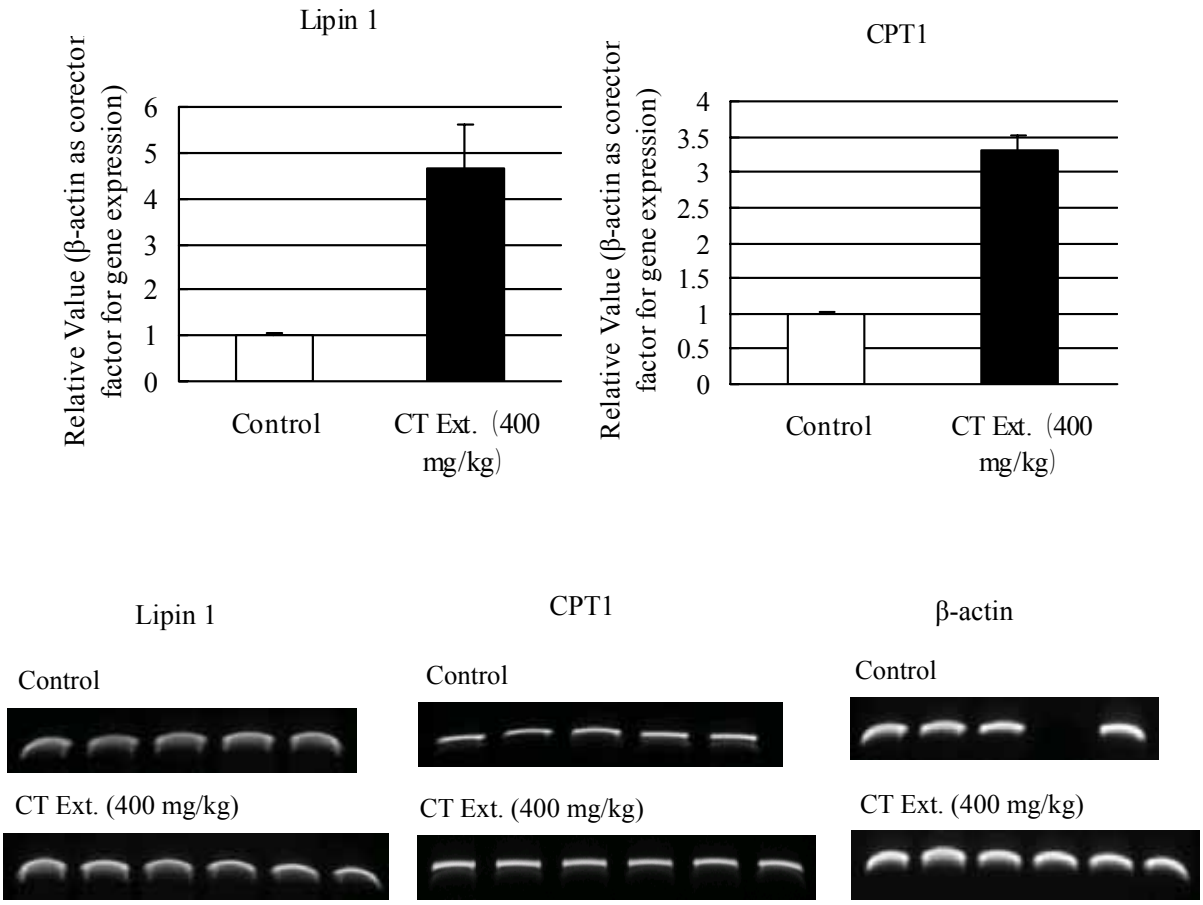


Fig. 42 RT-PCR analysis in Liver (enhanced of lipin 1 and CPT1, Mean±S.E., *n*=5-7)

