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ANTIOXIDANT EFFECTS OF NOOTKATONE ON ENHANCING PHYSICAL ENDURANCE IN MICE SUBJECTED TO TREADMILL EXERCISE.

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ABSTRACT: The study was designed to evaluate the anti-fatigue activity and biochemical effects of nootkatone in Balb-C mice. Thirty-six mice weighing 33-41g were randomly divided into six groups; 1) sedentary group, 2) sedentary group fed with 50 mg/kg nootkatone, 3) control group (exercise control), 4) exercise with 10mg/kg nootkatone group, 5) exercise with 25mg/kg nootkatone and 6) exercise with 50mg/kg nootkatone. Nootkatone was administered orally to mice from all groups. The anti-fatigue activity was assessed using a treadmill running test. Biochemical parameters were also evaluated. Nootkatone was found to increase run time on the treadmill and reverse the fatigue-induced reduction in liver/muscle glycogen, in addition to reducing lactate dehydrogenase (LDH) activity, blood urea nitrogen (BUN), and lactic acid. Moreover, nootkatone increases the activity of enzymes such as catalases, SOD and decreases lipid peroxidation. The data suggested that the nootkatone could extend the treadmill exercise duration in mice, as well as increase the tissue glycogen contents and decrease the malondialdehyde (MDA), lactic acid, BUN, CK and LDH levels. There was a significant increase in the antioxidant levels in the tissues such as SOD and CAT. These results also support that nootkatone with 50mg/body weight is the optimum concentration to act against fatigue. However, further studies are necessary to clarify the bioactive principles responsible for the mechanism involved in the anti-fatigue properties of nootkatone.

INTRODUCTION: One of the prime functions of the body is to produce energy from the food we eat. When biochemical energy production declines for any reason, the resulting symptom is fatigue. Fatigue is the basic indicator of an energy imbalance in the body. It is the most common complaint of the present generation.

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While it is not a disease, it is an early warning symptom for many potentially serious health conditions. There are enormous numbers of reasons for fatigue to occur. Fatigue not only affects physical health but also affects performance, behaviour and family dynamics.

During fatigue, the following symptoms can be observed: allergies, loss of memory, joint pain, lactic acid acidosis, hypoglycaemia, weakness in the muscle, poor digestion, low blood pressure, excessive sensitivity of stress, insomnia, mental depression. The above symptoms could also be extended to more serious conditions that have fatigue as an underlying cause.

Fatigue is the prime symptom of a deficiency of adequate biochemical energy production in the body cells. Armed force personnel as well as sports personnel experience high intensity physical activity leading to stress and fatigue, which may interfere in their maximal performance. It is therefore essential that they consume other supplements, apart from proper nutrition, which can eliminate or reduce the catabolic end products responsible for fatigue and enhance performances or endurances. Such substances that optimize physical performance are called ergogenic aids. Nootkatone [6-Isopropenyl-4, 4a-dimethyl-4, 4a, 5, 6, 7, 8 -hexahydro-3H-naphthalen-2-one], a natural aromatic compound, has been previously reported to increase physical endurance in balb-c mice and could be used as an ergogenic aid [1].

This sesquiterpenoid is generally found in grapefruit and is one of the main chemical components for its smell and flavor. Currently, nootkatone has been extensively used in the production of repellents for mosquitoes, bed bugs, lone star ticks, and head lice. Apart from this, it is also used as a food additive and flavoring [2, 3] and inhibiting the activity of acetylcholinesterases and cytochrome P450 monooxygenases CYP450 [4, 5].

Nootkatone has a significant antidepressant effect through modulation of oxidative stress induced by D-galactosamine administration [6]. In addition, nootkatone has the potential to be an efficacious repellent against adult Aedes mosquitoes [7]. Recently, stimulating effects by nootkatone on an adenosine monophosphate-activated protein kinase have also been claimed [1] and also the cardioprotective effects have been reported [8]. Nootkatone has been reported to have anti-inflammatory and antioxidant effects in a mouse model of systemic inflammation [9]. In continuation with the work of Murase *et al*. (2010), in the present study, we tried to elucidate the role of antioxidant effects of nootkatone on enhancing physical endurance on mice subjected to treadmill exercise.

