

Anti-fatigue activity of extract form the submerged fermentation of *Ganoderma Lucidum* using *Radix astragali* as substrate

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1 SUMMARY

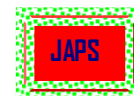
The objective of this study was to use *Radix astragali* as an alternative growth medium for cultivation of *Ganoderma lucidum* and to investigate anti-fatigue activity of its fermentation product. The results suggested that the growth of mycelial of *G. lucidum* can be inhibited when using *Radix astragali* as a substrate, but the extracellular polysaccharide in the fermentation production of *G. lucidum* increased significantly compared to that of general medium. Different doses of its product lengthened significantly the exhaustive swimming time, increased the contents of hepatic glycogen, and reduced significantly the levels of serum urea nitrogen (SUN) in blood compared to the control. However, the accumulation of blood lactic acid (BLA) or accelerate the clearance of BLA in the group which was administrated with the extract of submerged fermentation of *G. lucidum* using *Radix astragali* as substrate (GR) were significantly inhibited compared to the control. These results suggested that the extract of submerged fermentation of *G. lucidum* using *Radix astragali* as substrate might significantly alleviate physical fatigue of the mice *in vivo*.

2 INTRODUCTION

Ganoderma lucidum, which belongs to the family of Ganodermataceae of Polyporales, is known as 'Ling-zhi' in China. It has been widely used as a Traditional Chinese Medicine for more than 2000 years (Zhou et al. 2007). It has been reported to have a broad spectrum of medicinal properties for both health maintenance and treatment of disease. *G. lucidum* has received wide popularity as a medicine the Orient for the prevention and treatment of various types of diseases, such as cancer, hepatopathy, arthritis, hypertension, neurasthenia, chronic hepatitis (Boh et al. 2007; Sanodiya et al. 2009) and effective in modulating immune functions (Lin et al. 1999, 2000). Recently, ganoderic acids are

isolated from *G. lucidum* and its new biological activities such as the inhibitory effects on osteoclastic differentiation have been reported (Liu et al. 2010). Although many biological activities and pharmacological functions of *G. lucidum* have received a great deal of attention, researches in this area will be continued.

G. lucidum produces several metabolites with biological activity and therapeutic use, such as polysaccharides, triterpenoids, steroids, alkaloids, nucleotides, lactones, and fatty acids (Sanodiya et al. 2009). However, *G. lucidum* is traditionally cultivated in solid culture. This method usually takes several months to cultivate the fruiting body of *G. lucidum*, and it



is difficult to control the product quality during its cultivation. There is a great need to supply the market with a large amount of high-quality *G. lucidum* products. In addition, bioactive compounds in mushrooms may also be extracted from mycelia of these species without waiting for a full fruiting body to develop. Thus, submerged fermentation of *G. lucidum* has been considered as an efficient method for efficient production of its mycelium and valuable metabolites (Zhong and Tang 2004; Tang et al. 2007). To speed up the amount of mycelium and production of valuable metabolites, it is obviously necessary and important to develop a process for simultaneous production of these valuable metabolites in bioreactors.

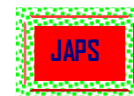
Traditional Chinese Medicine herbs have proven to be a very important as sources of drugs for modern medicine. *Radix astragali*, also known as *Huang Qi* in China, and is the dried root of *Astragalus membranaceus*. It has been used in Traditional Chinese Medicine for thousands of years and contains a number of active constituents, such as isoflavonoids, triterpene saponins and polysaccharides. These constituents have many bioactivity and functions, including hepatoprotection, neuroprotection against ischemic brain injury, immunologic properties, cardiogenic and anti-aging activities, gastroprotection, adjuvant, anti-tumor effects, anti-inflammatory effects,

stimulation of the growth of new tissues and treatment of chronic viral hepatitis (Wu and Chen 2004; Tang L.L. et al. 2009). Recently, reports suggested that the bio-activity constituents of *G. lucidum* fermented in drug-containing medium (containing *Radix astragali*) are significantly higher than those of in ordinary medium and selenium-rich drug-containing medium (Chen et al. 2004). In addition, Lin *et al.* (2008) reported that the product from culturing *C. militaris* in RA medium had a better anti-tumor activity than that culturing in synthetic medium. Hsu and Chiang (2009) reported natto-fermented *Radix astragali* can stimulate hyaluronic acid synthesis in primary human skin cells, and suggests that natto-fermented *Radix astragali* may play a promising role in anti-aging cosmetic applications. However, as far as we know, until now there are no reports on the product of submerged fermentation of *G. lucidum* using *Radix astragali* as substrate and its anti-fatigue activity. Therefore, the present study is to investigate the effects of the extracts of *Radix astragali* on mycelial growth and extra-cellular polysaccharide (EPS) production of *G. lucidum* in submerged fermentation. In addition, the anti-fatigue effects of the extract of the submerged fermentation of *G. lucidum* using *Radix astragali* as substrate are also investigated through swimming exercise of mice.