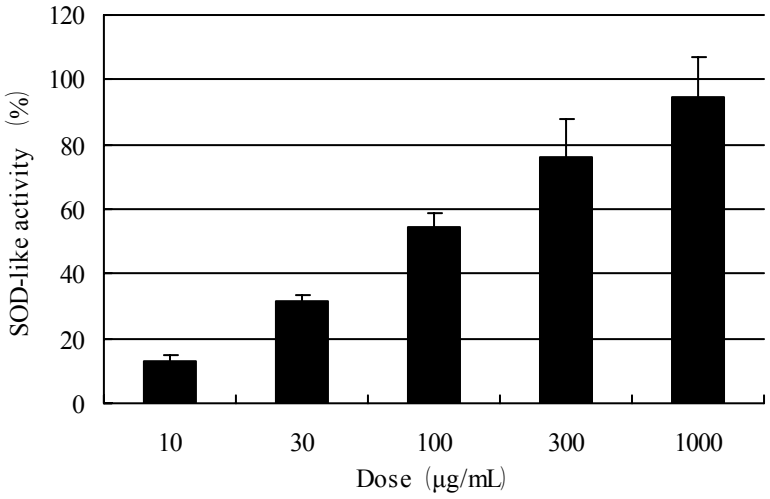
【Method】Cistanche Tubulosa extract (400 mg/kg) was orally administered to five month old, male ddY mice for two weeks. After stabilizing their liver tissue by "RNA later," RNA was derived using a kit manufactured by Quiagen. DNA microarray analysis was conducted on one specimen each of the control group and Cistanche Tubulosa extract administration group. Gene expression in other mice was evaluated by RT-PCR.

(8) Antioxidant Activities

SOD-like Activity and DPPH Radical Scavenger Activity (*in vitro*, ORYZA Data)

Free radicals are generated in our body in response to various endogenous metabolic reactions (e.g. stress). Free radicals such as reactive oxygen species (ROS) activate series of cells oxidation process leading to cell death and various degenerative diseases. Meanwhile, ageing process is accelerated with the increase in endogenous free radicals. The antioxidative effect of Cistanche Tubulosa Extract is evaluated using super oxide dismutase (SOD) model and 1,1-diphenyl 2-picryl-hyrazil (DPPH) radical scavenging model. As illustrated in Fig. 43, Cistanche Tubulosa Extract with high content of plant polyphenols demonstrated a dose-dependent antioxidative effect on SOD & DPPH radical scavenging models.

①SOD-like Activity



②DPPH Radical Scavenger Activity

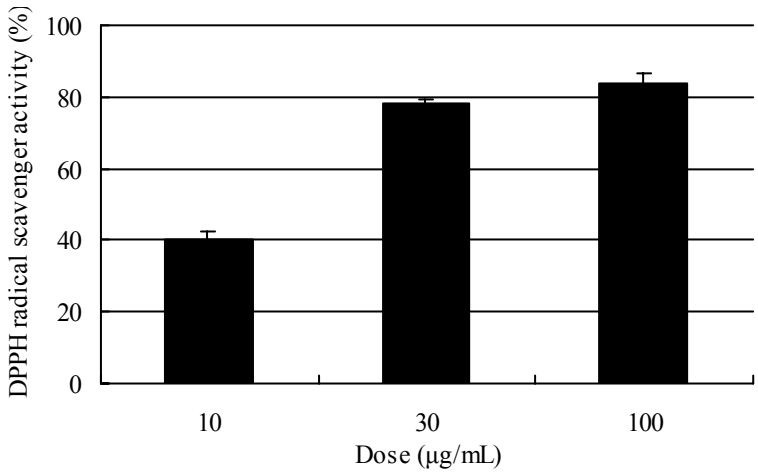


Fig. 43 Antioxidative Activity of Cistanche Tubulosa Extract (Mean±S.D., *n*=5)

4. Stability of Cistanche Tubulosa Extract

(1) Thermo stability

As illustrated in Fig. 44, echinacoside, acteoside and phenylethanoid glycosides content of Cistanche Tubulosa Extract is highly stable at 100°C and 120°C for 1 hour. It is stable at temperatures for processing food.

