

Original Article

EVALUATION OF THE PROTECTIVE EFFECT OF ETHANOLIC EXTRACT OF LEAVES OF *PUNICA GRANATUM* LINN. ON FORCED SWIMMING INDUCED CHRONIC FATIGUE SYNDROME IN MICE

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Received: 27 Jun 2016 Revised and Accepted: 24 Nov 2016

ABSTRACT

Objective: To study the protective effect of ethanolic extract of leaves of *Punica granatum* Linn. (EEPG) on forced swimming induced chronic fatigue syndrome (CFS) in mice.

Methods: Male albino mice of 25-40 grams were grouped into five groups taking 5 mice in each. Group A served as naïve control, Group B as stress control, Group C and D received EEPG at a dose of 100 mg/kg and 200 mg/kg respectively. Group E was given a standard drug (Imipramine 20 mg/kg). All animals received their respective agent orally daily for 7 d. Except for group A animals, animals in all other groups were subjected to force swimming 6 min daily for 7 d to induce a state of chronic fatigue. Animals were assessed for the duration of immobility on day 1st, 3rd, 5th and 7th. Level of anxiety (elevated plus maze and mirrored chamber test) and locomotor activity (open field test) were assessed 24 h after last force swimming which was followed by estimation of oxidative biomarkers in brain homogenate.

Results: Treatment with EEPG (100 mg/kg and 200 mg/kg) and imipramine resulted in a statistically significant ($p \leq 0.05$) reduction in anxiety and duration of immobility and there was a significant increase in locomotor activity when compared to stress control group. Significant reduction in MDA level and increase in catalase level were seen in EEPG and imipramine-treated group compared to stress control group.

Conclusion: The study confirmed that EEPG has protective action effect against experimentally induced CFS.

Keywords: *Punica granatum* Linn, Chronic fatigue, Forced swimming, Imipramine

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DOI: <http://dx.doi.org/10.22159/ijpps.2017v9i1.13712>

INTRODUCTION

Chronic fatigue syndrome also is known as myalgic encephalomyelitis was first defined in 1988 by US Centre for Disease Control and Prevention as "fatigue lasting more than 6 mo, usually acute in onset, associated with secondary symptoms and inducing 50% decreased the ability to participate in ordinary activities"[1]. CFS patients complain of a headache, gastrointestinal disturbance, paresthesia, cognitive dysfunction and neuropsychiatric problems including anxiety-like behaviour [2]. The etiology of CFS/ME is unknown. There are evidence to suggest that mitochondrial dysfunction plays a key role in CFS/ME etiology. Lowered ATP production, impaired oxidative phosphorylation and mitochondrial damage have been reported in patients with CFS/ME [3]. Studies suggest that chronic fatigue syndrome may be associated with altered carnitine homeostasis in the light of carnitine's role in mitochondrial energy production. Disturbance in carnitine is reflexive of a reduction in carnitine palmitoyl transferase 1 (CPT-1) activity, possibly a result of the accumulation of omega 6 fatty acid [4]. Also elevated levels of inflammatory mediators NFκB which regulates inflammatory and oxidative stress mediators have also been reported in CFS/ME [3]. Thus a wide range of potential mechanism has been suggested for the pathophysiological basis of peripheral muscle fatigue in CFS. However, the role of enhanced oxidative stress has been observed in many patients with CFS [5, 6]. Recent studies demonstrate that oxidative stress and NO are involved in the pathophysiology of CFS, which contributes to its pathology and appearance of clinical symptoms. CFS is reported to be initiated by different stressors including viral and bacterial infections, physical trauma, severe psychological stress, organic solvent exposure, exposure to pesticides, etc each of which increased the level of nitric oxide in the body by inducing the inducible nitric oxide synthase, iNOS. Elevated nitric oxide through its potent oxidant products such as peroxynitrites are thought to initiate a complex biochemical vicious cycle, the NO/ONOO-cycle which reflects the complex mechanism contributing to the disease

pathologies [7]. Elevated peroxynitrites level leads to mitochondrial dysfunction, Hypothalamus pituitary adrenal (HPA) dysfunction [5], single strand nicks on DNA, depletion of NAD/NADH pools, which produce lower oxygen utilisation in tissue. Also, it causes a decrease in NK cell function and other immune dysfunction [7]. In a mouse model of CFS, chronic fatigue was correlated with markers of oxidative stress [8].

