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Lycopene ameliorates Chronic Fatigue Syndrome in Murine Water Immersion Stress Model Mice

Shilpi Sachdeva[1,2], Anand Kamal Sachdeva[2], &Divya Yadav[1], Rakesh Yadav[4]*
1Department of Pharmacy, Banasthali University, Banasthali, Rajasthan, India.
2Sachdeva College of Pharmacy, Gharuan, Mohali, Punjab
3Idma Centre of Excellence for Research & Development, Idma Laboratories Ltd, Panchkula, India
4National Forensic Sciences University, Institute of National Importance, India
***Corresponding author's Email: rakesh_pu@yahoo.co.in**

ABSTRACT
Chronic fatigue syndrome (CFS) is characterized by profound fatigue, which substantially interferes with daily activities. CFS was produced in mice by subjecting them to forced swim 6 min daily for 15 days. Immobility period and post-swim fatigue was recorded. Lycopene and duloxetine were administered daily for 15 days, 30 min before forced swim session. On day 16th, various behavioral, biochemical and neurotransmitter estimations in the brain were carried out. There was a significant increase in immobility period and post-swim fatigue in vehicle treated mice on successive days. Chronic fatigue group exhibited significant behavioral alterations like anxiety response, hyperlocomotion, hyperalgesia, memory deficit, along with enhanced oxido-nitrosative stress, acetylcholinesterase activity as well as increased production of cytokines and corticosterone level as compared to naive group. The neurotransmitter estimations in the brain samples revealed a decrease in neurotransmitter levels on chronic exposure to forced swim for 15 days. Daily treatment with lycopene for 15 days produced a significant reduction in immobility period, post-swim fatigue and reversed various behavioral, biochemical, neurotransmitters and cytokine alterations induced by chronic fatigue. Lycopene could be of therapeutic potential in the treatment of chronic fatigue and thus may provide a new, effective and powerful strategy to treat CFS.
Keywords: *Chronic fatigue syndrome, lycopene, stress, depression, cytokines, forced swimming test*

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INTRODUCTION

Chronic fatigue syndrome (CFS) is characterized not only by severe fatigue, but also by impairment of immune, autonomic, neuroendocrine and cognitive functions suggesting the involvement of disorders in neuronal–endocrine–immune interactions. Stress and infection have been considered as major contributors in the development of CFS. However, the terms CFS and even chronic fatigue have been used to describe people with Myalgic Encephalomyelitis (ME) [37]. Exposure to a stressful stimulus or chronic fatigue (CF) is perceived as a threat to the organism's homeostasis and elicits a variety of symptoms involving behavioral, biochemical and neurochemical aspects. Syndromes of medically unexplained chronic fatigue may include chronic fatigue syndrome or idiopathic chronic fatigue [30].

A reduction in daily activity >50% for at least 6 months is a major criterion for diagnosis of CFS. There are no physical signs specific to CFS or CF, and there are no diagnostic tests to identify these syndromes. These syndromes are based on symptomatic complaints, and may possibly be characterized as heterogeneous with multiple etiologies involved. In fact, CF involves fewer symptoms than CFS.Besides, chronic stress has been known to associate with other behavioral dysfunctions such as cognitive deficits, anxiety, neurochemical alterations and induced oxidative. Stress related neuroendocrine mechanisms are proposed to be involved in the pathogenesis of affective disorders. The hypothalamic –pituitary–adrenal (HPA) axis plays a major role in the regulation of responses to stress. Hypofunctioning of the HPA axis could lead to an exaggerated stress response and a subsequently excessive release of proinflammatory cytokines. Increase stress level for long-term increase glucocorticoids and catecholamines levels, which over time suppress immune function [13]. Since there is no known cause of CFS, current treatment remains symptomatic, with a focus on management rather than cure.

Oxidative stress damage of erythrocytes by free radicals has been suggested to be involved in the pathogenesis of the CFS. Oxidative stress affects physical and mental function through various redox-

sensitive signaling systems [27]**.** CFS has been found to be associated with increased immune activation and inflammatory cytokine like Interleukin (IL)-1, IL-6 and TNF-α can signal the brain which triggers the activation of both central nervous system and hypothalamic-pituitary adrenal axis.Stress not only induces inflammatory reactions with an increased production of proinflammatory cytokines but also a prooxidant state and lipid peroxidation [34].

Lycopene is an aliphatic hydrocarbon carotenoid extracted from tomatoes, watermelons, and papayas, etc with powerful antioxidant having a singlet-oxygen-quenching capacity 47 and 100 times greater than that of β-carotene and vitamin E, respectively. Epidemiological studies have shown that lycopene can potent antiproliferative, anticancer, hypocholesterolemic agent and neuroprotective. Lycopene also reduces proinflammatory cytokine and chemokine expression macrophages [3]. With this background, the present study was undertaken to investigate the neuroprotective effect of lycopene on various behavioral and biochemical alterations in murine water immersion stress model of chronic fatigue.

MATERIAL AND METHODS

Animals

Healthy adult male healthy albino mice (32-38g) were used for the experiment. The animals were housed under standard laboratory conditions, maintained on a 12:12 h light: dark cycle and had free access to food and water. Animals were acclimatized in standard animal house environmental conditions for five days before the start of the experiment. The experimental protocols were approved by the Institutional Animal Ethics Committee and performed in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India on animal experimentation.

Drugs & treatment

Lycopene was obtained as a gift sample from Jagsonpal Pharmaceuticals, New Delhi, India, and duloxetine was obtained as gift sample from Torrent Pharmaceuticals Ltd., Ahmedabad, India. Tumor necrosis factor-α (TNF-α), tumor growth factor-β (TGF-β) and Interleukin 1β (IL-1β) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Minneapolis, MN, USA, while nuclear factor kappa light chain-enhancer of activated B cells (NF-kB) and caspase-3 ELISA kits were procured from Imegenex, San Diego, USA, and Biovision, USA, respectively. All other chemicals used for biochemical estimations were of analytical grade. Lycopene was dissolved in double-distilled water after triturating with 5% Tween 80. Drug solution was freshly prepared and administered in a constant volume of 10 ml/kg body weight. Lycopene (1 mg/kg, 2 mg/kg and 4 mg/kg) by oral gavage and duloxetine (20 mg/kg) was administered by intraperitoneally daily for 15 days.

Experimental design

Induction of chronic fatigue: Mice were individually forced to swim inside a rectangular glass jar (25×12×25 cm^3) containing 15 cm of water (22 ± 3^0C) for 6 min daily for a total period of 15 days [27].

Grouping of animals: The animals were divided into six groups, consisting of 10 animals in each group. Group I (control group) consisted of naive animals who were not subjected to forced swimming. Group II comprised of animals subjected to forced swimming, Group III, comprised of animals subjected to forced swimming and received duloxetine (20 mg/kg; i.p), Group IV, V and VI comprised of animals subjected to forced swimming and received lycopene (1, 2 and 4 mg/kg; oral gavage) for 15 days.

Immobility period and post-swim fatigue were assessed during schedule periods. Various behavioral parameters were assessed in mice 24 h after the last forced swim test on 15th day.

Behavioral assessment

Assessment of immobility period

Mice were individually forced to swim inside a rectangular glass jar (25×12×25 cm^3) containing 15 cm of water maintained at 22 ± 3^0C. Immobility period was recorded in last 4 min during 6 min forced swimming test with the help of stop-watch. The animal was judged to be immobile when it ceased struggling and remained floating motionless in water, making only those movements necessary to keep its head above water. The animals were forced to swim 6 min every day for total of 15 days, and the recording of immobility period was done on every alternate day [28].

Assessment of Post-swim fatigue

Post-swim activity was recorded immediately after the forced swim test. To assess post exercise fatigue, we recorded the time elapsed before mice-initiated grooming (licking and rubbing of the skin/fur) after a 6-min swim in water at 22 ± 3°C. Each mouse was removed from the forced swim test jar and placed in clear observation chamber. The time to grooming was recorded for each mouse on every alternative day [11].

Assessment of locomotor activity

Ambulatory activity was measured by using computerized actophotometer (IMCORP) for a period of 5 min. Mice were individually placed in a transparent plastic cage (30×23×22 cm^3) and were allowed to

acclimatize to the observation chamber for a period of 2 min. The locomotion was expressed in terms of total counts per 5 min per animal [1].

Assessment of grip strength in Rota-rod test

Mice were subjected to motor function evaluation by placing them individually on rota rod, which was adjusted to the speed of 25 rpm. The fall-off time was recorded for each mouse and the longest period any animal was kept on the rod was 300 [32].

Assessment of anxiety in the mirror chamber

The anxiety behavior was measured using the mirror chamber. During the 5 min test session the following parameters was noted (i) latency to enter the mirror chamber (ii) the total time spent in mirror chamber, and (iii) the number of entries animal made into the mirror chamber. Animals were placed individually at the distal corner of the mirror chamber at the beginning of the test. An anxiogenic response was defined as decreased number of entries and time spent in the mirror chamber [17].

Assessment of cognitive behavior using plus-maze test

Cognitive behavior was noted by using elevated plus-maze learning task [26]. Transfer latency (TL) that is the time taken by the animal to move from the open arm to enclosed arm, was considered as an index of learned task (memory process). The elevated plus maze consisted of two open arms (16 × 5 cm) and two closed arms (16 × 5 × 12 cm) with an open roof. The maze was elevated to a height of 25 cm from the floor. The animal was placed individually at the end of either of the open arms and the initial transfer latency was noted on the first day. If the animal did not enter an enclosed arm within 90 s, it was gently pushed in to the enclosed arm and the transfer latency was assigned as 90 s. To become acquainted with the maze, the animals were allowed to explore the plus maze for 20 s after reaching the closed arm and then returned to its home cage. Retention of the learned task was assessed 24 h after the 1st day trial and expressed as percentage of initial transfer latency.

Tail-suspension test (TST)

The total duration of immobility induced by tail suspension was measured according to the method of Steru *et al.* [33]. Mice both acoustically and visually isolated were suspended 50 cm above the floor by adhesive tape placed approximately 1 cm from the tip of the tail. The total immobility was measured for a period of 6-min with the help of stop-watch. A mouse was considered immobile, when it hangs passively and completely motionless.

Assessment of Stress-induced hyperalgesia/ allodynia

Thermal hyperalgesia: Stress-induced hyperalgesia was assessed by tail immersion test. Each mouse was placed individually in restrainer leaving the tail hanging out freely. The terminal 1 cm part of the tail was immersed in a water bath maintained at 52.5±0.5^{0}C. The withdrawal latency was defined as the time for the animal to withdraw its tail from water. A cut-off time of 15 s was used to prevent damage to the tail [29].