3 MATERIALS AND METHODS

3.1 Microorganism and culture conditions: *G. lucidum* used in the experiment was obtained from Sichuan Academy of Agriculture Science (Chengdu, China). The slant was inoculated and incubated in newly prepared PDA medium in a Petri dish at 27 °C for 7 days, then stored at 4 °C for about 2 weeks. Then transferred into the seed culture by punching out mycelia mat (ca. 1 cm²) from the Petri dish and incubated on a rotary shaker incubator at 150 rpm for 6 days at 27°C. The seeds were grown in 250 ml Erlenmeyer flasks containing 100 ml seed culture medium (Wei et al. 2007) , which is composed of potato extract 200 g/l, bran 15 g/l, sugar 20 g/l, peptone 2 g/l, yeast extract 1 g/l, KH₂PO₄ 1.5 g/l, MgSO₄ 5 g/l. The flask culture experiments were performed in 500 ml flasks containing 200 mL of

the medium after inoculating with 10% (v/v) of the seed culture. *Radix astragali* was purchased from a traditional chinese medicine market, Chengdu, China, and quality controlled by Sichuan Academy of Chinese Medicine Science where a voucher specimen was deposited (voucher specimen number: AM20091024). 100 g of *Radix astragali* was soaked in 1 L of double distilled water and then homogenized with a Waring blender. The whole preparation was boiled under reflux at 95 °C for 4 h. The supernatant was filtered and then condensed with water bath (95°C) to 100 ml (crude drug content was 1 g/ml) and stored at 4 °C until use. For comparison, *G. lucidum* was cultured in either base medium or *Radix astragali* medium. Base medium contained 1.5% bran extract, 0.2 %



soybean flour and 2 % sugar. The *Radix astragali* medium was similar to the base medium, but only with *Radix astragali* extract of 0.2%, 0.4% and 0.8%. The submerged fermentation experiments were carried out in a 500 ml flask containing 200 ml of the base or *Radix astragali* medium by inoculating with 10 % (v/v) of the seed culture. The fermentation was conducted at 27 °C on a rotary shaker incubator at 150 rpm for 5 days. Multiple flasks were performed at the same time, and three flasks were sacrificed at each sampling point.

3.2 Determination of mycelial biomass: The mycelial biomass was determined by measuring the dry cell weight (DCW). The mycelia were collected from fermentation broth every 12 h, and centrifuged for 15 min at $3000 \times g$. The resulting precipitate was filtered through a 40-mesh stainless sieve and washed repeatedly with distilled water. Then the mycelial pellets were dried at 60 °C until a constant weight was achieved to get the DCW measurement.

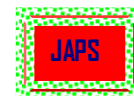
3.3 Measurements of extracellular polysaccharide: After removal of mycelia and the fermentation filtrate was dialyzed, the crude extracellular polysaccharide was precipitated with addition of 95 % (v/v) ethanol by about 4-5 times of volume, and then collected by centrifugation at 9000 rpm for 20 min. The crude extracellular polysaccharide was washed with 80 % (v/v) ethanol three times, and the insoluble components were suspended in 1 M NaOH at 60 °C for 1 h. The supernatant was determined by phenol/sulphuric acid method (Dubois et al. 1956).

3.4 Preparation of the extract of the fermentation product: After fermentation, the fermentation broth was collected and centrifuged for 10 min at $1157 \times g$. The resulting precipitate was washed repeatedly with distilled water and then the mycelial pellets were dried at 65 °C. The mycelial pellets was homogenized and decocted third by adding distilled water for 60 min, 10 times the volume of distilled water each time, combine the extractions, and then was condensed at 60°C under reduced pressure with vacuum evaporated to 5.12 g/L and stored at -20 °C for further experiments, the extract from the fermentation broth of *G. lucidum* using *Radix astragali* as substrate designed GR. Similarly, the fermentation broth of

G. lucidum in base medium (GB) and *Radix astragali* medium (RA) was also condensed with vacuum evaporated to 4.25 and 28.3 g/L, respectively. Finally, these fermentation broths and *Radix astragali* extract were dissolved into 20, 50 and 100 mg/ml with distilled water for further experiments.

