Chapter –IV

Screening of *C. rotundus*, *C. paniculatus* and *E. alba* for physical performance improving potential and evaluation of the anti-stress effects in C2C12 murine myoblasts
4.1. Introduction

Regular exercise and balanced diet are the effective strategies to maintain a healthy lifestyle. Moderate exercise improves muscle metabolism, cardiovascular function and mitochondrial biogenesis by fatty acid utilization, enhancing mitochondrial metabolic rate and proliferation through a series of transcription regulators and co-activators (Hood and Saleem, 2007). SIRT-1 is one such regulator which acetylates the transcription co-activator PGC-1α to enhance mitochondrial biogenesis and also stimulates the energy sensing pathway via phosphorylation of adenosine monophosphate activated protein kinase (AMPK). On the other hand glucose and lactate transporters such as Glut-4 and MCT-1, MCT-4 activity stimulates glucose and lactic acid supply. Further, endurance exercise stimulates PDK4 and VEGF activity thus enhances the fatty acid oxidation and angiogenesis (Hardie, 2004; Kumar et al., 2011). However, exercise at higher intensity can cause increased free radical production in the skeletal muscle and myocardium and induce muscle fatigue by increasing the lactic acid content leading to acidosis of muscle. Thus, prolonged exercise leads to oxidative imbalance and generates fatigue which is defined as difficulty in initiating or sustaining voluntary activity (Chaudhuri and Behan, 2004, Powers et al., 2011). Fatigue affects the overall performance of an individual by generating imbalance in body functions. The free radicals induced by intense exercise leads to impairment of muscular, as well as neuronal functions termed as physical and psychological fatigue, respectively. Therefore, antioxidant supplementation might be a valuable therapeutic approach in regulation of such fatigues.

Skeletal muscle cell damage has been implicated in several conditions such as fatigue, cachexia, unloading, bed rest, space flight and denervation (Andersen et al., 1999; Stein, 2002; Jackman and Kandarian, 2004; Reid, 2008). Muscle cells are highly susceptible to reactive oxygen species (ROS) induced oxidative stress that is particularly observed in strenuous exercise (Powers et al., 2011). The ROS induced muscle cell damage is routinely observed in athletes and sports personnel including patients with diabetes, cancer, cardiovascular, and HIV diseases (Singh, 1997; Barroso et al., 2003; Mafulli et al., 2011). Supplementation of a diet with natural antioxidants is an effective strategy to protect ROS induced cell damage.
4.2. Materials and Methods

4.2.1. Chemicals and Reagents

DMEM/F-12 and t-BHP were procured from Himedia (Bangalore, India) while penicillin and streptomycin solution, MTT, 2',7'-DCFH$_{2}$DA, rhodamine 123, RIPA buffer, protease and phosphate inhibitor cocktail were obtained from Sigma (St Louis, MO, USA). All other chemicals used for the experiments are of analytical grade and were procured from Merck, Bangalore, India.

4.2.2. Animals and grouping

Animal studies were conducted according to the guidelines from Institute Animal Ethical Committee and Committee for the Purpose of the Control and Supervision of Experiments on Animals; NO: 28/IAEC/CPCSEA. Male albino mice of Balb/C strain weighing 25 ± 2.0 g was selected from the stock colony, Defence Food Research Laboratory, Mysore, India, housed in an acrylic fibre cage in a temperature controlled room (temperature 25 ± 2 °C) and maintained in 12 h light/dark cycle. Food and water were provided ad libitum.

Eighty eight male Balb/C mice were randomly divided into the following eleven experimental groups (Table. 4.1). The herbal extracts were administered by gavage from one week before first swimming exercise and continued till last day of swimming exercise. Sedentary and swimming mice were fed orally with equal amounts of saline.

**Table 4.1.** Animals and grouping for forced swim test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Rats No., sex and type</th>
<th>Treatment and doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>8 Male, Balb/C</td>
<td>Sedentary + Vehicle control</td>
</tr>
<tr>
<td>Group II</td>
<td>8 Male, Balb/C</td>
<td>Sedentary + CPSE 100 mg/kg B. wt</td>
</tr>
<tr>
<td>Group III</td>
<td>8 Male, Balb/C</td>
<td>Sedentary + CRE 100 mg/kg B. wt</td>
</tr>
<tr>
<td>Group IV</td>
<td>8 Male, Balb/C</td>
<td>Sedentary + EAE 100 mg/kg B. wt</td>
</tr>
<tr>
<td>Group V</td>
<td>8 Male, Balb/C</td>
<td>Exercise + Vehicle control</td>
</tr>
<tr>
<td>Group VI</td>
<td>8 Male, Balb/C</td>
<td>Exercise + CPSE 50 mg/kg B. wt</td>
</tr>
<tr>
<td>Group VII</td>
<td>8 Male, Balb/C</td>
<td>Exercise + CPSE 100 mg/kg B. wt</td>
</tr>
<tr>
<td>Group VIII</td>
<td>8 Male, Balb/C</td>
<td>Exercise + CRE 50 mg/kg B. wt</td>
</tr>
<tr>
<td>Group IX</td>
<td>8 Male, Balb/C</td>
<td>Exercise + CRE 100 mg/kg B. wt</td>
</tr>
<tr>
<td>Group X</td>
<td>8 Male, Balb/C</td>
<td>Exercise + EAE 50 mg/kg B. wt</td>
</tr>
<tr>
<td>Group XI</td>
<td>8 Male, Balb/C</td>
<td>Exercise + EAE 100 mg/kg B. wt</td>
</tr>
</tbody>
</table>
4.2.3. Evaluation of swimming endurance

The weight-loaded endurance exercise was performed as described previously with slight modifications (Jung et al., 2004). The male Balb/C mice of V to XI groups were allowed to swim till exhaustion with additional constant loads (tagged to their tail base) equivalent to 10% of their body weight. The swimming exercise was carried out in a small tank with 30 cm deep water maintained at 25 ± 2 °C. Exhaustion was determined by observing the loss of coordinated movements and failure to return to surface within 10 sec (Wang et al., 2006). This swimming exercise was performed once in five days for a period of seven weeks.

