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Anti-stress and muscular endurance effects of natural specimens and laboratory cultured mycelia of *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung on rats and mice

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Ophiocordyceps sinensis (Berk.) G.H. Sung, J.M. Sung. is an entomogenous fungus, used as physical performance enhancer and herbal medicine for preventing or curing a number of human ailments. Owing to its high medicinal value and expensive market price, the present study was aimed to determine the anti-stress and muscular endurance effects of natural and laboratory cultured *O. sinensis* in animals. The natural specimens collected from 4000 – 5000 m altitude of Central Himalayan hills as well as the lab cultured mycelium were tested for anti-stress activity in rats and muscular endurance effect in mice with the help of Force Swim Test. Three doses (100, 300 and 500 mg/kg body wt.) of natural specimens and lab cultured *O. sinensis* were administered in test groups and Imipramine (15 mg/kg body wt.) in positive control group and 0.9 % NaCl solution in control group for 30 days.

Natural specimens (100, 300 and 500 mg) and lab cultured (300 and 500 mg) of *O. sinensis* showed anti-stress and muscular endurance effects significantly ($p<0.05$) following oral administration. Natural specimens and lab cultured *O. sinensis* at doses of 300 and 500 mg/kg significantly ($p<0.05$) inhibited the increase in LDH, cholesterol and BUN and decrease in ALP level of immobilized stress rats. Thus, both natural and lab cultured *O. sinensis* have almost equal anti-stress and muscular endurance effect.

Keywords: *Ophiocordyceps sinensis*, Anti-stress, Muscular endurance, Rat and Mice, Caterpillar Mushroom, Medicinal Fungus

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Introduction

Caterpillar Mushroom or *Ophiocordyceps sinensis* (Berk.) G.H. Sung, J.M. Sung. syn. *Cordyceps sinensis* (Berk) Sacc. is a high value medicinal fungus, which is being used as performance enhancer and traditional medicine in China for prevention and cure of several human ailments. It is a unique parasitic association of insect larva and fungus in the high altitude areas of Himalayan hills ranging from 4000 – 5000 m altitude from MSL. The fungus is endemic to the alpine habitats of the Tibetan Plateau above 3000 m in south-western China and there has been large-scale harvesting of the wild material from Nepal and India also. Fungus partner *O. sinensis* belongs to sub division Ascomycotina which parasitizes upon larva of high altitude moth named *Hepialus armoricanus* (Fam. Hepialidae). This high value medicinal fungus is commonly known as Yar-rsta- dgun- bu in Tibet meaning 'winter-worm and summer-grass' or 'worm-grass'[1], Dong Chong Xia Cao in China[2] and Yarsha Gamboo or Kira Ghas in India[3]. It is being used as Traditional Chinese Medicine (TCM) in China to promote longevity, increase athletic power and overall improvement in quality of life[4, 5] since a long time. *O. sinensis* contains various bioactive compounds, including cordycepin, adenosine, adenine, guanosine, ergosterol, uridine, uracil, hypoxanthine, mannitol and polysaccharides[6-12]. The first observation on its energetic effect was noticed about 1500 years ago in the Tibet mountain pasture, when a herdsman found that his cattle and livestock became energetic after consuming this mushroom and even older became vigorous[13]. This wonder drug of Himalayas got a wide popularity world over since 1993, when a group of Chinese women athletes shattered nine world records in world outdoor track and field championships in Germany. It was revealed that the vigour of these