MATERIALS AND METHODS:

Materials: The nootkatone was purchased from Alfa Aesar (A19166), pellet diet (Sri Venkateshwara enterprises, Bangalore, India), Superoxide dismutase kit (Randox, Cat no. SD.125 Canada), all the other reagents were of analytical grade obtained from SRL India.

Experimental Animals and Grouping: Male Balb-c mice weighing 30 to 40 g were maintained at a room temperature of 22 ± 2 ℃ with a 12-h light/ 12 h dark cycle and 45% - 55% relative humidity. The animals had free access to food and water in accordance with the guidelines approved by the Institutional Animal Ethics Committee. The mice were fed with a commercial pellet diet (Sri Venkateshwara enterprises, Bangalore, India) and water *ad libitum*. For the test groups, nootkatone was fed orally at three different concentrations. All the experiments were conducted between 09:00 and 14:00 h.

The animal study protocol was approved by the Institutional Animal Ethics Committee (IAEC) and by the committee for the control and supervision of experiments on animals (CPCSEA) NO: 28/IAEC/CPCSEA. The animals were housed in an acrylic fiber cage. After an adaptation period for a week, the mice were randomly divided into 6 groups, with 6 mice each. The first group was sedentary; the second group was fed with 50 mg nootkatone; the third group was exercise control; the fourth, fifth, and sixth groups were made to exercise, which were fed with 10, 25, 50 mg of nootkatone per Kg of body weight respectively. The nootkatone was purchased from Alfa Aesar (A19166), which was dissolved in sunflower oil. After oral feeding, the mice were rested for an hour and were made to run on the animal treadmill.

- **Group 1:** sedentary group.
- **Group 2:** 50 mg nootkatone group.
- **Group 3:** control group.
- **Group 4:** 10 mg/kg group. Exercise group.
- **Group 5:** 25 mg/kg group.
- **Group 6:** 50mg/kg group.

Treadmill Exercise: The mice fed with the different concentrations of nootkatone and the exercise control group were made to run on an animal treadmill.

The mice were subjected to two different types of training: adaptive training and intensive training. In adaptive training, the mice were made to run at a speed of 10 meters/min, 14 meters / min, 18 meters /min at 2.5 ºC inclination for 15 min (the slope was

kept constant) each day from 1st week to 3rd week and during intensive training the mice were made to run at a speed of 20 meters/min for 15 min at 2.5 ºC inclination. After the intensive training for one week, the mice were run until they got exhausted, and the time was noted to see the performance of the mice during exercise. After the test period, the mice were sacrificed under mild anesthesia, and blood, liver, and muscle tissues were collected for biochemical analysis.

MICE RUNNING ON TREADMILL

Estimation of Thiobarbituric acid Reactive Substances (TBARS): TBARS as malondialdehyde (MDA μmol/cm/g) was analyzed by Buege and Aust method (1978) with slight modification [10]. Liver and muscle tissues (100 mg) were homogenized in 2 mL of phosphate buffer (pH 7.0). TCA (10%), 0.5 and 2 mL of TBA mixture were added to tissue homogenate (0.5 mL).

The TBA mixture contained TBA (0.35%), SDS (0.2%), $FeCl_3$ (0.05 mM) and BHT in glycine-HCl buffer (100 mM, pH 3.6). The above reaction mixture was boiled at 100 °C for 30 min and then allowed to cool. The mixture was centrifuged at 8,000 rpm for 10 min, and the absorbance was measured at 532 nm.

Estimation of Lactic acid: Lactic acid content was measured according to Sawhney and Singh methods (2005) [11]. Tissue samples were homogenized in phosphate buffer (100 mM, pH 7.2), deproteinized with TCA (10%), and centrifuged at 5000 rpm for 15 min. To the supernatant, 1 ml of copper sulphate solution (20%) was added and diluted to 10 ml with distilled water.

