ANTI-FATIGUE EFFECTS OF *AGARICUS BISPORUS* EXTRACT IN RATS

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ABSTRACT

Mushroom antioxidants help in scavenging the free radicals formed during heavy exercise, so the addition of mushroom extract to curd may provide combinatorial effect. The present study was undertaken to investigate the anti-fatigue properties of *Agaricus bisporus* extract (ABE). Antioxidant properties of the extract were screened and the results showed increased free radical scavenging, reducing power and metal chelating property with increasing concentration of ABE. Total phenolic content was 7.5821 mg tannic acid equivalent /g extract (or) 27.8 mg quercetin/g of extract, and total flavanoid content was 2.2385 mg quercetin/g of extract. HPLC profile of the polyphenols in the mushroom extracts revealed the presence of chlorogenic acid and p-coumaric acid. The anti-fatigue activity of ABE was measured using animal treadmill exercise. Endurance exercises reduced the levels of glycogen in control group. However, ABE supplementation enhanced liver and muscle glycogen levels ($p<0.05$). Lactic acid (LA) levels in muscle tissue were significantly higher in control exercise group when compared to sedentary group ($p<0.05$). The ABE supplemented group showed lowered LA levels in muscle tissue when compared to control rats ($p<0.01$) showing the efficient usage of glucose during exercise by the rats. In control exercised group of rats, the values of malondialdehyde (MDA) concentration were significantly higher in muscle and liver when compared with sedentary group. The ABE treatment decreased the MDA levels in muscle (37.7%) and liver (16.2%) to that of control group showing the *in-vivo* antioxidant property of the extract. In conclusion, the extract will have beneficial effect *w.r.t* its anti-fatigue property.

Keywords: *Agaricus bisporus* extract (ABE), anti-fatigue, malondialdehyde, exercise

INTRODUCTION

Fatigue may be because of both physical and mental stress. Physical fatigue is the inability to continue to do work [1]. Severe and continuous exercise may elevate the formation of reactive oxygen species (ROS) elevating oxidative stress [2,3]. An elevated level of malondialdehyde (MDA), a lipid peroxidation product has been reported by many workers during the course of exercise [4]. The public have become much more health-conscious and an increased proportion of people now exercise routinely to prevent lifestyle-related diseases and to enhance their physical fitness. Moderate exercise is useful for preventing illness and mental stress, but over exercise itself can be a form of stress and cause fatigue or various types of damage to the organs. Fatigue is the symptom, which indicates that the health is either about or already subjected to harm [5]. Free radical formation and subsequent lipid peroxidation of bio-membranes might be noxious biochemical responses to either physical or emotional stress [6]. It has been demonstrated that exercise leads to increase in free radical formation thus causing oxidative damage to membranes (lipid peroxidation), thus, fatigue causes various disorders in relation to bio-regulatory, autonomic nervous, endocrine and immune system [7]. However, there is no detailed information available concerned with anti fatigue properties of mushrooms. The present study was undertaken to investigate the anti-fatigue properties of *Agaricus bisporus* extract by using animal treadmill and development of a fermented yogurt product using the same extract.

MATERIALS AND METHODS

Mushroom extract preparation

Preparation of raw mushroom powder: Mushrooms were purchased from the local market in Mysore, and were stored at 4 °C for 24 hours. The mushrooms were then cleaned, washed and cut into small pieces. The pieces were then freeze dried in alyophilizer until completely dry. The dried mushrooms were then powdered and sieved through a sieve (16 mesh screen). The mushroom powder was collected in sterile sample bags and stored in the dark at room temperature [8].

Preparation of mushroom extract powder: 5 g of the freeze-dried mushroom powder was added to 60 ml of 80% ethanol and heated to 60 °C using a water bath and left for extraction with shaking at regular intervals for 1 hour. Then the mixture was filtered through country filter paper and the residue was extracted with additional 60 ml of 80% Ethanol, as described above. The filtrate collected through each of the three extractions were pooled and well blended. The extract was then flash evaporated and freeze dried. The mushroom extract powder was collected in sterile sample bags and stored in dark at room temperature [8].

