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Effect of Asiaticoside Rich Extract from *Centella asiatica* (L.) Urb. on Physical Fatigue Induced by Weight-loaded Forced Swim Test

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ABSTRACT

The present study was undertaken to elucidate the mechanism responsible for ameliorating effect of asiaticoside rich *Centella asiatica* leaf extract (CAE) on fatigue. CAE at 10 mg kg⁻¹ b.wt. was administered orally to rats for 2 weeks and subjected to weight-loaded forced swim test (WFST) every alternative day for a period of three weeks to evaluate physical fatigue. CAE administrated rats prolonged the swimming time for exhaustion almost two fold compared to the control. Moreover, WFST also reduced the levels of glycogen content in muscle and liver tissues and activities of superoxide dismutase and catalase in liver and brain tissues. These effects were reverted back with the administration of CAE and therefore it is concluded that *C. asiatica* extract ameliorates the various impairments associated with physical fatigue in enhancing the physical endurance capacity.

Key words: *C. asiatica*, anti-fatigue, weight-loaded forced swim test (WFST), antioxidant enzymes, heat shock protein

INTRODUCTION

Centella asiatica (L.) Urb. is a herb belonging to the family Apiaceae, growing widely in various parts of world including eastern Asia, China and India (Yoo *et al.*, 2007). It is also being cultivated and used in several parts of south-India as a leafy vegetable. In classical Indian Ayurveda literature, it is considered to be one of the 'Rasayana' drugs for rejuvenation (Jayashree *et al.*, 2003). Leaves of this plant have been traditionally used in treating inflammations, diarrhea, asthma, tuberculosis and various skin lesions (Coldren *et al.*, 2003). *Centella asiatica* (CA) was studied as a neuroprotective agent to fight against oxidative damage caused by Reactive Oxygen Species (ROS) *in vitro* as well as towards neuronal cells which eventually lead to neurodegenerative diseases such as Alzheimer's and Parkinson disease (Anand *et al.*, 2010; Amid *et al.*, 2010). Major bioactive compounds of *C. asiatica* are triterpene glycosides such as centellasaponin, asiaticoside, madecassoside, sceafoleoside (Matsuda *et al.*, 2001), asiatic acid and madecassic acid (Inamdar *et al.*, 1996).

C. asiatica has been investigated for its health benefits and is attributed to triterpenoid saponin. These bioactive compounds have been shown to protect cortical neurons from exitotoxicity (Lee *et al.*, 2000), modulate neurotransmitter system in rat brain and enhance learning and memory process. It has been demonstrated that exhaustive exercise cause oxidative damage to membrane and leads to fatigue in relation to dysfunction of autonomic nervous,

endocrine and immune system (Maes *et al.*, 1998). Present generation of population rely on ethnological medicine for treatment of various diseases (Abdel-Azim *et al.*, 2011; Kumar *et al.*, 2011). Medicinal herbs have attained amplifying significance in development co-operation for recent years (Karim *et al.*, 2011; Malik *et al.*, 2011). In food and Pharmaceutical industry, there has been considerable interest in how to look for the natural materials, especially for proteins and peptides, to inhibit production of free radical and postpone the sports fatigue (Ali, 2011).

Moderate exercise is useful for preventing illness and mental stress but over exercise itself can be a form of stress and cause fatigue or various types of damage to the organs. Fatigue is the symptom, which indicates that the health is about or already subjected to harm (Yu *et al.*, 2008). Free radical formation and subsequent lipid peroxidation of bio-membranes might be noxious biochemical responses to either physical or emotional stress (Hoch *et al.*, 1988). It has been demonstrated that exercise leads to increase in free radical formations thus causing oxidative damage to membranes (lipid peroxidation), thus, fatigue causes various disorders in relation to bio-regulatory, autonomic nervous, endocrine and immune system (Maes *et al.*, 1998). However, there are no reports regarding the physical endurance properties of *C. asiatica*. In this study the leaf extract was evaluated for its anti-fatigue properties.