3.5 Animals and experimental process: BALB/c mice (20 ± 2 g, male and female were equal in number) were purchased from Institute of Chinese Traditional Medicine of Sichuan province, China. The animals were kept in a room at a controlled temperature of 20 ± 2 °C with a 12 /12-h light-dark cycle. The animals were treated according to the ethical guidelines of the Animal Center, Institute of Chinese Traditional Medicine of Sichuan province. The experimental protocol was approved by Institute of Chinese Traditional Medicine of Sichuan province. The animals were maintained on the following prescribed diet for a period of 20 days after 3 days of adaptation to the environment and to the standard diet. 100 mice were randomly divided into ten groups, each consisting of 10 mice. The first group designated as control group (Control) was administered with distilled water by gavage every day. The second, three and four group designated as GB group was administered with GB of 20, 50 and 100 mg/kg body weight day, respectively. The five, six and seven groups designated as GR group was administered with GR of 20, 50 and 100 mg/kg body weight day, respectively. The eight, nine and ten group designated as RA group was administered with RA of 20, 50 and 100 mg/kg body weight day, respectively. The administered groups with distilled water, GB, GR or RA were continued for 30 days.

3.6 Assay of exhaustive swim: For the anti-fatigue experiment, the swimming capacity of mice was studied with an adjustable-current water pool for mice. The details of this apparatus were reported by Kamakura et al. (2001) as the acrylic plastic pool (50 cm×50 cm×40 cm) filled 30 cm deep with water maintained at 25 ± 2 °C. Each mouse's tail was loaded with a bundle of lead pieces,



which was 10 % of its body weight. Mice were regarded as exhaustion when their noses were underwater for 10 sec, and the time was immediately recorded.

3.7 Assay of serum urea nitrogen: Mice in the three groups were continued for 30 days, and the mice were placed in the swimming pool for 90 min at 25 ± 2 °C. After resting for 60 min, blood was collected from orbital sinus for assaying of *serum urea nitrogen* (SUN). SUN was determined according to the procedures provided by the kits (Biosino Biotechnology & Science Inc., Beijing, China). The SUN content expressed as milimol nitrogen per litre bood serum.

3.8 Assay of hepatic glycogen: After swimming for 90 min, each mouse was anesthetized to death with high concentration aether in an acrylic plastic immobilizer and its liver was collected as soon as possible. The lives were washed three times with physiological saline solution and weighed. Hepatic glycogen (HG) was tested according to the procedures provided by the Hepatic Glycogen/Muscle Glycogen Detection kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu,

China). The HG content expressed as milligram hepatic glycogen per gram hepatic tissue.

3.9 Assay of blood lactic acid: The mice were continued for 30 days, and the mice were placed in the swimming pool for 90 min at 25 ± 2 °C. Blood was collected from the orbital sinus before and after swimming. After resting for 30min, 20- μ l blood was also collected from the orbital sinus, and combined with 40 μ l hypotonic buffer. Blood lactic acid (BLD) level was determined according to the procedures provided by Lactic Acid Detection Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The BLD level expressed as milimol blood lactic acid per litre bood. The accumulation/clearance ratio of BLD was calculated as flowing equation: Accumulation ratio $= (B-A)/A \times 100\%$. Clearance ratio $= (B-C)/B \times 100\%$

3.10 Statistical analysis: All treatments were arranged in a completely randomized design with three replicates. Data were expressed as means \pm SD. Statistical significance was evaluated with Student's t-test, and differences were considered significant if *P* values were 0.05.

