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ORIGINAL RESEARCH

Celastrus paniculatus Willd. mitigates t-BHP induced oxidative and apoptotic damage in C2C12 murine muscle cells

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Abstract Identification, exploration and scientific validation of antioxidant rich herbal extracts to mitigate the radical induced cell damage provide new insights in the field of ayurvedic research/therapies. In the present study, we evaluated the anti-oxidant and anti-apoptotic potential of *Celastrus paniculatus* seed extract (CPSE) against tertiary butyl hydroperoxide (t-BHP) induced mice muscle cell damage. The extract at a dose of 50 µg/ml protected the cells up to 70 % as evidenced by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell survival assay and also prevented LDH leakage against t-BHP induced cytotoxicity. CPSE showed potential antioxidant activity by restoring mitochondrial membrane potential and inhibited reactive oxygen species generation and lipid peroxidation. CPSE pretreatment also regulated the antioxidant markers such as superoxide dismutase and catalase enzymes content and proteins expression. Further CPSE showed anti-apoptotic effects by regulating cytochrome-C and heat shock protein-70 expression and also showed 43 % muscle cell DNA damage inhibitory activity against t-BHP challenge as observed by single cell gel electrophoresis assay. Overall the extract inhibits the muscle cell damage, thus explaining the possible anti-oxidant/anti-apoptotic defense status of the *C. paniculatus* seed extract.

Keywords Apoptosis · *Celastrus paniculatus* · DNA damage · Oxidative stress · ROS · t-BHP

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Abbreviations

CAT	Catalase
CPSE	*Celastrus paniculatus* seed extract
Cyt-C	Cytochrome-C
HSP-70	Heat shock protein-70
LDH	Lactate dehydrogenase
MMP	Mitochondrial membrane potential
ROS	Reactive oxygen species
SCGE	Single cell gel electrophoresis
SOD	Superoxide dismutase
t-BHP	Tertiary butyl hydroperoxide

Introduction

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; Maffulli et al. 2011). Supplementation of a diet with natural antioxidants is an effective strategy to protect ROS induced cell damage.

Celastrus paniculatus Willd. belongs to *Celastraceae* family, grows across India and is locally called Kariganne. Its active metabolites include triglycerides such as palmitooleopalmitin, palmitooleostearin, palmitodiolein, palmitooleolinolein, stearodiolein, triolein and dioleolinolein; sesquiterpene alkaloids such as celapanin, celapanigin, celapagin; quinone-methide and phenolic triterpenoids such as celastrol, pristimerin, zeylasterone and zeylasteral (Gamlath et al. 1990; Borbone et al. 2007; Ramadan et al. 2009; Hermanth Kumar et al. 2014b). *C. paniculatus* has been reported for its various pharmacological activities such as neurotropic, anti-inflammatory, hypolipidemic and antiarthritic activities (Parcha et al. 2003; Rajkumar et al. 2007; Harish et al. 2008). The relaxant effect of *C. paniculatus* extract in the rat and human ileum was reported by Borrelli et al. (2009). The hypolipidemic activity of *C. paniculatus* was observed by Patil et al. (2010). Of late Weng et al. (2013) has also reported the anti-cancerous property of *C. paniculatus* using MCF-7 breast cancer cells. Recently, we demonstrated the exercise enhancing effects of *C. paniculatus* seed extract in swimming endurance model of rats (Hermanth Kumar et al. 2014b).

Several studies have demonstrated the use of radicals/chemical toxicants such as hydrogen peroxide (H_2O_2), SIN-1 (3-morpholinosydnonimine), 2,2′-azo-bis-(2-amidinopropane)-dihydrochloride (AAPH) and tertiary butyl hydroperoxide (t-BHP) to study the cell damage and to assess the protective effects of herbal/chemical compounds against cell damage (Feng et al. 2011; Zhang et al. 2011; Kim et al. 2012; Hemanth Kumar et al. 2013). In previous studies Godkar et al. (2003, 2006) have reported the antioxidant effects of *C. paniculatus* against chemical mediated neuronal cell damage, but the mechanism of action is not clear. Hence the aim of the present investigation is to study the radical induced oxidative damage of muscle cells in vitro and to illustrate the protective mechanism of action of *C. paniculatus* seed extract (CPSE). In our recent study, we observed the protective effect of *C. paniculatus* against exercise mediated stress in mice (Hemanth Kumar et al. 2014b). Hence in the present study we used t-BHP to induce stress, which mimics fatigue/exercise induced oxidative/apoptotic damage of muscle cells and demonstrated that CPSE protects muscle cell damage by its antioxidant/antiapoptotic mechanisms of action.

Materials and methods

Chemicals and reagents

DMEM/F-12 and t-BHP were procured from Himedia (Bangalore, India) fetal bovine serum while penicillin and streptomycin solution, MTT, 2′,7′-DCFH$_{2DA}$, rhodamine 123, RIPA buffer, protease and phosphate inhibitor cocktail were obtained from Sigma (St Louis, MO, USA). All other chemicals used for the experiments were of analytical grade and were procured from Merck (Bangalore, India).

Plant material

Dry seeds of *C. paniculatus* Willd. were purchased from the local market and identified by Dr. K. Madhava Chetty, Botanist, Department of Botany, Sri Venkateswara University, Tirupati, India. Voucher specimen (Herbarium Accession Number 1062) was deposited in the herbarium, Department of Botany, S.V. University (Tirupati, India).

