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ANTI-FATIGUE EFFECT OF D-LIMONENE ON CHRONIC FATIGUE SYNDROME IN MICE

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Abstract

The objective of the present study was to evaluate the anti-fatigue effect of d-Limonene on Chronic Fatigue Syndrome in mice. Swiss albinomice were divided into six groups(n=6); Group 1:(normal control) received normal chow diet and vehicle, Group 2: (disease control) undergone forced swim test for 6 min/day for 7 days + normal chow diet, Group 3: (standard 1) received fluoxetine (10mg/kg/day p.o.) + FST for 6min/day for 7 days, Group 4: (standard 2) received carvedilol (5mg/kg/day p.o.) + FST for 6 min/day for days, Group 5 and 6 : (drug treated)received d-Limonene enriched pellet diet2% and 4%respectively + FST for 6 min/day for 7 days. During treatment period behavioural tests (despair swim test, marble burying test, three chambered test) were performed. At the end of the treatment period all the animals were sacrificed under ether anaesthesia and the brains were excised for biochemical analysis. Induction of CFS significantly increased the immobility time, number of marbles buried, and decreased sociability and social novelty by concurrent increase in the MDA, catalase levels and decrease in GSH and SOD levels in brain. Treatment with the d-Limonene (2% and 4%) chow diet and a potent antioxidant carvedilol (5 mg/kg/day p.o.) produced a significant reduction in immobility period. Fluoxetine (10 mg/kg/day p.o.), a selective serotonin reuptake inhibitor produced a significant effect only on first and second day of its treatment. Fluoxetine affected the biochemical variables not to the same extent as other treatments. Biochemical analysis revealed that chronic swimming significantly induced lipid peroxidation and decreased glutathione (GSH) levels in the brains of mice. The mice also showed decreased levels of antioxidant defence enzymes, superoxide dismutase (SOD), and catalase. Co-administration of antioxidants carvedilol, d-Limonene significantly reduced lipid peroxidation and restored the GSH levels decreased by chronic swimming in mice. In conclusion, the present study suggests that treatment with d-Limonene significantly ameliorated the FST induced CFS and this is due to itsantioxidant property.

Keywords: Chronic fatigue syndrome; Forced swimming test; d-Limonene; Oxidative stress;Lipid peroxidation.

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INTRODUCTION

Chronic fatigue syndrome (CFS) is characterized by profound, debilitating fatigue and a combination of several other symptoms resulting in substantial reduction in occupational, personal, social, and educational status. CFS is often misdiagnosed as depression. Chronic fatigue syndrome is a condition that can't be explained by underlying medical conditions that causes persistent fatigue and tiredness as well as low motivation and diminished mood that doesn't go away with sleep or rest [1,2] . It is fundamentally a psychiatric disorder and various neuro-endocrine and immune disturbances arise secondarily to it [3]. It is a type of heterogeneous disorder of unknown etiology characterized by debilitating fatigue with associated muscle aches, tender lymph nodes, joint pains, chills and post exertional malaise (a vague feeling of body discomfort [4-6]. Fatigue is a common symptom experienced by virtually everyone during the course of their lives [7]. It is a difficult entity to characterize and define as it encompasses complex interactions between biological, psychosocial and behavioral processes and has been defined medical as 'the state, following a period of mental or bodily activity, characterized by a lessened capacity for work and reduced efficiency of accomplishment, usually accompanied by a feeling of weariness, sleepiness or irritability'. A 2003 review states that studies have reported between 7 and 3,000 cases of CFS for every 100,000 adults.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Thirty six Swiss albino mice(20-25g)were obtained from National Centre for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad, India. They were housed individually in an environmentally controlled room with 12-h light/dark cycle and had free access to food and water. After a seven days acclimatization period, they were randomly selected for different experimental groups. All the experimental procedures were carried out in accordance with committee for the purpose of control and supervision of experiments on animal (320/CPCSCEA dated 03-01-2001) guidelines. The study was reviewed and approved by the Institutional Animal Ethics Committee (GPRCP/IAEC/07/15/02/PCL/AE-8-MICE-M-36), G. Pulla Reddy College of Pharmacy, Hyderabad, India.

DRUGS AND CHEMICALS: d- Limonene and TBA (thiobarbituric acid) were obtained from Sigma Aldrich. TCA (trichloroacetic acid) and Ellman's reagent were obtained from HIMEDIA.