4.2.4. Estimation of blood biochemical indices

Animals were sacrificed under mild anaesthesia immediately after the last exercise. Cardiac blood was collected using a heparinized syringe into centrifuge tubes. The plasma obtained after centrifugation was used for measurement of blood urea nitrogen (BUN), glucose, lactic acid, creatine kinase (CK) and lactate dehydrogenase (LDH) according to the kit supplier protocols (Biosystems and Agappe). Liver and muscle tissues were removed and frozen immediately at -80 °C for estimation of lipid peroxidation, lactic acid, glycogen and antioxidant enzyme/protein levels.

4.2.5. Estimation of glycogen content

The glycogen content of liver and muscle samples were determined by DNS method (Miller, 1972) and the results were expressed as mg/g of tissue.

4.2.6. Transmission electron microscope analysis

The muscle tissues were fixed in 2.5% glutaraldehyde (prepared in 0.1 mol/L PBS; pH 7.4) for 24 h at 4 °C. The samples were processed as described earlier in the previous chapter. The ultrastructural changes of muscle tissues were observed under a TEM (FEITECANIG2 Spirit BioTWIN, Netherland).

4.2.7. C2C12 cells differentiation and treatments

Murine C2C12 myoblasts were procured from National Centre for Cell Sciences, Pune, India. The cells were equally seeded into plates, flasks or dishes and
maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 \% fetal bovine serum and antibiotic and antimycotic solution in a humid atmosphere of 5 \% CO\textsubscript{2} and 95 \% air at 37 \degree C till confluence. To initiate differentiation, cells were changed to DMEM containing 2 \% horse serum (Invitrogen, Auckland, NZ) and cultured for five days. Before 12 h of treatments, the cells were transferred to serum free media. To initiate cell damage freshly prepared t-BHP was added for 24 h to the cells with or without pretreatment with CPSE for 2 h before any experiments.

4.2.8. Cell viability assay

C2C12 cells were cultured in 96 well plates at a density of 1\times10^{4} cell/ml, grown for 24 h and then subjected to the treatments of interest. Mitochondrial health was assessed by MTT assay as described in the previous chapter. The cell viability is expressed as the percentage of control.

4.2.9. LDH leakage assay

The C2C12 cells were plated at a density of 5\times10^{4} cells/ml on 24-well plates and after 24 h of growth the cells were subjected to the treatments of interest. Further, the cytotoxicity was quantified by plasma membrane damage estimation by LDH assay as described in the previous chapter.

4.2.10. Observations of cellular morphology

The cells were seeded in Petri dishes (1\times10^{6} cells/ml) followed by treatment with t-BHP for 24 h with or without pretreatment of CPSE for 2 h. The cellular morphology was observed and photographed using a phase contrast microscope (Olympus, Japan) equipped with Cool SNAP\textsuperscript{®} Pro color digital camera.

4.2.11. Estimation of mitochondrial membrane potential (MMP)

The cells were grown in 24 well plates for fluorometric analysis for 24 h. After the treatments, the protective effect of CPSE on mitochondrial damage of C2C12 cells induced by t-BHP was assessed by MMP assay using spectrofluorometer and the cellular morphology was analyzed using fluorescence microscope (Olympus, Japan) equipped with Cool SNAP\textsuperscript{®} Pro color digital camera as described in the previous chapter.
4.2.13. Estimation of intracellular ROS

The cells were seeded in 24-well plates at a density of 4.0×10^5 cells/ml, allowed to grow for 24 h and treated as described earlier. Followed by experiment treatments, the intracellular ROS formation was detected using spectrofluorometer and the cells were imaged using fluorescence microscope (Olympus, Japan) equipped with Cool SNAP® Pro color digital camera as described in the previous chapter.

4.2.14. Estimation of superoxide dismutase and catalase

The C2C12 cells (1×10^7 cells/ml) were seeded in 75 cm^2 flasks and treated as described earlier. The C2C12 cells and mice muscle tissues were lysed by sonication and the SOD, CAT and protein contents were estimated as described in the previous chapter and the results are expressed as U/mg of protein.

4.2.15. Estimation of lipid peroxidation

The C2C12 cells were seeded in 75 cm^2 flasks (1.0×10^7 cells/ml) and incubated at 37 °C till confluence and the cells were treated as described earlier. The C2C12 cells and mice muscle tissues were lysed by sonication and the lipid peroxidation was determined by measuring the malondialdehyde formed as described in the previous chapter.

4.2.16. Single cell gel electrophoresis assay

The protective effect of CPSE against exercise induced DNA damage in WBC of mice and t-BHP induced DNA damage in C2C12 cells was assessed using comet assay as described in the previous chapter. The DNA was visualized using a fluorescence microscope (Olympus, Japan) equipped with Cool SNAP® Pro color digital camera and the DNA damage was measured by RS Image® software to determine the tail length (µm). The results were expressed as percentage inhibition of tail length.