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athletes increased because of the regular consumption of *O. sinensis*, which in Traditional Chinese System of Medicine had been used for centuries to increase vigour and vitality of the people[14]. There are several reports on augmentation of performance of long distance runners who used cultured *O. sinensis* extract as supplementation in their meals[15]. Supplementation of cultured *O. sinensis* powder to healthy young volunteers (0.5 g/day for 2 weeks) during exhaustive running have resulted in significant augmentation of energy generation and antifatigue ability as compared to placebo controls[16-17]. In double blind, placebo controlled studies, supplementation of CS-4 for 6 weeks (3 g/day)[18] and 12 weeks (333 mg, 3 times a day)[19] to elderly healthy volunteers have significantly improved the metabolic and ventilator threshold and resistance to fatigue compared to placebo group. Previous workers have reported that oral supplementation of hot water fraction of *O. sinensis* mycelia (150 mg/kg/day, 8 days)[20] and polysaccharides (200 mg/kg/day, 21 days)[21] enhanced exhaustion time, reduced fatigue and stress in mouse during swim test. Observations were also made to prolong the exhaustive swimming time of mice significantly ($p<0.05$) with the supplementation of *O. guangdongensis* powder (0.455, 0.91 and 1.82g/kg daily for 30 days)[22] and polysaccharides from *O. sinensis* (100, 200 and 400mg/kg, daily for 28 days)[23]. *O. sinensis* supplementation with or without exercise was found to improve exercise endurance capacity by activating the skeletal muscle metabolic regulators as well as activation of NRF-2-ARE pathway limiting the oxidative stress[24].

Besides its uses as an antioxidant and anti-tumor agent[25] it is most popular TCM for liver and renal disorder[26], respiratory problems[27] and cerebro-vascular diseases[28]. Hot-water fraction of the submerged cultured mycelia of *O. sinensis* stimulates proliferation of bone marrow cells through Peyer's patch cells[29] and thus are useful in immunomodulatory function[11, 13, 30]. A preparation of *O. sinensis* stimulates proliferation of erythroid progenitor cells (CFU-E and BFU-E) in mouse bone marrow *in vivo* and *in vitro*[31]. In TCM this wonder drug is also used for its anti-aging effect[32].

Traditionally, *O. sinensis* is consumed after mixing with rice flour and boiled milk. It is generally used as a food supplement or nutraceuticals/additive to be cooked with meat[33]. The most common traditional use of the fungus is to mix with a variety of meats of chicken, duck and pork as medicinal soup[13, 34]. In Nepal, powder of *O. sinensis* is used with the orchid, *Dactylorhiza hatagirea* (D. Don) Soo [35], honey, ghee, alcohol, hot water and cow's milk as a combination for tonic and aphrodisiac[36-38]. Various *Ophiocordyceps* based products are available in the international markets as a health supplement or nutraceuticals. The market demand for *O. sinensis* has grown sharply in many countries[39] after its popularity by the sports women in the year 1993. The market price for wildly collected *O. sinensis* vary from 16,000 to 20,000 US$ per kg in India, while it is costing more than 25,000 US$ per kg in international markets. In 2008, price of *Cordyceps* in San Francisco and other major U.S. cities was as high as $75,000/kg[40].

The pure mycelium culture of *O. sinensis* was raised at DIBER Field Station, Pithoragarh (India) with an objective to develop health supplements or nutraceuticals from it. Study on efficacy and safety of lab cultured mycelia and natural specimens were also carried out in the laboratory. Present study is aimed to determine the anti-stress and muscular endurance effects of natural as well as lab cultured of *O. sinensis* in animals.

Materials and Methods

Biological materials

Natural specimens of *O. sinensis* were collected during the month of May-June from Johar valley (Longitutde: E-80° 10' 44.7"-18' 59.2", Latitude: N-30° 12' 28.5"-19' 44.4") in Munsyari region of Central Himalayan hills in district Pithoragarh, Uttarakhand (India). Sufficient numbers of natural specimens were collected from the natural habitat to conduct the desired study in the laboratory. Some of the natural specimens were utilized to raise the mycelium culture *in vitro.* Both the samples were freeze dried at -72 °C in lyophilizer (Model No. 038, NU LABCARE, New Delhi) and powdered with the help of mechanical grinder and stored in an air-tight container at -4°C. Both types of powders were suspended in vehicle (Saline) before administration to animals.

Drugs

Imipramine hydrochloride (Sigma-Aldrich®, USA) was dissolved in sterile isotonic saline and given by *ip* route. Specific diagnostic kits (AUTOPACK) of cholesterol, triglycerides, AST, ALT, ALP, BUN and LDH were purchased from SIEMENS, India.

