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Antifatigue Activity of Tissue Culture Extracts of *Saussurea involucrata*

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Abstract

The antifatigue effect of the tissue culture of *Saussurea involucrata* (TCSauI) KAR. *et* KIR. (Compositae) was investigated in mice on swimming time, hepatic glycogen content, serum lactic acid content, serum urea-nitrogen, and serum lactate dehydrogenase. The current study provides evidence that TCSauI has antifatigue activity, suggesting the potential of the tissue culture technique to substitute for wild *S. involucrata* (WSauI) in the pharmaceutical industry.

Keywords: Antifatigue, *Saussurea involucrata*, tissue culture.

Introduction

Saussurea involucrata Kar. *et* Kir. is also called snow lotus. It belongs to Saussurea DC, tribe Cynareae Less, and the family Compositae. *S. involucrata*, a typical alpine plant, grows near the snowline at the height of 2800–3400 m, one of the rare Chinese medicinal herbs on the verge of extinction. The seeds of *S. involucrata* germinate at the temperature of 0°C and grow at the temperature of 3–5°C. The seedling can even stand alive at a temperature of −21°C. In the natural environment, the plant blooms after 5 years of germination. The seeds are quite tiny and have a low germination rate, which makes sexual reproduction difficult.

S. involucrata is peculiar to high-mountain arcas and is rich in bioactive substances including flavonoids, alkaloids, polysaccharides, and several kinds of amino acid and mineral elements (Li et al., 2000). The pharmacologically active constituents are mainly flavonoids. It is widely used in Chinese folk medicine for the treatment of rheumatoid arthritis, cough with cold, stomachache, dysmenorrhea, and altitude sickness (Song & Jai, 1990). Pharmacological studies have demonstrated that *S. involucrata* and its constituents have anti-inflammatory (Li et al., 1980), cardiotonic (Razdan, 1974), abortifacient (Lin & Wang, 1986), anticancer (Han, 1995), antiradiation (Wang & Bai, 1996), and antifatigue actions (Huang & Zhao, 1996).

At present, plants resource distribution in the area below 3000 m altitude have become severely scarce. After four years, *S. involucrata* will be thoroughly extinct if collected at the current rate. Because of the specific characteristics of the environment in which it lives, there are many problems to be faced when carrying on artificial cultivation. For one thing, it is quite difficult, for another, it needs a long period to fulfill reproduction, and then, compared with that of wild plants, the contents of pharmacological substances are not stable. Thus it is difficult to manage a large-scale production under given conditions. In order to conserve the natural resource of *S. involucrata*, tissue culture of *S. involucrata* (TCSauI) has been developed, which might be used as a potential substitute for wild *S. involucrate* (WSauI) in the pharmaceutical industry.

TCSauI has been studied to determine its anti-inflammatory and analgesic activities in experimental animals (Jia et al., 2005).Similar to WSauI, TCSauI at doses of 75–300 mg/kg intragastrically for 7 days markedly inhibited hindpaw edema induced by carrageenin in rats, ear edema induced by dimethylbenzene, and increased capillary permeability in the mouse abdominal cavity induced by acetic acid. Moreover, TCSauI had inhibitory activities against the writhing reaction induced by acetic acid and the hot-plate reaction in mice. In the current study, therefore, the antifatigue activity of the product of tissue culture of *S. involucrata* was investigated.

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Materials and Methods

Animals

Swiss mice, weighing 18–22 g, were used in the studies. The Experimental Animal Center of Shenyang Pharmaceutical University supplied all animals. The animals were housed under standard conditions (22 ± 2°C, 50 ± 10% relative humidity, 12-h light/dark cycles, noise < 60 dB). Food and water were available *ad libitum*. During the experiments, 50% of animals were male and 50% were female in each group.

Plant material

WSauI used in this study was purchased from a local market in Xinjiang (China) and identified by Professor QiShi Sun (Shenyang Pharmaceutical University). The dried flowers (2.4 kg) of WSauI were ground and extracted three-times with 95% ethanol under reflux. The extracts were concentrated under vacuum evaporation to afford brown extracts (yield 6.5%). The content of total flavonoids in the dried extract was 1.12%.

Tissue culture material

Explants of flowers of WSauI were cultured on MS (Murashige & Skoog, 1962) medium with naphthylacetic acid 2.5 mg/L, 6-benzylaminopurine 1.5 mg/L, 2.5% sucrose, and 0.65% agar for the inducement of callus and tissue culture. The culture conditions were temperature, 24 ± 1°C; light intensity, 58.4 μmol·m^{-2}·s^{-2}; and light period, 12 h/day. The calli were dried at 60°C and ground through a 40-mesh sieve. TCSauI (5.0 kg) was extracted three-times with 95% ethanol. The extracts were concentrated under vacuum evaporation to afford brown extracts (450 g). The content of total flavonoids in the brown extracts was 3.2%. The quality stability of culture patches was inspected by determining the total flavonoids in TCSauI using spectrophotometric (Kulevanova et al., 2000) and HPLC methods (Chen et al., 2001).