4.2.17. Immunoblotting

Proteins extraction was carried out from muscle tissues in ice cold lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % SDS with protease and phosphatase inhibitor cocktail). The C2C12 cells (1×10^7 cells/ml)
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were cultured in 75 cm$^2$ flasks and treated with t-BHP with or without pretreatment of CPSE. After treatments, the protein extraction was carried out using RIPA buffer and protein contents were estimated as described in the previous chapter. The proteins were separated by 8-15 % SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked and incubated with primary antibodies namely α-tubulin (sc-5286), AMPK (SC 19128), Glut-4 (SC 7938), PDK4 (SC 130841), VEGF (SC 7269), PGC-1α (SC 13067), MCT-1 (SC19128), MCT-4 (SC 50329), SOD (sc-8637), CAT (sc-34280), HSP-70 (sc-66048), Cyt-C (sc-13156), (Santa Cruz Biotechnology, CA, USA) at 1:1,000 dilution and the immunoreactivity was detected using enhanced chemiluminescence peroxidase substrate kit (CPS-160, Sigma, St Louis, MO, USA) as described in chapter III.

4.2.18. Statistical analysis

The data are expressed as mean ± standard deviation. Data were analyzed using SPSS 17.0 software. Student’s $t$-test was performed and the differences were considered to be significant at $P<0.05$. For the cell culture data, statistical significance was analyzed with one-way analysis of variance followed by a Tukey’s HSD-post hoc test. Results with $P$ value less than 0.05 were considered statistically significant. The data is represented as the mean ± SD.

4.3. Results

4.3.1. Effect of CPSE, CRE and EAE on swimming time of mice

In the present investigation, the effect of CPSE, CRE and EAE on physical endurance activity was evaluated in mice subjected to swimming. An acute weight loaded (10 % of the body weight) swimming exercise for seven weeks was set with slight modification to the method described by Jung et al. (2004). A substantial increase in swimming time from the fifth day to the last day (35th day) was observed in CPSE administered group as compared to exercise control group ($P<0.05$). The CPSE 50 and 100 mg administered mice showed twofold increase in swimming time up to 24.2 ± 1.5 and 25.4 ± 1.65 min respectively compared to exercise group alone which showed 8.5 ± 0.58 min of swimming time on day 35. In contrast CRE 100 mg and EAE 100 mg fed mice showed one fold and half fold increase in swimming time up to 16.1 ± 1.25 and 13 ± 0.96 min respectively (Fig.4.1.).
Fig. 4.1. Effect of CPSE, CRE and EA administration on swimming endurance activity of mice. (n=8, # P<0.05 compared with the control group).

Mice supplemented with 50 mg of *C. paniculatus* showed maximum swimming activity compared to *C. rotundus* and *E. alba* extracts and therefore it was selected for further studies. There was no significant difference in body weight between sedentary and exercised groups with or without CPSE administration.

4.3.2. Effects of CPSE supplementation on serum biochemical indices

An increase in blood glucose level was observed with CPSE supplementation as compared to exercise group. Further, an increase in BUN, serum CK and LDH levels were recorded with swimming exercise which were significantly decreased with CPSE supplementation (P<0.05) (Table.4.2.).

**Table.4.2.** Effect of CPSE on serum biochemical parameters. Values are mean ± SEM (n = 8). * P<0.05, significantly different from the sedentary group, # P<0.05, significantly different from the exercise group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose (mg/dL)</th>
<th>BUN (mg/dL)</th>
<th>CK (U/L)</th>
<th>LDH (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>9.77 ± 0.82</td>
<td>14.36±1.3</td>
<td>130±11.5</td>
<td>325±29.2</td>
</tr>
<tr>
<td>CPSE 50</td>
<td>9.75 ± 0.8</td>
<td>14.18±1.2</td>
<td>125 ± 10.3</td>
<td>315 ± 27.4</td>
</tr>
<tr>
<td>Exe</td>
<td>7.15 ± 0.59</td>
<td>26.13±2.1</td>
<td>240±19.8</td>
<td>528±51.1</td>
</tr>
<tr>
<td>Exe + CPSE 50</td>
<td>8.81 ± 0.72</td>
<td>17.97±1.8</td>
<td>159±15.2</td>
<td>417±39.1</td>
</tr>
</tbody>
</table>
4.3.3. Effect of CPSE on liver and muscle lactic acid levels

Lactic acid is produced by anaerobic breakdown of carbohydrates as the end product of glycolysis, the energy source during exercise conditions. Its content in liver and muscle was significantly increased with swimming exercise as compared with the sedentary group. However, liver and muscle tissues of CPSE supplemented mice showed significantly reduced levels of lactic acid when compared with exercise group (P<0.05) as shown in Table. 4.3.

4.3.4. Effect of CPSE on liver and muscle glycogen

Glycogen content is one of the key parameters to assess the anti-fatigue activity. In the present investigation, a decrease in liver and muscle glycogen content was observed in the exercise group as compared with the sedentary group, whereas an increase in glycogen content was observed with CPSE supplementation (Table.4.3.) which demonstrates the regulation of metabolic activity by *C. paniculatus* administration.