*Mechanical hyperalgesia:*the nociceptive flexion reflex was quantified using the Randall Selitto paw pressure device (IITC, Woodland Hills, USA), which applies a linearly increasing mechanical force (in g) to the dorsum of the mouse's hindpaw [4].

Mechanical allodynia: mice were placed individually on an elevated mesh (1 cm^2 perforations) in a clear plastic cage and adapted to the testing environment for at least 15 min. von-Frey hairs (IITC, Woodland Hills, USA) with calibrated bending forces (in g) of different intensities were used to deliver punctuated mechanical stimuli of varying intensity. Starting with the lowest filament force, von-Frey hairs were applied from below the mesh floor to the plantar surface of the hindpaw, with sufficient force to cause slight bending against the paw, and held for 1s. Each stimulation was applied 5 times with an inter-stimulus interval of 4-5s. Care was taken to stimulate random locations on the plantar surface. A positive response was noted if the paw was robustly and immediately withdrawn. Paw-withdrawal threshold was defined as the minimum pressure required to elicit a withdrawal reflex of the paw, at least one time on the five trials. Voluntary movement associated with locomotion was not considered as a withdrawal response. Mechanical allodynia was defined as a significant decrease in withdrawal thresholds to von-Frey hair application.

Biochemical Estimations

After behavioral assessment, mice were sacrificed under deep anesthesia and brains were isolated and stored at 80°C for various biochemical estimations.

Measurement of Oxidative Stress

The malondialdehyde content, a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances by the method of [36]. Tissue protein was estimated using the Biuret method. Reduced glutathione was assayed by the method of Jollow *et al.* [14]. Cytosolic superoxide dismutase activity was assayed by the method of Kono [16]. Catalase activity was assayed by the method of Claiborne [5].

Measurement of Nitrosative stress

Nitric oxide (nitrate- nitrite) by product in brain tissue was determined using the standard total nitric oxide assay kit (Assay Design, Inc. USA). Nitrate was reduced to nitrite by 3h incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Nitrite was converted to a deep purple azo compound by the addition of Griess reagent. Total nitrite/nitrate concentration was calculated using sodium nitrate as standard. Results were expressed as micromoles/mg protein.

Acetylcholinesterase activity

Cholinergic dysfunction was assessed by AChE activity. The assay mixture contained 0.01 ml of supernatant, 0.6 ml of 0.01 M sodium phosphate buffer (pH 8), 0.02 ml of acetylthiocholine iodide and 0.02 ml 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman reagent). The change in absorbance was measured at 412 nm for 5 min. Results were calculated using molar extinction coefficient of chromophore (1.36×104 M^{-1} cm^{-1}) and expressed as percentage of control group Ellman *et al.* [8].

Measurement of monoamine oxidase (MAO) enzyme Activity

The MAO activity was assessed spectrophotometrically [31]. The buffer-washed brain samples were homogenized in 10 volume of sodium phosphate buffer (0.1 M, pH 7.4) and centrifuged (Remi Instruments, Mumbai) at 15,000 g for 20 min. Pellets were discarded. Supernatant was pipetted out and used for the estimation of MAO-A and MAO-B activity. For estimating MAO-A activity, 2.75 ml Tris buffer (0.1 M, pH 7.4) and 100 µl of 4 mM 5-hydroxytryptamine were mixed in quartz cuvette which was then placed in double beam spectrophotometer (Perkin Elmer, USA). This was followed by the addition of 150 µl solution of brain homogenate to initiate the enzymatic reaction, and the change in absorbance was recorded at wavelength of 280 nm for 5 min against the blank. For estimating MAO-B activity, 2.75 ml Tris buffer (0.1 M, pH 7.4) and 100 µl of 0.1 M benzylamine were mixed in quartz cuvette which was then placed in double beam spectrophotometer. This was followed by the addition of 150 µl solution of brain homogenate to initiate the enzymatic reaction, and the change in absorbance was recorded at wavelength of 249.5 nm for 5 min against the blank containing Tris buffer and 5-hydroxytryptamine. MAO activity was expressed as percent change in activity.

Assay of cytokines

Mouse TNF-α, IL-1β and TGF-β1 ELISA

The quantifications of TNF-α, IL-1β and TGF-β1 were done with the help of instructions provided by R&D Systems Quantikine mouse TNF-α, IL-1β and TGF-β1immunoassay kit. The Quantikinemouse TNF-α, IL-1β and TGF-β1 immunoassay is a 4.5-hsolid-phase ELISA designed to measure mousse TNF-α, IL-1β and TGF-β1 levels. The assay employs the sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse TNF-α, IL-1β and TGF-β1 has been precoated in the microplate. Standards, control and samples were pipetted into the wells, and any mouse TNF-α, IL-1β orTGF-β1 present is bound by the immobilized antibody. After washing away anyunbound substance, an enzyme-linked polyclonal antibody specific for mouse TNF-α, IL-1βand TGF-β1 is added to the wells. Following a wash to remove any unbound antibody enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of mouse TNF-α, IL-1β and TGF-β1 bound in the initial steps. The sample values are then read off the standard curve. Values were expressed as mean±S.E.M.

Quantification of NF-kB p65 unit

The NF-kB/p65 ActivELISA kit (Imgenex, USA) was used to measure NF-kB-free p65 in the nuclear fraction of different brain regions. The nuclear levels of p65 may correlate positively with the activation of the NF-kB pathway. The NF-kBActivELISA is a sandwich ELISA in which free p65 is captured by anti-p65 antibody-coated plates and the amount of bound p65 is detected by adding a second anti-p65 antibody followed by alkaline phosphatase conjugated secondary antibody using colorimetric detection in an ELISA plate reader at 405nm. The results were expressed as nanograms per milligram of protein.

Caspase-3 colorimetric assay

Caspase-3, also known as apopain, is an intracellular cysteine protease that exists as a proenzyme and becomes activated during the cascade of events associated with apoptosis. The tissue lysates/homogenates can then be tested for protease activity by the addition of a caspase-specific peptide that is conjugated to the color reporter molecule p-nitroanaline (pNA). The cleavage of the peptide by the caspase releases the chromophorep NA, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cytoplasmic fraction of different brain regions is directly proportional to the color reaction. The results were expressed as percentage of control.

Estimation of corticosterone

The quantifications of corticosterone levels were done with the help and instructions provided by R&D Systems kit.

Neurotransmitters estimation

Biogenic amines (dopamine, serotonin and norepinephrine) were estimated by HPLC with electrochemical detector. Waters standard system consisting of a high-pressure isocratic pump, a 20 ml sample injector valve, C18 reverse phase column and electrochemical detector were used. Data was recorded and analyzed with the help of empower software. Mobile phase consisting of sodium citrate buffer (pH 4.5)— acetonitrile (87:13, v/v). Sodium citrate buffer consist of 10 mM citric acid, 25 mM NaH_2HPO_4, 25 mM EDTA, and 2 mM of 1-heptane sulphonic acid(Patel *et al.*, 2005). Electrochemical conditions for the experiment were +0.75 V, sensitivity ranges from 5 to 50 nA. Separation was carried out at a flow rate of 0.8 ml/min. Samples (20 ml) were injected manually. On the day of experiment frozen brain samples were thawed and they were homogenized in homogenizing solution containing 0.2 M perchloric acid. After that samples were centrifuged at 12000 g for 5 min. The supernatant was further filtered through 0.22 mm nylon filters before injecting in the HPLC injection pump. Data was recorded and analyzed with the help of empower software.

Statistical Analysis

The results were expressed as mean±S.E.M. The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by Bonferroni's Multiple Comparison Test. Two-way ANOVA followed by Bonferroni posttest was employed to discover the intergroup variation in escape latency and path length data of Morris water maze by considering day of testing and treatment as two independent variables. Statistical significance was considered at $P<.05$. The statistical analysis was done using the Graph Pad Prism version 5.0.1.statistical software.

RESULTS

Effect of lycopene on immobility time, post-swim fatigue

Chronic exposure to forced swimming produced a significant increase in immobility period in control mice, the maximum response attained on day 13. Daily administration of lycopene (1, 2 and 4 mg/kg., p.o.) for 15 days 30 min prior to forced swim test (FST) reduced immobility period as well as post-swim fatigue in dose dependent manner as assessed on alternate days, i.e., 1, 3, 5, 7, 9, 11, 13 and 15th day of the study, respectively. Duloxetine 20 mg i.p dose also showed reduction in immobility period as well as post-swim fatigue. However, lycopene 4mg dose showed almost similar reduction (Figure 1A&1B).

Effect of lycopene on locomotor activity

Chronically exposed to forced swimming animal showed an increase in the locomotor activity compared to unstressed mice suggested that anxiety. However, chronic treatment with lycopene (1, 2, 4 mg/kg, p.o. for 15 days) decreased the ambulatory scores in chronic forced swim test (Figure1C).

Effect of lycopene on grip strength in Rota rod test

Similarly, chronically stressed mice showed a significant decrease in the fall-off time as compared to unstressed mice, thus displaying muscle in-coordination. Daily treatment with lycopene (1, 2, 4 mg/kg, p.o. for 15 days) before the exposure increased the mean fall off time as compared to chronic water immersion stress group (Figure1D).

Effect of lycopene treatment on anxiety by using mirror chamber

Chronic fatigue produced anxiety response in mice as the latency to enter the mirror chamber was significantly increased (Figure2A), decreased the number of entries (Figure2B), and also decreased the mean time spent in the mirror chamber (Figure2C) as compared to unstressed mice. Daily treatment with lycopene (1, 2 and 4 mg/kg, p.o. for 15 days) reversed these responses in chronically exposed mice.

Effect of lycopene on cognitive behavior using plus-maze test

Similarly, chronic fatigue significantly decreased percent retention of memory in mice as compared to unstressed mice which was reversed by chronic administration of lycopene (1, 2 and 4 mg/kg, p.o. for 15 days) (Figure2D).

Effect of lycopene on immobility period in the tail suspension test (TST)

The mice that were exposed to chronic forced swimming for 15 days showed a significant increase in the immobility period as compared to the control mice. Treatment with lycopene refurbished these alterations by significantly reducing the immobility time in mice. (Figure2E)

Effect of lycopene on stress-induced pain threshold

Thermal hyperalgesia

Animals chronically subjected to water immersion stress showed a significant decrease in tail withdrawal latency indicating hyperalgesia as compared to unstressed mice. Chronic treatment with lycopene (1, 2, 4 mg/kg, p.o. for 15 days) significantly attenuated the development of the hyperalgesia in chronically stressed animals (Figure3A).