In all tests, animals were randomly divided into four groups. TCSauI was intragastrically administered at the doses of 0.5, 1.0, and 1.5 g·kg^{-1}·day^{-1}, p.o. WSauI was administered at the dose of 1.0 mg/kg, p.o. Animals in blank control group were administered 0.5% CMC-Na intragastrically. TCSauI and WSauI were administered once a day for 15 days as pilot studies showed that acute treatment with TCSauI or WSauI has no obvious pharmacologic activity. All treatment was performed once a day for 15 days. Fifteen days after drug administration, the swimming time, liver starch content, serum lactic acid (LA) content, and serum urea-nitrogen of TCSauI groups and the control group were recorded.

Mice endurance capacity experiment

A piece of lead that weighed 5% of the weight of a mouse was fixed to the tail of each mouse 30 min after administering the test drug for the last time, and the mice were put in a box with water 30 ± 1 cm deep and temperature 25 ± 1°C, and then the water was agitated at intervals to make the mouse limbs keep moving. The duration of time from beginning of time swimming to exhaustion was recorded.

Determining the content of hepatic glycogen

The mice were killed 30 min after being given the test drug for the last time. Liver was taken out, washed, and blotted. Then, 200 mg of the liver tissue was added to 4.0 mL trichloroactic acid (TCA), homogenized for 1 min, centrifuged for 15 min at speed of 3000 rpm, and the upper layer of liquid was moved to another tube. The process was repeated, the liquid was mixed, and 1.0 mL was added to 4.0 mL 95% ethanol and mixed. After this, the mixture was centrifuged for 15 min at a speed of 3000 rpm, and the upper layer was cleared off completely. Then, 2.0 mL of distilled water was added to dissolve the hepatic glycogen. The tube from the blank group was filled with 2.0 mL of distilled water, and the tube from the standard group was filled with 0.5 mL of standard glucose solution and 1.5 mL distilled water. Then, 10.0 mL of anthracenone reagent was added into each tube, placed in cool water immediately, boiling water for 15 min, and cool water again. At room temperature, samples were assayed at a wavelength of 620 nm, zeroing with the blank tube by colorimetry, and the content of hepatic glycogen was calculated.

Determining serum LA concentrations

A piece of lead that weighed 2% of the weight of a mouse was fixed to the tail of each mouse 30 min after administering the test drug for the last time, and the mice were put in a box with water 30 ± 1 cm deep and temperature 25 ± 1°C. The water was agitated at intervals to make the mouse limbs keep moving. They were taken out 60 min later, allowed to rest for 15 min, and the eyeball was extirpated to take blood. A solution of 1% NaF solution (0.48 mL) was added into each tube, followed by 20 μL whole blood, and then 1.5 mL protein precipitator. After centrifugation for 10 min at a speed of 3000 rpm, 0.5 mL of the upper layer liquid was placed in a test tube. Sample from the standard group were treated with 0.5 mL of standard LA solution and samples of the blank group were treated with 0.5 mL of the mixture of NaF solution and the protein precipitator. Then, 0.1 mL 4% $CuSO_4$ and 3.0 mL concentrated sulfuric acid were added to each tube, mixed adequately, and placed into boiling water for 5 min, and into cool water for 10 min. Next, 0.1 mL L.5% hydroxydiphenyl was added to each tube, placed into 30 ± 1°C water for 30 min, into boiling water for 90 s, and refrigerated to room temperature. They were assayed at a wavelength of 560 nm, zeroing with the blank tube by colorimetry, and the serum LA concentrations were calculated.

Determining the content of serum urea-nitrogen and of serum LDH (lactate dehydrogenase)

The mice were weighed and placed into a box with water (30 ± 1 cm, 30 ± 1°C) 30 min after receiving the test drug for the last time. The water was agitated at intervals to make the mose limbs keep moving. One and one-half hour later, mice were taken out and their blood serum prepared and analyzed with COBAS MIRA (Roche Diagnostics Corporation, Indianapolis, IN, USA) automatic biochemistry analyzer at room temperature of 37 ± 1°C.

All the above methods used were described by Chen (1993) with slight modification.

All doses used in the experiments were the weight of the extract of the callus tissue. All samples were suspended in distilled water containing 0.5% carboxymethylcellulose sodium (CMC-Na) before use. Other reagents were purchased from commercial channels.

Statistical analysis

The results were expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Dunnett's test were used to compare the drug effects with the blank control group.

Results

Endurance capacity

The mean swimming time was obviously prolonged in the experiment group relative to the blank control group ($p < 0.05$). TCSauI, at doses of 1.5 and 1.0 g/kg p.o. for 15 days, showed a significant effect on prolonging swimming time of mice. TCSauI, at a dose of 0.5 g/kg p.o., showed no significant effect (Table 1).

Content of hepatic glycogen

At doses of 0.5, 1.0 and 1.5 g/kg p.o. for 15 days, TCSauI significantly increased the content of hepatic glycogen in mice (Table 2).

Table 1. Effect of extract on swimming time in mice.