Table.4.3. Effect of CPSE on tissue biochemical parameters. Values are mean ± SEM (n = 8). * P<0.05, significantly different from the sedentary group, # P<0.05, significantly different from the exercise group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glycogen (mg/g)</th>
<th>Lactic acid (mg/g)</th>
<th>Glycogen (mg/g)</th>
<th>Lactic acid (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Muscle</td>
<td>Liver</td>
<td>Muscle</td>
</tr>
<tr>
<td>Sedentary</td>
<td>9.21 ± 0.78</td>
<td>0.91 ± 0.07</td>
<td>3.22 ± 0.32</td>
<td>3.11 ± 0.21</td>
</tr>
<tr>
<td>CPSE 50</td>
<td>9.2 ± 0.75</td>
<td>0.90 ± 0.07</td>
<td>3.18 ± 0.33</td>
<td>3.08 ± 0.20</td>
</tr>
<tr>
<td>Exe</td>
<td>4.13 ± 0.38</td>
<td>1.93 ± 0.16</td>
<td>1.02 ± 0.11</td>
<td>5.92 ± 0.41</td>
</tr>
<tr>
<td>Exe + CPSE 50</td>
<td>7.72 ± 0.32</td>
<td>1.23 ± 0.11</td>
<td>1.89 ± 0.17</td>
<td>4.31 ± 0.32</td>
</tr>
</tbody>
</table>

4.3.5. Transmission electron microscope analysis of muscle tissue

The ultrastructural changes in the muscle with exercise alone or in combination with CPSE supplementation were assessed by TEM. In comparison with sedentary animals the muscle tissue of exercised animals showed dilation of sarcoplasmic reticulum which indicates muscle tissue damage, whereas CPSE supplementation showed the protective effect against exercise induced muscle tissue damage (Fig.4.2.).
4.3.6. Eukaryotic DNA damage inhibitory activity of CPSE

SCGE assay was performed to assess the protective effect of CPSE on exercise induced DNA damage in WBC isolated from mice blood cells. The fragmented DNA in the form of tail dispersion increased in exercised mice (Fig.4.3.). The tail length of sedentary animals was 8.0 ± 0.5 µm while in exercise group it was 55.74 ± 6.0 µm which was decreased to 18 ± 1.2 µm with CPSE supplementation. The results clearly indicate that exercise induced DNA damage was successfully overcome by the active compounds present in CPSE.

4.3.7. Protective effects of CPSE on antioxidant enzyme levels

An increase in SOD and CAT level was observed with exercise as compared with the sedentary mice whereas exercise with CPSE supplementation further increased the antioxidant status as observed by enzyme assays (Table.4.4.).
4.3.8. Effect of CPSE on lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid assay by estimating the content of malondialdehyde which is a by-product of peroxidation of polyunsaturated fatty acids (PUFA). The principle of the assay is that the removal of the hydrogen atom from the bis-allylic site of a PUFA generates lipid radical which forms peroxyl radical in reaction with oxygen. The peroxyl radical can propagate chain reaction with adjacent PUFA leading to lipid peroxidation (Deaton and Marlin, 2003). In the present investigation, an increase in lipid peroxidation was observed upon heavy exercise compared to that of sedentary animals which describe that exhaustive exercise generates lipid peroxidation, however CPSE administered mice showed a decrease in lipid peroxidation (Table.4.4).

Table.4.4. Effect of CPSE on antioxidant indicies in mice muscle tissue. Values are mean ± SEM (n = 8). * P<0.05, significantly different from the sedentary group, # P<0.05, significantly different from the exercise group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>SOD (U/mg protein)</th>
<th>CAT (U/mg protein)</th>
<th>MDA (M/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary</td>
<td>1.77 ± 0.12</td>
<td>19.36 ± 1.1</td>
<td>2.57 ± 0.2</td>
</tr>
<tr>
<td>CPSE 50</td>
<td>1.88 ± 0.12</td>
<td>21.75 ± 1.3</td>
<td>2.44 ± 0.2</td>
</tr>
<tr>
<td>Exercise</td>
<td>3.15 ± 0.29*</td>
<td>28.13 ± 2.1*</td>
<td>5.12 ± 0.5*</td>
</tr>
<tr>
<td>Exercise + CPSE 50</td>
<td>4.81 ± 0.32#</td>
<td>35.57 ± 2.8*</td>
<td>3.11 ± 0.3*</td>
</tr>
</tbody>
</table>

4.3.9. Effects of CPSE on exercise metabolic marker proteins

4.3.9.1. Effects of CPSE on metabolic regulators

AMPK is the mammalian homologue of the yeast SNF1 gene and is a heterotrimer composed of α, β and γ subunits of which α subunit contains a kinase domain and contributes to binding of AMP. AMPK is activated by a low ATP/AMP ratio which serves as a fuel gauge to protect against energy deprivation. In the present study, an increase in AMPK phosphorylation was observed with exercise which was further increased by CPSE supplementation (Fig.4.4.). The observed results demonstrate that C. paniculatus extract enhances phosphorylation of AMPK to meet the energy demands during exercise condition.
Glut-4 is a key transporter which plays an important role in supply of glucose to tissues in need during conditions of energy exhaustion. In the present study, an increase in Glut-4 expression was observed with exercise as well as exercise with CPSE supplementation which demonstrates that regular exercise and exercise with CPSE supplementation is beneficial which improves glucose uptake by enhanced Glut-4 expression. Similarly, the expression of PDK4 was increased with exercise whereas exercise along with CPSE supplementation further increased its level (Fig.4.4.).

4.3.9.2. Effects of CPSE on angiogenesis and mitochondrial biogenesis

Angiogenesis refers to remodelling of capillaries by sprouting or intussusceptions which enhance the blood flow and such adaptation is observed in athletes to satisfy the metabolic needs of the tissue as an adaptive response. In the current study, an increase in VEGF expression was observed with exercise which was further increased with *C. paniculatus* supplementation. Further, PGC-1α expression was increased with exercise indicating the muscle mitochondrial biogenesis which is even more increased with CPSE supplementation demonstrating the role of *C. paniculatus* seed extract to enhance muscle oxidative metabolism as shown in Fig.4.4.
4.3.9.3. Effects of CPSE on Lactate transporters MCT-1 and MCT-4

In view of cell-cell lactate shuttle hypothesis by Brooks (2009) and other researchers, the lactate clearance and metabolism occurs by lactic acid transporters, MCT-1 and MCT-4 from cytosol to mitochondria. In the current study, an increase in MCT-1 content but not MCT-4 in exercised mice alone was observed whereas further increase in MCT-1 but not MCT-4 was observed in the exercise with CPSE supplemented group (Fig. 4.4.). The results clearly demonstrate that CPSE enhances lactic acid transport through MCT-1.