Mechanical hyperalgesia

Chronic forced swimming produced a significant decrease in paw-withdrawal threshold in Randall Selitto paw pressure device as compared to control group (Figure3B). Lycopene (1, 2 and 4 mg/kg), significantly and dose-dependently increased the paw-withdrawal threshold in chronic forced swimming mice.

Mechanical allodynia

In von-Frey hair test, chronic forced swimming mice showed significant increase in pain sensitivity to non-noxious stimulus as compared to control mice (Figure3C). Lycopene (1, 2and 4 mg/kg) produced significant and dose-dependent increase in paw-withdrawal threshold in response to von- Frey hair stimulation.

Effect of lycopene treatment on biochemical alterations

Malonaldehyde (MDA) and nitrite levels were significantly increased in the brain of chronic fatigue mice as compared to control group. Chronic treatment with lycopene produced significant ($p < 0.05$) and dose-dependent reduction in MDA and nitrite levels in brain of chronic forced swimming mice. Reduced glutathione levels and enzyme activity of superoxide dismutase and catalase significantly decreased in the brains of chronic forced swimming mice as compared to control group mice. This reduction was significantly and dose dependently restored by the treatment with lycopene in the brain of chronic forced swimming mice (Figure 4).

Effect of lycopene on monoamine oxidase (MAO) activity

Chronic forced swimming procedure resulted in significant increase in monoamine oxidase (MAO-A and MAO-B) enzymatic activity (Figure 5). Chronic lycopene (1, 2 and 4 mg/kg) significantly reduced MAO-A and MAO-B enzymatic activity in fatigued mice as compared to control mice. Duloxetine (20 mg/kg) did not affect MAO activity in forced swim mice.

Effect of lycopene on TNF-α, TGF-β and IL-1β level

TNF-α, TGF-β and IL-1β levels were significantly increased in the brain of chronic forced swimming mice suggesting involvement of neuroinflammation. Treatment with lycopene (1, 2 and 4 mg/kg) significantly and dose dependently inhibited these alterations (Figure6A, 6B & 6C).

Effect of lycopene on serum corticosterone levels

Forced swimming stress significantly increased the serum corticosterone levels. Treatment with lycopene (1, 2and 4 mg/kg) 30 min before forced swimming resulted in a significant decrease in corticosterone levels compared to alone stress groups (Figure6D).

Effect of lycopene treatment on NFκβ and caspase-3 activity

NF-κβ p65 subunit and caspase-3 levels were significantly elevated in chronic forced swimming mice brain. Lycopene (1, 2 and 4 mg/kg) treatment significantly inhibited enhanced NF-κβ p65 subunit expression in the nuclear fraction and caspase in dose-dependent manner (Figure 6E & 6F).

Effect of lycopene on neurotransmitter levels

Chronic forced swimming fatigue resulted into decreased levels of dopamine (41%), norepinephrine (69%) and serotonin (61%) (Figure 7) which was dose dependently replenished by lycopene (1, 2 and 4 mg/kg). Lycopene 4 mg/kg produced a significant increase in the dopamine (56%), norepinephrine (121%) and serotonin (109%) in chronic forced swimming fatigued mice brain.

DISCUSSION

CFS represents a multifactorial etiological disorder in which its specific pathophysiological mechanisms cannot be fully understood.It is an atypical manifestation of major depression. The high rates of depression in CFS might be a result of overlying symptoms, viral or immune changes, an emotional response to disabling fatigue, or alterations in brain physiology. The antidepressants drugs are commonly used for treating CFS [9].

In the present study, CFS was induced in mice by forced swimming daily for 6 min over a total period of 15 days. The results of the present study confirmed that water immersion stress induces several fatigue symptoms such as increased immobility period and post-swim fatigue, anxiety and depression. Immobility period increased to maximum on day 13 and persisted upto day 15. Moreover, chronically fatigued mice showed decreased muscle grip and increased anxiety response in mirror chamber as well as decreased pain threshold in Vonfrey hair and Randall-Selitto test. In addition to pain, animals also exhibit depression and memory impairment signs in tail suspension test and elevated plus maze test respectively. Mice treated with duloxetine (antidepressant drug) or lycopene (1, 2 and 4 mg/kg) 30 min before forced swimming for 15 days showed significant reduction in immobility period and post-swim fatigue as well as rise in mean tail withdrawal latency and increased muscle strength as compare to control group.

Oxidative stress is an emerging focus of research, in view of various findings that it contributes to the pathology and clinical symptoms of CFS. Reactive Oxygen Species (ROS) has been suggested to play a

pivotal role in the pathophysiology of CFS. Oxidative stress is caused by an increase in the generation of ROS. On the other hand, the condition may be driven by a decline in the efficiency of antioxidant enzyme systems.

Oxidative stress is marked by an elevation of the lipid peroxidation product malondialdehyde (MDA), and by a reduction of the enzymatic levels of glutathione and of superoxide dismutase (SOD). Both of the two enzymes are important factors in cellular protection [35]. Similarly, in present study, the biochemical evidences indicated 15 days of forced swimming amplified oxidative stress (increased lipid peroxidation, nitrite level as well as decreased glutathione, SOD and catalase activity).

Stress also stimulates the brain monoamine oxidase (MAO-A and MAO-B) enzyme activity which may further result in the depletion of brain monoamine levels. Various antidepressant drugs, either by inhibiting MAO enzyme or by inhibiting reuptake mechanism, increase the central monoamine levels and reverse the stress-induced depressive-like behavior [20].

In this study, animals with 15 days chronic FST exhibited increased activity of monoamine oxidase (MAO) as well as decreased serotonin, norepinephrine and dopamine levels in the brain. It has been suggested that chronic fatigue syndrome is associated with a decrease of central synaptic serotonin neurotransmitters. Serotonin is known to exert a protective effect in the hippocampus and attenuate the behavioral consequences of stress by activating 5-HT1A serotonin receptors and may work as an innate antioxidant defense mechanism in the CNS [20]. Noradrenaline is also works in protection of cortical neurons against microglial-induced cell death. It has been shown that noradrenaline can protect neurons from Aβ-induced damage, and suggest that its actions by increases in glutathione production. Intracellular reactive oxygen species (ROS) drastically reduced by treatment with noradrenaline. These studies indicate that the neurotransmitter itself acted as an antioxidant. Increased oxidative stress damage dopaminergic neurons in the brain. Patients with dopaminergic deficits show symptomatic mental fatigue resulting from a failure to maintain adequate levels of dopaminergic transmission resulting in impaired cognitive control [19].

Some other studies have shown that lycopene pretreatment can reverse LPS-induced depression-like behavior in the forced swim test and the tail suspension test in mice [38]. Chronic lycopene treatment (21 days) has been shown to prevent haloperidol-induced increase in MDA, NO, homovanillic acid (HVA), and 3,4-dihydroxyphenylacetic acid (DOPAC) levels, as well as haloperidol-induced decrease in GSH, DA, 5-HT, norepinephrine, and 5-hydroxy indole acetic acid (5HIAA) levels in the striatum [3]. Similarly, present study also suggested that lycopene may be a potential antidepressant confirmed by decreased immobility period in tail suspension test as well as decreased MAO activity and increased serotonin, norepinephrine and dopamine levels in chronic forced swimming mice brain.

Usually, the patients with chronic fatigue syndrome suffer from the comorbid anxiety or mood disorder. Long-term stressed mice enhanced sensitivity to amphetamine-induced hyperlocomotion [7]. In our study, , the locomotor activity in mice was increased, 24 h after the last episode of forced swim test, which may be due to the reason that animals are more anxious. Our findings are in concurrence with the previous report [6], here we also found that chronic stress could cause anxiety-like behavior. When exposed to the mirror chamber, the chronic forced swim animals exhibited anxiety as revealed by a decrease in the number of entries and time spent in mirror chamber. This indicates that chronic stress might considerably induce anxiety-like behavior in animals. Lycopene treatment significantly refurbished anxiety and hyperlocomotion in the chronic fatigued mice in a dose-dependent manner.

In present study, we also found a significant diminishing in memory of chronic fatigued mice in elevated plus maze test and this finding is supported by the results of Haig-Ferguson *et al.* [12], found a significant deficit in memory and attention of children with CFS. Lycopene treatment significantly increased percent initial transfer latency in elevated plus maze. Our results were correlated with previous studies stating chronic lycopene treatment (2.5 or 5 mg/kg) has been shown to reverse colchicine-induced memory impairment and increase in AchE (Prakash and Kumar, 2013).

Patients afflicted with chronic fatigue syndrome have also exhibited increased cytokine levels which may be produced in the brain in response to stressors.

Stress stimulates prostaglandin synthesis by releasing pro-inflammatory cytokines, which, in turn, initiate and modulate many aspects of the stress response, leading to the development of various behavioral symptoms associated with CFS problems such as anxiety and depression. These cytokines can activate a cascade of events, including synthesis of nitric oxide, reactive oxygen species, leukotrienes, prostaglandins, and platelet activating factor (PAF) and these might be responsible for causing nonspecific symptoms of fatigue and pain [10]. Increased levels of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6), and interferon-g (IFN-g), stimulate the production of elevated levels of inducible nitric oxide synthase (iNOS), which in turn produce increased amounts of nitric oxide (NO). Nitric oxide reacts rapidly with superoxide to form

peroxynitrite, which is a potent oxidant. Once peroxynitrite levels increased, various positive feedback mechanisms intensify to sustain these levels and a self-perpetuating vicious cycle is generated. This condition produced a chronic pathological state in response to a comparatively short-term triggering event. The raised peroxynitrite levels also cause HPA axis dysfunction and thus cause fatigue symptoms. Thus, it can be projected that pharmacological agent that attenuates this amplified peroxynitrite level or inhibits the action of iNOS may be helpful in treating CFS. Studies have shown that nuclear factor-kβ (NF-kβ) levels are augmented in chronic fatigue a patient who mediates intracellular inflammatory response in white blood cells (WBC) that activates many inflammatory genes that encode for pro-inflammatory cytokines, chemokines that selectively induce the inflammatory enzymes such as Cyclo-oxygenase-2, inducible nitric oxide synthase (iNOS) and adhesion molecules. All the released mediators thus contribute to the expression of inflammation and hyperalgesia [21].

Ordinary physical activity (commuting) was associated with significantly elevated plasma TGF-β protein concentrations, and that aerobic exercise was associated with significantly elevated serum tumour necrosis factor (TNF) protein concentrations in CFS cases compared to healthy controls [23]. In present study higher TGF-β level in chronic forced swimming group also defend previous studies.