MATERIALS AND METHODS

Isolation of bioactive components: *C. asiatica* (L.) Urb. was purchased from local market of Mysore and identified with the help of herbarium collection, Department of Botany, Mysore University, Mysore, India. The material was shade dried, powdered and soaked overnight in autoclaved distilled water followed by macerating with 90% ethanol. The filtrate of ethanolic extract was flash evaporated under reduced pressure (Heidolph, Germany). The lyophilized powder of CAE in distilled water was administered orally to the animals at 10 mg kg⁻¹ b.wt. per day for 14 days.

Qualitative analysis of alcoholic extract by HPLC: The extract was qualitatively analyzed by HPLC (JASCO HPLC system) using a reverse phase C₁₈ column (150×4.5 mm) as per the method described by Verma *et al.* (1999) with minor modifications. Twenty microliters of the sample and asiaticoside standard were injected into the column and the asiaticoside peak was detected at 220 nm using UV detector. The composition of mobile phase used was water (containing 1% trifluoroacetic acid): methanol (30:70, v/v) at the flow rate of 0.5 mL min⁻¹.

Experimental design: Animal studies were conducted according to the institute animal ethical committee regulations approved by the committee for the purpose of the control and supervision of experiments on animals (CPCSEA). Eighteen male albino rats of Wistar strain weighing 100-120 g (3-4 week old) were selected from the stock colony of Defence Food Research Laboratory, Mysore, India, housed in an acrylic fiber cage in a temperature controlled room (25±2°C) and was maintained at 12 h light/dark cycle. The rats were randomly divided into the following 3 groups: sedentary group, control group and treatment group (CAE). The treatment group rats were administered orally with CAE extract (10 mg kg⁻¹ of b.wt. day⁻¹) for a period of two weeks. Sedentary and control rats were orally administered with equal amount of distilled water. The animals had free access to diet and water.

Weight-loaded forced swim test (WFST): The weight-loaded forced swim test (WFST) was performed as described previously with some minor modifications (Jung *et al.*, 2007). The rats of CAE administered group and control group were allowed to swim with constant loads (tagged to the

tail base) corresponding to 5% of their body weight. The swimming exercise was carried out in small tank with 30 cm deep water maintained at $25\pm 2^\circ\text{C}$. Exhaustion time was determined by observing loss of coordinated movements and failure to return to the surface within 10 sec (Wang *et al.*, 2006). This was repeated every alternate day for a period of three weeks. At the end, animals were sacrificed under mild anesthesia blood, liver, brain and gastrocnemius muscle tissues were analyzed for various biochemical parameters.

Determination of superoxide dismutase (SOD) and catalase (CAT) activity: Liver tissues were homogenized in 50 mM phosphate buffer saline (pH 7.4). SOD activity of was determined with a commercially available kit (Randox, Cat No. SD. 125, Canada). CAT was determined by measuring the decay of 6 mM H_2O_2 solution at 240 nm by spectrophotometric degradation method (Cohen *et al.*, 1970).

Determination of serum blood urea nitrogen (BUN) and creatine kinase (CK): Serum BUN and CK were determined according to the procedures provided by the kits (Biosystems, India and Agappe, India, respectively).

Determination of thiobarbituric acid-reactive substances (TBARS): TBARS as malondialdehyde (MDA $\mu\text{mol}/\text{cm}/\text{g}$) was analyzed by Buege and Aust (1978). Brain, 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 contains 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 (Girotti and Deziel, 1983).

Determination of glycogen: Liver and muscle tissues were digested with 2 mL of KOH (30%) and boiled in water bath for 30 min with occasional shaking and then allowed to cool at 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 (Miller, 1972) was followed to determine hydrolyzed product of glycogen.

Determination of lactic acid: Lactic acid content was measured accordingly (Sawhney and Singh, 2005). Tissue samples were homogenised in phosphate buffer (100 mM, pH 7.2) and 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. One hundred micro liters of p-hydroxy-diphenyl reagent was added to the above sample mixture and incubated at 37°C for 30 min. The absorbance was measured at 560 nm.