Preparation of CPSE

The hydroalcoholic extract was prepared by maceration keeping the powdered seeds and solvent ratio of 1:10 (w/v). The extract was filtered through Whatman No.1 filter paper and the filtrate was concentrated using a rotary vacuum evaporator. The concentrate was finally freeze dried and used for the experiments.

C2C12 cell 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 (Thermoscientific, Bangalore, India) and penicillin and streptomycin solution (at 10 ml/l, Sigma, Bangalore, India) in a humid atmosphere of 5 % CO_2 and 95 % air at 37 °C till confluence. To initiate differentiation, cells were changed to DMEM containing 2 % horse serum (Invitrogen, Auckland, NZ) and cultured for 5 days. Before 12 h of treatments, the cells were transferred to serum-free medium (DMEM). 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.

Cell viability assay

Mitochondrial health was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which is based on the cleavage of tetrazolium salts by mitochondrial succinate reductase in viable cells to form formazan dye (Mosmann 1983). C2C12 cells were cultured in 96-well plates at a density of 1×10^4 cell/ml, grown for 24 h and then subjected to the treatments of interest. Followed by treatments, MTT (0.5 mg/ml) was added to each well and incubated for 2 h at 37 °C. Later, the formazan crystals formed were dissolved in DMSO. The absorbance was measured at 540 nm using a VERSA max Hidex plate chameleon^{TM} V (Turku, Finland) and the cell viability were expressed as the percent of control.

LDH leakage assay

Further, the cytotoxicity was quantified by plasma membrane damage estimation by means of LDH-estimation kit (Agappe-11407002, Mysore, India) according to the manufacturer's instructions. The principle of the assay is such that the leakage of cytosolic LDH increases following plasma membrane damage. Here, the enzyme activity was measured through the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD^+) at a wavelength of 340 nm. The rate of increase in enzyme activity due to the formation of reduced nicotinamide adenine dinucleotide (NADH) is directly proportional to the LDH activity in the sample. To do the assay, the C2C12 cells were plated at a density of 5×10^4 cells/ml in 24-well plates and after 24 h of growth the cells were subjected to the treatments of interest. After the treatments, 10 µL of cell lysis solution (2 % Triton ×-100) was added to the untreated cells, which were selected as the total LDH activity. The cells were separated by centrifugation at $2{,}500 \times g$ for 5 min at 4 °C and the supernatant was used for LDH activity.

Observations of cellular morphology

The cells were seeded in petri dishes (1×10^6 cells/ml) followed by treatment with 250 µM t-BHP for 24 h with or without pretreatment of 50 µg/ml CPSE for 2 h. The cellular morphology was observed and photographed using a phase contrast microscope (Olympus, Tokyo, Japan) equipped with Cool SNAP® Pro color digital camera.

Estimation of mitochondrial membrane potential (MMP)

Further, the protective effect of CPSE on mitochondrial damage of C2C12 cells induced by t-BHP was assessed by mitochondrial membrane potential (MMP) assay using the fluorescent dye rhodamine 123. The cells were grown in 24 well plates for fluorimetric analysis for 24 h. After the treatments, rhodamine 123 (10 µg/ml) was added to the cells and incubated for 1 h at 37 °C. Followed by washing twice with PBS, the cells were collected and the fluorescence was detected using Hidex plate chameleon^{TM} V at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For imaging, the cells were cultured on coverslips that were coated with poly-L-lysine. (A poly-lysine solution was prepared in sterile tissue culture grade water at a concentration of 100 mg/100 ml. The coverslips were coated with poly-L-lysine and incubated for 2 h at 37 °C. The poly-L-lysine solution was aspirated and the petri plates with coverslips were washed twice with sterile water and were used for further experiments). After experimental treatments, the cells were incubated with rhodamine 123 as mentioned above and excess dye was washed out with PBS. The cells were imaged using fluorescence microscope (Olympus) equipped with Cool SNAP® Pro color digital camera.

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. Then

the cells were treated with 50 μg/ml CPSE for 2 h followed by treatment with 250 μM t-BHP for 24 h and the ROS was estimated as described. This was followed by experimental treatments, the oxidation-sensitive dye 2′,7′-DCFH$_{2DA}$ was added to the cells and incubated for 30 min. Later, the cells were collected after washing twice with PBS and the intracellular ROS formation was detected using Hidex plate chameleon^{TM} V at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For imaging, the cells were cultured on coverslips that were coated with poly-L-lysine. After experimental treatments, the cells were incubated with 2′,7′-DCFH$_{2DA}$ as mentioned above and excess dye was washed out with PBS. The cells were imaged using fluorescence microscope (Olympus) equipped with Cool SNAP® Pro color digital camera.

Estimation of TBA in C2C12 cells

Lipid peroxidation was determined by measuring the malondialdehyde formed following the method of Ohkawa et al. (1979) with slight modifications. 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 cells were lysed by sonication in 1.15 % KCl with 1 % Triton × -100 at 4 °C. Aliquots (100 μl) of the cell lysates were mixed with 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid (pH 3.5), 1.5 ml of 0.8 % thiobarbituric acid and the volume was brought up to 4.0 ml using distilled water. The mixtures were boiled for 2 h to develop the color, followed by cooling. The contents were centrifuged at 1,500×g for 10 min and absorbance of the supernatants was measured at 532 nm.