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Doubled distilled water, H_2O_2, EDTA, fluoxetine, carvedilol and other chemicals were obtained locally.

INDUCTION OF CHRONIC FATIGUE SYNDROME IN MICE

Swiss albino mice weighing about 20-25 g were used. Chronic Fatigue Syndrome was induced by Forced swimming test model (FST). FST was performed as six min session for seven days after half an hour of injection. The animals were forced to swim individually in a glass jar containing water at room temperature and water level was maintained constant throughout the experiment.

EXPERIMENTAL STUDY DESIGN

Swiss albino mice were divided into six groups (n=6); Group 1: (normal control) received normal chow diet, Group 2: (disease control) undergone forced swim test 6 min/day, for 7 days + normal chow diet, Group 3: (standard 1) received fluoxetine (10mg/kg/day p.o.) + FST for 6min session for 7 days, Group 4: (standard 2) received carvedilol (5mg/kg/day p.o.) + FST for 6 min session for 7 days, Group 5 and 6 : (drug treated) received d-Limonene enriched pellet diet 2% and 4% respectively + FST for 6 min/day for 7 days. During treatment period behavioral tests (duration of immobility, marble burying test, three chambered test) were performed. At the end of the treatment period all the animals were sacrificed under ether anesthesia and the brains were excised for biochemical analysis.

EXPERIMENTAL ANIMAL MODEL AND STUDY DESIGN

In order to evaluate the anti-fatigue effect of d-Limonene on Chronic Fatigue Syndrome in mice.

i. Induction of chronic fatigue syndrome in mice: Swiss albino mice weighing about 20-25 g were used. Chronic Fatigue Syndrome was induced by Forced swimming test model (FST). FST was performed as six min session for seven days after half an hour of injection. The animals were forced to swim individually in a glass jar containing water at room temperature and water level was maintained constant throughout the experiment.

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treatment period all the animals were sacrificed under ether anesthesia and the brains were excised for biochemical analysis.

BEHAVIORAL STUDIES

Duration of immobility behavior: During six minute FST session [8,9], the mice were judged immobile, when they ceased struggling and made minimal limb movements to keep the head above water level.This procedure is followed for 7 days.This chronic forced swimming produced depression and fatigue resembling CFS.

MARBLE BURYING TEST

The marble burying test is a useful model of anxiety [10], neophobia [11] and OCD. This test also have a predictive validity of testing anti-depressants [12], anxiolytics [13] and antipsychotics [14-15]. Mice are placed in an individual cages (30*20 cm).The cage is filled with wood chip bedding or husk approximately to 5cm depth, and lightly tamped down to make a flat, even surface.Now place a regular pattern of glass marbles on the surface, evenly spaced, each about 4cm apart.The total number of marbles buried (to 2/3 their depth) were counted during a period of 30min. The mice should be undisturbed.

THREE CHAMBERED TEST [16-18]

Sociability testing is a more specific study of social behavior and only focuses on the behavior of one animal towards others.A variety of neuropsychiatric disorders are characterized by disruptions in social behavior and social recognition, including depression, autism Spectrum disorders, bipolar disorders, obsessive-compulsive disorders, and schizophrenia. The apparatus is called as “Crawley’s sociability test apparatus”.Social behavior test box, where a mouse is given a choice between staying in the center chamber, spending time in the side chamber with an unfamiliar mouse (stranger 1), or spending time in the side chamber with either a novel object or a newly introduced mouse (stranger 2) during social preference tests.Stranger mice are enclosed in wire cages.(Fig:13)

The following parameters were measured:

- Duration of occupancy in center-chamber.
- Time spent in chamber with empty cage (a novel object).
- Time spent in chamber with stranger 1 (a novel mouse).
- Time spent sniffing cage with stranger 1.
- Number of entries to chamber with empty cage.

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- Number of entries to chamber with stranger 1 in cage.
- Time spent in chamber with stranger 2 (a second novel mouse).
- Time spent sniffing cage with novel stranger 2.
- Number of entries to chamber with novel stranger 2 in cage.

BIOCHEMICAL ESTIMATIONS

Estimation of malondialdehyde / thiobarbituric acid reactive substances:

TBARS level in tissue is a measure of lipid peroxidation [19].