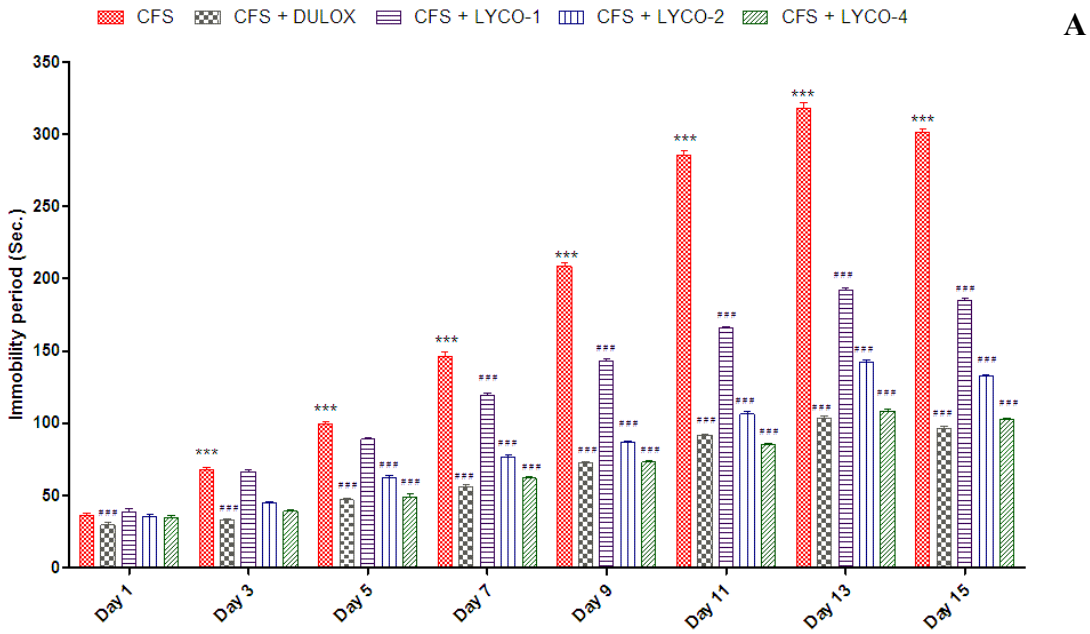
Logan and Wong (2001) discuss the role of nutritional supplements (vitamins E and C) as well as dietary modification in humans and emphasize their valuable potential in chronic fatigue syndrome. Muzandu *et al.* [23] also demonstrated that intracellular lycopene and β-carotene are capable of protecting cells against DNA damage caused by peroxynitrite formed from the simultaneous generation of superoxide and nitric oxide. Muzandu *et al.* [23] also established that lycopene and β-carotene are capable of shielding cells against DNA damage caused by peroxynitrite.

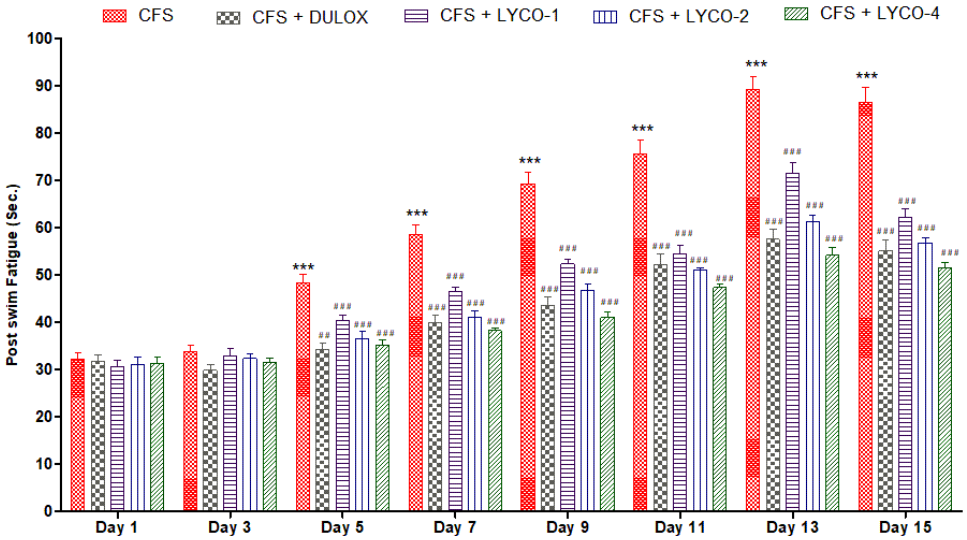
Lycopene treatment significantly reduced of neuroinflammatory markers TNF-α and IL-1β levels in striatum of haloperidol-induced orofacial dyskinesia rat which is attributed to the potent anti-inflammatory properties of lycopene. TNF-α is also acknowledged to depress food intake by a centrally mediated effect leading to weight loss [29, 7]. We also found that mice subjected to forced-swim showed considerable reduction in body weight, food and water consumption.

The current findings are further supported by results from Gouranton *et al.* [11] who found a significant decrease in pro-inflammatory cytokines on treatment with lycopene. We observed a significant increase in levels of NFkb and caspase-3 in the brain of chronic fatigued mice suggesting a possible role of apoptotic pathway. Our findings are supported by observations from Morris and Maes [22] who suggested activated NF-kb level in CFS.

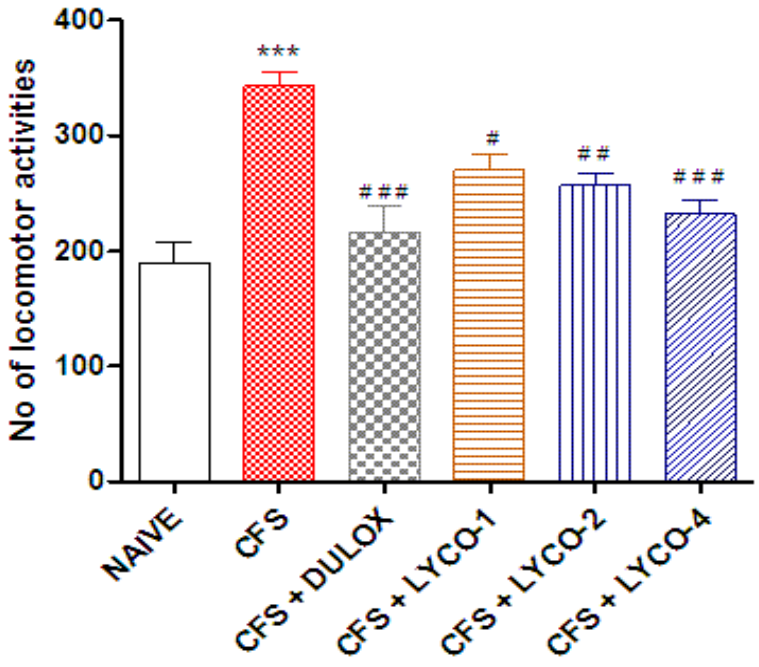
Kipp and Rivers [15] demonstrated that oxidative stress also stimulated the hypothalamic–pituitary–adrenal, resulting in increased production of corticosterone. Our findings are in concurrence with previous studies such as stress has been reported to increased corticosterone levels, which is correlated with anxiety-like behavior and painful response in human [2].

The core finding of present study is that lycopene attenuates various behavioral, biochemical and neurochemical alterations due to chronic fatigue caused by daily exposures of mice to forced swim for 15 days.




CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4
Post swim Fatigue (Sec.)
Day 1
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15