Western blot analysis for HSP-70: Liver tissue samples were processed for SDS-PAGE followed by western blot for the analysis of HSP-70 expression. Tissue samples were homogenized with 5-10 volumes of lysis buffer pH 7.4 (10 mM HEPES 42 mM KCl, 50 mM MgCl_2 , 0.1 mM EDTA,

0.1 mM EGTA, 5 mM DTT, 2 mM PMSF, 1X protease inhibitor cocktail). Homogenate was centrifuged at 10,000 rpm for 20 min and supernatants were used as whole protein extract and the total protein was estimated by Lowry *et al.* (1951). Protein from each sample (100 µg) was separated on SDS-PAGE and transferred onto a nitrocellulose membrane using an electro blotting apparatus (Bio-Rad, USA). After transfer, the membranes were probed with α -tubulin B-7 (sc-5286) and anti HSP-70 C92F3A (sc-66048) antibodies, 1: 1000 and incubated at room temperature for 3 h. The membranes were then washed four times in TBST for 15 min followed by incubation for 2 h in horse radish peroxidase conjugated rabbit anti-mouse secondary antibody (Dako) used at 1: 10,000 dilution. The membranes were washed again and developed using an enhanced chemiluminescent detection system (ProteoQwest[®], Sigma). Developed membranes were exposed to X-ray film and the developed band intensity was captured (Jayaraj *et al.*, 2006). Western blot band intensity was measured using NIH image analysis software.

Statistical analysis: The data are expressed as Mean \pm standard deviation of the mean (SD). Data was analyzed using one-way ANOVA. Differences at $p < 0.05$ were considered to be significant.

RESULTS

HPLC analysis of aqueous extract: In the present study Asiaticoside, a trisaccharide triterpene, was identified as a major peak at 5.4 min based on the retention time (Fig. 1).

Effect of CAE on body weight and swimming time: The swimming exercise with 5% tail load was carried out for three weeks. On day one, no significant difference in swimming time between control and CAE treated group was observed. However, the swimming time of rats gradually increased with CAE treatment compared to that of control group. Maximum swimming time was recorded on day 13 of the CAE treatment with 21 \pm 4 minutes to that of control group 9 \pm 1.1 min $p < 0.05$ (Fig. 2). There was no change in body weight gain among the groups.

Effect of CAE on antioxidant enzyme activities: In the control group exhaustive swimming decreased the activities of SOD and CAT in liver (22, 28%, respectively) and in brain (34 and 39%, respectively) compared with sedentary group ($p < 0.01$). However, CAE administration significantly increased the activities of SOD and CAT (Table 1).

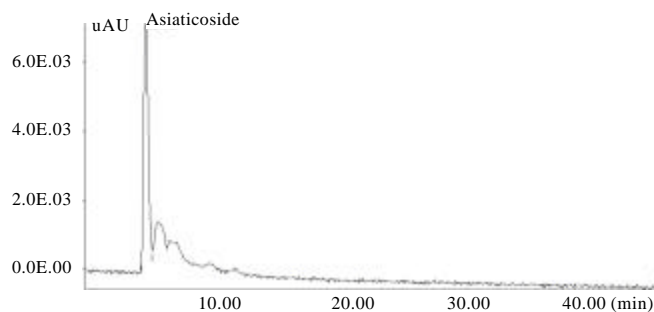


Fig. 1: HPLC chromatogram of alcoholic extract of *Centella asiatica*

Table 1: Effect of CAE on biochemical parameters after the weight-loaded forced swim test

Group	BUN (mg dL ⁻¹)	CK (U L ⁻¹)	SOD (U mg ⁻¹ protein)		CAT (U mg ⁻¹ protein)	
	Serum	Serum	Liver	Brain	Liver	Brain
Sedentary	17.7±1.5	172±30.72	6.31±0.9	1.51±0.03	29.0±2.0	9.04±0.6
Control	21.1±2.0 ^B	199±25.2 ^A	4.91±1.0 ^C	1.00±0.02 ^C	20.8±3.3 ^C	5.54±0.4 ^C
CAE	19.4±2.3 ^a	180±12.2 ^a	5.50±0.7 ^b	1.25±0.02 ^b	26.2±2.4 ^B	6.49±0.4 ^a

Data express the Mean±SD for 6 rats, Sedentary: Rat unexposed to the WFST and treated with distilled water, Control: Rat exposed to the WFST and treated with distilled water, CAE: Rat exposed to the WFST and treated with *C. asiatica* extract (10 mg/ kg b.wt./day) for 14 days, Different letters indicates statistically significant differences, ^Ap<0.05, ^Bp<0.01, ^Cp<0.001 vs. sedentary; ^ap<0.05, ^bp 0.01, vs. control. BUN: Blood urea nitrogen, CK: Creatine phosphokinase, CAT: Catalase, SOD: Superoxide dismutase