Procedure:

- 500µl of 2% tissue homogenate in 0.15mol/L Kcl was mixed with 200µl 8.1% SDS and then incubated at room temperature for 5 min
- Add 1.5ml of 20% acetic acid (pH 3.5) & 1.5ml 0.8% thiobarbituric acid. The reaction mixture was heated at 95ºc for 90 minutes.
- Cool the mixture and add 1ml of distilled water & 5ml butanol/pyridine (15:1) solution under agitation using a vortex.
- This solution was centrifuged at 1000xg for 15min & the resultant colored layer was separated and measured at 532nm using spectrophotometer.

Calculations:

The concentration of MDA = $\frac{Absorbance}{L} * \frac{1}{\varepsilon} * D$

L: Light path (cm)

ε : Extinction coefficient (1.56×10^5 M^{-1} .Cm^{-1})

D: Dilution factor

Units: nmol of MDA/ mg tissue.

MEASUREMENT OF CATALASE ACTIVITY

Catalase activity of pancreatic tissue was measured by the method of Aebi (1984)[20].

Procedure

- 0.1ml of 2% tissue homogenate in 0.1M phosphate buffer pH 7.4was added to cuvette containing 1.9ml of 50mM phosphate buffer (pH 7.0)
- Reaction was started by the addition of 1.0ml of freshly prepared 30mM H_2O_2.
- The absorbance was read at 15 and 30 seconds at 240 nm.

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Calculations

Activity of Catalase (K/ml) = $\frac{Vt}{vs} * \frac{2.303}{\Delta t} * log \frac{A1}{A2} * 60$

Δt: (time 2 - time 1) = 15 seconds.

A_1: First absorbance at 15 seconds.

A_2: Second absorbance at 30 seconds.

V_t: Total volume = 3 ml.

Vs: Sample volume = 2 ml.

K: First order rate constant. It expresses the activity of Catalase.

Units: K/ml = Specific activity.

MEASUREMENT OF SOD ACTIVITY

SOD level was measured in pancreas using the method described by Misra et al.(1972)[21].

Procedure

- 0.5ml of supernatant (2% tissue homogenate in 0.1 M phosphate buffer, pH 7.4) was taken.
- 1.5ml of carbonate buffer (pH 10.2) was added.
- 0.5ml of 0.1mM EDTA was added.
- 0.4ml of epinephrine was added just before taking the optical density.
- Measure the optical density spectro photometrically at 480 nm.

MEASUREMENT OF GLUTATHIONE

Reduced glutathione (GSH) level was measured in pancreas using the method described by Ellman (1959)[22].

Procedure:

- Tissue was taken and homogenized in 0.1M phosphate buffer pH 7.4.
- The homogenate was added with equal volume of 20% trichloro acetic acid containing 1mM EDTA to precipitate the tissue proteins.
- Mixture was allowed to stand for 5 minutes.
- Centrifugation was done at 200 rpm for 10 minutes.
- Supernatant (200 µl) was taken in fresh tubes
- 1.8ml of the Ellman's reagent (0.1mM) was prepared in 0.3M phosphate buffer with
- 1% sodium citrate solution.
- The test tubes were made up to 2 ml.

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- After completion of total reaction, solution was read at 412 nm against blank.

Calculations

The concentration of GSH = $\frac{Absorbance}{L} * \frac{1}{\varepsilon} * D$

L: Light bath (cm)

ε : Extinction coefficient (14150 M^{-1} .Cm^{-1})

D: Dilution factor.

Units: µmol / gm tissue

STATISTICAL ANALYSIS

Results are expressed as mean ± SEM. Statistical Analysis was performed by one way ANOVA followed by Dunnett's as post hoc test using Graph pad Prism5 for comparison of more than two groups. Differences were considered to be statistically significant when$p< 0.05$.

RESULTS

BEHAVIOURAL STUDIES

Duration of immobility in chronic fatigue syndrome mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. As shown in table 1 FST for 6 min session per day for 7 days produced depression and fatigue in disease control animals resembling CFS, as indicated by increase in the duration of immobility in disease control groups. Treatment with fluoxetine once daily prior to the exposure to FST significantly reduced the immobility time on first and second day, but the effect disappeared on other days with a little effect on last day. Carvedilol and d-Limonene when administered once daily prior to the exposure to FST significantly reduced the time of immobility on all days of the treatment. Marble burying behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. As shown in table 2 Mice exhibit a spontaneous behaviour of burying aversive materials present in their environment, being characterised as a defensive behaviour reflecting the anxiety of animals. The marble burying assay is characterised by evaluating the animal behaviour in relation to attempt to bury potentially dangerous objects, and this is reduced or suppressed by action of anxiolytic drugs. Induction of CFS significantly increased number of marbles buried in specific period of time (30 min), when compared to normal control group. Treatment with d-Limonene (4% and 2%w/w), significantly decreased the number of marbles buried when compared to disease control group. However, these changes were restored to normal values in 4% fed limonene group and carvedilol treated group. Fluoxetine treated group didn't