Imipramine and citalopram have a neuroprotective effect against CFS induced behavioural and biochemical alterations with possible involvement of NO pathway [3]. A number of antioxidants were found to be useful in the treatment of CFS [9]. In the light of proven antioxidant nature of leaves of *Punica granatum* Linn. and the definite role of oxidative stress in the pathogenesis of CFS, this animal experimental study is performed to evaluate the potential therapeutic benefit of ethanolic extract of leaves of *P. granatum* Linn in CFS.

Punica granatum Linn (Family Punicaceae) an ancient, mystical and highly distinctive fruit, is a small tree or large shrub, grows 12-18 feet and has spiky branches. Leaves are glossy and lanced shaped. Flowers are large, red, funnellform and borne terminally on clusters of 1-5. Fruit 2 ½-5 inches wide, has tough leathery skin or rind, basically yellow or more or less overlaid with light or deep pink or rich red. The interior is separated by membranous walls into compartments packed with transparent sacs filled with juicy pink or whitish pulp. It is a native of the Himalayas in northern India to Iran but has been cultivated over the entire Mediterranean region of Asia, Africa and Europe [10].

The most therapeutically beneficial constituents are ellagic acid, ellagitannins, puniceic acid, flavonoids, anthocyanins, anthocyanins and estrogenic flavonols and flavones. Ellagic acid exhibits powerful anti-carcinogenic and antioxidant properties. In the past decade, numerous studies show antioxidant, anti-inflammatory, antiatherogenic, hypoglycemic and anti-hyperglycaemic effect focusing on the treatment and prevention of cancer, CV disease,

diabetes, erectile dysfunction, bacterial infection and antibiotic resistance, UV radiation-induced skin damage. Traditionally, the plant and rind of fruit are used for the treatment of, hepatic damage, ulcer, snake bite, infant brain ischaemia, Alzheimer's disease, arthritis, obesity, breast carcinoma and cerebral malaria [8, 10]. The leaves of *Punica granatum* Linn. are reported to have an anti-anxiety and anticonvulsant effect [8].

MATERIALS AND METHODS

Collection and authentication of plant material

Leaves of *Punica granatum* Linn were collected from the local market in Dibrugarh and identified by Dr L. R. Saikia, Professor, Department of Life Science, Dibrugarh University (Voucher specimen No DUL. Sc.460/2013). A voucher specimen was deposited in the Herbarium of the institute.

Preparation of plant extract

Leaves of *Punica granatum* Linn were collected, air-dried, powdered. About 850 grams of powder was obtained which was then packed into a Soxhlet apparatus, and extraction was done by continuous hot percolation using ethanol (95% v/v) as a solvent. The extract was concentrated using a rotary evaporator. It was further concentrated and dried in desiccators. The final yield of ethanolic extract was found to be 7.24% (W/W).

Phytochemical analysis

EEPG was subjected to qualitative phytochemical analysis of alkaloids, flavonoids, tannins, saponins, sterols, terpenoids and others as per standard methods [11].

Drugs and chemicals

Imipramine was bought from Abbott healthcare pvt. Ltd (solan). Diazepam was procured from Ranbaxy Laboratories Limited (Solan). Ethanol was procured from Merck (Mumbai, India). Hydrogen peroxide and tricarboxylic acid were procured from Sigma-Aldrich Private Limited, Bangalore. TBA reagent was bought from Himedia Laboratories Private limited.

Experimental animals

Healthy male Swiss albino mice (25-40 grams) were taken from the Central Animal House, Assam Medical College (registration no. 634/02/a/CPCSEA dated 19/05/02). The animals were housed in standard cages under standard conditions of 12 h light and dark cycle and normal room temperature. Animals were fed with normal diet and water *ad libitum*. Before starting the study permission from the Institutional Animal Ethics Committee was taken. The study was conducted according to CPCSEA guidelines.