Estimation of SOD and catalase from C2C12 cells

The C2C12 cells (1×10^7 cells/ml) were seeded in 75 cm^2 flasks and treated as described above. The cells were collected and sonicated in ice-cold lysis buffer (50 mM potassium phosphate buffer, pH 7.4 with 2 mM EDTA and 0.1 % Triton ×-100). The homogenates were centrifuged at 13,000×g for 10 min at 4 °C to remove cell debris and the resulting supernatants were analyzed for protein contents by Bradford method (1976). Superoxide dismutase (SOD) was estimated according to the kit supplier protocol (Randox, Cat no. SD. 125, Mississauga, ON, Canada) while catalase (CAT) was estimated manually by measuring the decay of 6 mM H_2O_2 solution at 240 nm by the spectrophotometric degradation method (Cohen et al. 1970).

Immunoblotting

The cells (1×10^7 cells/ml) were cultured in 75 cm^2 flasks and treated with t-BHP with or without pretreatment of CPSE. After treatments, the cells were washed twice with PBS and lysed in ice-cold RIPA buffer with protease and phosphatase inhibitor cocktail (Sigma) at a concentration of 10 μg/ml. The C2C12 cell lysates were centrifuged for 10 min at 12,000×g at 4 °C, supernatants were collected and protein contents were estimated as mentioned earlier. The proteins were separated by 8–15 % SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4 °C with 5 % (v/v) non-fat dry milk in tris-buffered saline with Tween-20 (TBS-T) (10 mM Tris–HCl, 150 mM NaCl and 0.1 % Tween-20, pH 7.5) and incubated with primary antibodies namely α-tubulin (sc-5286), α-SOD (sc-8637), α-CAT (sc-34280), α-Cyt-C (sc-13156), α-HSP-70 (sc-66048) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1,000 dilution for 2 h with shaking. The membranes were then washed in TBS-T followed by incubation for 2 h at room temperature in dark with horseradish peroxidase (HRP) conjugated rabbit anti-goat, goat anti-mouse and goat anti-rabbit secondary antibodies (DAKO, Glostrup, Denmark) at 1:10,000 dilutions. The membranes were washed again and the immunoreactivity was detected using enhanced chemiluminescence peroxidase substrate kit (CPS-160, Sigma, St Louis, MO, USA).

Single cell gel electrophoresis (SCGE) assay

The protective effect of CPSE against t-BHP induced DNA damage in C2C12 muscle cells was assessed using alkaline comet assay. The muscle cells (4×10^5 cells/ml) were seeded in 75 cm^2 flasks which were treated as mentioned earlier and the assay was performed as described by Singh et al. (1988). The DNA was visualized using a fluorescence microscope (Olympus) 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 percent inhibition of tail length.

Statistical analysis

The data were represented as the mean ± SD. Statistical significance was analyzed with one-way analysis of variance followed by a Tukey's HSD-post hoc test. Results with P value <0.05 were considered statistically significant.

Results

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 percent cell viability was determined by MTT assay that was found same as that of untreated control cells. The free radical-induced muscle cytotoxicity was evaluated by treatment with different concentrations of t-BHP (0–1,000 μM) for 24 h and a dose-dependent cell death was observed (Fig. 1a). 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. 1b, CPSE pre-treatments significantly ameliorated t-BHP-induced cell death.

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. 1c. The C2C12 cells were pre-treated with 25 and 50 μg/ml of CPSE for 2 h followed by 250 μM t-BHP treatment 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. 1d).

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. 1e).

CPSE inhibits t-BHP induced loss of MMP

MMP is used as an index to measure the mitochondrial health and a collapse of MMP indicates 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. 2a); this was further confirmed by fluorescence microscope analysis (Fig. 2b).

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 a nonionic, nonpolar dye that crosses cell membranes and is 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. 3a, b).