Animals

7-8 weeks old wistar male rats (180-220g body wt) and 6-8 weeks old albino mice (22-30g body wt), were selected and randomly kept in different groups (n=5) for studies. Animals were kept in the cages for at least 7 days prior to the start of the study for acclimatization under the prevailing lab conditions. All the animals were kept under constant room temperature (25±3 °C), relative humidity (50–70%), and 12h/12h dark/light cycle for the whole experimental period. Animals were allowed to chew the food and drinking water *ad libitum*. All the procedures were followed under strict compliance of approved directions of Institutional Animal Ethics Committee (IAEC), DIBER, Field Station Pithorgarh (Utarakhand) India, which is a registered committee with CPCSEA, Ministry of Environment and Forest, Government of India, New Dehli.

Acute toxicity study

Fine uniform suspension of natural specimens and lab cultured *O. sinensis* were prepared in distilled water and administered at different doses, viz. 100, 500, 1000 and 2000 mg/kg body weight orally to different groups of mice for acute toxicity. Acute toxicity study was carried out according to the OECD Guideline 423. The lethal dose of natural specimens and lab cultured *O. sinensis* was not found up to 2000 mg/kg body weight. Anti-stress and muscular endurance were studied by using one fourth of the highest dose as per OECD Guidelines 2001.

Muscular endurance effect

The selected mice were divided into eight groups of 5 in each to evaluate the muscular endurance effect. Vehicle (0.5 mL/animal, oral, n=5) and Imipramine (15 mg/kg, *ip*) were administered for 30 successive days in group I and II, respectively. Three doses of 100, 300 and 500 mg/kg of lab cultured and natural specimens were given orally to the mice for 30 days in test groups III, IV, V, VI, VII and VIII, respectively. After 1 h of last administration, animals were subjected to Force Swimming Test (FST) for recording of data related to muscular endurance. Swimming capacity of animals was studied in acrylic plastic water pool (45 cm X 20 cm) filled 35 cm deep water level, maintained at 28±1°C. The mice were loaded with a lead ball corresponding to 4 % of their body weight attached to the tails[41]. The swimming time to exhaustion was used as the index of the forced swimming capacity. Animals were judged to be fatigued when they failed to rise to the water surface to breath within a 7s period as the index of swimming capacity[41].

Anti-stress activity

The selected rats were divided into eight groups (5 in each) to evaluate anti-stress activity. Vehicle (0.5 mL/animal, oral, n=5) and Imipramine (15 mg/kg, ip) were administered for 30 successive days in group I and II, respectively. Three doses of 100, 300 and 500 mg/kg of lab cultured and three doses of 100, 300 and 500 mg/kg natural specimens were given orally for 30 days in III, IV, V, VI, VII and VIII test groups of rats, respectively. After 1 h of last administration, animals were subjected to FST for recording activity.

Anti-stress activity of rats was determined by FST, in which immobility time of animals was studied. Before experiment, rats were placed in the water pool for 15 min. in the water and removed to dry in a heated enclosure (32°C) before being returned to their home cages. After 24 h they were again placed in the water pool and total duration of immobility was measured during a 5 min. test. The immobilized stress was given for the last 48 h of the experiment. The immobilized-stress technique was carried out by the modified method of Brekhman and Dardymov (1969)[42] and Watanabe and Ayugase (2008)[43]. Food intake, body weight and biochemical parameters were also recorded in rats.

At the end of the experiment, blood samples were collected from orbital sinus for serological analysis. Rats were kept under overnight fasting before taking blood in sterilized disposable syringes (22 gauge needle) after anaesthetizing the rat with anesthetic ether. Blood samples were transferred to non heparinized tubes. The serum was separated and stored at -20°C in clean vial labelled with date and sample number. Biochemical parameters like cholesterol, triglycerides, AST, ALT, ALP, BUN and LDH were determined at the end of the study in all groups of rats. The study was carried out with the help of semi auto chemical analyzer (RA-50, BAYAR) and specific diagnostic kits from SIEMENS (AUTOPAK), India.