Acute oral toxicity test

Acute oral toxicity test was done following OECD guidelines 425 (up and down Method). EEGP was found safe at 2000 mg/kg dose [12]. Two arbitrary doses 100 mg/kg and 200 mg/kg were selected for the study.

Experimental design

Animals were randomly assigned to five groups with 5 animals in each. (n=5)

Group A-Naïve animals (neither subjected to stress nor given any drug or extract)

Group B-Subjected to force swimming (to induce CFS) for 7 d (stress control).

Group C-Subjected to forced swimming+EEPG (100 mg/kg) for 7 d,

Group D-Subjected to forced swimming+EEPG (200 mg/kg) for 7 d,

Group E-Subjected to forced swimming+standard drug (imipramine 20 mg/kg) for 7 d.

Imipramine (20 mg/kg) was taken as the reference standard [13]. EEGP and imipramine were administered orally 1 hour prior to force swimming.

Induction of CFS: forced swimming

Forced swimming for 7 d is a well-validated animal model of CFS [2]. The animals were forced to swim individually in a glass jar measuring 25 × 12 × 25 cm, filled with water at room temperature (22 °C ± 3 °C). The depth of water was kept constant at 15 cm throughout the experiment. Generally, after an initial period of vigorous activity, the animals assume a typical immobile posture. During the 6 min forced swimming period, the total duration of immobility was measured. The animals were judged immobile when they ceased struggling movement of their limbs to keep their head above water. The increase in immobility period induced by continued forced swimming is considered as a situation analogous to CFS [5]. The duration of forced swimming to induce CFS is taken as 6 min daily for 7 d. Immobility period was measured on day 1st, 3rd, 5th and 7th [5, 8].

Elevated plus maze (EPM) test

This paradigm is suitable for assessing unconditioned anxiety state in rodents [14]. The elevated plus maze apparatus consisted of two covered arms (16 cm × 5 cm × 12 cm) two and two open arms (16 cm × 5 cm). The arms extended from a central platform (5 cm × 5 cm). The maze was kept at the height of 25 cm from the floor [15]. Each mouse was placed individually at the centre of the EPM with their heads facing towards the open arm. During the 5 min test, parameters observed were a) latency to enter into open arm, b) time spent in open arm [16] c) Number of entries into open arm (one entry was counted when there was all four paw entry into that arm) [13]. Readings were taken after 24 h of last forced swimming [5].

Mirror chamber test

The mirror chamber apparatus consists of a mirrored cube (30 cm × 30 cm × 30 cm) open on one side constructed of 5 pieces of the mirror with one side mirrored and opposite side painted dark brown. The container box (40 cm × 40 cm × 40 cm) has opaque black walls and white floor. Placement of the mirrored cube into the container box forms a five-centimeter corridor which completely surrounds the mirrored chamber. A sixth mirror is placed on the wall of the container box in such a way that it faces the single open side of the mirrored chamber [16]. Luminance inside mirror chamber was 100 lux and in the corridor surrounding the mirror chamber was 200 lux [17].

Each mouse was placed individually in a fixed corner outside the mirror chamber. During the 5 min test session, parameters noted were: (a) latency to enter into mirror chamber, (b) number of entry into mirror chamber, (c) total time spent in the mirror chamber and d) average time per entry (time/entry) in mirror chamber [16].

Assessment of locomotor activity: open field test

The open field apparatus was made of plywood and measured 72 cm × 72 cm with 36 cm high walls. The floor was divided into sixteen 18×18 cm squares by drawing blue lines with a marker. A central square of 18 cm × 18 cm was drawn in the middle of the apparatus. Each mouse was placed into one of the four corners of the open field, facing the centre. The mouse was then allowed to explore the apparatus for 5 min. The behaviours scored were: 1. Line crossing: numbers of the line crossed with all four limbs. 2. Rearing: number of times the mouse stood on its hind legs in the open field [18].