B


No of locomotor activities
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

C

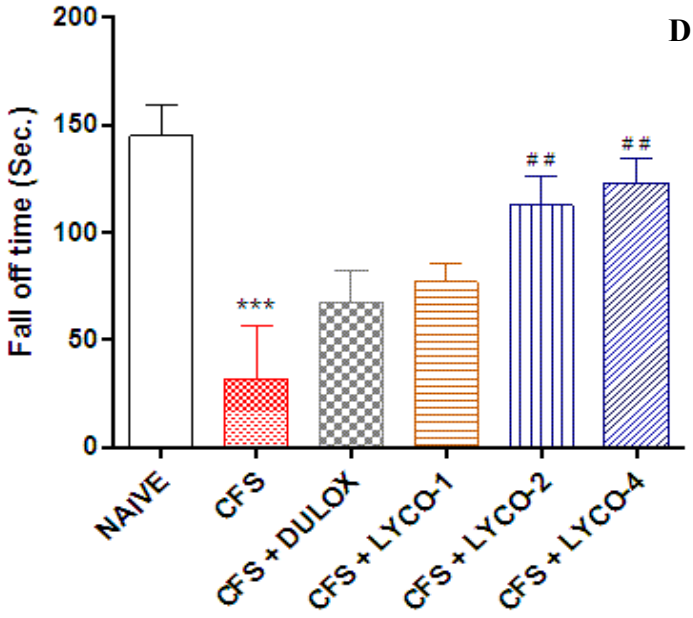
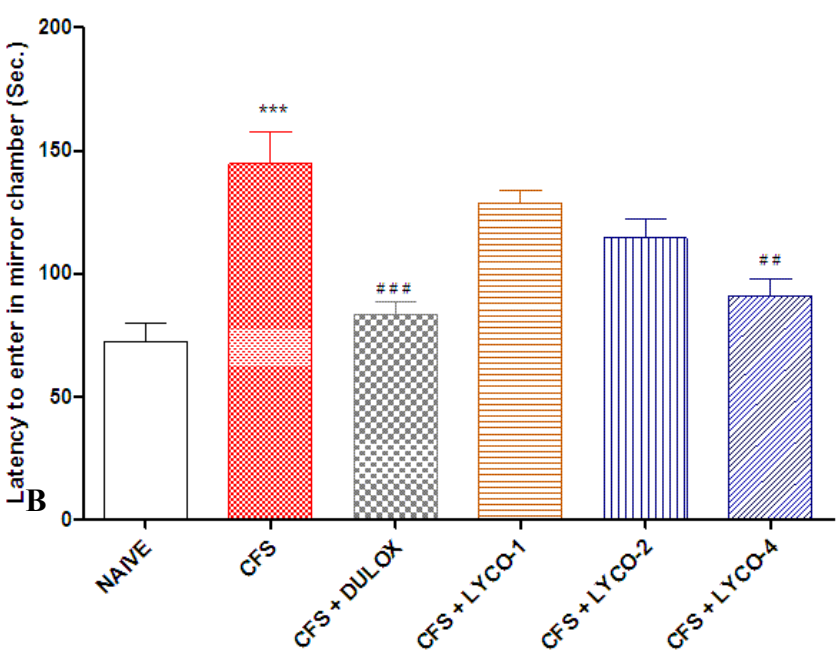
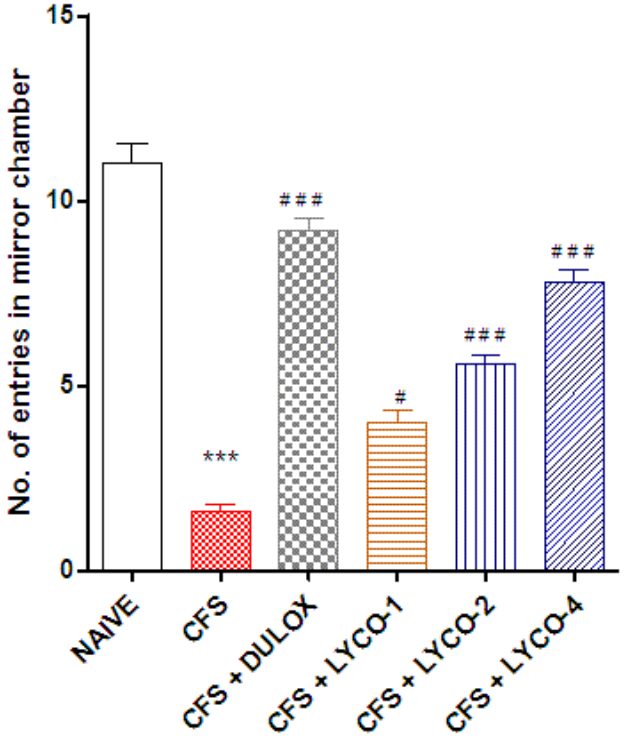
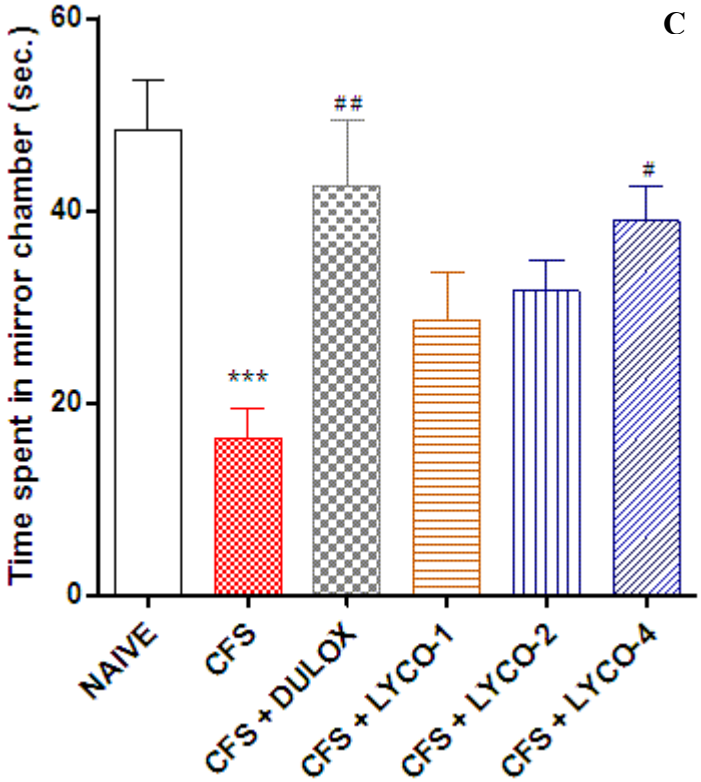


Figure 1: Effect of treatment with lycopene (1, 2 and 4 mg/kg) on immobility (A), post-swim fatigue (B), locomotor activity (C) and rota rod (B) in mouse model of chronic fatigue syndrome. Values were expressed as mean± S.E.M. The intergroup variation was measured by two-way ANOVA followed by Bonferroni posttests. CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial. *** p<0.001 as compared to day 1 CFS group; # p<0.05 as compared to CFS group on respective day; ### p<0.001 as compared to CFS group on respective day.




B
No. of entries in mirror chamber
15
10
5
0
***
###
#
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4


C
Time spent in mirror chamber (sec.)
60
40
20
0
***
##
#
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

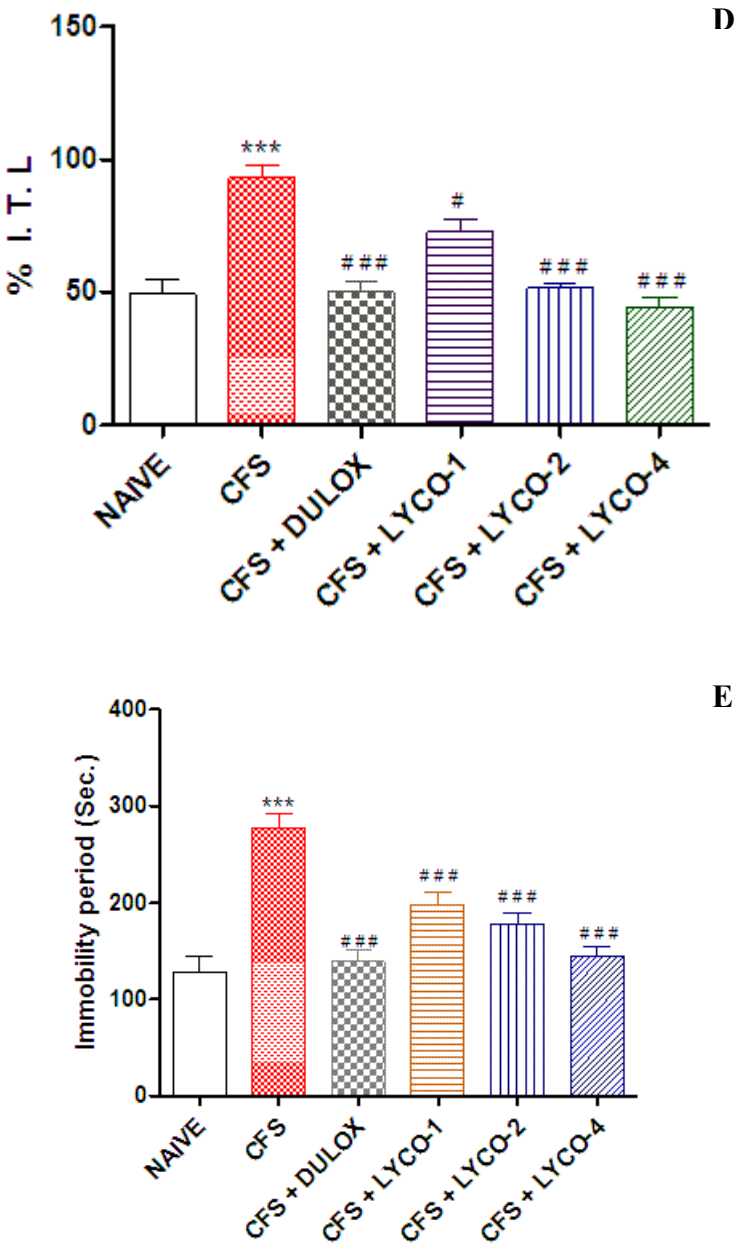
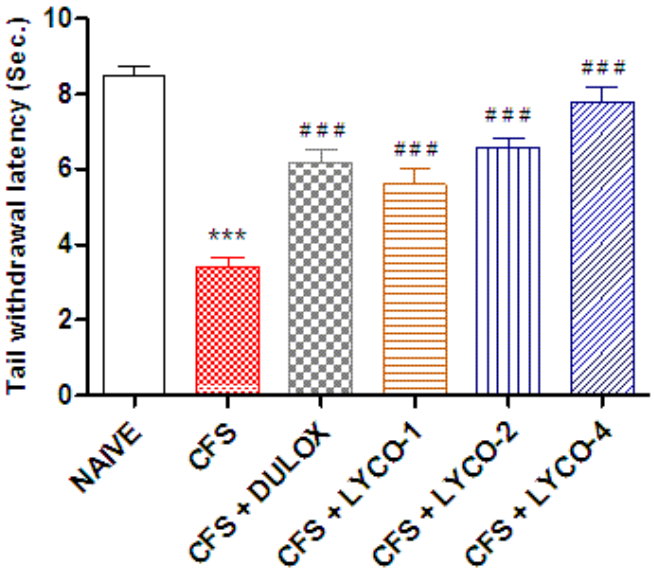
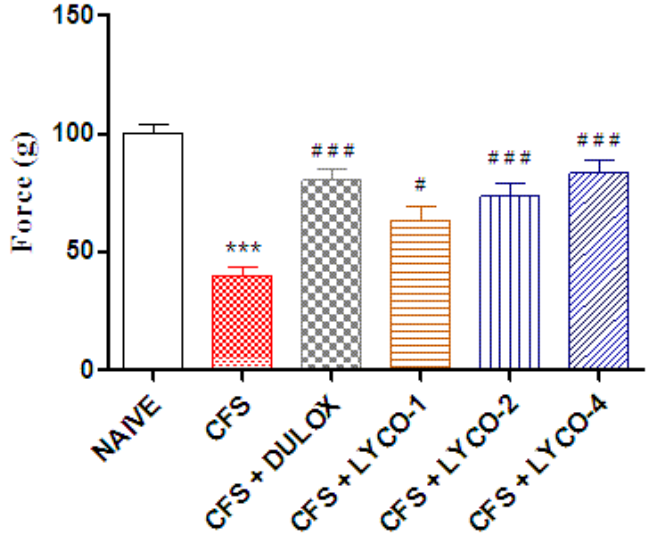


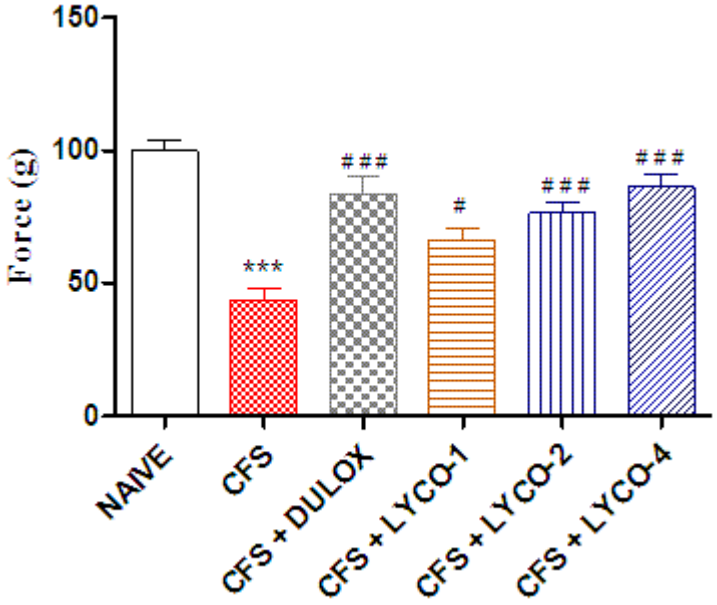
Figure 2: Effect of lycopene on latency to enter the mirror chamber (A), number of entries (B), mean time spent in the mirror chamber (C), plus maze (D) and tail suspension test (E) in mouse model of chronic fatigue syndrome.Values were expressed as mean± S.E.M.CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial.. *** $p<0.001$ as compared to day 1 CFS group; # $p<0.05$ as compared to CFS group on respective day; ### $p<0.001$ as compared to CFS group on respective day.* $p<0.05$ as compared to control group, # $p<0.05$ as compared to chronic fatigue group.