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show much significant change in the number of marbles buried indicates that the use of potent antioxidants may be more beneficial in treatment of CFS than antidepressant drugs. Sociability and social novelty behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. Sociability testing is a more specific study of social behaviour and only focuses on the behaviour of one animal towards others.The first session of the test allows estimation of socialaffiliation and motivation of the subject mouse. If animal spends significantly more time in the compartment with Stranger 1 compared to the compartment with empty cup, indicating normal sociability, social motivation and affiliation. If there is no significant difference between the time spent with empty cup compared to the cup containing stranger, this indicates impaired sociability in these animal models.As shown in the tables 3a, 3b andfigures: 19, 20,upon FST the time spent in the chamber and sniffing frequency with stranger 1 decreased when compared to the normal group mice. Treatment with d-Limonene (4% and 2%), significantly increased the time spent and sniffing frequency when compared to disease control group.The second session of the test is designed to estimate social novelty and social memory. If animal spends tends to spend more time with the newly encountered mouse, indicating intactsocial memory and predilection for novel experiences. If there is no significant difference between the times spent with the newly encountered mouse and familiar mouse that indicates decreased social motivation and novelty.As shown in tables3c, 3d andfigures: 21, 22, upon FST disease control mice significantly decreased the time spentand sniffing frequency in comparison with normal control group was observed. Treatment with d-Limonene (4% and 2%), significantly increased the time spent and sniffing frequency when compared to disease control group.However, these changes were restored to normal levels in 4% fed limonene group and carvedilol treated group. Fluoxetine treated group didn't show much significant changes.

BIOCHEMICAL PARAMETERS

Whole brain level of MDA in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. As shown in the table 4 and figure: 1, Induction of CFS significantly increased MDA levels in whole brain of disease control group when compared to normal control group. Treatment with d-Limonene (4% and 2%), significantly decreased MDA levels when compared to disease control group. However, these changes were restored to normal levels in 4% fed limonene group and carvedilol treated group. Fluoxetine treated group didn't show much significant changes in the enzyme levels. Whole brain

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level of GSH in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. As shown in the table 4 and figure: 2, Induction of CFS significantly decreased GSH levels in whole brain of disease control group when compared to normal control group. Treatment with d-Limonene (4% and 2%), significantly increased GSH levels when compared to disease control group. However, these changes were restored to normal levels in 4% fed limonene groupand carvedilol treated group. Fluoxetine treated group didn't show much significant changes in the enzyme levels. Whole brain level of SOD in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. As shown in the table 4 and figure: 3,Induction of CFS significantly decreased SOD levels in whole brain of disease control group when compared to normal control group. Treatment with d-Limonene(4% and 2%), significantly increased SOD levels when compared to disease control group. However, these changes restored to normal levels in 4% fed limonene groupand carvedilol treated group. Fluoxetine treated group didn't show much significant changes in the enzyme levels. Whole brain level of CATALASE in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days. As shown in the table 4 and figure: 4, Inductionof CFS significantly increased Catalase levels in whole brain of disease control group when compared to normal control group. Treatment with d-Limonene (4% and 2%), significantly decreased Catalase levels when compared to disease control group. However, these changes restored to normal levels in 4% fed limonene groupand carvedilol treated group. Fluoxetine treated group didn't show much significant changes in the enzyme levels.

Table 1: Duration of immobility in chronic fatigue syndrome mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

DAYS	Disease	d-Limonene 2%	d-Limonene 4%	Fluoxetine	Carvedilol
Duration of immobility (in sec)					
Day1	47.83±1.11	63.16±1.94*	65.3±2.44*	61.83±1.48*	65.33±1.03*
Day2	93.33±2.84	85.16±2.33*	74±1.69*	74.66±1.78*	65.5±1.83*
Day3	198.3±13	99±9.34*	90.5±10.22*	70.16±10.39*	52.1±3.18*
Day4	234.6±3.46	94.83±1.24*	84.6±1.71*	68.5±2.15*	37.16±2.23*
Day5	165.33±12	77.83±3.5*	62.33±4.87*	99±7.12*	38.33±3.01*
Day6	241.16±2.32	57.66±1.41*	52.83±1.69*	104.66±1.97*	41.16±1.54*
Day7	270.33±2.37	49.33±1.09*	39.33±1.26*	111.83±2.05*	34.66±1.12*

Data expressed as mean ± SEM (n = 6).