Statistical analysis

The results are presented as mean (±) standard deviation. Statistical analysis of the data was done using Dunnett multiple comparisons test method for comparison among groups. All data were reported as mean±SEM and Significance was assumed at $P < 0.05$.

Results

Effect on swimming time of mice

Table 1 depicts the effect of subacute administered imipramine, lab cultured and natural *O. sinensis* on swimming time of mice. All doses of both lab cultured and natural specimens increased swimming time in dose dependent manner. Imipramine (15 mg/kg), natural specimens (100-500 mg/kg) and lab cultured mycelium (100-500 mg/kg body wt., oral) enhanced the swimming time in mice significantly. All these three types of samples were found superior over their control.

Effect on immobility time in rat

Table 2 depicts the effect of subacute administered imipramine, lab cultured and natural *O. sinensis* on immobility time of rats. All doses of both lab cultured and natural specimens of *O. sinensis* decreased immobility time in dose dependent manner. Application of Dunnet's test revealed that, natural specimens of *O. sinensis* (100-500 mg/kg, oral) significantly decreased the immobility time of test rats similar to imipramine and were superior over control. Lab cultured mycelium also reduced the immobility time at higher concentrations (300-500 mg/kg). However, lower dose (100 mg/kg) failed to exhibit significant effect on immobility time of the rats.

Effect on food intake and body weight of rats

Subacute treatment with all the three types of samples showed hyperphagia and progressive increased in body weight of the animals. Lab cultured mycelium and natural samples along with imipramine increased the food intake from day 20 onwards at the dose of 300 to 500 mg/kg (Fig. 1) (Table 3).

Table 1—Effect of imipramine, natural or laboratory cultured *O. sinensis* on swimming time of mice

		Swim Time (Min.) ±SEM	
Groups	Dose (mg/kg)	1st Day	30th Day
Control (0.9% NaCl)		23.82±3.42	24.2±3.42
Imipramine (mg/kg)	15	29.5±4.95	56.7±4.95*
Lab Cultured *O. sinensis* (mg/kg)	100	26.2±1.84	36.8±6.12*
	300	27.4±2.78	41.2±10.37*
	500	30.6±3.42	53.5±5.98*
Natural *O. sinensis* (mg/kg)	100	26.6±2.06	39.8±8.13*
	300	28.6±2.01	47.5±6.94*
	500	31.8±1.72	59.2±6.52*

Different groups of mice were administered with imipramine (15mg/kg, body wt., ip), natural *O. sinensis* (100-500mg/kg body wt., oral) and lab cultured *O. sinensis* (100-500mg/kg body wt., oral), while control animals received saline (0.5mL/mouse, oral). Swimming time of mice was measured 1h post injection time-points. The data represents mean±SEM for each group at respective time-points. *p<0.05 vs. control.

Table 2—Effect of imipramine, laboratory cultured or natural *O. sinensis* on immobility time of rats

		Immobility Time (Sec) ±SEM	
Groups	Dose (mg/kg)	1st Day	30th Day
Control (0.9% NaCl)		235.6±15.39	227.2±17.37
Imipramine (mg/kg)	15	216.4±13.15	131.8±12.28*
Lab Cultured *O. sinensis* (mg/kg)	100	224.2±12.85	200.6±19.73*
	300	217.4±14.38	171.2±16.66*
	500	215.8±11.03	132.4±13.35*
Natural *O. sinensis* (mg/kg)	100	221.4±11.26	192.8±17.24*
	300	217.8±13.74	156.4±16.33*
	500	214.4±12.62	108.2±19.72*

Different groups of rats were administered with imipramine (15mg/kg, body wt., ip), natural *O. sinensis* (100-500mg/kg body wt., oral) and lab cultured *O. sinensis* (100-500mg/kg body wt., oral), while control animals received saline (0.5mL/rat, oral). Immobility time of rats was measured 1h post injection time-points. The data represents mean±SEM. for each group at respective time-points. *p<0.05 vs. control.

Statistical test revealed that significant increase in the body weight was achieved at a dose of 300 to 500 mg/kg for all the three types of samples. However, lower dose (100 mg/kg) of lab cultured and natural *O. sinensis* failed to exhibit significant effect on food intake and body weight of rats.