HPLC analysis of mushroom phenolics in hydro-alcoholic extract

Phenolic acids of hydro-alcoholic extract were analyzed according to the method of Wulf and Nagel on a reversed phase Shimpak C18 column (4.6 x 250 mm), using a diode array detector (operating at 650 nm). The Shimpak C18 HPLC column was obtained from Shimadzu Corp. A solvent system consisting of water/acetic acid/methanol (80:5:15) (v/v/v) was used as mobile phase at a flow rate of 1 ml/min. Phenolic acid standards such as gallic acid, *p*-coumaric acid, chlorogenic acid, sinapic acid, protocatechol acid, ellagic acid, and rutin were used for the identification of phenolic acids present in the hydro-alcoholic extract of mushroom. Quantification of phenolic acids was achieved by the absorbance recorded in the chromatograms related to external standards at 650 nm.

***In-vitro* antioxidant assays**

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity: The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of DPPH. This spectrophotometer assay uses the stable radical DPPH as a reagent. The DPPH free radical scavenging activity of the extracts was measured according to the method of Brand-Williams *et al.* [9] . Stock solution of each mushroom extract (50 mg/mL) was diluted to a concentration in the range of 0.1 to 50 mg/ml. For the test, 3.9 ml of 0.06 mM DPPH radical (Sigma) was added to 0.1 ml of mushroom extract. Reaction mixture was vortexed and absorbance was measured at 515 nm using a spectrophotometer with methanol as the blank. The decrease in absorbance was monitored at 0 min, 1 min, 2 min, and every 15 min until the reaction has reached a plateau. The time taken to reach the steady state was determined by one-way analysis of variance (ANOVA). The DPPH free radical scavenging activity, expressed as percentage of radical scavenging activity, was calculated as follows:

Radical scavenging activity (SA) = $\{(A_0 - As)/A_0\} * 100$

Where, A_0 is the absorbance of 0.06 mM methanolic DPPH only whereas *As* is the absorbance of the reaction mixture.

Fe^{2+} Metal-chelating ability: Metal- chelating ability of mushroom extract was assessed using the method of Decker and Welch [10]. Mushroom extract (1mg/ml) was mixed with 3.7 mL of distilled water. It was then reacted with a solution containing 0.1 ml 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. After 10 min, the absorbance of the reaction mixture was measured at 562 nm.

% Metal-chelating ability = [1-(O.D. of the sample/ O.D. of the control)] x100

Reducing power ability: Reducing power of the mushroom extracts was determined according to the method of Athukorala *et al*. [11]. The reducing power can be determined by the method of 1.0 ml extract was mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50 °C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of $FeCl_3$ (6 mM) and absorbance was measured at 700 nm. Ascorbic acid, butylated hydroxyanisole (BHA), á-tocopherol, trolox [12] or butylated hydroxytoluene (BHT) [13] can be used as positive control.

Determination of total phenolic compounds: Total phenolic compounds in the ethanol extracts were determined using Folin–Ciocalteu method [14]. One ml of the extract was added to 10.0 ml distilled water and 2.0 ml of Folin-Ciocalteu phenol reagent. The mixture was allowed to stand at room temperature for 5 min and then 2.0 ml of 20% sodium

carbonate was added to the mixture. The resulting blue complex was measured at 680 nm. Catechin was used as a standard for the calibration curve. The phenol compound was calibrated using the linear equation based on the calibration curve. The contents of the phenolic compound were expressed as mg catechin equivalent/g dry weight.

Determination of total flavonoid concentration: The $AlCl_3$ method [15] was used for determination of the total flavanoid content of the sample extracts. Aliquots of 1.5 ml of extracts were added to equal volumes of a solution of 2% $AlCl_3$ (2 g in 100 ml methanol). The mixture was vigorously shaken, and absorbance at 367 nm was read after 10 min of incubation. Quercetin was used as a standard for the calibration curve. The flavonoid compound was calibrated using the linear equation based on the calibration curve. The contents of the flavarnoid compound were expressed as mg quercetin equivalent/g dry weight.