4.3.9.4. Protective effects of CPSE on oxidative stress biomarkers

The antioxidant status was further supported by immunoblot analysis (Fig. 4.4.). The SOD and CAT protein expression was up-regulated in muscle tissues of exercised mice which was further up-regulated with CPSE supplementation.

Heat shock proteins (HSPs) are a class of molecular chaperons constitutively expressed in a variety of tissues including muscle tissue. In the present study, an
increase in HSP-70 level was recorded with exercise which elucidates the stress generation in muscle tissues. However, a decrease in its expression was observed with CPSE administration demonstrating the anti-stress effect of *C. paniculatus* seed extract as observed in Fig.4.4.

4.3.10. Protective effects of CPSE against t-BHP induced stress in C2C12 murine myoblasts

4.3.10.1. Protective effects of CPSE against t-BHP induced cytotoxicity

The C2C12 muscle cells were treated with different doses of CPSE (5, 10, 25 and 50 µg/ml) for 24 h and the percentage cell viability was determined by MTT assay and found to be same as that of untreated control cells. The free radical-induced muscle cytotoxicity was evaluated by treatment with different concentrations of t-BHP (0-400 µM) for 24 h and a dose-dependent cell death was observed (Fig.4.5.A). About 43% of the cells survived after 250 µM t-BHP challenge and the same concentration was used in subsequent experiments. The protective effect of CPSE against t-BHP induced muscle cell damage was evaluated by pre-treatment with 5-50 µg/ml of CPSE for a period of 2 h followed by treatment with 250 µM t-BHP for 24 h. As shown in Fig.4.5.B., CPSE pre-treatments significantly ameliorated t-BHP-induced cell death.

![Fig.4.5.A.](image)

![Fig.4.5.B.](image)

**Fig.4.5.A.** Cytotoxic effects of tertiary butyl hydroperoxide (t-BHP) on C2C12 muscle cell. * P<0.05 versus control cells without any treatment. **Fig.4.5.B.** Dose dependent protective effect of pre-treatment of *Celastrus paniculatus* seed extract (CPSE) on t-BHP induced cytotoxicity in C2C12 cells, the cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. # P<0.05 versus control group * P<0.05 versus control cells and 250 µM t-BHP treated group.
Further, the cytotoxicity of t-BHP and protective effect of CPSE against t-BHP induced cell damage was assessed by LDH assay that is based on the leakage of cytosolic LDH to the media as the number of dead cells increases. LDH leakage was dose dependently increased with t-BHP challenge of C2C12 muscle cells; up to ~45% LDH leakage was observed with 250 µM t-BHP treatment as shown in Fig.4.5.C. The C2C12 cells pre-treated with 25 and 50 µg/ml of CPSE for 2 h followed by 250 µM t-BHP treatments for 24 h and analyzed by LDH leakage assay. CPSE pre-treated cells exhibited a decrease in LDH release as compared with t-BHP treated cells (Fig.4.5.D.).

Fig.4.5.C. Cytotoxic effects of t-BHP and protective effect of CPSE in C2C12 muscle cells analyzed by lactate dehydrogenase (LDH) leakage assay. * P<0.05 versus control cells. Fig.4.5.D. Protective effect of CPSE pretreatment on t-BHP induced cytotoxicity by LDH leakage assay. # P<0.05 versus control group * P<0.05 versus control cells.

The protective effect of CPSE against t-BHP treatment was confirmed by morphological analysis of C2C12 muscle cells. The muscle cells treated with t-BHP showed cellular damage of murine myotubes observed as detached floating cells. However, the cellular morphology was restored with CPSE pretreatment as evidenced by bright field images (Fig.4.6.).

Fig.4.6. Effects of CPSE pretreatment on t-BHP induced morphological alterations in C2C12 muscle cells by phase-contrast microscopy.
4.3.10.2. CPSE inhibits t-BHP induced loss of MMP

Mitochondrial membrane potential (MMP) is used as an index to measure the mitochondrial health and a collapse of MMP indicates the mitochondrial damage (Feng et al., 2011). To estimate whether t-BHP challenged apoptosis and its protection by CPSE involve MMP pathway, measurement of MMP was carried out using rhodamine 123 in C2C12 muscle cells. Rhodamine 123, a cationic and lipophilic fluorescent dye, partitions into active mitochondria based on highly negative MMP where its diffusion and accumulation is proportional to the degree of MMP (Ubl et al., 1996). Mitochondrial membrane depolarization causes loss of dye from mitochondria and subsequent decrease in intracellular fluorescence. The results of the present investigation demonstrate that cells treated with 250 µM t-BHP for 24 h exhibited loss of membrane polarization as MMP decreased to 45 % in comparison to that of control cells. However, the cells pre-treated with CPSE prior to t-BHP treatment showed a regain in the fluorescence intensity to 82 % (Fig.4.7.A.); this was further confirmed by fluorescence microscope analysis (Fig.4.7.B.).

**Fig.4.7.A.**
Fig. 4.7.A. Estimation of mitochondrial membrane potential in C2C12 muscle cells with pre-treatment of CPSE on t-BHP challenge. The fluorescence intensity was determined using spectrofluorimeter. Fig. 4.7.B. The membrane potential was monitored by fluorescent microscope (Olympus, Japan) Control cells without any treatment (a), 50 µg/ml CPSE treatment for 2 h (b), 250 µM t-BHP treatment for 24 h (c), and cells pre-treated with 50 µg/ml CPSE for 2 h then treated with 250 µM t-BHP for 24 h (d). The data are represented as means ± SD of three independent experiments. # P<0.05 versus control group, * P<0.01 versus control cells and * P<0.05 versus 250 µM t-BHP treated group. Scale bar = 50 µm.