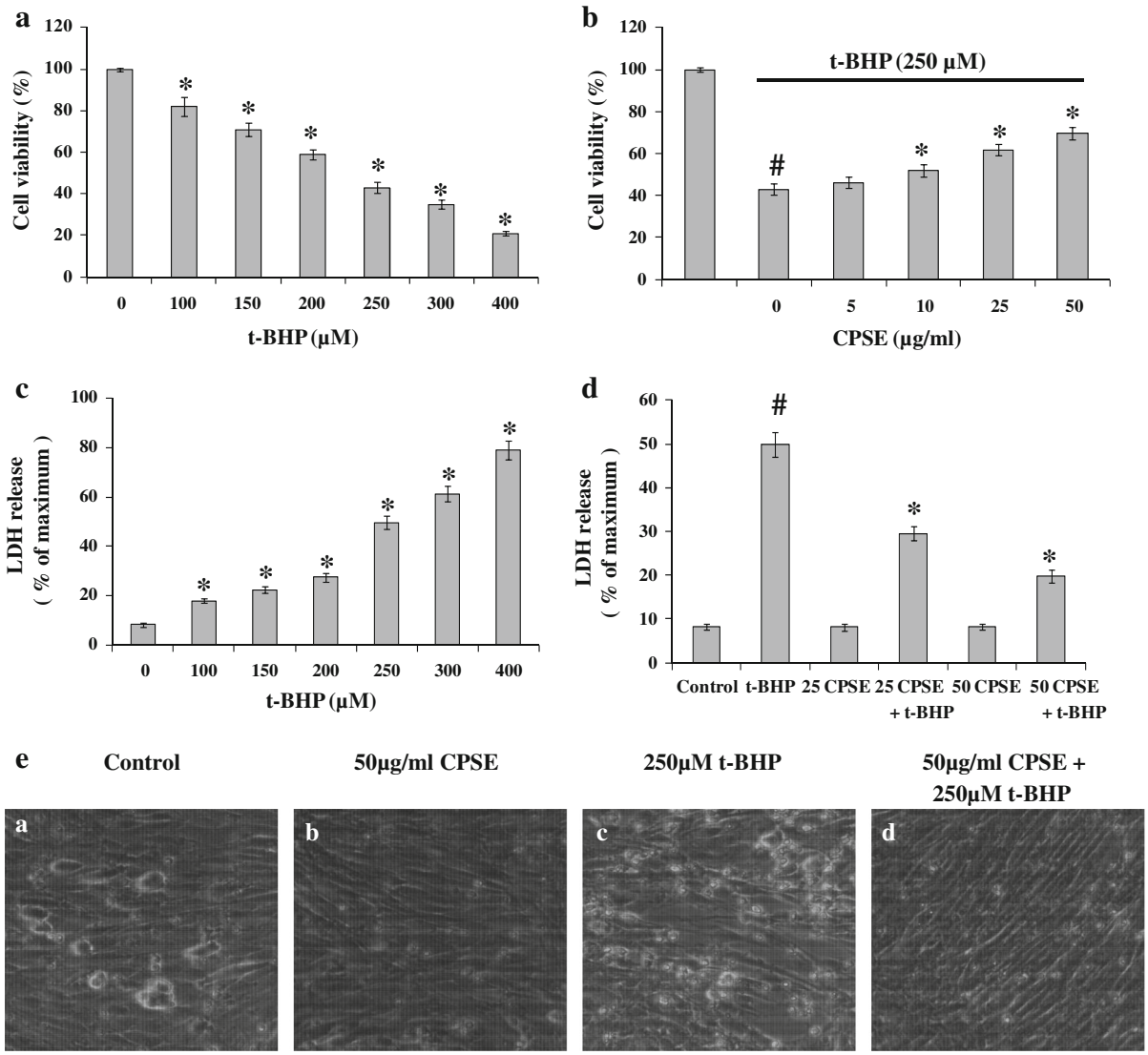

Fig. 1 **a** Cytotoxic effects of teritiary butyl hydroperoxide (t-BHP) on C2C12 muscle cells. $*P < 0.01$ versus control cells without any treatment. **b** Dose dependent protective effect of treatment with *Celastrus paniculatus* seed extract (CPSE) on t-BHP induced cytotoxicity in C2C12 cells, the cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. $*P < 0.01$ versus control cells and 250 μM t-BHP treated group. **c** Cytotoxic effects of t-BHP on C2C12 muscle cell analyzed by lactate dehydrogenase (LDH) leakage assay. $\#P < 0.01$ versus control group $*P < 0.01$ versus control cells. **d** Protective effect of CPSE pretreatment on t-BHP (250 μm) induced cytotoxicity by LDH leakage assay. $*P < 0.01$ versus control cells and 250 μM t-BHP treated group. $\#P < 0.01$ versus control group. **e** Effects of CPSE treated on t-BHP induced morphological alterations in C2C12 muscle cells by phase-contrast microscopy. The data are represented as mean ± SD of three independent experiments

Effect of CPSE on lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid by estimating the content of malondialdehyde that is a by-product of peroxidation of polyunsaturated fatty acids (PUFA). The principle of the assay is that, the removal of hydrogen atoms from the bis-allylic site of PUFA generates lipid radicals that form peroxyl radicals in reaction with oxygen. The peroxyl radicals can propagate chain reactions with adjacent PUFA leading to lipid peroxidation (Deaton and Marlin 2003). In the present investigation, we observed an