Assessment of oxidative stress

24 h after the last forced swimming experimental animals were sacrificed by decapitation and the whole brain was removed. 10% (w/v) brain homogenate was prepared in 0.1 M phosphate buffer at pH 7.4. The post-nuclear fraction was obtained by centrifugation of the homogenate at 1000g for 15 min at 4 °C and it was used for catalase assay. For malondialdehyde assay, brain homogenate was centrifuged at 12000g for 60 min at 4 °C [2].

Biochemical assessment of brain homogenate included the following

Catalase (CAT) assay

The CAT activity assay was carried out by Beers RF Jr *et al.* [19]. 2.5 ml of phosphate buffer (pH 7.8, 65 μM) was added to 0.1 ml of

supernatant and incubated for 30 min at 25 °C. After transferring to a cuvette, absorbance was measured at 240 nm by spectrophotometer. After that 650 µl of hydrogen peroxide solution (7.5 mmol) was added to initiate the reaction. Change of absorbance was measured for three minutes. Values were expressed as µmol of H₂O₂/min/mg of proteins.

Assessment of lipid peroxidation

Malondialdehyde (MDA) level was estimated as described by Satoh K *et al.* [20]. 75 mg of Thiobarbituric acid (TBA) was dissolved in 15% TCA. To this 2.08 ml of 0.2N HCL was added. The final volume was made up to 100 ml using 15% TCA. 3.0 ml of this reagent was then added to 0.75 ml of brain homogenate. The test tubes were then kept in a boiling water bath for 15 min. They were cooled and centrifuged for 10 min at 10,000 rpm. The absorbance of the supernatant was read against the blank at 535 nm. The results were expressed in nmol/mg of protein.

Protein estimation

Amount of protein was estimated according to Lowry *et al.* Here bovine serum albumin was used as standard [21].

Statistical analysis

The statistical significance between groups was analysed by using One-way analysis of variance (ANOVA), followed by Dunnett's

multiple comparison test. 'p' values of <0.05 were considered as significant.

RESULTS

Acute toxicity study

Acute oral toxicity test was done following OECD guidelines 425 (up and down Method). EEPG was found safe at 2000 mg/kg dose [12].

Phytochemical analysis

Phytochemical analysis of Leaves of *Punica granatum* Linn has revealed the presence of alkaloids, flavonoids, glycosides, saponins, tannins and terpenoids.

Effect of extract treatment on duration of immobility in forced swimming test

The results of Forced swimming test are shown in table 1. Duration of immobility was significantly increased on day 3rd, 5th and 7th in group B (stress control) when compared to group A (p<0.05). EEPG (both 100 and 200 mg/kg) showed significantly (P<0.05) reduction in immobility period when compared to stress control group (group B). A similar reduction in the duration of immobility was also observed in imipramine-treated animals. There was no statistically significant difference between extract and Imipramine treated animals.

Table 1: Shows effect of extract treatment on duration of immobility of chronically fatigued animals at different intervals of time during 7 d study

Group	Treatment	Duration of immobility (Sec)			
		Day 1	Day 3	Day 5	Day 7
A	Naïve	145.2±0.02	153.3±0.5	156±0.21	156.4±0.14
B	Stress control	144.3±0.93	199.7±0.2 ^a	205.3±1.2 ^a	208.8±0.14 ^a
C	EEPG 100 mg/kg	146.3±0.12	147.5±0.17 ^b	143.3±2.82 ^b	138.7±1.21 ^b
D	EEPG 200 mg/kg	145.1±0.31	141.3±0.9 ^b	136.7±0.61 ^b	131.7±0.18 ^b
E	Imipramine 20 mg/kg	147.3±1.37	139.1±1.3 ^b	133.2±0.14 ^b	129.0±0.14 ^b

All values are expressed in mean±SEM. Analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. a=p<0.05 when compared to naïve group. b=p<0.05 when compared to stress control group.