4.3.10.3. CPSE inhibits t-BHP induced ROS generation

The oxidative damage of muscle cells induced by t-BHP has been found to be associated with increased ROS generation. 2′,7′-DCFH-2DA is nonionic, nonpolar dye that crosses cell membranes and enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH that is oxidized to highly fluorescent dichlorofluorescein (DCF) in the presence of ROS (LeBel et al., 1992). Hence, total ROS was quantified by estimation of the intracellular DCF fluorescence, where the emitted fluorescence is directly proportional to the concentration of ROS. The fluorescence intensity of muscle cells was ~ 160 % with 250 µM t-BHP challenge as compared with the control group. In cells pre-treated with CPSE followed by t-BHP treatment, the fluorescence intensity was decreased up to ~ 120 % that was further confirmed by fluorescence imaging (Fig. 4.8.A. and 4.8.B.).
4.3.10.4. Effect of CPSE on lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid assay by estimating the content of malondialdehyde that is a by-product of peroxidation of polyunsaturated fatty acids (PUFA). In the present investigation, an increase in lipid peroxidation was observed in muscle cells with t-BHP challenge that was significantly decreased by CPSE pre-treatment (Fig.4.9.).
Fig. 4.9. Estimation of lipid peroxidation products by thiobarbituric acid assay in C2C12 muscle cells with pre-treatment of CPSE on t-BHP challenge. The data are represented as means ± SD of three independent experiments. # P<0.05 versus control group, * P<0.05 versus control cells and 250 µM t-BHP treated group.

4.3.10.5. Protective effects of CPSE on oxidative stress biomarkers

A decrease in SOD and CAT antioxidant enzyme levels were recorded with t-BHP treated muscle cells. However, CPSE pre-treatment significantly restored the antioxidant level of muscle cells (Fig. 10A and Fig. 10B). Similarly, the SOD and CAT antioxidant marker proteins expression were down-regulated in t-BHP challenged muscle cells, which was significantly restored with CPSE pre-treated muscle cells which demonstrate the antioxidant defence of C. paniculatus seed extract (Fig.4.10.A and 4.10.B.).

Fig.4.10.A and B Pre-treatment of CPSE on restoration of SOD and catalase enzyme activities in C2C12 muscle cells challenged with t-BHP. The data are represented as means ± SD of three independent experiments. # P<0.05 versus control group, * P<0.01 versus control cells and * P<0.05 versus 250 µM t-BHP treated group.
4.3.10.6. Protective effect of CPSE on apoptotic biomarkers

Apoptosis is the key event of cell death and can be evaluated by expression analysis of apoptotic marker proteins. Cytochrome-C release from mitochondria to cytosol was observed with t-BHP treatment that is more likely to be a result of changed oxidative condition of the cells. However, CPSE pre-treatment significantly reduced cytochrome-C release (Fig.4.11.).

An increase in HSP-70 level was observed with t-BHP treatment that elucidates the stress generation in murine muscle cells. However, a decrease in its expression was observed with CPSE pre-treatment demonstrating the anti-stress effect of *C. paniculatus* extract (Fig.4.11.).

**Fig.4.11.**

<table>
<thead>
<tr>
<th>a</th>
<th>CPSE(50µg/ml)</th>
<th>t-BHP (250µM)</th>
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<tr>
<td>SOD</td>
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<td>— + +</td>
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<tr>
<td>CAT</td>
<td></td>
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<tr>
<td>Cyt-C</td>
<td></td>
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<tr>
<td>HSP-70</td>
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<td>α- Tubulin</td>
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**Fig.4.11.a.** The protective effect of pre-treatment of CPSE on t-BHP induced expression of oxidative stress marker proteins SOD, CAT and apoptotic marker proteins Cytochrome-C and HSP-70 analyzed by immunobloting. **Fig.4.11.b-e.** The band intensity is calculated by Image-J software. The data are represented as means ± SD of three independent experiments. # P<0.05 versus control group, * P<0.01 versus control cells and * P<0.05 versus 250 µM t-BHP treated group.
4.3.10.7. Eukaryotic DNA damage inhibitory activity of CPSE

SCGE assay was performed to assess the DNA damage protective effect of CPSE against t-BHP induced stress in C2C12 muscle cells. The tail length of the comet was measured as an index of DNA damage in the cell. The fragmented DNA in the form of tail dispersion increased with 250 µM t-BHP challenge of C2C12 muscle cells. The tail length of C2C12 control cells was 9.5 ± 1.5 µm while, in the case of 250 µM t-BHP, it was increased to 78.14 ± 6.5 µm which demonstrates that t-BHP induces DNA damage which was further decreased to 29.74 ± 3.0 µm with 50 µg/ml CPSE pre-treatment (Fig. 4.12.A. and Fig. 4.12.B.).

Fig. 4.12.A.

Fig. 4.12.B.