Data is analyzed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means. *$p<0.001$ when compared to disease control.

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Table 2: Marble burying behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Groups	Treatment	No. of marbles buried during 30 min period on 7th day
N.C	Vehicle	10.78±0.58
D.C	Vehicle + FST 6 min/day for 7 days	20.42±0.82 [a]
d-Limonene 2%	d-Limonene 2%(pellet feed) + FST 6min/day	16.54±0.74 [α,b]
d-Limonene 4%	d-Limonene 4%(pellet feed) + FST 6min/day	12.60±0.67 [β]
Fluoxetine	10mg/kg (p.o.) + FST 6 min/day	17.45±0.46 [γ,b]
Carvedilol	5mg/kg (p.o.) + FST 6 min/day	12.95±0.77 [α]

Data expressed as mean ± SEM (n = 6).
Data is analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.
[a]p<0.001, [b]p<0.01 when compared to normal control.
[α]p<0.001, [β]p<0.01, [γ]p<0.05 when compared to disease control.

Table 3a: Sociability behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Chambers	Normal	Disease control	d-Limonene 2% (pellet feed)	d-Limonene 4% (pellet feed)	Fluoxetine (10 mg/kg p.o.)	Carvedilol (5 mg/kg p.o.)
Time spent in each chamber (in sec) during 10 min period.						
Stranger 1	360±0.85	100±0.11[c]	240±0.62[b,β]	360±0.75[α]	290±0.85[b,β]	375±0.76[α]
Centre	120±0.57	240±0.66[a]	150±0.51[b,β]	130±0.49[γ]	140±0.51[b,β]	110±0.42[γ]
Empty	120±0.61	260±0.71[a]	110±0.64[b,β]	110±0.55[γ]	170±0.59[b,β]	115±0.61[γ]

Data expressed as mean ± SEM (n = 6).
Data is analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.
[a]p<0.001, [b]p<0,01, [c]p<0.05 when compared to normal control.
[α]p<0.001, [β]p<0.01, [γ]p<0.05 when compared to disease control

Table 3b:Sniffing behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Groups	Treatment	Sniffing frequency
N.C	Vehicle	4±0.59
D.C	FST for 6 min	1±0.23 [b]
d-Limonene 2%	2%(pellet feed) + FST 6min	2±0.47 [a,β]
d-Limonene 4%	4%(pellet feed) + FST 6min	3.6±0.56 [α]
Fluoxetine	10 mg + FST 6 min	2.6±0.49 [a,β]
Carvedilol	5mg + FST 6 min	3.8±0.57 [α]

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Data expressed as mean ± SEM (n = 6).

Data is analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.

[a]p<0.001, [b]p<0,01 when compared to normal control.

[α]p<0.001, [β]p<0.01 when compared to disease control.

Table 3c: Social novelty behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Chambers	Normal	Disease control	d-Limonene 2% (pellet feed)	d-Limonene 4% (pellet feed)	Fluoxetine(10 mg/kg p.o.)	Carvedilo(5 mg/kg p.o.)
Time spent in each chamber (in sec) during 10 min period.						
Stranger 1	170±0.85	230±0.11 [a]	140±0.62 [b,β]	160±0.75 [β]	135±0.85 [b,β]	165±0.76 [β]
Centre	100±0.57	240±0.66 [a]	150±0.51 [c,β]	110±0.49 [γ]	150±0.51 [c,β]	105±0.42 [γ]
Stranger 2	330±0.61	130±0.71 [c]	310±0.64 [a,β]	330±0.55 [α]	315±0.59 [a,β]	330±0.61 [α]

Data expressed as mean ± SEM (n = 6).

Data is analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.

[a]p<0.001, [b]p<0,01, [c]p<0.05 when compared to normal control.

[α]p<0.001, [β]p<0.01, [γ]p<0.05 when compared to disease control.