4 RESULTS AND DISCUSSION

4.1 Effects of Radix astragali extract on mycelial biomass and extracellular polysaccharide of G. lucidum: Extract of *Radix astragali* (0.2%, 0.4 % and 0.8%) were added into the basic medium of *G. lucidum* to investigate their effects on the mycelial biomass and extracellular polysaccharide. As shown in Fig.1, the mycelial biomass at 0, 0.2%, 0.4 % and 0.8% of *Radix astragali* extract increased gradually with culture time up 120, 108, 120 and 108 h, and reached 18, 16.5, 12.9 and 12 g/L, respectively. These results showed that different concentrations of *Radix astragali* extract could lead to decrease in biomass production compared to the control. However, *Radix astragali* extract showed stimulatory effects on EPS production, and the highest yield of EPS increased by 23.7%, 46.9% and 77.4% at the addition of 0.2%, 0.4 % and 0.8% *Radix astragali* extract compared to the control, respectively (Fig.2). To accelerate mycelial biomass and extracellular polysaccharide production in *G. lucidum* by

submerged fermentation, the effects of culture conditions, addition of extract and fed-batch fermentation, etc have been reported (Fang and Zhong 2002; Wagner et al. 2003; Tang et al. 2007). Earlier studies showed that using *Radix astragali* as the medium could enhance the anti-tumor activity of the fermentation broth of *C. militaris* as compared to the synthetic medium (Lin and Chiang 2008). Studies have reported that many types of extracts from Traditional Chinese Medicine herbs, such as *Ligusticum*, *Ganodermataceae* and *Astragalus membranaceus*, displayed various pharmacological effects (Sinclair, 1998; Wu and Chen, 2004; Zhou *et al.*, 2007). In the present study, the addition of *Radix astragali* extract at 0.4 g/L led to a significant decrease in biomass production, but it was of great advantage to polysaccharides production of *G. lucidum* compared to the control (Fig.2). Our results suggested that *Radix astragali* extract could stimulate the EPS production.

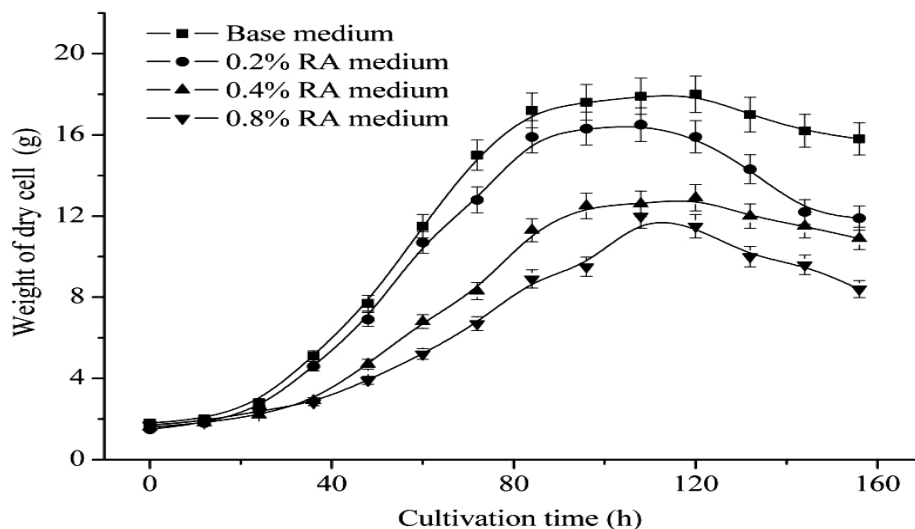


Figure 1: Time-course of the mycelial growth of *G. lucidum* with the addition of 0.2%, 0.4% and 0.8% *Radix astragali* extract in shak-flask cultures. The values given are the averages of three experiments.

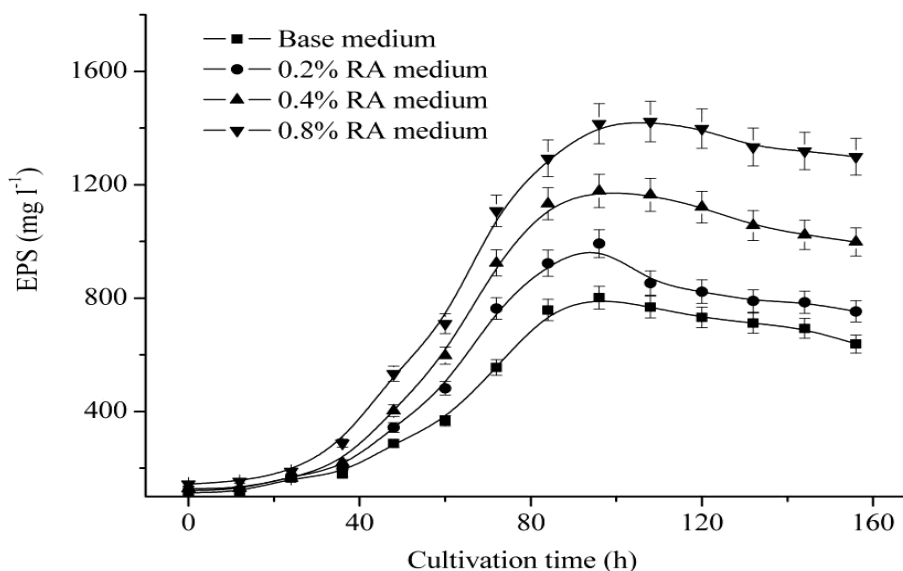
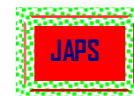