Effect of extract treatment on the level of anxiety in elevated plus maze test

The results are shown in table 2. There was a significant decrease in the number of entries into the open arm and total time spent in open arm in stress control group (Group B) when compared to naïve group (p<0.05). EEPG and imipramine treatment significantly increased the number of entries and total time spent in the open arm when compared to stress control group (Group B). There was no

significant difference between EEPG and imipramine-treated animals with regards to both these parameters.

Latency to enter into the open arm was increased in the stress control group when compared to naïve group (p<0.05). EEPG and imipramine-treated animals showed a significant (p<0.05) decrease in the latency period when compared to Group B (stress control group). There was no significant difference between imipramine and extract treated animals.

Table 2: Shows effect of extract treatment on the performance of chronically fatigued animals in elevated plus maze test

Group	Treatment	No of entries to open arm	Time spent in open arm (sec)	Latency to enter open arm (sec)
A	Naïve	4.0±0.2	31.2±1.2	112.7±0.41
B	Stress control	1.2±0.2 ^a	8.2±0.31 ^a	243.7±0.71 ^a
C	EEPG 100 mg/kg	3±0.4 ^b	25.5±0.32 ^b	106.3±0.2 ^b
D	EECF 200 mg/kg	3.6±0.4 ^b	28.7±0.13 ^b	99.2±0.11 ^b
E	Imipramine 20 mg/kg	3.8±0.4 ^b	28.9±0.21 ^b	91.1±0.61 ^b

All values are expressed in mean±SEM. Analysed by one-way ANOVA followed by Dunnett's multiple comparison tests. a=p<0.05 when compared to naïve group. b=p<0.05 when compared to stress control group

Effect of extract treatment on level of anxiety in animals tested in mirror chamber test

Results are shown in table 3. Latency to enter the mirrored chamber increased significantly (p<0.05) in stress control group compared to Group A (naïve). EEPG treated animals showed significant (p<0.05) decrease in latency to enter the mirror chamber when compared to Group B (stress control group). There was no significant difference between extract and imipramine-treated animals with regards to latency to enter the mirror chamber.

But there was a significant decrease in the number of entries, total time spent in mirrored chamber and average time per entry in Group B (stress control group) compared to Group A (naïve).

All these parameters were significantly increased (p<0.05) in EEPG and imipramine-treated animals when compared to Group B (stress control group).

There was no significant between EEPG and imipramine-treated animals with regards to these parameters.

Table 3: Shows effect of extract on performance of chronically fatigued animals in mirror chamber test

Group	Treatment	Latency (Sec)	No of entry	Total time spent in mirror chamber (Sec)	Average time spent in mirror chamber (Sec)
A	Naïve	130.4±0.2	3±0.2	41.6±0.8	13.7±1.21
B	Stress control	199.2±2.1 ^a	1.2±0.4 ^a	9.1±0.1 ^a	7.9±0.4 ^a
C	EEPG 100 mg/kg	134.4±0.3 ^b	2.6±0.2 ^b	34.7±1.04 ^b	13.1±1.2 ^b
D	EEPG 200 mg/kg	129.7±0.1 ^b	3±0.4 ^b	40.8±0.7 ^b	13.8±1.41 ^b
E	Imipramine 20 mg/kg	122.6±0.4 ^b	3±0.4 ^b	41.4±1.8 ^b	13.9±0.12 ^b

All values are expressed in mean±SEM. Analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. a=p<0.05 when compared to the naïve group. b=p<0.05 when compared to stress control group.

Assessment of locomotor activity by open field test

Results are shown in table 4. A significant decrease (p<0.05) in total line cross and rearing was seen in Group B (stress control group)

when compared to Group A (naïve). EEGP and imipramine treatment significantly increased (p<0.05) both these parameters of ambulatory activity when compared to group B (p<0.05). There was no significant difference seen between EEGP and imipramine-treated animals.