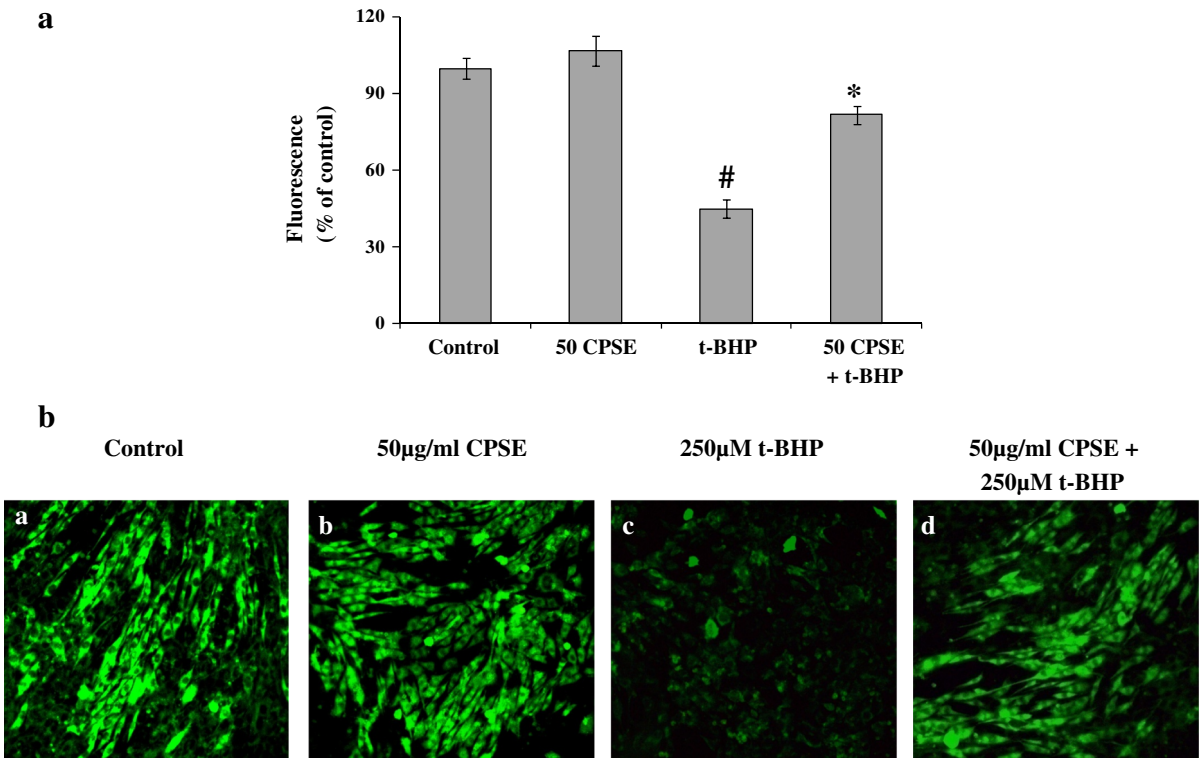

Fig. 2 **a** Estimation of mitochondrial membrane potential in C2C12 muscle cells with pre-treatment of CPSE on t-BHP challenge. The fluorescence intensity was determined using a spectrofluorimeter. **b** The membrane potential was monitored by fluorescent microscope (Olympus) Control cells without any 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 mean ± SD of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.01$ versus control cells and *$P < 0.01$ versus 250 µM t-BHP treated group

increase in lipid peroxidation in muscle cells with t-BHP challenge that was significantly decreased by CPSE pre-treatment (Fig. 4).

Protective effects of CPSE on oxidative stress biomarkers

In the current study, we observed a decrease in SOD and CAT antioxidant enzyme levels with t-BHP treated muscle cells. However, CPSE pre-treatment significantly restored the antioxidant level of muscle cells (Fig. 5a, b). Similarly, theexpression of the antioxidant marker proteins SOD and CAT was downregulated in t-BHP challenged muscle cells, which was significantly restored in CPSE pre-treated muscle cells which demonstrated the antioxidant defence of CPSE (Fig. 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. In the current study, we observed cytochrome-C (Cyt-C) release from mitochondria to cytosol with t-BHP treatment that is more likely to be a result of modified oxidative condition of the cells. However, CPSE pre-treatment significantly reduced Cyt-C release (Fig. 6).

Heat shock proteins (HSPs) are a class of molecular chaperones constitutively expressed in a variety of tissues including muscle tissue and play a protective role particularly during oxidative stress conditions. In the present study, we found an increase in HSP-70 level with t-BHP treatment that elucidates the stress generation in murine muscle cells. However, a decrease in its expression was observed with CPSE