Table 3d: Sniffing behaviour in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Groups	Treatment	Sniffing frequency	
		Stranger 1	Stranger 2
N.C	Vehicle	4±0.43	7±0.96
D.C	FST for 6 min	1±0.21 [c]	2±0.48[c]
d-Limonene 2%	2%(pellet feed) + FST 6min	2±0.49[b,β]	4.3±0.55[b,β]
d-Limonene 4%	4%(pellet feed) + FST 6min	4±0.43[β]	6.7±0.86[γ]
Fluoxetine	10 mg + FST 6 min	1.8±0.48[b,α]	4.8±0.67[b,β]
Carvedilol	5mg + FST 6 min	4±0.44[β]	6.8±0.89 [γ]

Data expressed as mean ± SEM (n = 6).

Data is analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.

[b]p<0,01, [c]p<0.05 when compared to normal control.

[α]p<0.001, [β]p<0.01, [γ]p<0.05 when compared to disease control.

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Table 4: Whole brain levels MDA, GSH, SOD, CATALASE in normal and chronic fatigue syndromeinduced mice treated with vehicle, d-Limonene (4% and 2%), carvedilol (5mg/kg p.o.), and fluoxetine (10mg/kg p.o.) for 7 days.

Groups	Treatment	Brain MDA (nmol/mg tissue)	Brain GSH (µmol/g tissue)	Brain catalase (K / ml)	Brain SOD (µmol/g tissue)
N.C	Vehicle	14.64±0.32	11.1±0.16	11.54±0.07	4.84±0.43
D.C	FST 6 min	24.23±0.61[a]	4.81±0.06 [a]	16.1±0.1[a]	1.33±1.27 [b]
Limonene 2%	2%(pellet feed) + FST 6min	11.13±0.6 [α]	8.9±0.17 [α]	10.21±0.06 [β,b]	2.05±0.41 [β,b]
Limonene 4%	4%(pellet feed) + FST 6min	8.93±0.4 [α]	10.8±0.17 [β,a]	11.48±0.07 [α]	4.18±0.55 [α]
Fluoxetine	10 mg + FST 6 min	10.93±0.42 [α,a]	8.3±0.17 [β,a]	10.01±0.06 [β,b]	2.11±0.55 [β,c]
Carvedilol	5mg + FST 6 min	8.12±0.8 [α]	10.62±0.17 [α]	11.41±0.05 [α]	4.39±0.57 [α]

Data expressed as mean ± SEM (n = 6).
Data analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.
[a]p<0.001, [b]p<0.01, [c]p<0.05 when compared to normal control.
[α]p<0.001, [β]p<0.01 when compared to disease control.

BIOCHEMICAL ESTIMATIONS

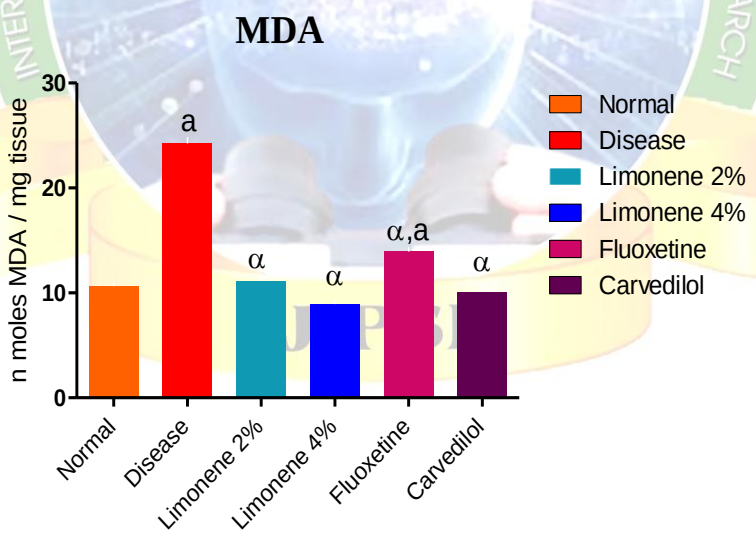

Fig. 1: Whole brain levels of MDA in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Data is expressed as Mean ± SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.
[a]p<0.001 compared to normal control group;
[α]p<0.001 compared to Disease control group.