Table 4: Showing effect of extract treatment on the locomotor activity of chronically fatigued animals in open field test

Group	Treatment	Total lines crossed	Rearing
A	Naïve	69.5±2.32	10.6±1.02
B	Stress control	20.2±0.41 ^a	3.2±0.2 ^a
C	EEPG 100 mg/kg	70.8±0.62 ^b	9.2±0.03 ^b
D	EEPG 200 mg/kg	76.1±0.6 ^b	9.8±0.2 ^b
E	Imipramine 20 mg/kg	78.2±0.6 ^b	9.8±0.64 ^b

All values are expressed in mean±SEM. Analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. a=p<0.05 when compared to naïve group. b=p<0.05 when compared to stress control group

Biochemical estimation of effect of extract treatment on level of Catalase and MDA in mice brain

The results are shown in table 5. Animals of stress control group recorded a significant decrease (p<0.05) in catalase levels when compared to Naïve (group A). Significant elevation of catalase level was seen in EEGP and imipramine-treated animals when compared to Group B (stress control group). There was no significant

difference in brain homogenate catalase levels between EEGP and imipramine-treated animals.

Again MDA level was significantly high in Group B compared to Group A (P<0.05). EEGP and imipramine-treated animals showed a significant reduction in the level of MDA when compared to stress control group (Group B). There was no significant difference between EEGP and imipramine-treated animals.

Table 5: Showing effect of extract treatment on levels of Catalase and MDA in brain tissue

Group	Treatment	Catalase (μmol/min/mg of proteins)	MDA (nmol/mg of proteins)
A	Naïve	2.62±0.21	0.201±0.29
B	Stress control	1.11±0.23 ^a	0.959±0.32 ^a
C	EECF 200 mg/kg	2.82±0.26 ^b	0.315±0.36 ^b
D	EECF 400 mg/kg	2.94±0.08 ^b	0.248±0.82 ^b
E	Imipramine 20 mg/kg	3.02±0.13 ^b	0.223±0.039 ^b

All values are expressed in mean±SEM. Analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests. a=p<0.05 when compared to naïve group. b=p<0.05 when compared to stress control group

DISCUSSION

Chronic fatigue syndrome is a disorder of unknown etiology with unexplained, incapacitating and persisting physical and mental fatigue and widespread pain. Various studies have now focused on the involvement of reactive oxygen species in the pathophysiology of fatigue. There is an increase in lipid peroxidation and decreased levels of antioxidant enzymes in the brain in the mouse model of Chronic fatigue syndrome.

In the present study, we observed that continued swimming of 6 minutes daily for 7 d in the stress control group (group B) resulted in significant changes in behavioural parameters such as increased immobility period, elevation of anxiety level and decreased locomotor activity. In brain homogenate decrease in catalase and increased lipid peroxidation markers (MDA) were seen which indicates increased oxidative stress. So, we can definitely say that continuously forced swimming for 7 d produced a CFS-like condition in mice.

The method of 7 d exposure of mouse to forced swimming is a well-validated animal model of CFS [17]. In this model, mice are

chronically exposed to the aversive situation (swimming) from which there is no possibility of escape. The animals eventually stop struggling and assume a typical immobile posture that is suggestive of behavioural depression and fatigue [13]. This experimental model helps to evaluate the drugs that would be useful in counteracting CFS. In our present study chronic forced swimming for seven days significantly increased immobility period (in forced swimming test) and decreased locomotor activity (in open field test) in the stress control group. However, extract and imipramine-treated animals showed a significant increase in locomotor activity and decrease in immobility period when compared to stress control group. Pall suggests the level of the oxidant peroxynitrite is important in CFS patients. Elevated peroxynitrite causes mitochondrial dysfunction, lipid peroxidation and by way of positive feedback elevated cytokine level. The cytokines, in turn, cause the formation of nitric oxide that combines with superoxide to form the potent peroxynitrite thus continuing the cycle. Thus, a vicious biochemical cycle is initiated and maintained that produces the chronic nature of CFS. Increased fatigue seen in CFS may be attributed to mitochondrial energy metabolism dysfunction due to attack by peroxynitrite on several

mitochondrial proteins leading to oxidation of cardiolipin molecules in the inner mitochondrial membrane by superoxide, leading to lower complex I, III and IV activity. This results in lowered oxygen utilisation in the tissues [7]. Also, Peroxynitrite oxidises a compound Tetrahydrobiopterin (BH₄, a cofactor for nitric oxide synthase and also plays a role in the production of Catecholamines and Serotonin/Melatonin. Melatonin stimulates endogenous anti-oxidant enzymes and scavenges peroxynitrite. Also, it inhibits nitric oxide synthase [17]. Peroxynitrite, superoxide and nitric oxide also deplete ATP [7]. The protective action of EEPG seems to be due to the presence of polyphenols and flavonoids which scavenge peroxynitrite and superoxide radical and thus help to protect cells against oxidative damage caused by free radicals [22].