Groups	Dose ($g\ kg^{-1}day^{-1}$ p.o.)	No. of animals	Swimming time (min) (Mean ± SD)
Control	—	10	26.00 ± 3.03
TCSauI	0.5	10	47.50 ± 7.43
	1.0	10	76.20 ± 13.04*
	1.5	10	84.70 ± 14.66*
WSauI	1.0	10	80.50 ± 12.52*

* $p < 0.05$ vs. control. TCSauI and WSauI in the table refer to ethanol extracts.

Table 2. Effect of extract on the content of hepatic glycogen in mice.

Groups	Dose ($g\ kg^{-1}day^{-1}$p.o.)	No. of animals	Content of hepatic glycogen (mg/100g) (Mean ± SD)
Control	—	10	2135.11 ± 328.22
TCSauI	0.5	10	2582.31 ± 271.32*
	1.0	10	2653.74 ± 351.33*
	1.5	10	2861.78 ± 395.62*
WSauI	1.0	10	2791.44 ± 260.58*

* $p < 0.05$ vs. control. TCSauI and WSauI in the table refer to ethanol extracts.

Table 3. Effect of extract on the content of serum LA concentrations in mice.

Groups	Dose ($g\ kg^{-1}\ day^{-1}$ p.o.)	No. of animals	LA concentrations of mice (mg/100 mL) (Mean ± SD)
Control	—	10	66.09±10.04
TCSauI	0.5	10	60.24 ± 13.07
	1.0	10	48.36 ±14.93*
	1.5	10	47.01 ± 13.02**
WSauI	1.0	10	50.72 ± 12.21*

* $p < 0.05$, ** $p < 0.01$ vs. control. TCSauI and WSauI in the tables refer to ethanol extracts.

Serum LA concentrations

The results showed that at doses of 1.0 and 1.5 g/kg p.o. for 15 days, TCSauI significantly reduced serum LA concentrations in mice. TCSauI at 1.5 g/kg p.o. showed even greater effects ($p < 0.01$) (Table 3).

Content of serum urea-nitrogen

At a doses of 1.0 g/kg p.o. for 15 days, TCSauI, slightly but significantly reduced the content of serum urea-nitrogen ($p < 0.05$). TSaul, at the dose of 1.5 g/kg p.o., showed an

Table 4. Effect of extract on the content of serum urea-nitrogen in mice.

Groups	Dose ($g\ kg^{-1}\ day^{-1}$ p.o.)	No. of animals	Content of serum urea-nitrogen (mg/100 mL) (Mean ± SD)
Control	—	10	10.86 ± 0.516
TCSauI	0.5	10	10.95 ± 0.604
	1.0	10	8.43 ± 0.846*
	1.5	10	7.96 ± 0.853**
WSauI	1.0	10	8.58 ± 0.741*

** $P < 0.01$ vs. control. TCSauI and WSauI in the tables refer to ethanol extracts.

Table 5. Effect if extract on the content of serum LDH in mice.

Groups	Dose (g kg^{-1} day^{-1} p.o.)	No. of animals	Content of serum LDH (U/L) (Mean ± SD)
Control	—	10	1655.56 ± 102.46
TCSauI	0.5	10	1678.93 ± 101.37
	1.0	10	1806.72 ± 60.08**
	1.5	10	1909.60 ± 125.23**
WSauI	1.0	10	1938.45 ± 110.82**

**P < 0.01 vs. control. TCSauI and WSauI in the table refer to ethanol extracts.

even greater effect. TCSauI, at dose of 0.5 g/kg p.o. showed no significant effect (Table 4).

Content of serum LDH

Compared with the control group, at doses of 1.0 and 1.5 g/kg p.o. for 15 days, TCSauI significantly increased the activity of serum LDH ($p < 0.01$) (Table 5).

Discussion

The cumulation of serum LA concentrations in blood is one of the important causes inducing fatigue (Cady, 1989). When in a state of intense exercise, O_2 and pyruvic acid are reduced by LDH to LA, which decreases the pH, affects the functions of the cardiocirculating system, and the skeletal muscle system, decreases the contractive strength of muscle, and finally induces fatigue. Change in the contents of serum urea-nitrogen in blood is used to reflect the decomposition of protein, which also can induce the decrease of contractive strength of muscle and fatigue. The contents of hepatin is another index reflecting the degree of fatigue, and the action of hepatin is to complement the consumption of blood glucose and maintain blood glucose in the physiologic range. Fatigue will happen when hepatin is consumed largely.

Previous studies have shown that the ethanol extract and flavones of wild *S. involucrata* could be used for antifatigue purposes (Zheng et al., 1993). The current study, by using different animal models, firstly demonstrated that TCSauI could significantly prolong the duration of swimming ($p < 0.01$) in mice, reduce serum LA content, serum urea-nitrogen content ($p < 0.01$), and enhance liver starch content ($p < 0.01$). The study demonstrated that both TCSauI and the extract of wild *S. involucrata* have similar antifatigue effects. Tissue culture has advantages of resource conservation and industrial potential. These results support the potential of using tissue culture as a substitute for the exhausted resource of wild *S. involucrata* in the pharmaceutical industry.

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