Animal studies

Experimental design: Animal experiment was carried out as per guidelines of Institutional Animal Ethics Committee. Twenty five male mice of body weight 35-40 g were selected from the stock colony of this institution based on uniform food intake and body wt gain, placed individually in SS cages, exposed to 12 hr dark and light cycle maintained at 27$\pm$ 2 ^{o}C with free access to drinking water and synthetic diet (15% protein, 10% fat from peanut oil with recommended levels of minerals and vitamins). These animals were divided into five groups consisting of five mice each, group I sedentary control; group II exposed to exercise but without feeding mushroom extract; group III sedentary controls, but forced fed with 100 mg equivalent of mushroom extract/mouse/day, daily in the morning at 12 Noon after fasting the mice for 3 hours (the diet cups were removed at 9 AM), group IV and group V were forced fed with 15 mg and 100 mg equivalent of mushroom extract, respectively for 21 days and subjected to treadmill exercise, for a fixed time.

Tread mill experiment: The mice were subjected to treadmill experiment for a period of 2-3 minutes daily. The speed was adjusted based on the ability of the mice to maintain its running pace constant.The speed and inclination was increased gradually to max.15 m/min and max. 5^{o}, respectively, based on the performance of the mice.

Determination of number of shocks: After the mice have been trained to run on tread mill, they were evaluated for their performance under pre- determined set of conditions. The mice were made to run on the tread mill at a specific speed (10 min/min), inclination (5 V) and time duration (7 min). The mice which get tired and unable to run, stops running and sits on the track, which then hits the shock grid and receives a shock of 1 V, every time it comes into contact with the grid. The number of shocks received by each mouse for a particular period of time was recorded, which can be related to the fatigue experienced by mouse.

Determination of biochemical variables: On 28thday, a 7 min treadmill exercise was performed by group III and IV mice, 1 hr after the last treatment administration and were sacrificed immediately after exercise by cervical dislocation. Blood samples were collected from the retro orbital sinus. Blood glucose was determined immediately using a portable glucose analyser. Serum was separated after centrifuging the blood at 1000 rpm for 5 minutes at 4 ^{o}C. The effects of mushroom extract on liver and muscle tissues were analyzed for TBARS.

Thiobarbituric acid reactive substances (TBARS): The liver and muscle tissues were homogenized in ice-cold Tris–HCl buffer (pH 7.4, 20 mM) to produce a 1:2 (w/v) tissue homogenate, which was centrifuged at 3000*g* for 10 min. An aliquot (0.5 ml) of the supernatant was incubated with trichloroacetic acid (10 % w/v, 0.5 ml), followed by thiobarbituric acid (TBA, 2%, 2 ml), and the mixture was then heated at 80°C for 30 min. The reaction mixture was cooled and centrifuged at 8000 *g* for 20 min to remove the precipitated protein. The colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at $\ddot{e}$ = 532 nm. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = [(A\text{-}B)/A] \times 100$$

Where, *A* and *B* were the absorbance of the control and the compound solution, respectively.

RESULTS AND DISCUSSION

Antioxidant properties of *Agaricus bisporus* extract

DPPH scavenging activity: DPPH is nitrogen cantered free radical that shows strong absorbance at 517 nm. Deep violet coloured of DPPH solution changes to yellow in presence of DPPH radical scavengers. DPPH radical accepts an electron to become a stable diamagnetic molecule. Extent of DPPH radical scavenged was determined by the decrease in intensity of violet color in the form of EC_{50} values. Lower EC_{50} value represents higher antioxidant activity. To evaluate the free radical scavenging activity, the mushroom extracts were subjected to scavenge DPPH by donating an electron. Results confess that scavenging activity increased with increasing concentration of *A. bisporus* extract. The results of DPPH radical scavenging activity revealed that % inhibition of all extracts increased with increasing concentration. The results are given in Table 1.