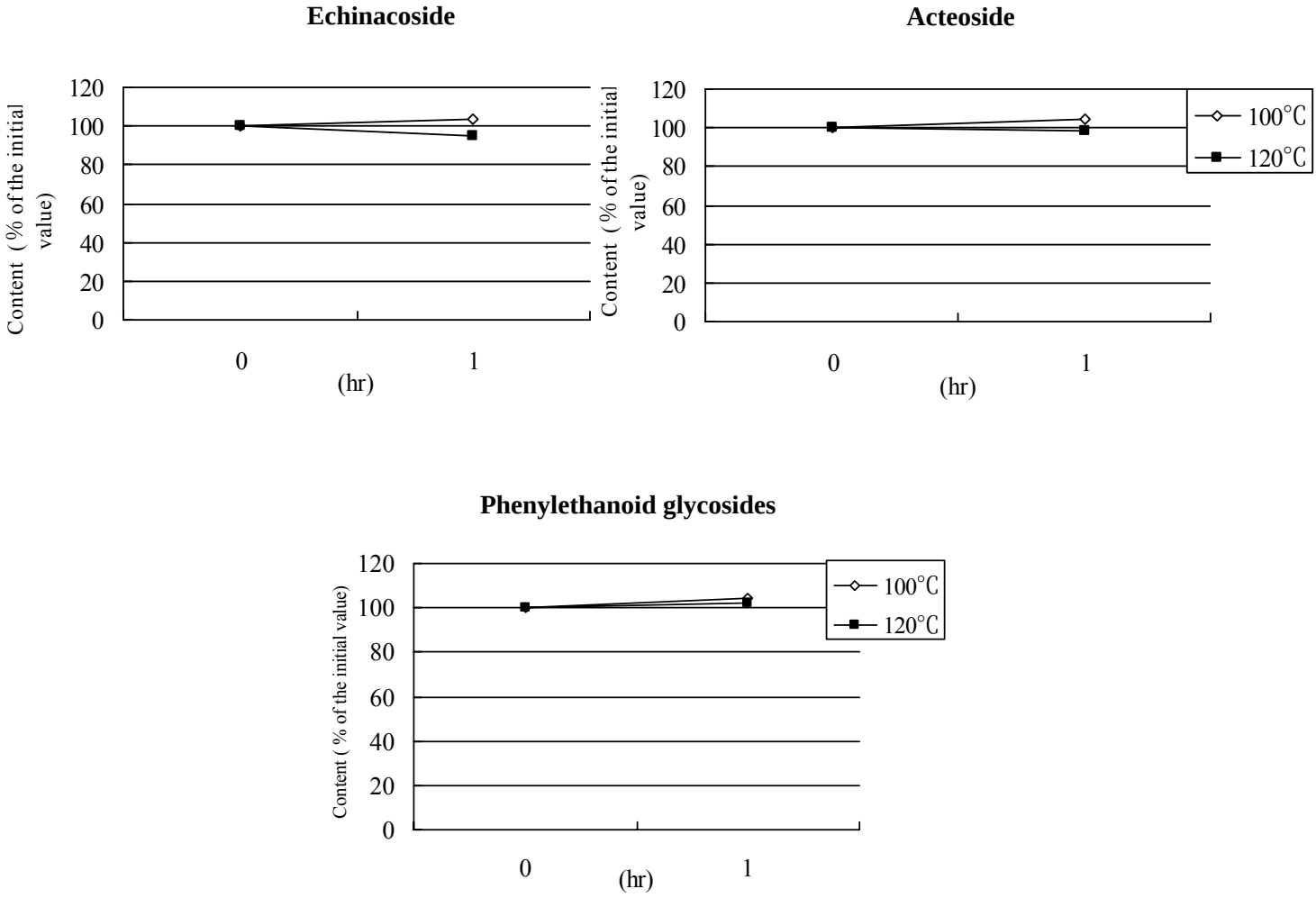
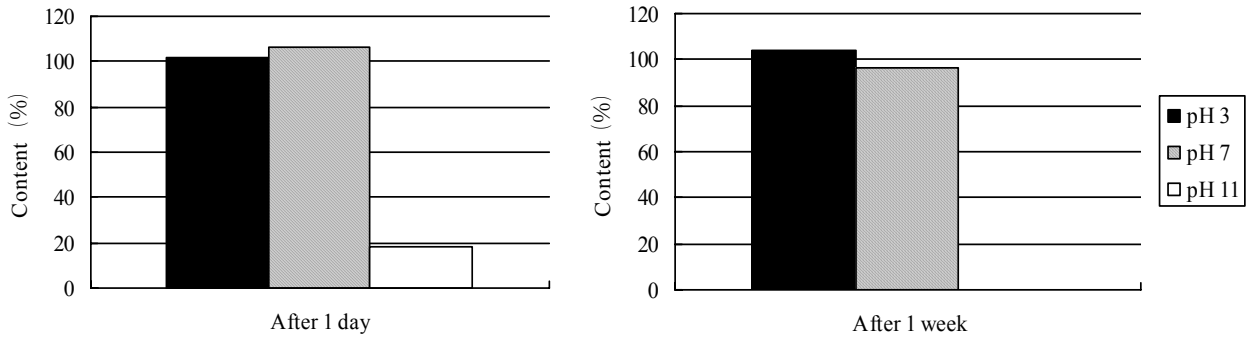