Tail withdrawal latency (Sec.)
10
8
6
4
2
0
***
###
###
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

A

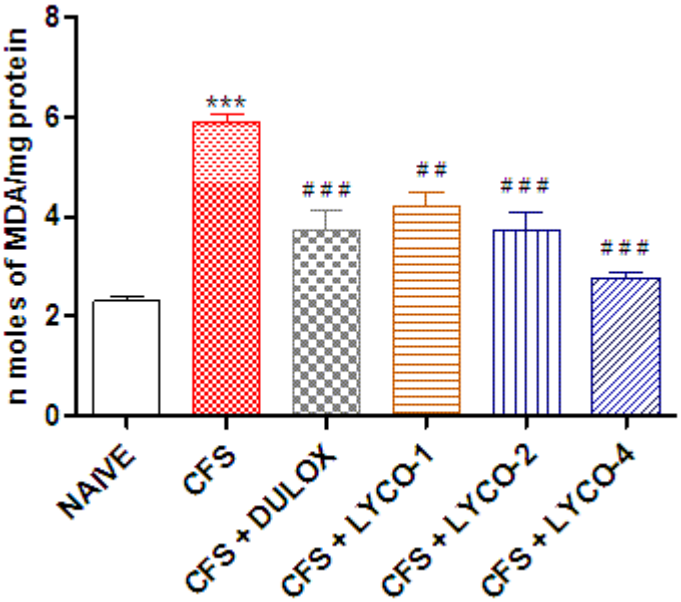

Force (g)
150
100
50
0
***
###
#
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

B



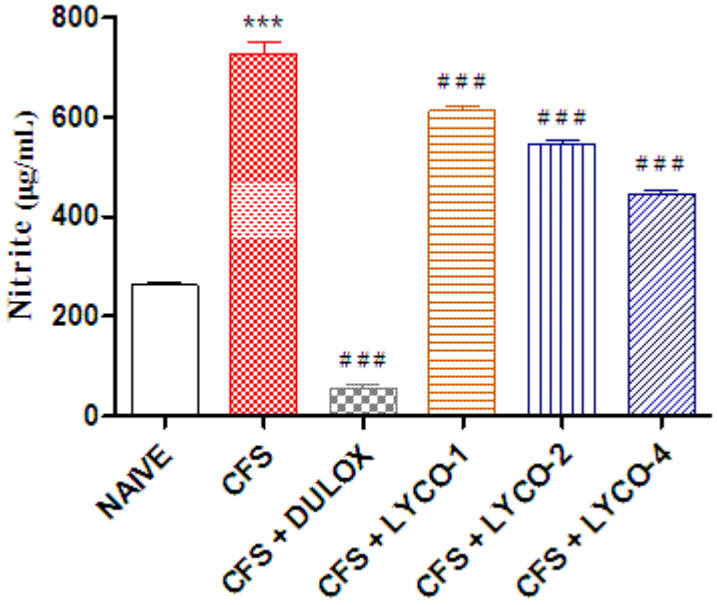
C

Figure 3 Effect of treatment with lycopene (1, 2 and 4 mg/kg) on Thermal hyperalgesia (A), Randall Selitto paw pressure device-induced mechanical hyperalgesia (B) and Mechanical allodynia by von- Frey hair(C) in mouse model of chronic fatigue syndrome. Values were expressed as mean± S.E.M.CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial. * p<0.05 as compared to control group, # p<0.05 as compared to chronic fatigue group.

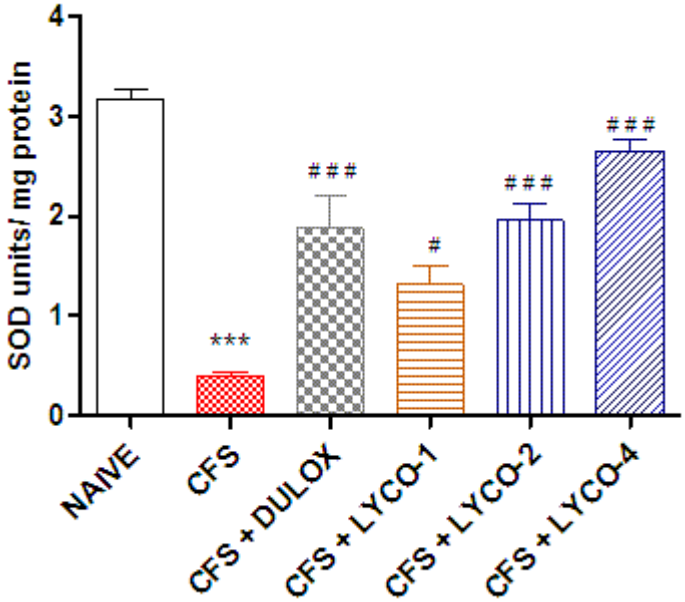


A

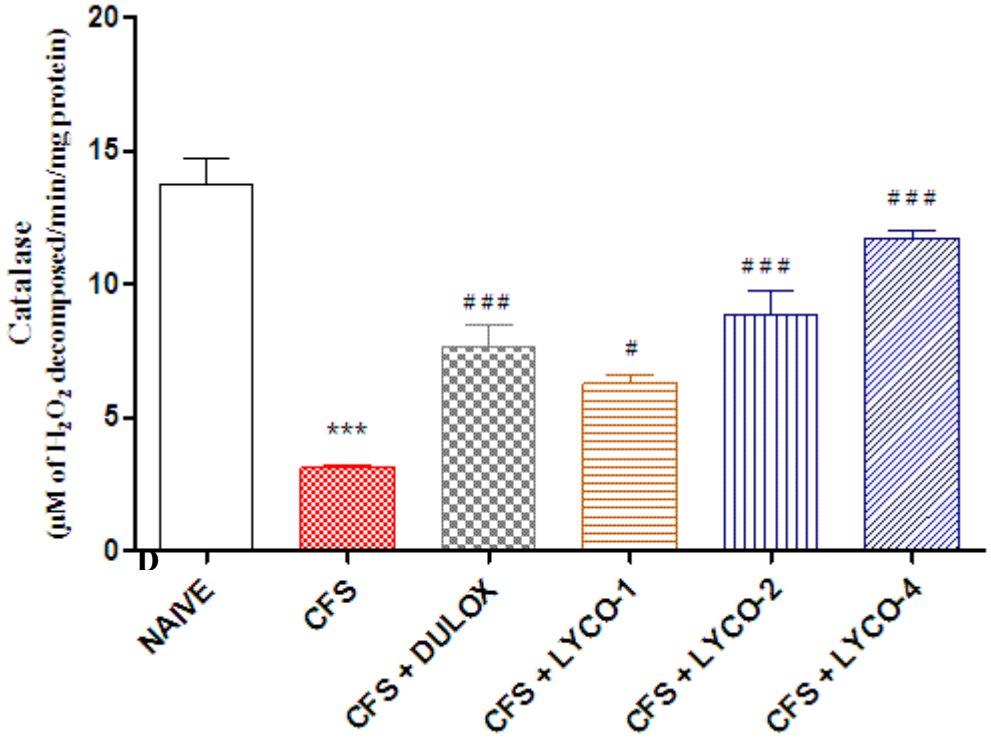
B


Nitrite (µg/mL)
800
600
400
200
0
***
###
###
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

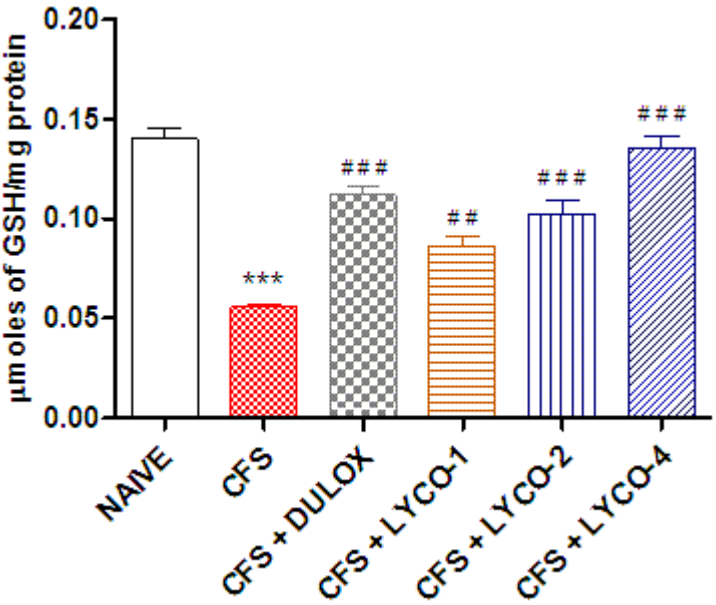
C


SOD units/ mg protein
4
3
2
1
0
***
###
#
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

D


Catalase
(µM of H_2O_2 decomposed/min/mg protein)
20
15
10
5
0
***
###
#
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

E


µmoles of GSH/mg protein
0.20
0.15
0.10
0.05
0.00
***
###
##
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