Fig. 4.12.A. Protective effect of CPSE on DNA damage induced by t-BHP in C2C12 cells. Control cells without any treatment (a), Cells with 250 µM t-BHP treatment for 24 h (b), C2C12 cells were pre-treated with CPSE for 2 h at 50 µg assay and induced with 250 µM t-BHP (c). Fig. 4.12.B. Tail length (µm): Bars. Inhibitory effect of CPSE on DNA damage: −→∪→. # P<0.05 versus control group. * P<0.05 versus 250 µM t-BHP treated group.
4.4. Discussion

Moderate exercise elicits beneficial effects by enhancing the metabolic activities, whereas strenuous exercise leads to impairment in body functions due to generation of excessive free radicals which induce damage of muscle tissues in particular and whole body in general. Improving exercise performance has been a wide research to decipher the anti-fatigue phenomena and to identify the alternate therapies to enhance exercise performance which may improve athletic performance as well as regulate the exercise induced stress effects.

In the present study the extracts of three plants *C. paniculatus*, *C. rotunuds* and *E. alba* were studied to see their effects on the endurance, it was observed that CPSE supplementation of mice for seven weeks enhances swimming endurance about two fold compared to CRE and EAE supplementation which also enhanced the swimming activity to one fold and half fold respectively. The increase in swimming activity of mice with CPSE administration is concomitant with restoration of glucose and glycogen levels as well as inhibition of LDH, lactic acid, urea and creatine kinase (CK) formation and inhibition of exercise induced oxidative stress. Further, the study explores the molecular mechanism of action of the anti-fatigue activity of CPSE by its effects on energy sensing, angiogenesis, mitochondrial biogenesis, glucose and lactate transport.

Glucose plays a pivotal role in enhancing the exercise activity. A decrease in brain glucose level, a condition called hypoglycemia, plays an inhibitory role in preventing the exercise activity. Protein and amino acid catabolism generate BUN as metabolic end product. Hence BUN estimation is a key parameter to assess the exercise induced renal function. Whereas 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 body is normally present in muscle and an increase in the amount of CK in the blood indicates muscle damage. CK levels parallel the increase in myoglobin and are used clinically as surrogate marker of muscle injury (Clarkson et al., 2006). Hence blood CK estimation is used as a marker to measure the muscle damage. Similarly, LDH which catalyses the interconversion of lactate to pyruvate is present in muscle and its appearance in the blood indicates muscle damage. The restoration of glucose
levels and decrease in BUN, CK and LDH levels were recorded with CPSE supplementation. Thus, it may be inferred that CPSE plays a protective role against muscle damage. The observed results are in agreement with a recent study by You et al. (2011) who demonstrated that papain digestion of loach peptides inhibits the anti-fatigue activity by modulation of biochemical and antioxidant enzyme levels.

The excess calorie is stored in liver and muscle as glycogen, of which liver glycogen is used as a body fuel in the exercise condition. 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 level; particularly carbohydrate supplementation has been shown to enhance glycogen content after exhaustive exercise. In a recent report Kanda et al. (2012) reported that whey protein hydrolysates enhance glycogen levels via glycogen synthase activity in exercised mice. On the other hand marked increase in lactic acid level was observed as a marker of fatigue to meet ATP demand during exercise conditions (Robergs et al., 2004). The results showed a decrease in lactic acid level which are in correlation with the recent report that demonstrate that decapeptide CMS001 supplementation decreases the lactic acid content in swimming endurance mice (Wang et al., 2008).

Low energy / high AMP level leads to phosphorylation of AMPK, which help to meet the energy demand by inhibiting ATP-consuming pathways and activating ATP-producing pathways (Wang and Zang, 2010). The observed findings are in corroboration with an earlier report that demonstrates the AMPK phosphorylation by Nootkatone, a characteristic constituent of grapefruit, stimulates energy metabolism (Murase et al., 2010). Glut-4 is located in membrane vesicles, which is activated by GLUT-4 enhancing factor (GEF) and myocyte enhancer factor-2 (MEF-2), where it translocates to cell membrane and facilitates glucose transport during exercise (Oshel et al., 2000). In earlier reports Goodyear (1998) and Henriksen (2002), had described the pivotal role of Glut-4 on insulin resistance during diabetes and exercise conditions. Fatty acid synthesis takes place through conversion of carbohydrates by pyruvate to Acetyl-CoA by regulation of PDK4 complex in resting conditions. In contrast the upregulation of PDK4 activity during exercise conditions leads to inhibition of fatty acid synthesis and utilization of carbohydrates as the energy (Pilegaard and Neufer, 2004).
A variety of regulatory genes plays a role in angiogenesis, among which VEGF, a glycoprotein that binds to the cells of blood and lymphatic vessels and stimulates endothelial cell proliferation, migration, and tubule formation (Prior et al., 2004; Ahmetov et al., 2008). Previous reports by several authors elucidated the increase in VEGF level after exercise both in human and rat subjects to facilitate the metabolic adaptation (Breen et al., 1996; Richardson et al., 1999). The observed effects elucidate the angiogenic activity of the extract and it can be concluded that regular exercise along with CPSE administration will enhance the cardiac metabolism. The peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) is a 798 amino acid protein and is a transcription coactivator which interacts with nuclear receptor PPARγ. PGC-1α activity by acetylation of lysine during deprived energy conditions helps to maintain energy homeostasis by mitochondrial fatty acid oxidation (Gerhart-Hines et al., 2007). The observed results are in accordance with earlier studies by Lagouge et al. (2006), who demonstrated that the resveratrol improves mitochondrial function by activating PGC-1α complex.

Lactic acid is produced as a metabolic byproduct by glycolysis pathway by conversion of NAD+ by pyruvate, thus plays a role in energy homeostasis by breakdown of glucose. In strenuous exercise, exaggerated glycolysis and production of lactic acid by anaerobic conditions generates energy by lactic acid, but simultaneous increase in lactate content leads to acidosis of muscle. In an earlier study Dubouchaud et al. (2000) have reported a significant increase in MCT-1 content with training but not MCT-4 in muscle homogenates. In a recent study Kumar et al. (2011) demonstrated that Cordyceps sinensis enhances lactic acid transport through MCT-1 in exercised mice.