Table 1. DPPH scavenging activity

Sample	IC_{50} (mg)
BHA	0.030
BHT	0.025
Vitamin C	0.045
Agaricus bisporus extract	0.968

Table 2. Metal chelating efficiency

Sample	IC_{50} (mg)
EDTA	0.130
Agaricus bisporus extract	0.221

Metal chelating efficiency: Iron is known to generate free radicals through the Fenton and Haber–Weiss reaction. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalysing transition metal in lipid peroxidation. It is reported that chelating agents, which form *s*-bonds with a metal, are effective as secondary antioxidants since they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. Metal chelating efficiency of the extracts was estimated over a wide range of concentration (0-500 ppm) and also compared with EDTA, the standard chelating agent (Table 2.). EDTA showed 98% chelation at ≥ 400 ppm. The results of Fe^{2+} chelating ability revealed that % inhibition increased with increasing concentration. The results are:

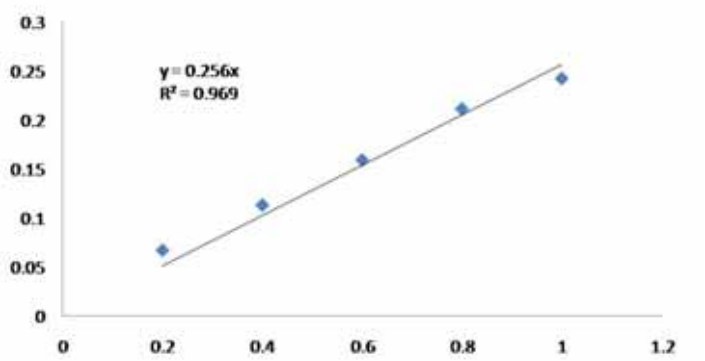

Figure 1. Reducing power ability of hydro-alcoholic extract of *A.bisporus*

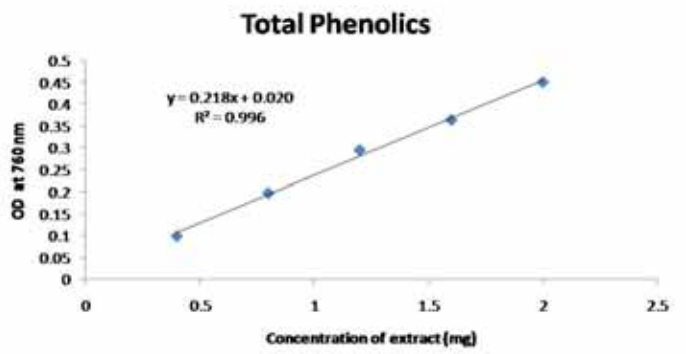

Figure 2. Total phenolic content of hydro-alcoholic extract of *A.bisporus*

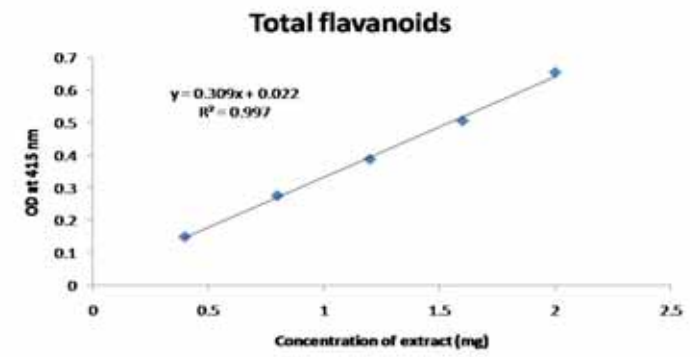

Fig. 3. Total flavanoid content of hydro-alcoholic extract of *A.bisporus*

Reducing power ability: Reducing power assay method is based on the principle that substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm. Reducing power was 46.84 mg Ascorbic acid equivalent / g of mushroom extract. The reducing power of the *A. bisporus* extract increases with the increase in concentration (Fig. 1). All the methods have proven the effectiveness of *A. bisporus* extract compared to the standard antioxidant, ascorbic acid.

Phenolics and flavanoid contents in *A. bisporus* extract: Total phenolic content was 7.5821 mg Tannic acid equivalent / g extract (or) 27.8 mg quercetin/ g of extract (Fig. 2) and total flavanoid content =2.2385 mg quercetin / g of extract (Fig. 3).

Determination of polyphenol contents in *Agaricus bisporus* extract by HPLC: HPLC profile of the polyphenols in the mushroom extracts revealed the presence of chlorogenic acid and p-coumaric acid (Figs. 4 and 5).