Figure 2: Time-course of EPS production of *G. lucidum* with the addition of 0.2%, 0.4% and 0.8% *Radix astragali* extract in shak-flask cultures. The values given are the averages of three experiments.

4.2 Effectss of test substances on swimming time and body weights of weight-burdening mice: The anti-fatigue effects of *Radix astragali* extract were investigated by exhaustive swimming

test. As shown in Table 1, the longest swimming times of mice in 20, 50 and 100 mg/kg GR groups were significantly prolonged compared to the control groups, and the longest times increased by



18.5%, 28.5% and 11%, respectively. However, the longest swimming times of mice in GB and RA groups showed no significant changes compared to the control groups. The body weights of mice in GB, RA and GR groups increased significantly compared to the control groups. The highest increments in GB, RA and GR groups increased by 88.9%, 89% and 82.1% at doses of 100, 20 and 20 mg/kg compared to the control, respectively. It is well accepted that the most important physiological effect of fatigue is on the energy metabolism of muscular activity, and the improvement of exercise

endurance is the most powerful representation of anti-fatigue enhancement (Belluardo et al. 2001). The forced-swimming test is commonly used in the anti-fatigue tests. As far as we known, the anti-fatigue effects of submerged fermentation of *G. lucidum* was not reported. In the present study, the anti-fatigue effects of the GR were investigated using an adjustable current swimming pool for mice. Our results suggested that different doses of GR might significantly prolong the exhaustive swim time, which showed that GR might elevate the exercise tolerance of mice.

Table 1: Effects of GB, RA and GR on body weight of mice in different groups

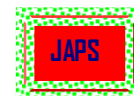
Groups	Doses	Swim times (Sec)	Before experiment	After experiment
Control	20 ml/kg	572 ± 82	19.72 ± 1.36	36.42 ± 3.75
	20 mg/kg	563 ± 272	19.83 ± 1.25	35.61 ± 2.85
GB	50 mg/kg	589 ± 167	20.92 ± 2.55	37.62 ± 3.55
	100 mg/kg	582 ± 134	20.25 ± 1.18	38.15 ± 5.74
RA	20 mg/kg	538 ± 131	19.67 ± 2.67	37.16 ± 2.74
	50 mg/kg	552 ± 121	20.00 ± 3.06	36.51 ± 3.58
	100 mg/kg	589 ± 159	20.65 ± 1.72	37.23 ± 2.58
GR	20 mg/kg	678 ± 114*	20.43 ± 2.63	36.94 ± 3.18
	50 mg/kg	735 ± 199*	21.18 ± 3.63	36.25 ± 3.32
	100 mg/kg	635 ± 197*	20.21 ± 1.43	35.76 ± 3.52

Values are expressed as mean ± SD (n = 3) of 10 mice per group. * $P < 0.05$

4.3 Effects of GR on serum urea nitrogen, hepatic glycogen and lactic acid levels of mice:

SUN, hepatic glycogen (HG) and lactic acid are representative blood biochemical parameters related to fatigue. In the present study, the levels of SUN, HG and lactic acid were determined after swimming. As shown in Table 2, the SUN level (7.25 mM) in the normal group was significantly lower than that of the control group (9.81 mM). The SUN levels in 20, 50 and 100 mg/kg GR groups were 9.43 ± 0.89 , 7.92 ± 0.77 and 8.19 ± 0.57 mM, respectively, which were lower than that of the control group. SUN is a sensitive index to evaluate the bearing capability when human bodies suffer from a physical load and caused by catabolism of proteins and amino acids. Protein and amino acids have a stronger katabolic metabolism when body cannot obtain enough energy by sugar and fat catabolic metabolism. Therefore, there is a positive