To this, 1 g of calcium hydroxide was added, mixed well, allowed to stand for 30 min, and centrifuged at 5,000 rpm for 10 min to 1 ml of supernatant, 0.05 ml of copper sulphate solution (4%), and 6 ml of H_2SO_4 (conc) were added and kept in boiling water bath for 5 min and allowed to cool. 100 microliters of p-hydroxy-diphenyl reagent were added to the above sample mixture and incubated at 37°C for 30 min. The absorbance was measured at 560 nm.

Estimation of Glycogen: Liver and muscle tissues were digested with 2 ml of KOH (30%), boiled in the water bath for 30 min with occasional shaking, and then allowed to cool to room temperature. Saturated Na_2SO_4 solution was added to the mixture and stirred well. Glycogen was precipitated by adding 5 ml of ice-cold ethanol to the sample mixture and centrifuged at 10,000 rpm for 10 min. One milliliter of HCl (1.2 N) was added to the supernatant (1:1 v/v) and incubated at 90 °C for 2 h and then allowed to cool and neutralized with 0.5 M NaOH. DNS method was followed to determine the hydrolyzed product of glycogen [12].

Estimation of Superoxide Dismutase (SOD) and Catalases (CAT): The antioxidant enzymes of liver tissues such as SOD were estimated according to the kit supplier protocol (Randox, Cat no. SD.125 Canada), while CAT estimation was carried out by measuring the decay of 6 mM H_2O_2 solution at 240 nm using spectrophotometer [13]. Protein content was estimated by the Bradford method, and the results were expressed as U/mg of protein [14].

Estimation of Creatinine Kinase (CK), Lactate Dehydrogenase (LDH), Blood Urea Nitrogen (BUN): The serum obtained after centrifugation was used for the measurement of BUN, CK, and LDH according to the assay kit supplier protocols (Bio systems and Agappe).

HPLC analysis of Nootkatone: The purity of nootkatone was checked by HPLC. The integrated high-performance liquid chromatography with a UV detector was used for the analysis.

The mobile phase used was an acetonitrile-water mixture (60:40) pumped at a flow rate of 1ml/min, and the sample was chromatographed at a wavelength of 245 nm and the run time was 30 min.

All the solvent and the mobile phase were of HPLC grade, and water was purified on the Millipore Milli-Q system [15].

Statistical Analysis: Data were expressed in mean ± SD. One-way ANOVA assessed a significant difference between the groups. $P<0.05$ was considered statistically significant by using Graph Pad Prism 5 software.

RESULTS AND DISCUSSION:

HPLC Analysis of Nootkatone: A single peak of nootkatone was observed at a retention time of 30 mins at 245nm with a flow rate of 1.0 ml per minute **Fig. 1.** The chromatogram shows no other molecules present in the sample, so the nootkatone used for the study was pure.