Fig. 44 Thermo stability of Cistanche Tubulosa Extract

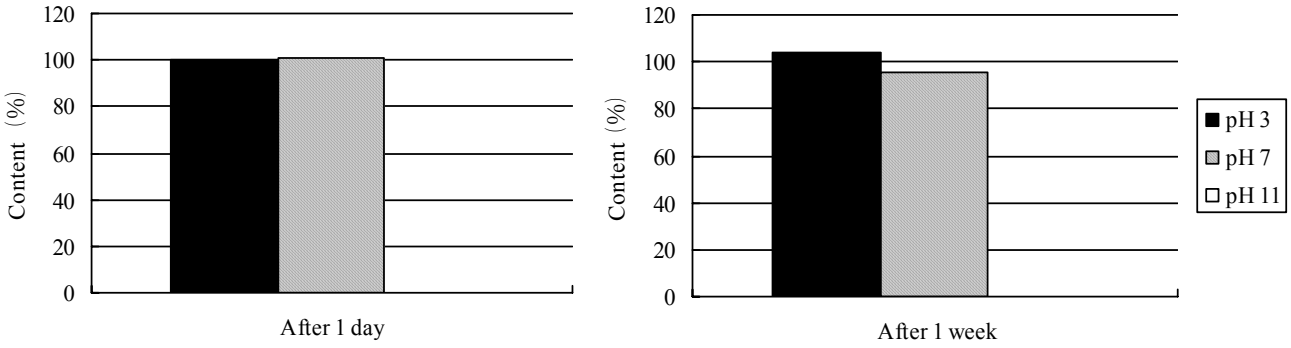
(2) pH stability

Cistanche Tubulosa Extract was dissolved in 30% ethanol, adjusted to its pH and stored at room temperature for 1 day and 1 week respectively. Echinacoside, acteoside and phenylethanoid glycosides content of Cistanche Tubulosa Extract was measured and results showed (Fig. 45) that echinacoside, acteoside and phenylethanoid glycosides content remained stable at acidic condition.

Echinacoside



Acteoside



Phenylethanoid glycosides

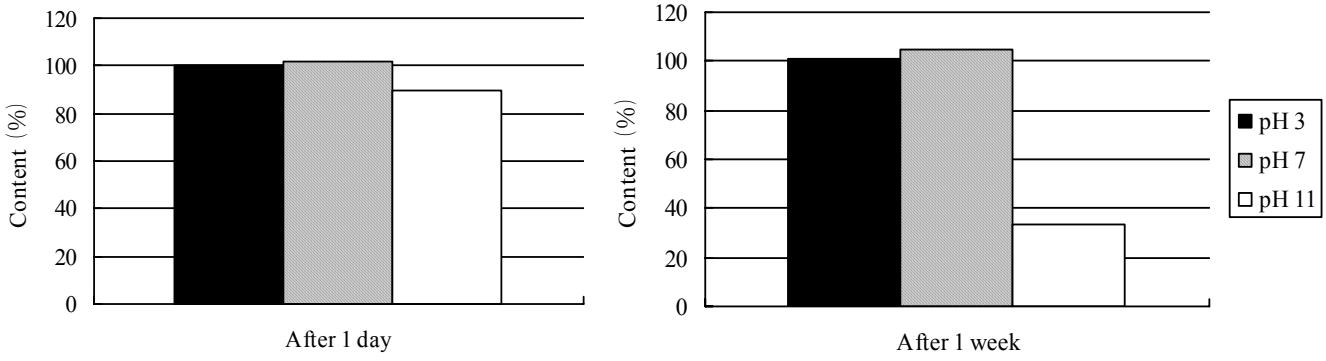


Fig. 45 pH stability of Cistanche Tubulosa Extract（100% as initial value）

(3) Stability in Aqueous Solution

A 0.4% solution (pH 3.5) of Cistanche Tubulosa Extract-P25 (powder, water soluble) was prepared and stored at room temperature (with and without light), 40°C (without light) & 5°C (without light) for 2 weeks. Visual observation on precipitation, turbidity and color change was conducted. As tabulated below, Cistanche Tubulosa Extract-P25 is highly in aqueous condition.