Effect on biochemical parameters in rats

Biochemical parameters like AST, ALT, ALP, triglycerides, LDH, BUN and cholesterol level were estimated in serum of all rats (Table 4). Levels of AST, LDH, BUN and total cholesterol were significantly increased and ALP level decreased significantly in

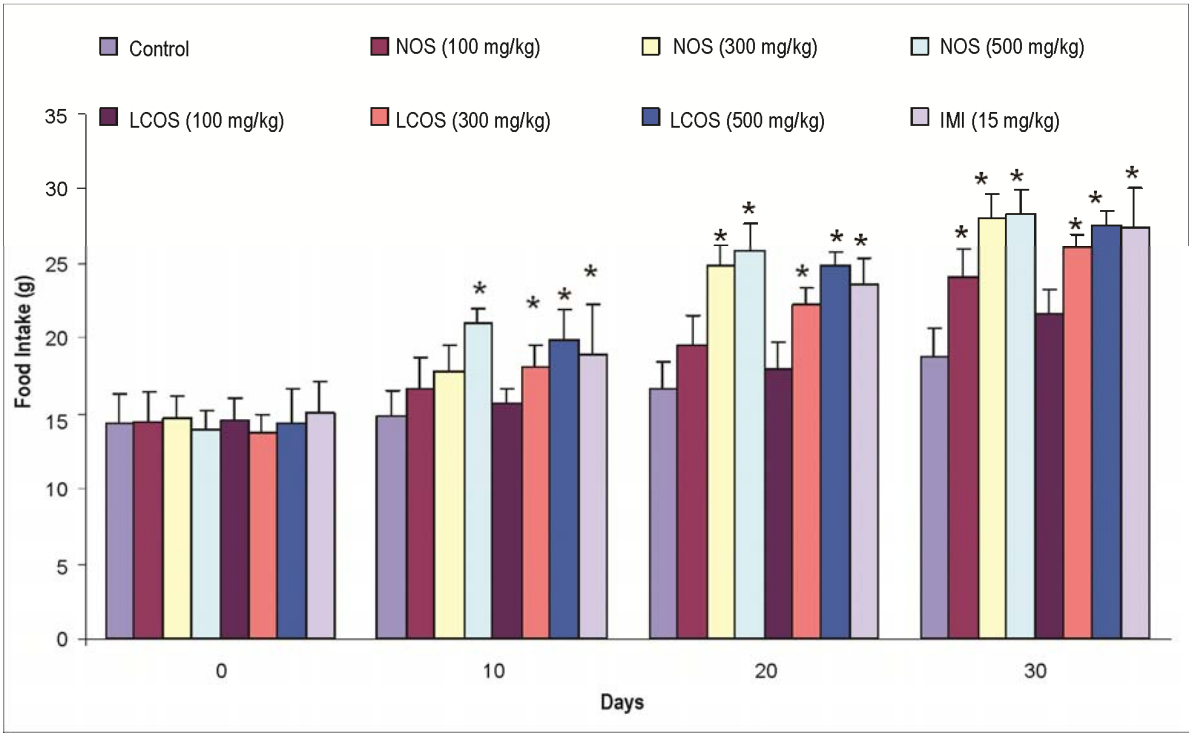

Fig. 1—Effect of imipramine, laboratory cultured and natural *O. sinensis* on food intake in rat IMI=Imipramine, NOS=Natural *O. sinensis*, LCOS=Laboratory Cultured *O. sinensis*. Different groups of rats were administered with imipramine (15mg/kg, body wt., ip), natural *O. sinensis* (100-500mg/kg body wt., oral) and lab cultured *O. sinensis* (100-500mg/kg body wt., oral), while control animals received saline (0.5mL/rat, oral). The data represents mean±SEM for each group at respective time-points. *p<0.05 vs. control.