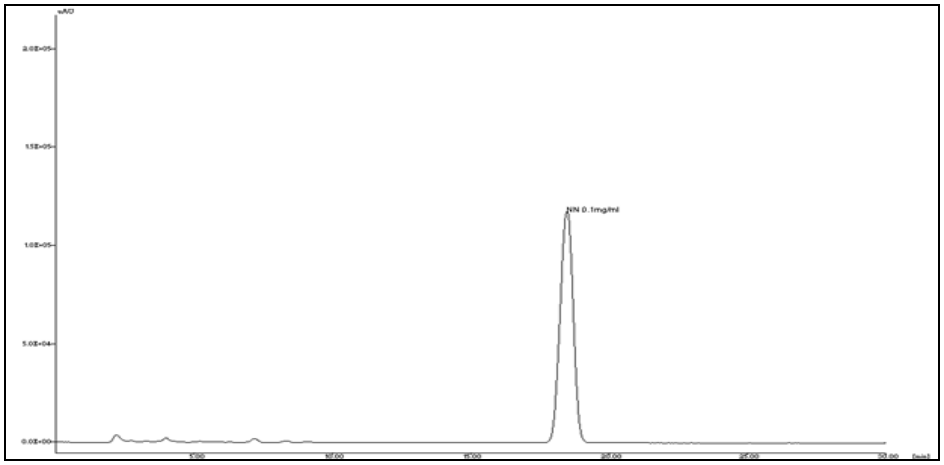

FIG. 1: HPLC CHROMATOGRAM OF NOOTKATONE AT 245 NM. THE CHROMATOGRAM SHOWED A SINGLE PEAK INDICATING THAT THE NOOTKATONE USED FOR THE STUDY WAS DEVOID OF ANY IMPURITIES.

Effect of Nootkatone on Food Intake and Body Weight Gain: Feeding nootkatone did not show any significant change in body weight gain compared to the control and it had no effect on food and water intake **Fig. 2A.**

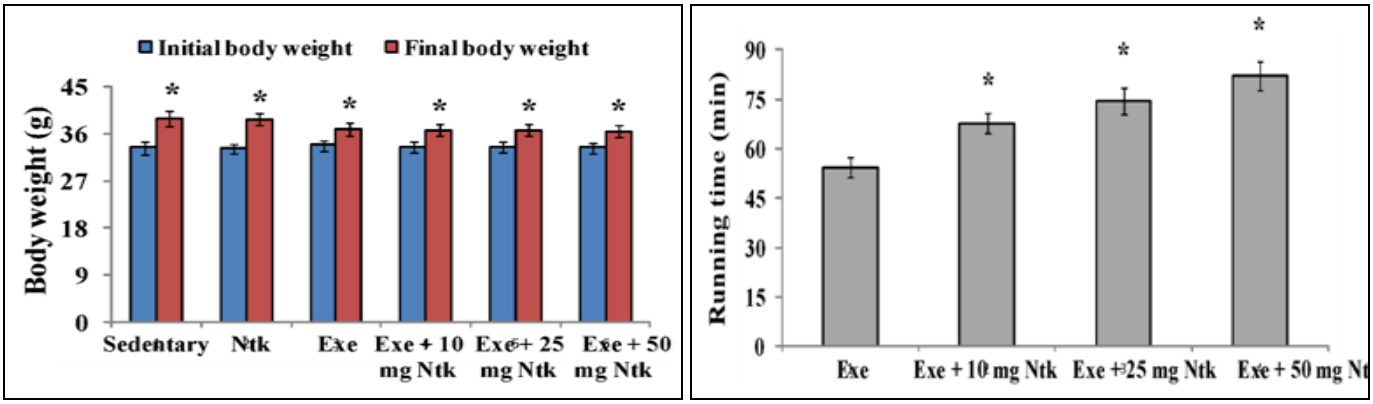

FIG. 2A: EFFECT OF NOOTKATONE SUPPLEMENTATION ON BODY WEIGHT OF MICE, BEFORE AND AFTER TREADMILL EXERCISE (N = 8, *P < 0.01). B) EFFECT OF NOOTKATONE ADMINISTRATION ON ENDURANCE ACTIVITY OF MICE. (N = 8, *P < 0.05 VERSUS EXERCISE GROUP).

Effect of Nootkatone on Running Time: The treadmill running time of nootkatone-treated groups was increased overall. In other words, the time taken to be exhausted by each of the mice fed with nootkatone was increased compared to the exercising control group.

The 10 mg, 25 mg, 50 mg nootkatone fed group showed an increase of 8%, 17%, 28% running time, respectively, compared to the control group. This indicates that nootkatone has increased the endurance of the mice, as well as the increase in endurance, was concentration dependent. The higher the nootkatone fed, the more was the endurance **Fig. 2B.**

Effect of Nootkatone on Serum Biochemical Indices: Serum glucose was found to be depleted in the control group when compared to the sedentary group. 10 mg, 25 mg and 50 mg nootkatone fed groups showed improvement (29%, 53% and 64%, respectively) in glucose level. 50 mg fed nootkatone groups showed a maximum increase in the level of glucose in serum compared to other groups fed with lower concentrations of nootkatone **Table 1.**