	Liquid stability（0.4% solution、pH 3.5）			
	Room temperature (light shielding)	25℃ (without light shielding)	40℃ (without light shielding)	5℃ (without light shielding)
Precipitation, turbidity	Negative	Negative	Negative	Negative
Color changes	Negative	Negative	Negative	Negative

5. Nutrition Information (Cistanche Tubulosa Extract-P25)

Description	Amount	Note	Analytical Method
Moisture	3.2g/100g		Heat-drying at atmospheric pressure
Protein	1.9g/100g	1	Kjeldahl Method
Fat	1.0g/100g		Acid degradation
Ash	2.9g/100g		Direct Incineration
Carbohydrate	91.0g/100g	2	
Energy	381kcal/100g	3	Atwater Method (Revised)
Dietary fiber	0.0g/100g		Prosky Method
Sodium	250mg/100g		Atomic absorption spectrophotometory
Sodium	0.6g/100g		Sodium Equiv. value

1. Nitrogen, protein conversion factor: 6.25
2. Carbohydrate expression standard (Ministry of Health and Welfare's announcement No. 176)
   Calculation: 50 – (water + protein + fat + ash)
3. Energy expression standard (Ministry of Health and Welfare's announcement No. 176)
   Conversion factor: Protein 4, fat 9, sugar 4; dietary fiber 2

Test trustee: SRL, Inc
Date of analysis: December 13, 2006
Test No.: 200611300029

6. Cistanche Tubulosa Extract – Product Safety Profile

(1) Residual Agricultural Chemicals

Cistanche Tubulosa Extract is conformed to regulation stipulated for 447 residual agricultural chemical compounds. No residual agricultural chemicals were detected as confirmed by test trustee.

Test trustee：Masis Co. Ltd.

Data： January 16, 2007

Report No.：9444

(2) Organic Certification

Cistanche Tubulosa Extract is an Organic Certified product. (Ref: COFCC-R-0704-0096)

CERTIFICATE

(Conversion)

China Organic Food Certification Center

This is to certify that

The Products in the Annex

produced by

Hetian Tianli Pharmaceutical Development Co.,Ltd

conform to

GB/T 19630.1-.4

"Organic Products"

(Production, Processing, Labeling and Marketing, Management System)

and are certified to be organic products

Category of certification: Production, Processing and Marketing

Production base: Kazinuke Development Zone(3000mu), Yutian County

Certificate serial number: COFCC-R-0704-0096

Issue date: 2007.4.26(Valid until 2008.4.25)

(3) Acute toxicity test（LD_{50}）

Acute Toxicity test was conducted according to the Guidelines for Single-Dose Toxicity Tests for Pharmaceutical Products. Cistanche Tubulosa Extract was orally administered to male and female mice at 26.4 g/kg and kept for 8 days. No abnormalities and fatal event observed at 26.4 g/kg. Upon autopsy no abnormalities were observed. Thus, LD_{50} of Cistanche Tubulosa Extract is deduced to be >26.4 g/kg in both male and female mice.

Furthermore, LD_{50} of Cistanche Tubulosa Extract is deduced to be >17.6 g/kg in both male and female rats.

(4) Genotoxicity

① Ames test

Ames test showed no difference of the colony counting in TA97, TA98, TA100 and TA102 strains with or without Cistanche Tubulosa Extract (8-5000 μg/plate).

② Micronucleus test

Micronucleus test of polychromatic erythrocyte in mice marrow showed that Cistanche Tubulosa Extract (2.5-10 g/kg) has no damage effects to bone marrow cells.

③ Teratogenicity test

Teratogenic test showed Cistanche Tubulosa Extract (2.5-10 g/kg) has no teratogenesis to mice spermatozoon.