correlation between the urea nitrogen *in vivo* and the exercise tolerance (Tsopanakis and Tsopanakis 1998). The present results showed that the levels of SUN in GR groups were lower than control group, suggesting that GR may reduce catabolic decomposition of protein for energy. Energy for exercise is derived initially from the breakdown of glycogen, after strenuous exercise muscle glycogen will exhaust, and later, energy will from circulating glucose released by the liver (Suh et al. 2007). Thus, the glycogen contents are sensitive parameters related to fatigue. As shown in table 2, our results suggested that the HG levels in the GR groups, especially 50 mg/kg, were significantly higher than that of the control group, suggesting that GR can significantly increase the levels of hepatic glycogen of mice after swimming. In the GR treated groups, the HG levels were higher than that of the control group. The reason is that GR may increase the HG



content of mice post exercise by improving glycogen reserve, or reducing the glycogen consume during exercise, or both. However, this detailed mechanism is not clear and needs the further studies. As shown in Table 3, there were no significant differences in the blood lactic acid levels in the GR groups compared to the control groups before swimming. After swimming, the blood lactic acid contents in 20, 50 and 100 mg/kg GR groups were lower than that of the control group, especially in 20 mg/kg GR. Blood lactate acid is the glycolysis

product of carbohydrate under an anaerobic condition, and glycolysis is the main energy source for intense exercise in a short time. The accumulation of BLA is a reason of fatigue during the physic excise, and if medicine may inhibit the accumulation of BLA and accelerate the clearance of BLA, which it have the anti-fatigue activity (Cairns 2006). In the present study, GR can inhibit the increase of blood lactic acid of mice after swimming, which suggested that GR have anti-fatigue activity.

Table 2: Effects of GR on serum urea *nitrogen* and hepatic glycogen of mice in different groups.

Groups	SUN (mM)	Hepatic glycogen (mg/g)
Control	9.81 ± 1.57	14.50 ± 7.67
Normal	7.25 ± 0.53 **	44.95 ± 9.24**
20 mg/kg	9.43 ± 0.89	16.13 ± 8.15
50 mg/kg	7.92 ± 0.77 **	22.73 ± 9.54*
100 mg/kg	8.19 ± 0.57*	15.13 ± 6.52

Values are expressed as mean ± SD (n = 3) of 10 mice per group. * $P < 0.05$, ** $P < 0.01$

Table 3: Effects of GR on blood lactate level of mice in different groups.

Group	BLD level (mM)			Accumulation ratio	Clearance ratio
	pre-exercise	post-exercise	post-rest	(B-A)/A*100%	(B-C)/B*100%
Control	0.91 ± 0.23	3.30 ± 0.56	1.16 ± 0.28	262.6 ± 50.3	64.9 ± 28.3
20 mg/kg	0.92 ± 0.18	2.83 ± 0.38	0.93 ± 0.25	207.6 ± 66.8	67.1 ± 29.6
50 mg/kg	0.89 ± 0.24	3.15 ± 0.78	1.15 ± 0.58	253.9 ± 69.54	63.5 ± 22.1
100mg/kg	0.90 ± 0.28	3.25 ± 0.92	1.09 ± 0.49	261.1 ± 43.52	66.5 ± 19.3

Data are expressed as mean ± SD (n = 3) of 10 mice per group.

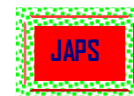
5 CONCLUSION

In conclusion, *Radix astragali* extract was of great advantage to EPS production of *G. lucidum*, and its extract can be used as easily available stimulators for polysaccharide production by submerged fermentation of *G. lucidum*. In addition, the extract of the submerged fermentation of *G. lucidum* using *Radix astragali* as substrate may prolong the exhaustive swimming time, as well as increase the body weight and hepatic glycogen, but decrease the

serum urea and blood lactic acid levels. These results suggested that the extract of submerged fermentation have significant anti-fatigue effects on mice. However, for the evaluation of pharmacological application of the product of submerged fermentation, further studies to clarify the detailed mechanisms involved in the anti-fatigue properties of the product of submerged fermentation are necessary.

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