In addition, BUN, CK and LDH levels in serum were found to be higher in the control group compared to the sedentary group and 10 mg, 25 mg, 50 mg nootkatone fed groups **Table 1.**

TABLE 1: EFFECT OF NOOTKATONE ON SERUM BIOCHEMICAL PARAMETERS. VALUES ARE MEAN ± SEM (N = 8). P<0.05, SIGNIFICANTLY DIFFERENT FROM THE SEDENTARY GROUP AND HOMOGENEOUS SUB GROUPS SHARE A COMMON LETTER.

Groups	Glucose (mg/dl)	BUN(mg/dl)	CK (U/l)	LDH (U/mg protein)
Sedentary	8.11±0.74[a]	18.59 ± 1.25[a]	103 ± 8.3	314 ± 24.6
50 mg Nootkatone	8.05 ± 0.72[a]	18.21 ± 1.18[a]	102 ± 7.8 [a]	308 ± 22.5
Exercise	4.39 ± 0.38[b]	29.12 ± 1.4[b]	195 ± 17.4[b]	611 ± 57.6[b]
Exercise + 10 mg Nootkatone	5.71 ± 0.48[a]	24.14 ± 1.25[a]	172 ± 16.3[a]	521 ± 48.5[a]
Exercise + 25 mg Nootkatone	6.85 ± 0.52[a]	22.53 ± 1.2[a]	149 ± 12.8 [a]	446 ± 41.8[a]
Exercise + 50 mg Nootkatone	7.05 ± 0.61[a]	21.91 ± 1.19[a]	137 ± 12.1 [a]	381 ± 34.5[a]

Effect of Nootkatone on Muscle and Liver Glycogen: Glycogen is one of the important biochemical parameters to investigate the anti-fatigue activity. In the present investigation, we observed that liver and muscle glycogen was depleted in the exercise control group compared to the sedentary group. 10 mg, 25 mg, 50 mg nootkatone groups showed a slight improvement in the muscle and liver glycogen **Table 2.**

TABLE 2: EFFECT OF NOOTKATONE ON TISSUE BIOCHEMICAL INDICES. VALUES ARE MEAN ± SEM (N = 6). P<0.05, SIGNIFICANTLY DIFFERENT FROM THE SEDENTARY GROUP AND HOMOGENEOUS SUBGROUPS SHARE A COMMON LETTER

Groups	Liver		Muscle	
	Glycogen (mg/g)	Lactic acid (mg/g)	Glycogen (mg/g)	Lactic acid (mg/g)
Sedentary	9.15 ± 0.85[a]	0.85 ± 0.08[a]	3.22 ± 0.32[a]	3.25 ± 0.27[a]
50 mg nootkatone	9.14 ± 0. 83[a]	0.82 ± 0.15[a]	3.25 ± 0.3[a]	3.12 ± 0.28[a]
Exe	5.85 ± 0.31[b]	1.35 ± 0.12[b]	1.43 ± 0.16[b]	6.2 ± 0.5[b]
Exe + 10 mg nootkatone	6.24 ± 0.65[a]	1.22± 0.11[a]	1.85± 0.16[a]	5.44 ± 0.42[a]
Exe + 25 mg nootkatone	7.32 ± 0.68[a]	1.12 ± 0.1[a]	2.13 ± 0.18[a]	5.12 ± 0.38[a]
Exe + 50 mg nootkatone	7.85 ± 0.72[a]	0.95 ± 0.08[a]	2.53 ± 0.19[a]	4.75 ± 0.32[a]

Effect of Nootkatone on Muscle and Liver Lactic Acid: Lactic acid is the metabolic end product of anaerobic glycolysis, which serves as the energy source during exercise conditions.