Chronic swimming also led to increasing anxiety behaviour of animals in the elevated plus maze and mirror chamber. Elevated plus maze is a sensitive behavioural test that reveals animal's neophobia or anxiety [16]. In elevated plus maze, the number of entries to open arm, total time spent in the open arm were significantly increased in the extract treated and standard treated groups when compared to stress control group. The mirror chamber method is another rapid, sensitive and quantitative method which is used to evaluate animal's anxiety. Many animals exhibit approach-avoidance conflict upon placement of a mirror within their environment [16]. In this test, latency to enter the mirror chamber was significantly increased while the number of entries to mirror chamber, total time in the mirror chamber and average time spent in the mirror chamber were significantly decreased in the stress control group.

However, extract and imipramine treatment significantly reversed these parameters when compared to stress control group. This indicates decreased the anxiety of the extract and imipramine-treated animals compared to stress control group. This anti-anxiety activity of *Punica granatum* Linn. may be attributed to the flavonoids present in the extract. Flavonoids modulate GABA_A receptors. Different flavonoids potentiate the therapeutic effect of diazepam. This synergistic effect of flavonoids is due to their binding affinity with GABA_A receptor through the high-affinity flumazenil sensitive site or low-affinity, flumazenil-insensitive site [23]. In intact animals activation of this receptor is associated with anti-anxiety actions [16].

Lipid peroxidation provides a further supply of free radicals, which initiate further peroxidation leading to the breakdown of erythrocyte membranes and oxidation of protein and DNA [13]. Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation [24]. It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine [25, 26]. Evidence of oxidative damage to DNA and lipids in the vastus lateralis muscle points towards oxidative stress in CFS [15]. Catalase is a tetrameric ubiquitous heme protein [13]. It directs degradation of hydrogen peroxide to water and molecular oxygen [27] thereby acting as an antioxidant. In our experiment, Stress control group recorded a significant decrease in catalase level and an increase in MDA level compared to Naïve group which is suggestive of oxidative stress. Treatment with imipramine and EEPG resulted in significant increase in the level of catalase and a decrease in the level of MDA when compared to stress control group. This may be attributed to the antioxidant property of the extract which protected the animals from forced swimming induced oxidative stress.

Studies in our lab and many other *in vitro* and *in vivo* studies showed that EEPG has potent antioxidant property [28-30]. The antioxidant property of *Punica granatum* Linn. may be due to the presence of phenols and flavonoids. Flavonoids are chain-breaking antioxidants, scavenges peroxynitrite and superoxide. They also lower NF-kappa B activity, help to restore Tetrahydrobiopterin (BH₄) level [9]. Thus flavonoid content of EEPG may be responsible for the beneficial effects of EEPG in CFS.

CONCLUSION

The present study concludes that the ethanolic Extract of leaves of *Punica granatum* Linn. Possesses significant protective effect against

CFS. Further study is required for isolation and identification of active constituents and to confirm exact mechanism.

ACKNOWLEDGEMENT

The authors are thankful to Mr Bipul Thakuria, Mr Mechikanta Mech and Dr Phulen Sarma for their constant encouragement and technical support.

CONFLICT OF INTERESTS

Declared none

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How to cite this article

- Tarali Devi, Swarnamoni Das. Evaluation of the protective effect of Ethanolic extract of leaves of *Punica granatum* linn. on forced swimming induced chronic fatigue syndrome in mice. *Int J Pharm Pharm Sci* 2017;9(1):207-212.