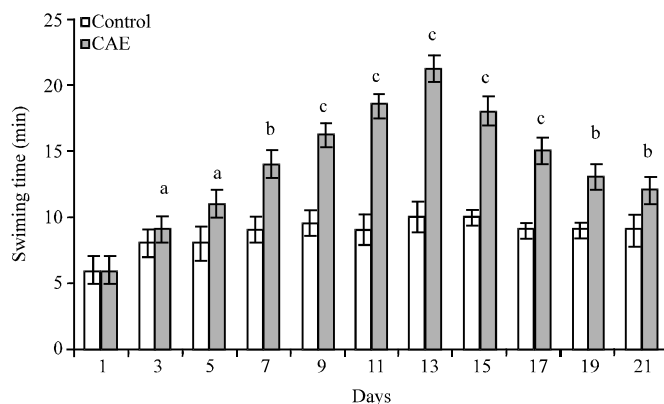


Fig. 2: Effect of CAE on the weight-loaded forced swim test. Data express the Mean±SD for 6 rats, Sedentary: Rat unexposed to the WFST and treated with distilled water, Control: Rat exposed to the WFST and treated with distilled water, CAE: Rat exposed to the WFST and treated with *C. asiatica* extract (10 mg/ kg b.wt./day) for 14 days, Different letters indicates statistically significant differences, ^ap<0.05, ^bp<0.01, ^cp<0.001 vs. control

Effect of CAE on serum BUN and creatine phosphokinase: BUN and CK are biomarkers to evaluate the bearing capability when animals suffer from a physical stress. The swimming exercise significantly increased the levels of BUN and CK activity by 19.2 and 15.6%, respectively to that of sedentary group, (p<0.05) and was reduced to 8 and 9.5%, respectively in CAE administered group (Table 1).

Effect of CAE on lipid peroxidation, glycogen and lactic acid levels: Thio-barbituric acid reactive substances (TBARS) measured as malondialdehyde (MDA μmol/cm/g) represents lipid peroxidation in liver, brain and muscle tissues and results are shown in Fig. 3. Swimming exercise significantly increased MDA concentration in liver (p<0.001) and muscle (p<0.01) when compared to the sedentary group. However, supplementation of CAE decreased lipid peroxidation in liver 0.6% (p>0.05) and in muscle tissues 4.3% (p<0.01), compared to control group. Results of liver and muscle glycogen were shown in Fig. 4. The utilization of muscle and liver glycogen due to WFST was significantly lower in CAE group than control group (13.8±0.2 and 4.2±0.14 mg g⁻¹, respectively, p<0.001). The results of the lactic acid levels are presented in Fig. 5. These LA levels were also significantly reduced by CAE treatment in both the tissues (p<0.01).

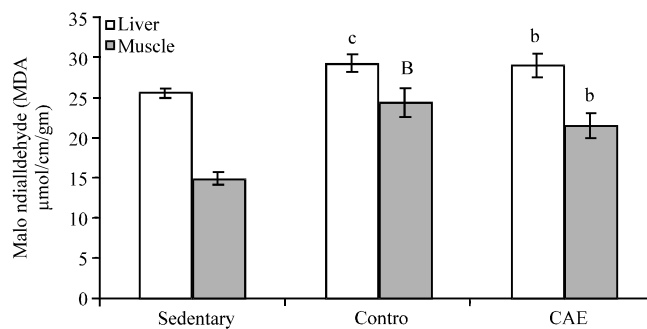


Fig. 3: Effect of CAE on lipid peroxidation, Data express the Mean±SD for 6 rats, Sedentary: Rat unexposed to the WFST and treated with distilled water, Control: Rat exposed to the WFST and treated with distilled water, CAE: Rat exposed to the WFST and treated with *C. asiatica* extract (10 mg kg⁻¹ b.wt.) for 14 days, Different letters above the bar indicates statistically significant differences, ^cp<0.001, ^Bp<0.01 vs. sedentary and ^bp<0.01 vs. control