Table 3—Effect of imipramine, laboratory cultured or natural *O. sinensis* on body weight of rat

	Body weight (g)			
Groups	1st Day	10th Day	20th Day	30th Day
Control (0.9% NaCl)	139.2±8.93	166.0±8.28	209.2±13.46	262.4±8.59
Imipramine (mg/kg)	141.4±11.40	178.4±14.36	237.6±12.20*	288.2±15.22*
Lab Cultured *O. sinensis* (mg/kg)				
100	142.6±14.12	171.0±18.26	212.6±13.43	266.0±9.03
300	139.8±9.36	180.8±14.86	229.6±10.45	292.8±11.84*
500	143.6±10.53	176.4±12.58	234.8±8.64*	294.8±12.79*
Natural *O. sinensis* (mg/kg)				
100	143.8±13.27	174.2±16.42	217.4±13.29	275.8±12.36
300	140.6±10.31	170.6±12.62	234.2±11.26*	291.2±10.48*
500	138.2±9.63	180.8±9.52	242.8±10.43*	305.6±14.43*

Different groups of rats were administered with imipramine (15mg/kg, body wt., ip), natural *O. sinensis* (100-500mg/kg body wt., oral) and lab cultured *O. sinensis* (100-500mg/kg body wt., oral), while control animals received saline (0.5mL/rat, oral). The data represents mean±SEM for each group at respective time-points. *p<0.05 vs. control.

Table 4—Effect of imipramine, laboratory cultured or natural *O. sinensis* on biochemical parameters

Groups	Biochemical Parameters						
	AST (U/L)	ALT (U/L)	ALP (U/L)	Cholesterol (mg/dl)	Triglycerides (mg/dl)	LDH (U/L)	BUN (mg/dl)
Control (0.9% NaCl)	55.8±10.89	47.8±12.93	151.6±16.05	82.2±19.24	65.6±12.36	361.8±20.99	14.9±2.67
Stress Control	91.4±16.06#	54.6±8.35	106.8±15.55#	125.6±16.21#	67.5±9.94	495.8±23.16#	26.5±3.55#
Imipramine (mg/kg)							
15	82.4±13.18	50.8±10.62	136.4±15.50*	118.6±12.01	69.4±13.96	468.8±36.77	18.2±4.78*
Lab Cultured *O. sinensis* (mg/kg)							
100	82.6±11.28	51.8±5.93	127.6±16.75	116.2±16.01	66.5±9.88	493.2±35.02	23.4±4.00
300	71.6±16.83	45.6±10.62	134.2±9.75	94.5±12.68*	65.1±14.01	424.6±18.85*	19.2±3.62*
500	62.8±12.73*	40.2±10.42	140.8±14.09*	85.64±10.55*	62.8±21.03	386.2 ±27.71*	16.4±4.77*
Natural *O. sinensis* (mg/kg)							
100	79.4±14.57	50.2±5.93	133.4±11.72*	112.4±17.25	65.8±12.00	482.6±32.51	20.4±5.03
300	70.6±13.72	43.6±10.62	139.8±14.68*	92.1±15.00*	63.9±14.74	396.4±20.81*	17.7±3.45*
500	51.4±9.93*	38.4±9.20*	144.0±18.74*	75.6±18.06*	58.8±18.49	352.2±17.11*	15.2±3.17*

AST=Aspartate Aminotransferase, ALT=Alanine Aminotransferase, ALKP=Alkaline Phosphatase, LDH=Lactate Dehydrogenase.
Different groups of rats were administered with imipramine (15mg/kg, body wt., ip), natural *O. sinensis* (100-500mg/kg body wt., oral) and lab cultured *O. sinensis* (100-500mg/kg body wt., oral), while control animals received saline (0.5mL/rat, oral). The data represents mean±SEM for each group at respective time-points. *p<0.05 vs. stress control, #p<0.05 vs. control.

immobilized stress control rats. However, there was no significant increase in ALT level. Treatment with lab cultured mycelium and natural specimens at the dose of 300 and 500 mg/kg was found inhibiting significantly ($p < 0.05$) to the stress induced LDH, BUN, total cholesterol level and ALP. However, there was no significant change in AST level at the dose of 100 and 300 mg/ kg in case of both the samples. Whereas, triglycerides and ALT levels in serum were not changed significantly in test animals as compared to control. Decreased ALP level of immobilized stress rats was also inhibited significantly at the lower dose (100 mg/kg) of natural specimens only.