(5) Sub-acute Toxicity

Cistanche Tubulosa Extract was orally administered to male and female rats at 0.65-1.30 g/kg and kept for 30 days. No abnormalities and fatal event were observed at 0.65-1.30 g/kg. Upon autopsy no abnormalities were observed.

(6) Long-term toxicity

Cistanche Tubulosa Extract was orally administered to male and female rats at 1.65 g/kg and kept for 180 days. No abnormalities and fatal event were observed at 1.65 g/kg. Upon autopsy no abnormalities were observed.

Furthermore, Cistanche Tubulosa Extract was orally administered to male and female beagle dogs at 1.50 g/kg and kept for 180 days. No abnormalities and fatal event were observed at 1.50 g/kg. Upon autopsy no abnormalities were observed.

7．Recommended Daily Dosage

The recommended daily dosage for Cistanche Tubulosa Extract–P25 is 100-400 mg/day.

8．Applications

	Applications	Claims	Examples
Foods	Brain function improving food Tonic food Beauty food	1) Improve of brain function 2) Tonic 3) Anti-aging 4) Anti- fatigue 5) Aphrodisiac 6) Beauty	Beverages, hard & soft capsules, tablets, candies, chewing gums, chocolates, wafers, jellies etc
Cosmetics	Beauty cosmetic		Body lotions, body gel etc.

9．Packaging

CISTANCHE TUBULOSA EXTRACT-P25（Water-soluble, for food）
CISTANCHE TUBULOSA EXTRACT-PC25（Water-soluble, for cosmetic）
5kg Interior packaging：Aluminum bag
Exterior packaging：Cardboard

10．Storage

Store in cool and dry dark place.

11．Expression

＜Food＞
CISTANCHE TUBULOSA EXTRACT-P25
Expression: CISTANCHE TUBULOSA EXTRACT

PRODUCT STANDARD
PRODUCT NAME

CISTANCHE TUBULOSA EXTRACT-P25

FOOD

This product is extracted from Cistanche tubulosa with aqueous ethanol. It guarantees minimum of 25.0 % echinacoside, 9.0 % acteoside and 50.0 % phenylethanoid glycosides. This product is water-soluble.

Appearance	Brown-light brown powder with light unique smell.	
Echinacoside	Min. 25.0 %	(HPLC)
Acteoside	Min. 9.0 %	(HPLC)
Phenylethanoid glycosides	Min. 50.0 %	(UV)
Loss on Drying	Max. 10.0 %	(Analysis for Hygienic Chemists, 1 g, 105℃, 2 h)
Purity Test		
(1)Heavy Metals	Max. 10 ppm	(The Japanese Standards for Food Additives)
(2)Arsenic	Max. 1 ppm	(Standard Methods of Analysis in Food Safety Regulation)
Standard Plate Counts	Max. 1×10^3 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Cistanche Tubulosa Extract	100 %

PRODUCT STANDARD
PRODUCT NAME

CISTANCHE TUBULOSA EXTRACT-PC25

COSMETIC

This product is extracted from Cistanche tubulosa with aqueous ethanol. It guarantees minimum of 25.0 % echinacoside, 9.0 % acteoside and 50.0 % phenylethanoid glycosides. This product is water-soluble.

Appearance	Brown-light brown powder with light unique smell.	
Echinacoside	Min. 25.0 %	(HPLC)
Acteoside	Min. 9.0 %	(HPLC)
Phenylethanoid glycosides	Min. 50.0 %	(UV)
Loss on Drying	Max. 10.0 %	(1 g, 105℃, 2 h)
Purity Test		
(1)Heavy Metals	Max. 10 ppm	(The Second Method)
(2)Arsenic	Max. 1 ppm	(The Third Method)
Standard Plate Counts	Max. 1×10^3 cfu/g	(Analysis for Hygienic Chemists)
Moulds and Yeasts	Max. 1×10^2 cfu/g	(Analysis for Hygienic Chemists)
Coliforms	Negative	(Analysis for Hygienic Chemists)

Composition

Ingredients	Contents
Cistanche Tubulosa Extract	100 %

Ref: The Japanese Standards of Quasi-Drug Ingredients.

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

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

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ORYZA OIL & FAT CHEMICAL CO., LTD.