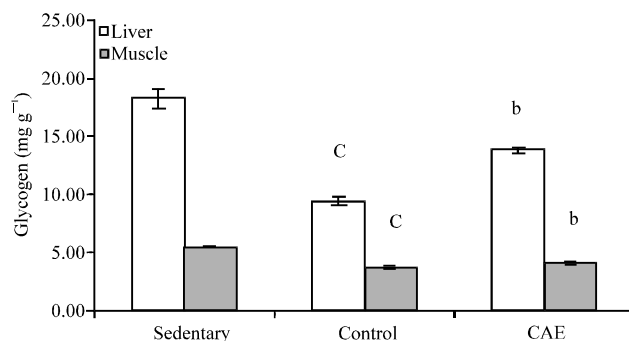


Fig. 4: Effect of CAE on liver and muscle glycogen, Data express the Mean±SD for 6 rats, Sedentary: Rat unexposed to the WFST and treated with distilled water, Control: Rat exposed to the WFST and treated with distilled water, CAE: Rat exposed to the WFST and treated with *C. asiatica* extract (10 mg kg⁻¹ b.wt.) for 14 days, Different letters above the bar indicates statistically significant differences, ^Cp<0.001 vs. sedentary and ^bp<0.01 vs. control

Effect of CAE on HSP-70 expression: In exercised control rats, HSP-70 expression was significantly increased when compared to the sedentary group. CAE treated rats showed reduced levels of HSP-70 expression (Fig. 6).

DISCUSSION

Many health benefits of *C. asiatica* have been investigated. Leaf extract of *C. asiatica* rich in triterpenoid saponin has been shown to protect cortical neurons from glutamate induced excitotoxicity (Lee *et al.*, 2000; Schaneberg *et al.*, 2003). This bioactive compound has been reported to have a number of pharmacological properties such as antioxidant; immunological effects etc., (Anand *et al.*, 2010; Maes *et al.*, 1998; Matsuda *et al.*, 2001). An aqueous extract of *C. asiatica* leaf had shown a modulation on dopamine, 5-hydroxytryptamine (5-HT) and nor-epinephrine systems in rat brain. The extract also proved to enhance the learning and memory process *in vivo* (Jung *et al.*, 2004). Earlier reports proved that forced swimming induces stress and leads to fatigue

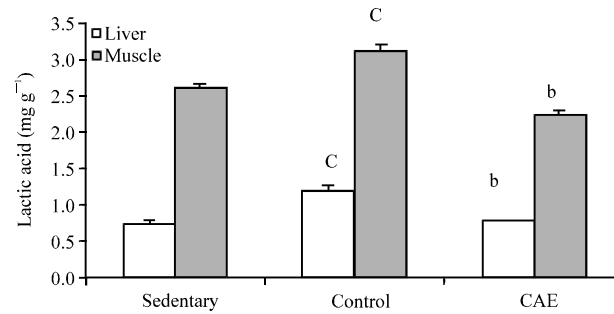


Fig. 5: Effect of CAE on liver and muscle lactic acid, Data express the Mean \pm SD for 6 rats, Sedentary: Rat unexposed to the WFST and treated with distilled water, Control: Rat exposed to the WFST and treated with distilled water, CAE: Rat exposed to the WFST and treated with *C. asiatica* extract (10 mg kg⁻¹ b.wt.) for 14 days, Different letters above the bar indicates statistically significant differences, ^cp<0.001 vs. sedentary and ^bp<0.01 vs. control

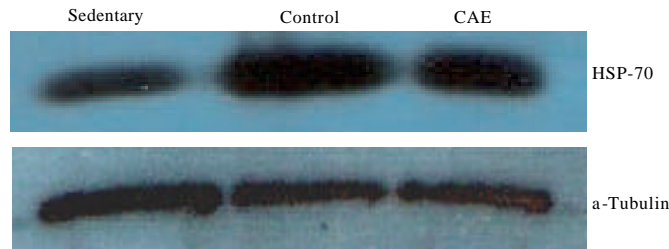


Fig. 6: Effect of CAE on heat shock protein (HSP-70) protein expression, Percent increase or decrease in HSP-70 expression was plotted after normalization of the values against sedentary, Control: Rats exposed to the WFST and treated with distilled water, CAE: Rats exposed to the WFST and treated with CAE (10 mg kg⁻¹ b.wt.) for 14 days, α -tubulin used as protein loading control. Representative of typical data of six experiments