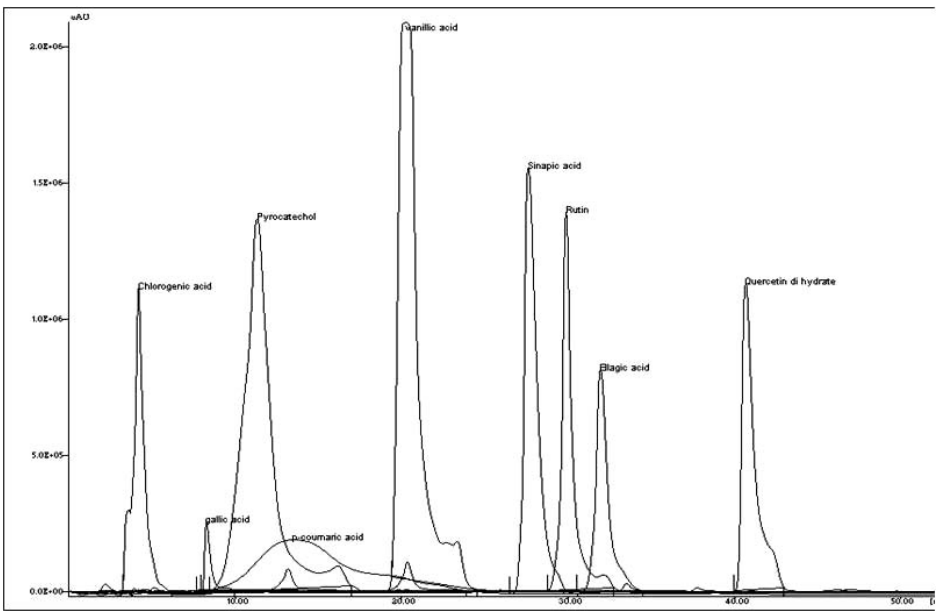

Figure 4. HPLC profile of standard polyphenols at 650 nm

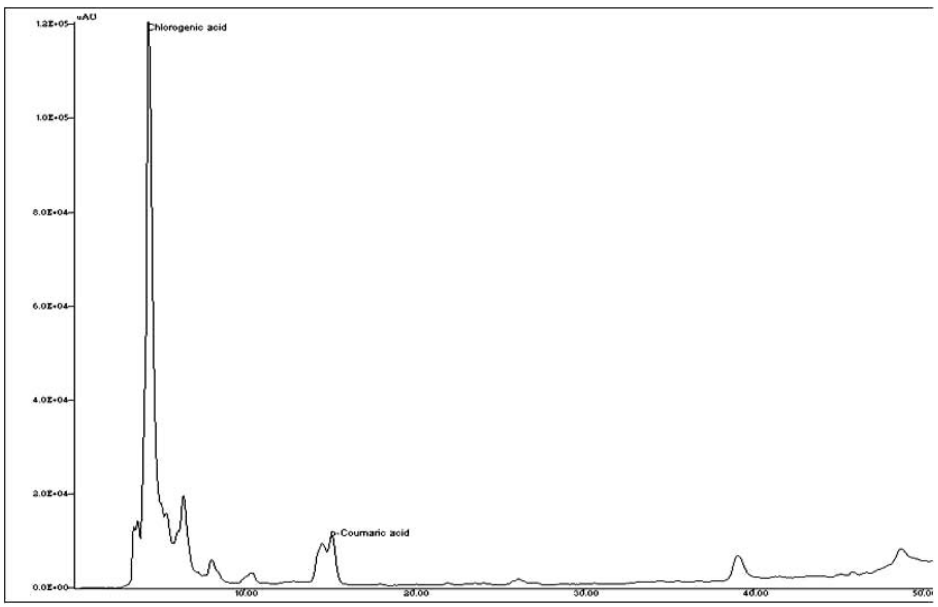

Figure 5. HPLC profile of *Agaricus bisporus* extract at 650 nm

Anti-fatigue properties of *Agaricus bisporus* extract

Effect of *Agaricus bisporus* extract on physical endurance: The anti-fatigue activity of *Agaricus bisporus* extract was measured by calculating the number of shocks received by the animal during its treadmill exercise. This is because only the animals with less endurance capacity will stop running and receives more number of shocks; i.e., animals receiving less number of shocks are having more endurance capacity. The numbers of shocks received by the animals gradually decreased from day 1 to day 6 with *A. bisporus* extract treatment when compared to control group ($p < 0.05$). Minimum number of shocks were recorded on day 6 of the *A. bisporus* extract treatment (2 numbers of total shocks) to that of control group (12 numbers of total shocks), (Table 3).