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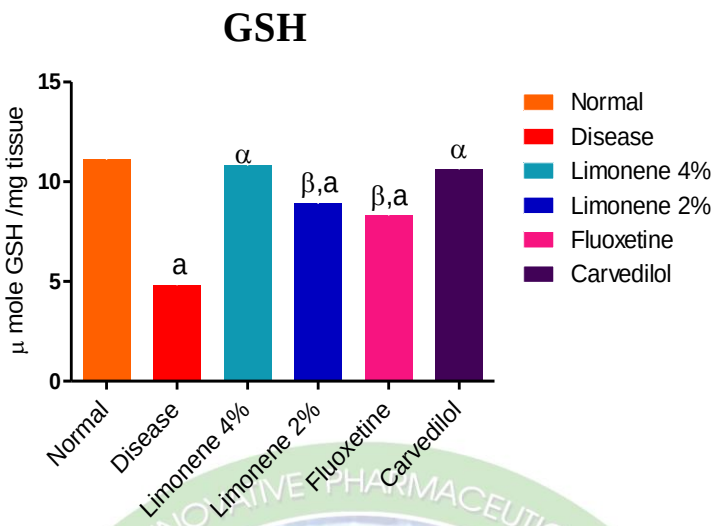

Fig.2: Whole brain levels of GSH in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Data is expressed as Mean ± SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.
[a]p<0.001 compared to normal control group;
[α]p<0.001, [β]p<0.01 compared to Disease control group.

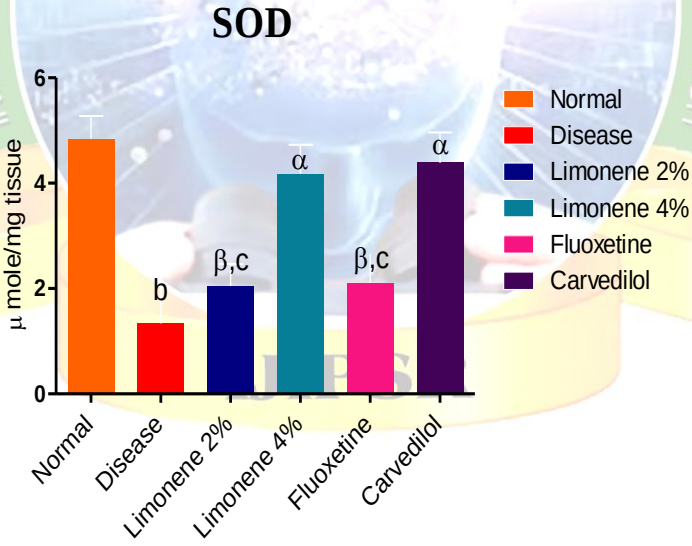

Fig. 3: Whole brain levels of SOD in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Data is expressed as Mean ± SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Dunnett's test for the comparison of means.
[b]p<0.01, [c]p<0.005compared to normal control group;
[α]p<0.001, [β]p<0.01, compared to Disease control group.

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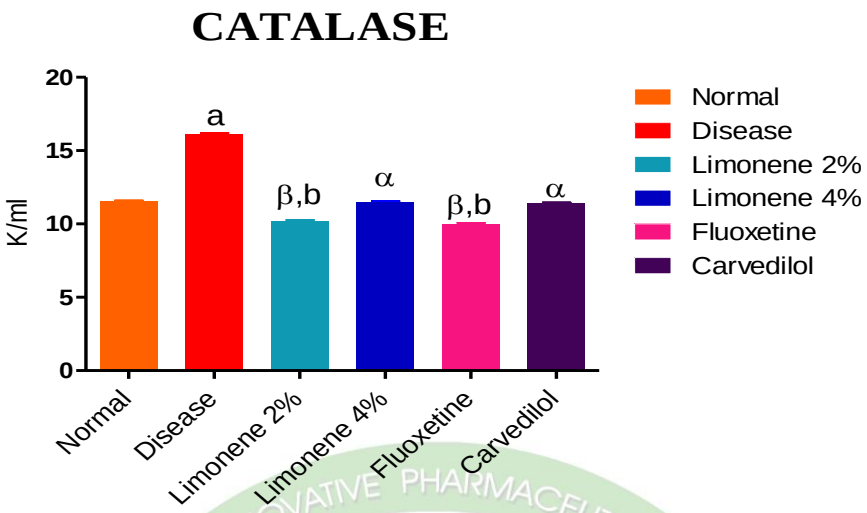

Fig. 4: Whole brain levels of CATALASE in normal and chronic fatigue syndrome induced mice treated with vehicle, d-limonene (2% & 4%), fluoxetine & carvedilol for 7 days.