Muscle and liver lactic acid was found to be increased in the control group when compared to the sedentary group. 10 mg, 25 mg, 50 mg nootkatone groups showed a reduced level of lactic acid in muscle **Table 2.**

Effect of Nootkatone on Antioxidant Enzymes: Superoxide dismutase and catalase are antioxidant enzymes that play a fundamental role in protecting the biological systems against free radical attack [16]. In the current study, we observed that the level of above said antioxidant enzymes in the liver sample was found to be increased in the exercise control group compared to the sedentary group.

In addition, nootkatone-fed groups showed an increased SOD and CAT levels than the controls in a concentration-dependent manner. The increased amount of nootkatone feeding resulted in a greater level of antioxidant enzymes **Table 3.**

TABLE 3: EFFECT OF NOOTKATONE ON ANTIOXIDANT PARAMETERS IN MICE LIVER TISSUE. VALUES ARE MEAN ± SEM (N = 8). P<0.05, SIGNIFICANTLY DIFFERENT FROM THE SEDENTARY GROUP AND HOMOGENEOUS SUBGROUPS SHARE A COMMON LETTER.

Groups	SOD (U/mg protein)	CAT (U/mg protein)	MDA (M/mg protein)
Sedentary	1.62 ± 0.13[a]	16.22 ± 1.1[a]	2.68 ± 0.25[a]
50 mg nootkatone	1.93 ± 0.15[a]	18.75 ± 1.25[a]	2.24 ± 0.22[b]
Exe	2.12 ± 0.18[b]	22.15 ± 1.6[b]	5.77 ± 0.52[a]
Exe + 10 mg nootkatone	2.25 ± 0.18[a]	26.78 ± 1.8[a]	4.78 ± 0.48[a]
Exe + 25 mg nootkatone	2.37 ± 0.18[a]	29.22 ± 1.9[a]	4.18 ± 0.37[a]
Exe + 50 mg nootkatone	2.42 ± 0.18[a]	32.12 ± 2.3[a]	3.43 ± 0.31[a]

Effect of Nootkatone on Thiobarbutaric Acid Reactive Oxygen Species (TBARS): Lipid peroxidation was measured by TBARS by estimating the content of malondialdehyde (MDA), , which is a by-product of the peroxidation of polyunsaturated fatty acid (PUFA). In the present investigation, we observed that MDA level was found to be increased in the exercise control group compared to the sedentary group. Exercise with 10 mg, 25 mg and 50 mg nootkatone fed groups showed a reduced level of MDA in an inverse concentration-dependent manner as the higher the nootkatone fed lesser the MDA levels were **Table 3.**

DISCUSSION: Normally, moderate exercise shows beneficial effects by enhancing metabolic activities, but intensive exercise leads to impairment in the body functions due to the generation of free radicals, which results in damaging the muscle tissues and the whole body in general. Improving exercise performance has been a wide research topic to decipher the anti-fatigue phenomenon and to identify alternate supplements to enhance exercise performance as well as to reduce the exercise-induced stress effects [17]. In the present study, nootkatone supplementation to mice for 4 weeks enhanced run time on the treadmill. This is concomitant with the restoration of glucose and glycogen level as well as inhibition of lactate dehydrogenase activity, decreases in the levels of lactic acid and blood urea nitrogen and decreases in the activity of creatinine kinase. Furthermore, treadmill exercise increased the activity of superoxide dismutase and catalases in the control group compared to that in the sedentary group. Nootkatone supplementation further increased the activities of these antioxidant enzymes; also as there was a decreased level of lipid peroxidation when compared to the control group. Nootkatone sesquiterpenoid constituent of grapefruit was keen to stimulate energy metabolism by activating AMP kinase in muscle and liver [1]. AMP-activated protein kinase (AMPK or AMP kinase) is a metabolic stress-sensing protein kinase responsible for coordinating metabolism and decreased energy. Exercise accelerates fatty acid metabolism in rodents, enhances glucose uptake, and stimulates nitric oxide (NO) production in skeletal muscles. AMPK phosphorylates and inhibits Acetyl CoA carboxylase, and enhances GLUT-4 translocation and thereby ensures continuous energy supply. AMP kinase is also known to stimulate fatty acid oxidation and mitochondrial biogenesis [18]. It is quite likely that nootkatone exerts its activity through this route.