F

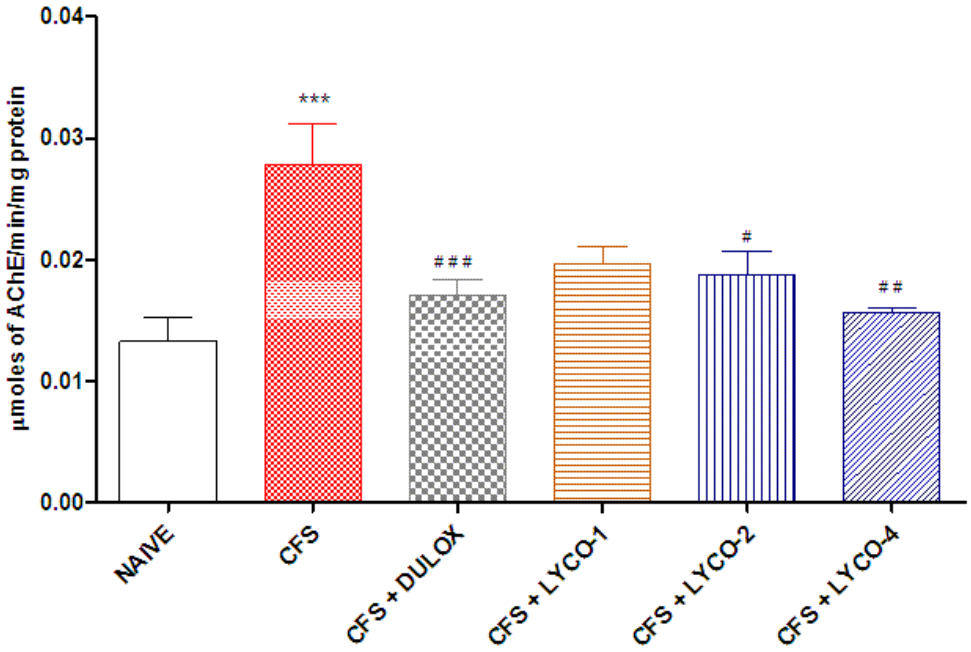
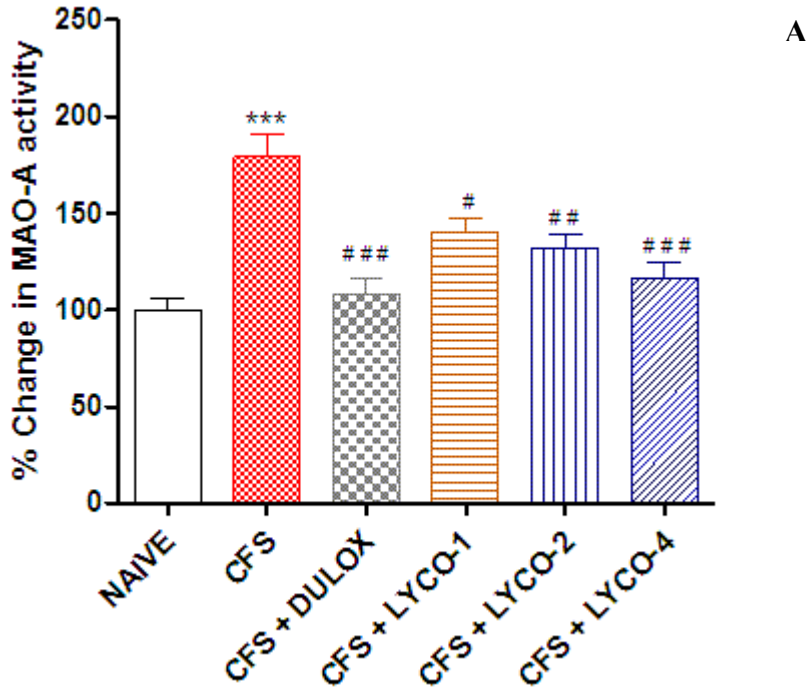


Figure 4: Effect of treatment with lycopene (1, 2 and 4 mg/kg) on lipid peroxides (A), nitrite levels (B), superoxide dismutase (C), catalase (D), reduced glutathione (E) and acetylcholine esterase (F) in mouse model of chronic fatigue syndrome. Values were expressed as mean± S.E.M.CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial. The intergroup variation was measured by one-way ANOVA followed by Tukey's test. * $p<0.05$ as compared to control group, # $p<0.05$ as compared to chronic fatigue group.

A



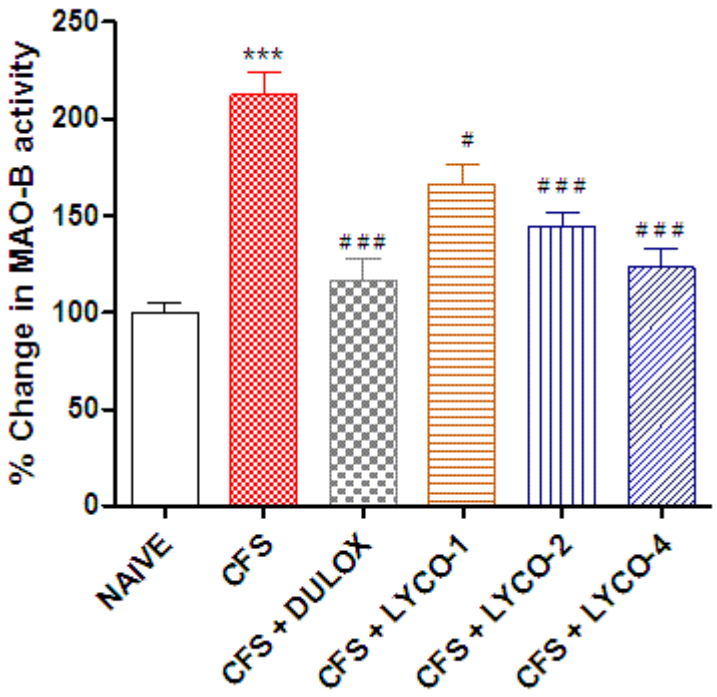
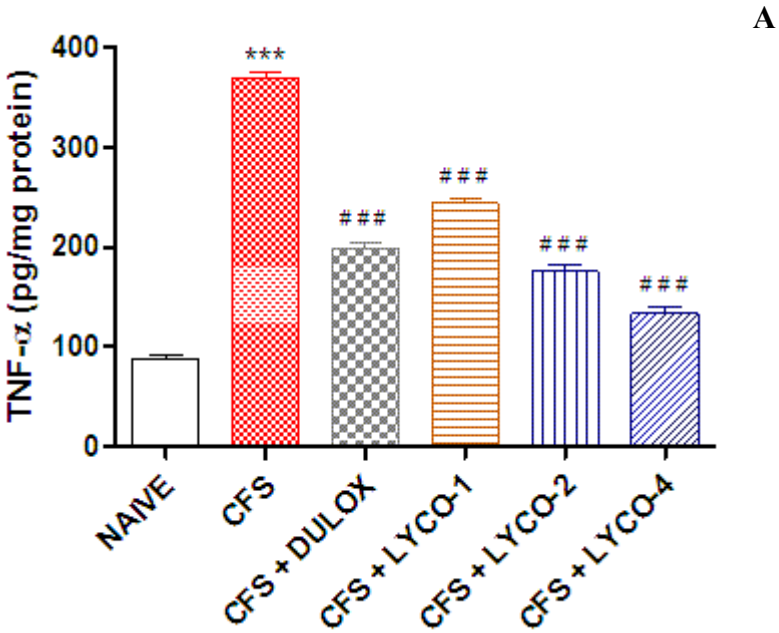
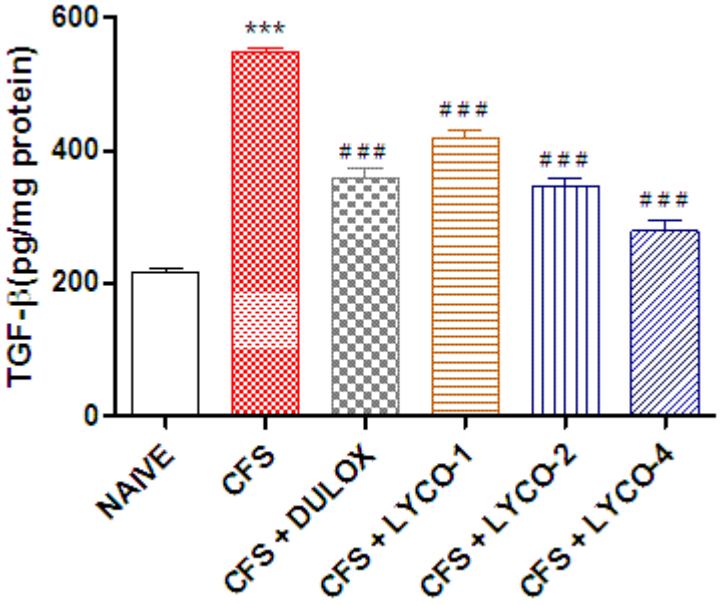


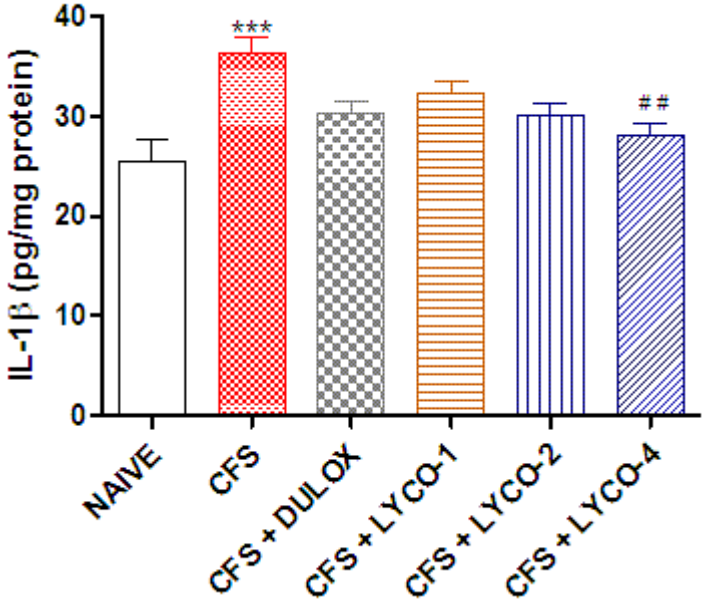
Figure 5: Effect of lycopene (1, 2 and 4 mg/kg) on MAO (A) and MAO-B enzymatic activityin mouse model of chronic fatigue syndrome. Values were expressed as mean± S.E.M.CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial.