Data is expressed as Mean ± SEM (n=6) and analysed by one way analysis of variance (ANOVA) followed by Dunnett's test as post hoc test for the comparison of means.
[a]p<0.001, [b]p<0.01 compared to normal control group;
[α]p<0.001, [β]p<0.01, [γ]p<0.005 compared to Disease control group.

DISCUSSION

It is well known that involvement of oxidative stress and inflammatory mediators are the major causative factors for the initiation and progression of CFS. In accordance with previous reports, in the present study induction of CFS significantly increased the whole brain MDA and catalase and decreased the GSH and SOD levels. Marble burying test in animals can be used for drug screening with anxiolytic properties. One of the characteristic behaviors of rodents, either in their natural habitat or both in laboratory conditions, is called bury defensive (defensive-burying) that refers to the behavior of moving the material that covers the surface of the cage, with characteristic movement forelegs, so as to cover the localized sources of aversive stimulus or potential danger. This behavior is also exhibited in the presence of seemingly harmless objects, such as marbles used in the behavioral test, which has led several authors to question the defensive nature of this behavior. MDA, a marker for lipid peroxidation was elevated in FST treated mice could be attributed to the accumulation of free radicals proposed to be generated which, initiate lipid peroxidation of the membrane bound polyunsaturated fatty acids, leading to impairment of the membrane functional and structural integrity [23-24]. The observed significant decrease in GSH, and SOD levels indicates induction of CFS. Increased ROS, attacks the active

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site of antioxidant enzymes and make them to loss their function to scavenge ROS. So ROS levels get increased and antioxidant enzyme levels get further decreased in chronic disease conditions.Treatment with d-Limonene alleviated increased MDA levels. These findings suggest that d-Limonene may be beneficial in preventing fatigue as a complication of CFS due to its anti-oxidant property. The primary source for the formation of reactive oxygen species (ROS) is the increased influx of pyruvate and oxygen in mitochondria and the increased production of ROS (mainly superoxide) in oxidative processes in the electron transport chain. The first line of defence against oxidative damage are the endogenous enzyme antioxidants superoxide dismutase (SOD), catalase (CAT) and glutathione system, among them, glutathione reductase (GSH-Rd) and glutathione peroxidase (GSH-Px). There are also the non-enzymatic antioxidants that are lipid soluble (tocopherols, carotenoids, quinones and bilirubin) and water soluble (ascorbic acid, uric acid and proteins bound to metals[25]. The present study showed that the d-limonene was able to significantly increase superoxide dismutase and decrease catalase activity, demonstrating a change in defence neuroprotective brain. Superoxide dismutase (SOD) is a metalloenzyme that catalyses the dismutation of O_2 radical. The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to oxidative stress. The less reactive compound, H_2O_2, can be degraded by enzymes GPx and CAT. The main forms of SOD of the biological system are: Cu/Zn-SOD and Mn-SOD. The Cu/Zn-SOD is the major isoform involved in the removal of superoxide anions from the cytoplasm and possibly also from the peroxisome, whereas the physiological function of Mn-SOD seems to protect mitochondria of superoxide generated during breathing. Catalase is a cytoplasmic hemeprotein with NADPH dependent activity, which was catalysed by reduction of H_2O_2 to H_2O and O_2. This reaction is important since H_2O_2 is harmful to cell due to their ability to attack the unsaturated fatty acid chains present in the membranes, and other macromolecules, such as proteins, as well as by Haber–Weiss and Fenton reactions through the participation of iron and copper metals. This culminates in the generation of OHradical dot radical, against which there is no enzyme defencesystem. This enzyme is present in animals, plants, bacteria and fungi. It may be found in blood, bone marrow, mucosae, liver and kidney.

CONCLUSION

In summary, treatment with d-Limonene has shown significant anti-fatigue effect on FST induced Chronic fatigue syndrome characterized by:

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1. Decreased immobility time.
2. Decreased number of marbles buried.
3. Increased sociability and social novelty.
4. Increased antioxidant enzyme profile.
5. Decreased levels of ROS.

In conclusion, the present study suggests that treatment with d-Limonene has shown anti- fatigue effect on FST induced chronic fatigue syndrome in mice, may be due to its antioxidant property.

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