Glucose plays an important and vital role in enhancing exercise activity. The increases in glucose stores in the liver and muscle as glycogen is an advantage to enhance the physical endurance capacity [19]. Energy for exercise is derived initially from the breakdown of glycogen in muscle. After strenuous exercise, glycogen is depleted, and at the later stages, the required energy may be derived from liver glycogen. The exhaustive treadmill exercise depleted liver and muscle glycogen levels in control mice, whereas, in the 10 mg, 25 mg 50 mg nootkatone fed groups, the depletion in glucose level was lesser. It is quite likely that in this case, the body might have resorted to fats for its energy requirements. Protein and amino acid catabolism generate urea nitrogen estimation of which in blood is a key index to assess the exercise-induced protein catabolism and renal function as well. At the same time, Creatinine kinase (CK) adds a phosphate group to creatine and converts it into a high-energy molecule phosphocreatine, which is used as an immediate energy source by muscle cells during high-energy demand. Most of the CK in the body is normally present in muscle, and therefore an increase in the amount of CK in blood

indicates muscle damage. Hence, CK estimation is used as a marker to measure muscle damage. Similarly, LDH, which catalyzes the interconversion of lactate to pyruvate, is present in muscle, and its appearance in blood indicates muscle damage. The findings showed there was an increase in the level of CK, BUN, and LDH in the control mice during the treadmill exercise test. However, their content was decreased in the nootkatone groups, with 50 mg nootkatone showing the least LDH, BUN, and CK compared to the other two doses. Thus, it may be inferred that nootkatone plays a protective role against muscle damage.

The excess calorie is stored in the liver and muscle as glycogen, which is used as a body fuel in exercise conditions. It has been reported that depletion of muscle glycogen leads to fatigue due to loss of energy source. It is demonstrated that herbal supplementation enhances glycogen levels. Similar effects have been reported by Kanda *et al.* (2012) [19] with whey protein hydrolysates that enhances the glycogen levels *via* glycogen synthase activity in mice subjected to swimming exercise.

Lactic acid is produced as a metabolic by-product of glycolysis. In strenuous exercise conditions, exaggerated glycolysis and production of lactic acid under anaerobic conditions lead to muscle acidosis. There was a significant decrease in the concentration of lactic acid in all nootkatone groups (10 mg, 25 mg, 50 mg groups) compared to the control group in muscle and liver tissues. However, liver and muscle tissue of animals fed with 50 mg nootkatone-exercise group treated mice showed significantly reduced levels of lactic acid when compared with the other two doses of treatments of nootkatone.

Lipid peroxidation products in liver and muscle increase after severe exercise [20]. There is evidence that mitochondrial dysfunction is directly related to fatigue in humans. Nootkatone (50 mg) group treatment reduced lipid peroxidation significantly. SOD and catalase are the key antioxidant enzymes that play a role in the antioxidant defense mechanism to protect the cell from free radical-mediated damage. Several studies decipher the increase in antioxidant status with exercise [21, 22]. In the present study, it was observed that there was an increase in antioxidant level in exercise as well as exercise with nootkatone supplementation. The nootkatone with 50 mg group treatment increased the antioxidant levels *i.e.*, SOD and CAT when compared with the other two groups of nootkatone. Therefore, this study showed that nootkatone has a very good antioxidant property.

CONCLUSION: In conclusion, the data suggested that the nootkatone could extend the treadmill exercise duration in mice, as well as increase the tissue glycogen contents and decrease the malondialdehyde (MDA), Lactic acid, BUN, CK, and LDH levels. There was a significant increase in the antioxidant levels in the tissue, such as SOD and CAT. These results also support that nootkatone with 50mg/body weight is the optimum concentration to act against fatigue. However, further studies are necessary to clarify the bioactive principles responsible for the mechanism involved in the antifatigue properties of nootkatone.

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CONFLICTS OF INTEREST: Authors declare that we do not have any conflict of interest.

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