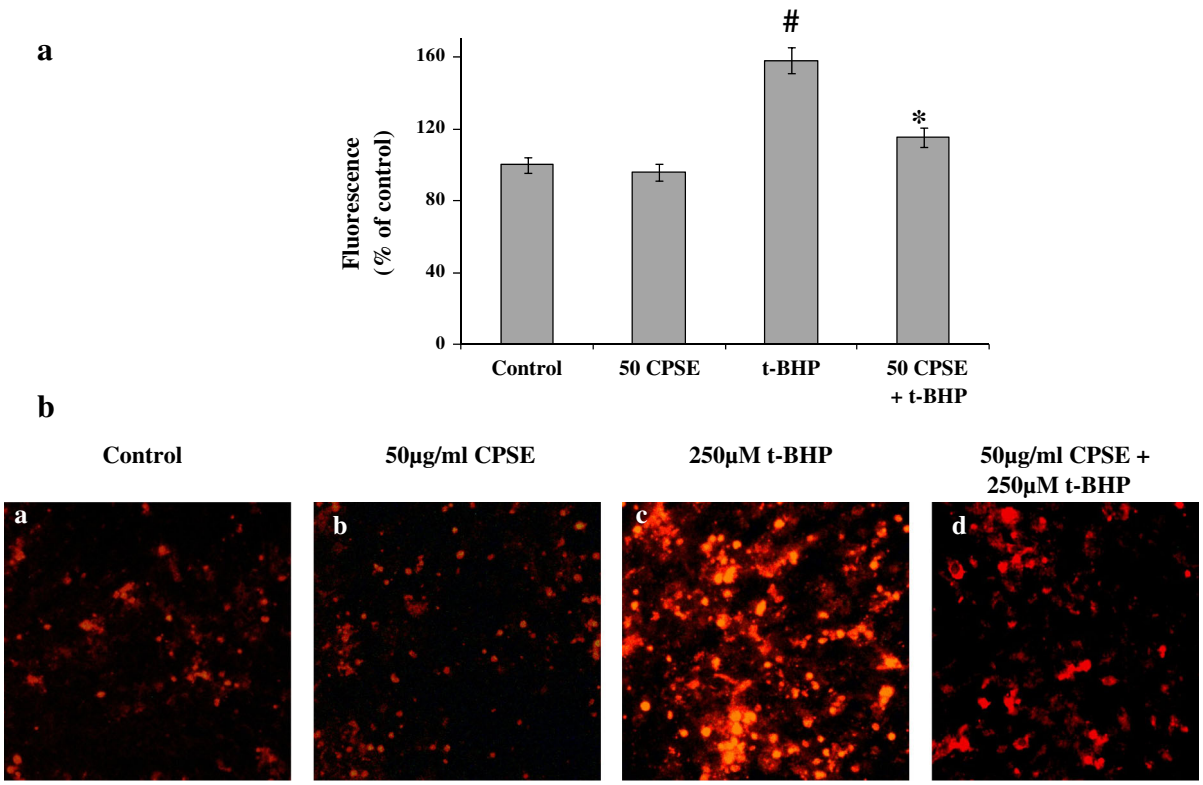

Fig. 3 **a** Estimation of intracellular ROS production using 2′,7′,$DCFH_{2DA}$ in C2C12 muscle cells pre-treated with CPSE on t-BHP challenge by spectrofluorimeter. **b** The membrane potential was monitored by fluorescence microscope (Olympus). Control cells without any treatment (*a*), 50 µg/ml CPSE 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 mean ± SD of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.01$ versus control cells and *$P < 0.01$ versus 250 µM t-BHP treated group

pre-treatment demonstrating the anti-stress effect of CPSE (Fig. 6).

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 principle of the assay is that the undamaged DNA retains a highly organized association with the protein matrix in the nucleus and when damaged this organization is disrupted. When an electric field is applied the disrupted and smaller DNA fragments move faster and are visualized in the form of a comet whereas undamaged DNA strands, being too large, do not leave the cavity. The tail length of the comet is 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 case of 250 µM t-BHP, it was increased to 78.14 ± 6.5 µm which demonstrates that t-BHP induced DNA damage which was further decreased to 29.74 ± 3.0 µm with 50 µg/ml CPSE pre-treatment (Fig. 7).

Discussion

Improving exercise performance has been a wide 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_2O_2 generation

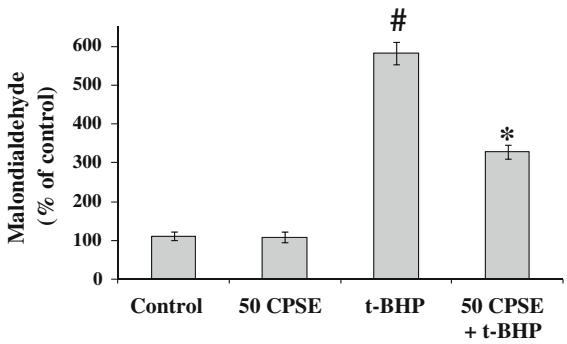

Fig. 4 Estimation of lipid peroxidation products by thiobarbituric acid (*TBARS*) assay in C2C12 muscle cells with pre-treatment of CPSE on t-BHP challenge. The data are represented as mean ± SD of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.01$ versus control cells and 250 µM t-BHP treated group

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 we used t-BHP due to its stability compared with H_2O_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. Our observations of cytoprotective effects are in accordance with aforementioned neuronal cell protective effects by *C. paniculatus* against H_2O_2 induced cell damage (Godkar et al. 2003). Of late Choi et al. (2014) also demonstrated that celastrol, an active metabolite isolated from the root bark of *Tripterygium wilfordii* Hook F. of the *Celastraceae* family, protected SH-SY5Y cells against rotenone induced neurotoxicity.

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. Our findings are in line with Wang et al. (2013) who have also demonstrated the mitochondrial damage protection and ROS scavenging activity of (*M*)-bicelaphanol, a trinorditerpene isolated from *Celastrus orbiculatus* against H_2O_2 induced oxidative stress of SHSY5Y human neuronal cells. Lipid peroxidation has been widely used as a marker to estimate the stress induced oxidative damage. In the present investigation, we observed that CPSE effectively inhibited t-BHP induced MDA generation in C2C12 cells. Our observations are in accordance with Patel and Prasanna (2013) as well who reported that *C. paniculatus* seed oil administration exerts protective effects against chronic fatigue of rats by inhibition of lipid peroxidation.