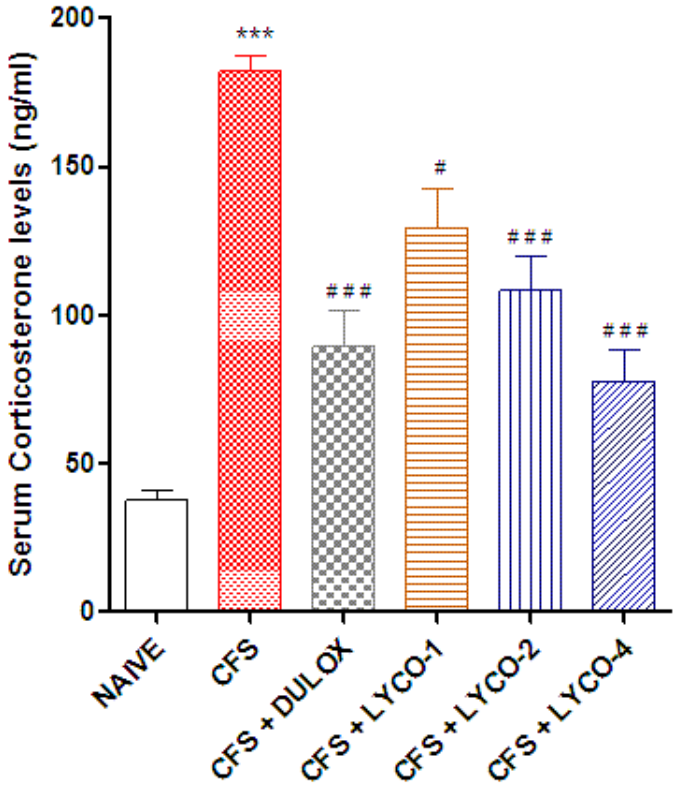
B


TGF-β(pg/mg protein)
600
400
200
0
***
###
###
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

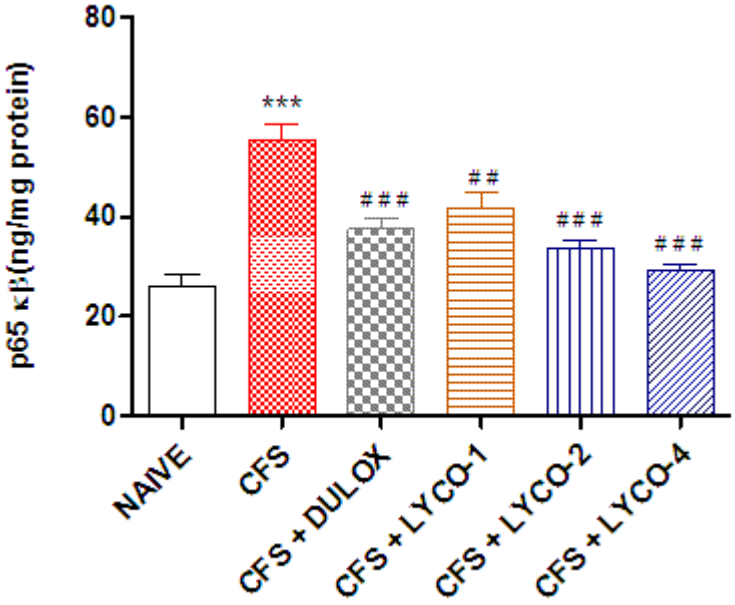
C


IL-1β (pg/mg protein)
40
30
20
10
0
***
##
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

D


Serum Corticosterone levels (ng/ml)
200
150
100
50
0
***
###
#
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

E


p65 κβ(ng/mg protein)
80
60
40
20
0
***
###
##
###
###
NAIVE
CFS
CFS + DULOX
CFS + LYCO-1
CFS + LYCO-2
CFS + LYCO-4

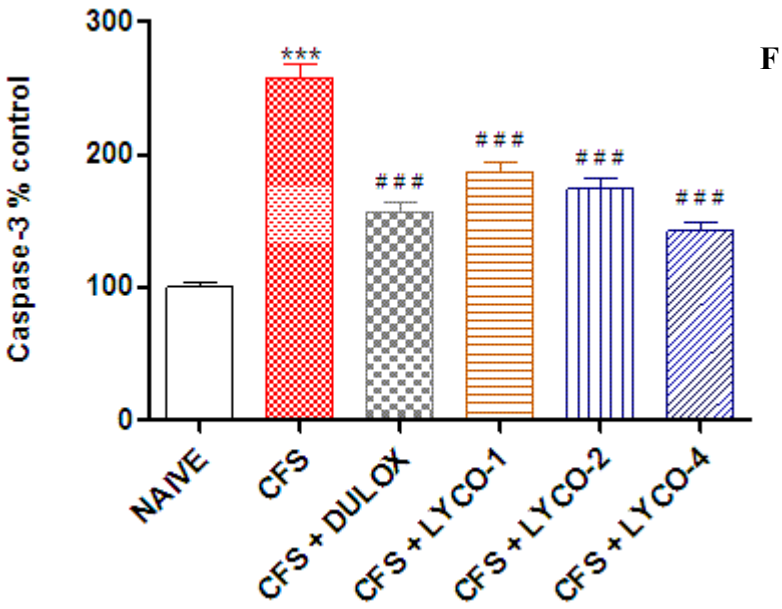
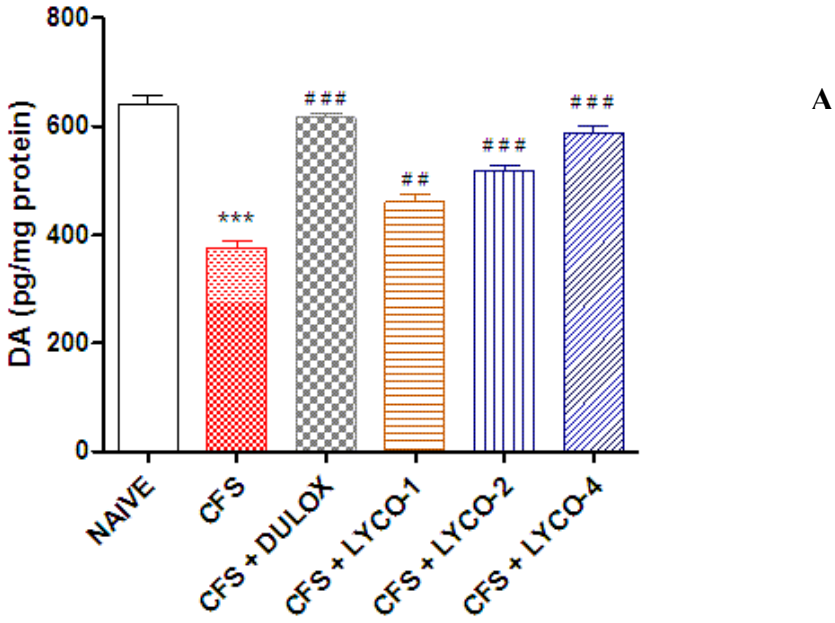
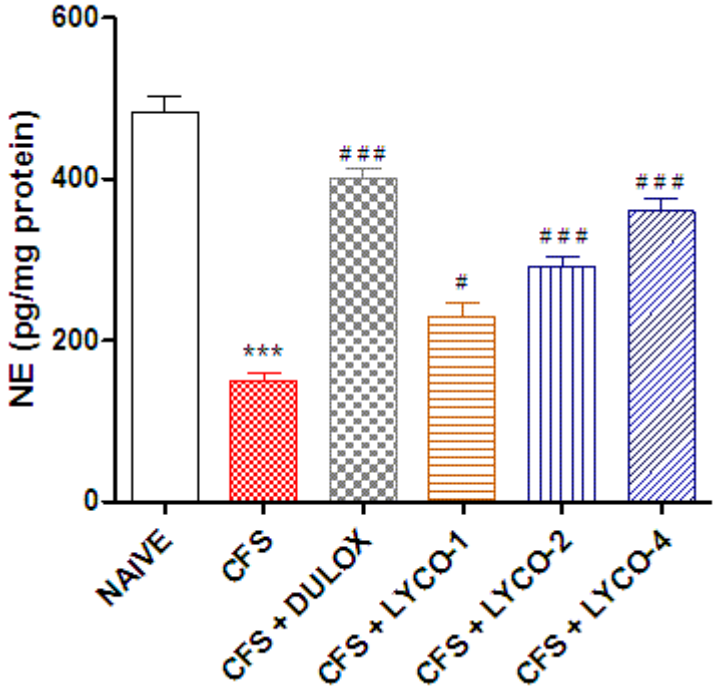
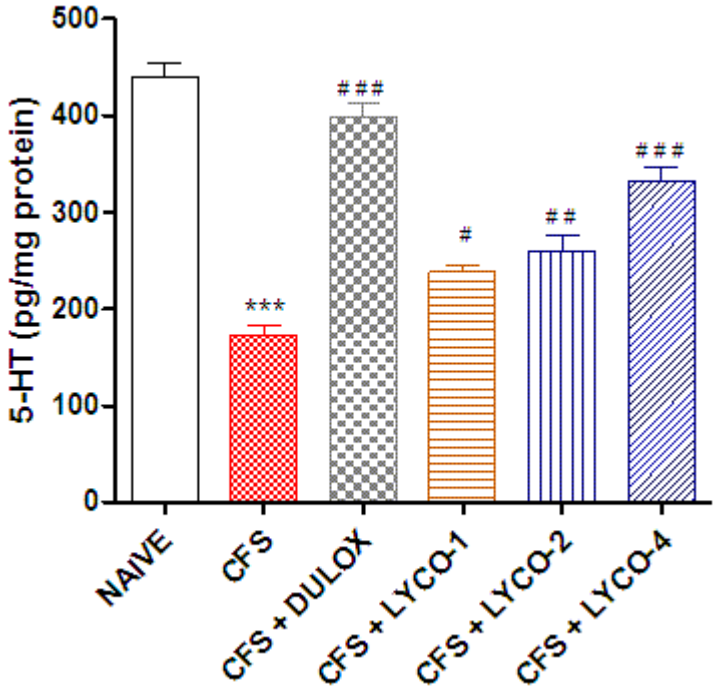


Figure 6: Effect of lycopene (1, 2 and 4 mg/kg) on TNF-α (A), TGF-β (B), IL-1β (C), corticosterone (D), NFκβ (E) and caspase-3 (F) activitylevels in mouse model of chronic fatigue syndrome. Values were expressed as mean± S.E.M.CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial.



B





C

Figure 7: Effect of lycopene (1, 2 and 4 mg/kg)treatment on brain neurotransmitter levelsdopamine (A), norepinephrine (B) and serotonin (C). Values were expressed as mean± S.E.M.CFS, Chronic Fatigue Syndrome (water immersion chronic stress); LYCO 1, lycopene 1mg/kg oral gavage; LYCO2, lycopene 2 mg/kg oral gavage; Lyco 4,lycopene 4 mg/kg oral gavage; DULOX, Duloxetine 20 mg/kg intraperitonial.

CONFLICT OF INTEREST

We have no conflict of interest.

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