Discussion

Present study concludes that subacute administration of powder of lab cultured *O. sinensis* shortened immobility time of rats and enhanced swimming time of mice significantly. Thus potent anti-stress and muscular endurance effect of lab cultured mycelium was well validated.

FST has been used to evaluate the anti-stress and muscular endurance effects of various compounds[20, 44]. The sub acute administration of powder of natural *O. sinensis* (100, 300 and 500 mg/kg, oral) was found responsive to increase swimming time of mice significantly and reduce the immobility time of rats in dose dependent manner. The findings are similar to previous studies on administration of supercritical fluid extract of *O. sinensis*, in shortening of immobility times of mouse in dose dependent manner in tail suspension test[45] and oral administration of the water extract of *O. sinensis* for 5 days in improving the swimming time of mice[46]. *O. sinensis* has been found useful in the improvement of metabolic threshold, anti-fatique and anti-stress activity[19, 21]. The sub acute administration of lab cultured mycelium powder of *O. sinensis* (100, 300 and 500 mg/kg, oral) increased swimming time of mice significantly and reduced immobility time of rats in dose dependent manner similar to the natural specimens. This effect of *O. sinensis* may be due to the presence of cordycepin, polysaccharides and cordyglucan which are reported to be responsible to enhance the endurance and energy metabolism[21]. Our study is well supported by the previous work on enhancement of the endurance[20] and minimal genetic variability in *in vitro* propagated mycelium[47].

Changes in the body weight and food intake are critical for the evaluation of any product on test animals which is indicative of the enhanced growth and anti-stress like activity in the animals[48-49]. In the present study, animals treated with natural *O. sinensis* (300 and 500mg/kg, oral) showed significant increases in food intake ($p<0.05$) and body weight ($p<0.05$) as compared to control animals. Similarly, supplementation of both CS powder and CS-4 along with exercise increases the metabolic threshold of

young and elderly human volunteers[17-19]. Manabe *et al* (2000)[50] reported that increase in the ratio of ATP to inorganic phosphate (ATP:Pi) in the treated mice with mycelial extract of cultured *O. sinensis*, represented an increase in cellular energy state. Increased food intake may promote the energy production in the form of ATP in animals. Zhu and Rippe (2004)[51] have suggested that the *O. sinensis* significantly increases energy output and oxygen capacity. Similarly, in anaemic mice model, supplementation of cultured O. *sinensis* mycelia (200 mg/kg/day, orally for 4 weeks) increased hepatic blood flow and energy metabolism in both normal and anaemic mice without any toxic effect[50]**.** Our findings are well validated by the work of Mounnissamy *et al* (2010)[52] who have reported that progressive increase in body weight and organ weight of rats during 28 days study, resulting from administration of ethanol extract of *C. rheedii* may indicate the improvement in the nutritional state of the animal. Subacute administration of lab cultured *O. sinensis* (300 and 500 mg/kg body wt, oral) increased the food intake ($p<0.05$) and gain in body weight ($p<0.05$) significantly by the animals as compared to control.

AST, ALT, ALP, LDH, cholesterol, BUN and triglycerides are the most important and common blood parameters used for the evaluation of anti-stress like effect in animals[20, 53]. Immobilization stress was induced to mark the increase in serum LDH, AST and total cholesterol levels and decrease in ALP level[20, 54]. The BUN value was found to increase significantly after exercise[55]. In the present study, lab cultured *O. sinensis* significantly inhibited the increase in the level of AST, LDH, total cholesterol and BUN and the decrease in ALP level of immobilized stress rats similar to natural *O. sinensis* which are the indications of anti-stress effect.

Conclusion

In view of the above finding, it could be concluded that powder of both natural and lab cultured *O. sinensis* have anti-stress and muscular endurance effects like activity. Lab cultured *O. sinensis* powder can be used in product development as performance enhancer nutraceuticals for improving human health and quality of life substituting to the natural specimens of *O. sinensis* which are otherwise highly expensive and available in scant.

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