SOD and catalase are the key antioxidant enzymes which play a role in the antioxidant defence mechanism in order to protect the cells from radical mediated damage. The decrease in antioxidant enzymes level has been reported in chemical/oxidative stress challenge, where the antioxidant system stabilizes the

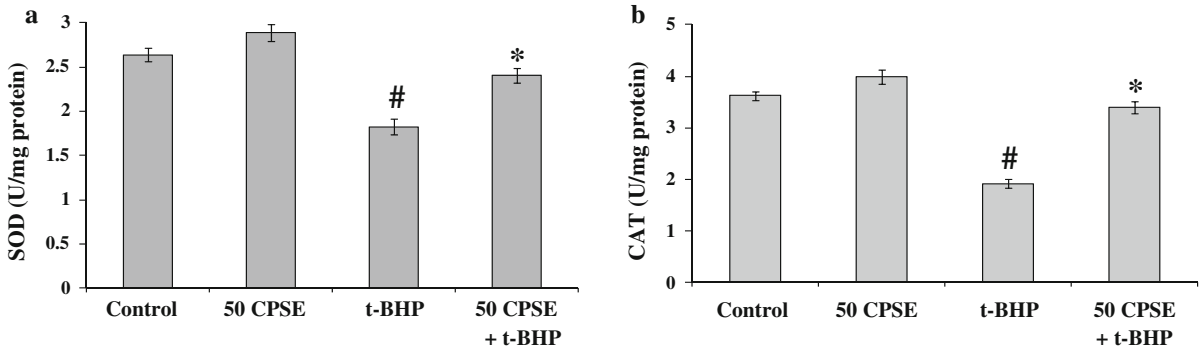

Fig. 5 Pre-treatment of CPSE on restoration of SOD and catalase enzyme activities in C2C12 muscle cells challenged with t-BHP. The data are represented as mean ± SD of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.01$ versus control cells and *$P < 0.01$ versus 250 µM t-BHP treated group

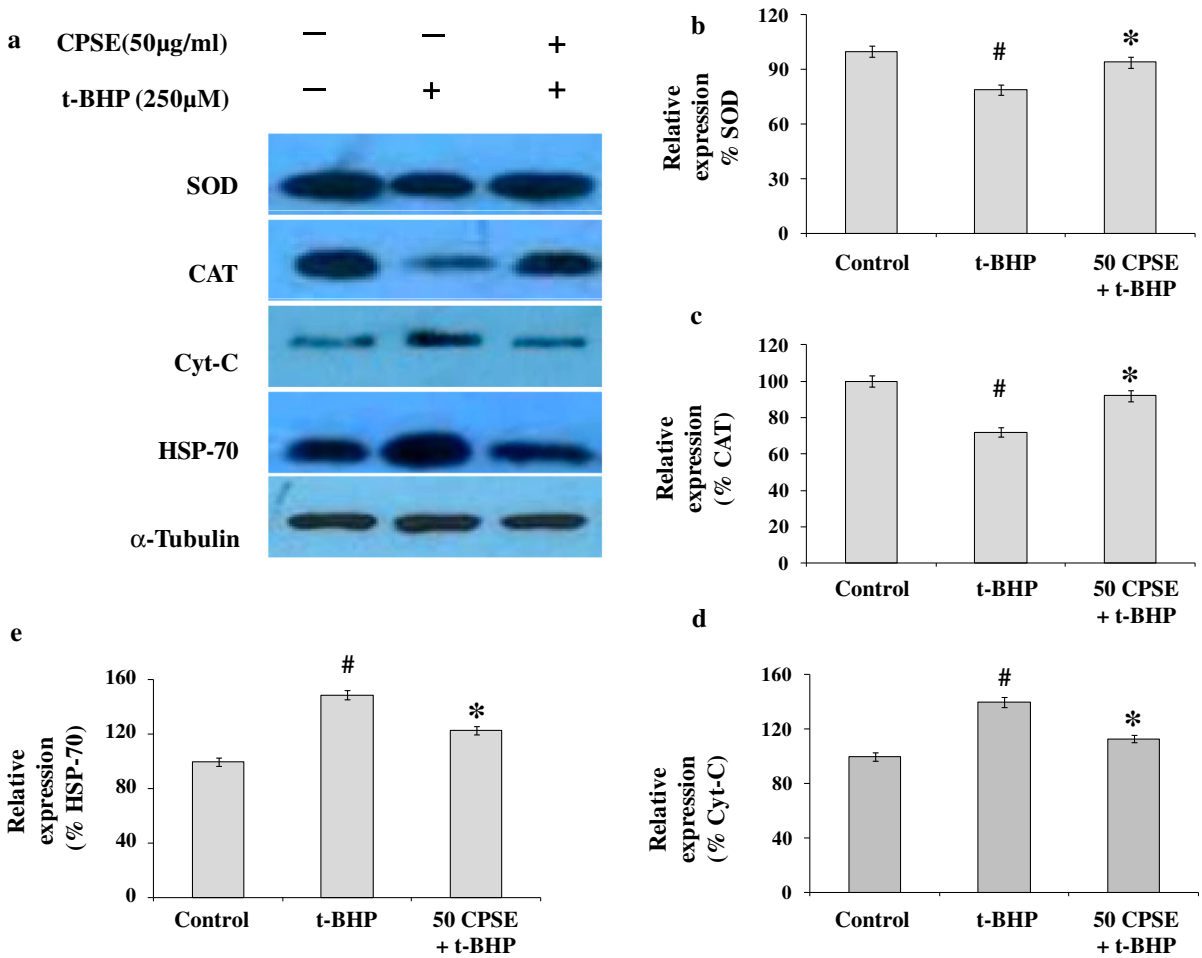

Fig. 6 **a** The protective effect of pre-treatment of CPSE on t-BHP induced expression of oxidative stress marker proteins SOD, CAT and apoptotic marker proteins Cytochrome-c and HSP-70 analyzed by immunobloting. **b–e** The band intensity is calculated by Image-J software. The data are represented as mean ± SD of three independent experiments. #$P < 0.01$ versus control group, *$P < 0.01$ versus control cells and *$P < 0.01$ versus 250 µM t-BHP treated group

generated free radicals (Tiwari and Kakkar 2009; Hemanth Kumar et al. 2014a). Similarly in the present investigation we observed a decrease in the antioxidant level in C2C12 muscle cells with t-BHP challenge that was restored with CPSE supplementation. These results are in line with an earlier study by Godkar et al. (2006) who demonstrated the antioxidant activity of *C. paniculatus* in neuronal cultures.