(Liu *et al.*, 2011; Prasad and Khanum, 2012). In the present study, the potential of CAE was evaluated to prevent various types of damages from exercise, to enhance the physical endurance which has not been reported so far. The forced swim test is one of the most commonly used animal models for evaluating the anti-fatigue properties (Powers *et al.*, 2004). The data on WFST observations showed that administration of CAE has evidently extended the swimming time significantly, indicating anti-fatigue activity, with no change in body weight gain (Fig. 1).

The overall anti-oxidant defense mechanism weakens during chronic fatigue conditions. In the present study, swimming reduced the activities of SOD and CAT in control group compared to that of that of sedentary group. In CAE group increased activities of these enzymes was observed (Table 1), indicating CAE has neutralized/inhibited the formation of ROS generation during swimming exercise. Growing evidence indicate that ROS are responsible for exercise-induced protein degradation and contribute negatively to muscle anti-fatigue (Powers *et al.*, 2004). BUN represents normally the renal function, however, there are many factors like, protein break down, dehydration, stress and fatigue etc can cause alteration in BUN levels and CK activity

(Wang *et al.*, 2006). In this study the results indicate that the levels of BUN and CK activity in CAE group were lower than the control group ($p < 0.05$). Mitochondrial membrane glycolipids, fatty acids and essential lipids are damaged by oxidation process (Schulte-Mattler *et al.*, 2003). Earlier study reported that CAE inhibit lipid peroxidation in aged animals (Subathra *et al.*, 2005). In the present study, control rats showed higher lipid peroxidation levels to that of sedentary group which was significantly inhibited by CAE supplementation ($p < 0.01$), in both liver and muscle (Fig. 2).

It is known that endurance capacity decreases if the available energy is reduced. Glycogen is one of the sources of preferred energy during exercise; the increase in glycogen store in liver is an advantage to enhance the physical endurance capacity (Yu *et al.*, 2008). Energy for exercise is derived initially from the breakdown of glycogen in muscle, after strenuous exercise may be depleted and at the later stages the required energy may be derived from liver glycogen (Suh *et al.*, 2007). As shown in Fig. 3, the exhaustive swimming exercises depleted liver and muscle glycogen levels in control rats (47.8 and 32%, respectively) whereas the depletion was only 24.1 and 23.8%, respectively, in CAE supplemented group compared to sedentary group. LA is one of the glycolysis metabolic products of carbohydrate metabolism under anaerobic condition and is considered as one of the biomarkers for evaluating the degree of fatigue (Wang *et al.*, 2006; Yu *et al.*, 2008). Significant decrease in LA concentration in CAE treated group was observed to that of control group (Fig. 4) indicating aerobic metabolism of carbohydrates leading to enhanced formation of ATP indicating again the anti-fatigue property of *C. asiatica*.

The heat shock proteins (HSPs) are a group of protein that has a high degree of identity at amino acid level of different organisms (Peng *et al.*, 2000). Chemical induced stress elevated the HSP-70 expression was reported in earlier studies *in vitro* (Venkatraman *et al.*, 2006). Present study we have noticed the increasing level of HSP-70 expression in control rats may attribute to oxidative effect of fatigue that enhances the expression of HSP-70 as a cellular defense mechanism. The results of immune blot analysis of HSP-70 expression showed that the CAE treated animals showed lower levels of protein than control group indicating further suppression of fatigue (Fig. 5).

CONCLUSIONS

The present study demonstrated that the administration of CAE extract significantly increased the swimming time as well as anti-oxidant status by increasing the activities of anti-oxidant enzymes and glycogen stores by utilizing the energy derived from aerobic condition. Moreover, the extract also significantly reduced the lipid peroxidation, lactic acid levels and HSP-70 protein expression. This observed anti-fatigue property of the extract could be correlated with the presence of major amount of asiaticoside. Hence, it may be concluded that CAE can be used as a herbal supplement to enhance the physical endurance.

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