Table 3. Endurance capacity of *A. bisporus* extract assessed by number of shocks during the period of treadmill test

	Total Number of shocks from all 6 mice					
	1st day	**2nd day**	**3rd day**	**4th day**	**5th day**	**6th day**
Group II (Exercise control)	26	27	18	16	16	12
Group IV (15mg extract + exercise)	33	19	15	6	4	2
Group V (100mg extract + exercise)	16	7	5	3	2	2

Effects of *Agaricus bisporus* extract on lipid peroxidation: Thiobarbituric acid-reactive substances (TBARS)/ MDA, represents lipid peroxidation. In control exercise group the values significantly increased MDA concentration in muscle (43.7%) and liver (14.6%) when compared with sedentary group. The *A. bisporus* extract treatment decreased the MDA levels in muscle (37.7%) and liver (16.2%) to that of control group (Table 4).

Table 4. Determination of TBARS in Muscle and Liver

Group	**Feeding and Exercise pattern**	**Muscle TBARS (x 10^{-5} m MDA/g)**	**Liver TBARS (x 10^{-5} m MDA/g)**
Group I	Control	31.28	3.47
Group II	Exercise	52.16	5.36
Group III	Extract 100 mg	32.54	4.04
Group IV	Test 15 mg + Exercise	42.38	4.75
Group V	Test 100 mg + Exercise	23.38	3.3

CONCLUSION

Anti-fatigue natural agents may be essential for persons involved in heavy exercise, such as athletes and other sports personnel, soldiers, industrial employees engaged in severe physical work prompting to utilization of available/stored energy. Continuous physical work may lead to anaerobic condition resulting in accumulation of blood lactic acid, increased toxic products of lipid peroxidation in blood and liver, which in turn may reduce overall physical performance. Dietary macronutrients especially the carbohydrates definitely provide the immediate energy required for carrying out the physical activity, however, it can lead to the accumulation of lactic acid [16, 17]. It is known that severe and prolonged physical activity increases oxidative stress causing elevated free radicals / reactive oxygen species (ROS) concentration that may decrease cellular function / integrity. Endogenous antioxidants namely vitamins A, E and C may neutralize these radicals under normal condition. However, supplementation of these vitamins is essential to neutralise excessive ROS. On the other hand polyphenol with many phenolic functional groups may possess better antioxidant activity compared to that of vitamin A, E and C. As such there is a paucity of information regarding the role of *Agaricus bisporus* in enhancing the physical endurance. In the present study we force fed *A. bisporus* extract to groups of animals and subjected them to exercise for evaluating the antioxidant potency. The results obtained demonstrated that the elevated level of free radicals as indicated by increased levels of lipid peroxidation [18-19] in liver of group III animals was reversed in group IV and V animals, which were force fed with *A. bisporus* extract suggesting an anti-oxidant role of the ingested polyphenols [20,21,22].

Accumulation of lactic acid in blood has been shown to decrease the physical efficacy [17]. In aerobic glycolysis NADH may be thought of a high energy compound whereas in anaerobic glycolysis its free energy oxidation is dissipated as heat [17]. Results clearly show significant decrease in number of total shocks from day one onwards in group IV and V to that of group III. As the exercise days continued, animals received less number of shocks in both the groups, which may be attributed to learning process of animals. This endurance might be attributed to *A. bisporus* extract supplementation. During the course of severe exercise, glycogenolysis may be predominant for adequate supply of energy [17]. From the present study the antifatigue property of *A. bisporus* extract was clearly established. This was achieved through decreased levels of lipid peroxidation in tissue which also support the antifatigue property of the *A. bisporus* extract.

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