The mitochondria mediated apoptosis leads to cell death by DNA damage that initiates with bax activation and Cyt-C release with further increase in activation of caspases that play a role in 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. Our data demonstrate that *C. paniculatus* extract bolsters muscle mitochondria against t-BHP mediated apoptosis by regulating Cyt-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 *T. 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,

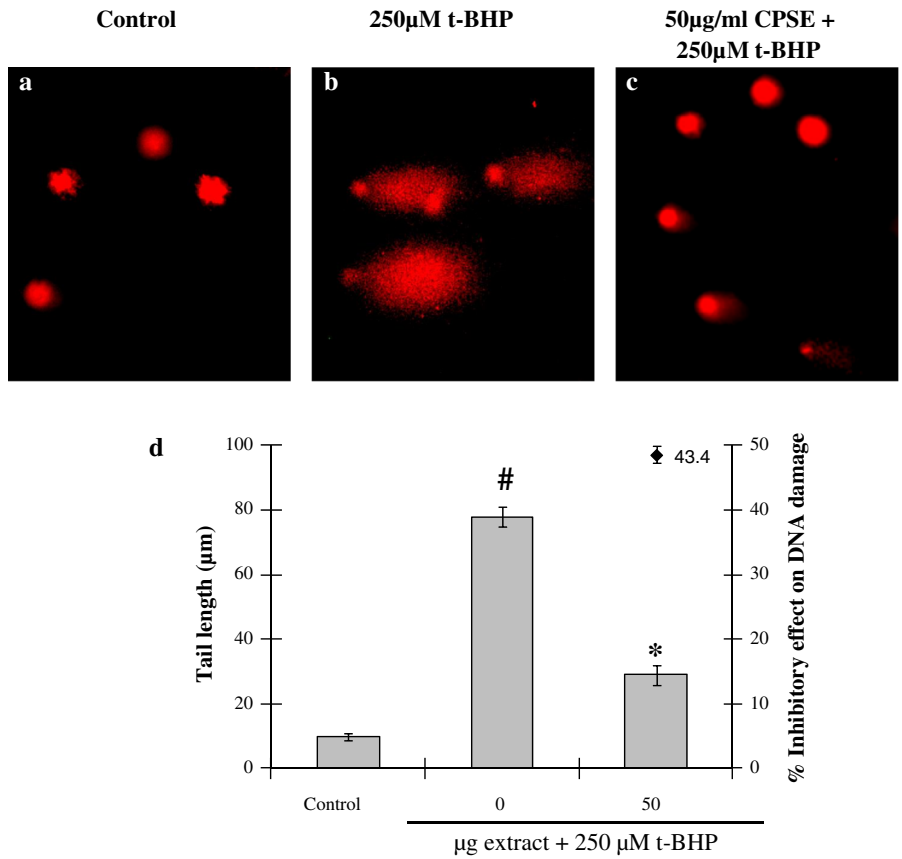

Fig. 7 Protective effect of CPSE on DNA damage induced by t-BHP in C2C12 cells. Control cells without any treatment (**a**), Cells with 250 µM t-BHP treatment for 24 h (**b**), C2C12 cells were pre-treated with CPSE for 2 h at 50 µg/assay and treated with 250 µM t-BHP (duration: 24 h) (**c**). Tail length (µm) *Bars*. Inhibitory effect of CPSE on DNA damage *diamond* (**d**)

aggregated proteins and induces proteolytic degradation of denaturated proteins (Gupta et al. 2010). Increased expression of HSP-70 has been reported in radical induced oxidative stress condition (Jung et al. 2011; Kumar and Khanum 2013). The protective effect of *C. paniculatus* against t-BHP induced HSP-70 down-regulation further confirms the anti-stress effects. Stress induced ROS generation has been implicated to cause DNA damage that can be regulated by antioxidant supplementation (Venuprasad et al. 2013). The observed C2C12 cells DNA damage protective effects are in agreement with our recent study that also demonstrated the DNA damage protective effects of *C. paniculatus* against exercise mediated oxidative DNA damage in mice (Hemanth Kumar et al. 2014b). 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 t-BHP induced muscle cell damage demonstrates that CPSE could be used as an antioxidant supplement during fatigue/exercise induced stress and to inhibit free radical-induced oxidative damage of cells.

Conclusion

Natural antioxidant supplements have wide applications to treat radical mediated stress conditions. In the present investigation we observed that *C. paniculatus* seed extract inhibited t-BHP induced muscle cell cytotoxicity, apoptosis, mitochondrial and DNA damage and restored the antioxidant status. Our observed results demonstrate the possible application of CPSE to treat strenuous exercise/fatigue induced oxidative damage of muscle cells. However, further studies are necessary to better clarify the active principles/compounds of *C. paniculatus* responsible and detailed anti-stress mechanism of action at cellular level.

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