Improving exercise performance has been a wide area of research to decipher the anti-fatigue phenomena and to identify the alternative therapies/supplements to enhance exercise performance that may improve athletic performance as well as regulate the exercise induced stress effects. There are no reports on the detection of exercise induced H$_2$O$_2$ generation because of its immediate decomposition which is further an inducer of oxidative stress during fatigue/intense exercise. For the present study, to mimic the in vivo exercise generated oxidative stress t-BHP was used due to its stability compared with H$_2$O$_2$ in C2C12 mice muscle cells as demonstrated in a
recent study by Feng et al. (2011). The study provides significant insight into protective effects of CPSE on modulation of t-BHP induced oxidative and apoptotic stress of C2C12 mice muscle cells.

The protective effect of CPSE against t-BHP induced cytotoxicity in C2C12 cells as determined by MTT assay corroborates to the LDH assay. The observations of cytoprotective effects are in accordance with aforementioned neuronal cell protective effects by *C. paniculatus* against H$_2$O$_2$ induced cell damage (Godkar et al., 2003). Recently, Choi et al. (2014) also demonstrated that Celastrol an active metabolite isolated from the root bark of *Tripterygium wilfordii* Hook F. of Celastraceae family protects SH-SY5Y cells against rotenone induced neurotoxicity.

SOD and catalase are the key antioxidant enzymes which play a role in antioxidant defence mechanism in order to protect the cell from radical mediated damage. Several studies decipher the increase in antioxidant status with exercise (Lee et al., 2009; Feng et al., 2011). In contrast the decrease in antioxidant enzymes level has been reported in chemical/oxidative stress challenge, where the antioxidant system stabilizes the generated free radicals (Tiwari and Kakkar, 2009). In the present study, an increase in antioxidant level was observed in exercise as well as exercise with CPSE supplementation. In contrast, a decrease in antioxidant level was recorded in C2C12 muscle cells with t-BHP challenge that was restored with CPSE supplementation. Effects observed in the present study are in accordance with previous studies which demonstrate that *C. paniculatus* and *C. sinensis* promote antioxidant status in neuronal cultures and exercised mice (Godkar et al., 2006; Kumar et al., 2011).

Lipid peroxidation has been widely used as a marker to estimate the exercise/stress induced oxidative damage. In the present investigation, CPSE effectively inhibited exercise/ t-BHP induced MDA generation in mice muscle and C2C12 cells. The observed results are in accordance with the recent report by Wang et al. (2010) and Patel and Prasanna et al. (2013) as well who reported that *Panax ginseng* and *C. paniculatus* seed oil administration exerts protective effects against exercise and chronic fatigue of rats by inhibition of lipid peroxidation. Further, the transmission electron microscope analysis demonstrates that CPSE supplementation protects exercise induced muscle cell damage.
The mitochondrial mediated apoptosis leads to cell death by DNA damage that initiates with bax activation and cytochrome-C release with further increase in activation of caspases that play a role in the cleavage of the various enzymes. These changes lead to decrease in ATP with ATPase and glucose transporters inactivation and concurrent calcium influx and mitochondrial damage with decrease in bcl-2 expression. The data demonstrates that *C. paniculatus* extract bolsters muscle mitochondria against t-BHP mediated apoptosis by regulating cytochrome-C release from mitochondria. These results are in line with a recent study by Choi et al. (2014) who demonstrated the protective effect of celastrol isolated from the *Tripterygium wilfordii* Hook F. of *Celastraceae* family against rotenone induced expression of apoptosis biomarkers.

HSP-70 is overexpressed in stress conditions in order to prevent the appearance of misfolded, aggregated proteins and induces proteolytic degradation of denaturated proteins (Gupta et al., 2010). Increased expression of HSP-70 has been reported in swimming endurance and radical induced oxidative stress condition (Tarricone et al., 2008; Jung et al., 2011). In the present investigation, a decrease in HSP-70 expression was observed in exercise animals with CPSE supplementation. Similarly, the protective effect of *C. paniculatus* against t-BHP induced HSP-70 down-regulation further confirms the anti-stress effects.

Mitochondrial metabolism is an important cellular activity for all cells in general and muscle cells in particular where the mitochondrial damage induced stress generation has been implicated in strenuous exercise conditions. The present study provides evidence that CPSE regulates t-BHP induced mitochondrial membrane potential and ROS generation. These findings are in line with Wang et al. (2013) who have also demonstrated the mitochondrial damage protective and ROS scavenging activity of (M)-bicelaphanol, a trinorditerpene isolated from *Celastrus orbiculatus* against H$_2$O$_2$ induced oxidative stress of SHSY5Y human neuronal cells. Exercise induced ROS generation has been implicated to cause DNA damage of neutrophils which can be regulated by antioxidant supplementation (Wierzba et al., 2006). The findings on DNA damage of WBC are in agreement with Hartmann et al. (1995) who reported the inhibitory effect of vitamin E against exercise mediated oxidative DNA
damage. The results clearly indicate that t-BHP induced murine C2C12 cells damage was successfully overcome by the active compounds present in CPSE.

The regulatory effect of CPSE against exercise and t-BHP induced muscle cell damage demonstrates that CPSE could be used as an effective supplement during fatigue/exercise induced stress and to inhibit free radical-induced oxidative damage of cells (Fig. 4.13 and Fig. 4.14).

**Fig. 4.13.** Exercise enhancing effects of *C. paniculatus*. 
Fig. 4.14. Protective effects of *C. paniculatus* on t-BHP induced stress in C2C12 muscle cells.