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Evaluation of the protective role of the seed coat of *Caesalpinia bonduc* L. Roxb. Against chronic fatigue syndrome induced by forced swimming in rats

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ABSTRACT

Introduction: *Chronic Fatigue Syndrome (CFS), also known as myalgic encephalomyelitis, is a complex, multisystem disorder characterized by persistent and unexplained fatigue, cognitive impairment, sleep disturbances, autonomic dysfunction, and post-exertional malaise. These symptoms markedly reduce patients' functional capacity and quality of life. Early diagnosis and effective intervention are crucial to minimizing morbidity and long-term disability.*

Objective: *The present study aimed to evaluate the therapeutic potential of the ethanolic extract of the seed coat of Caesalpinia bonduc L. Roxb. (EESCB) against forced swimming-induced CFS in experimental rats and to investigate its possible underlying mechanisms, with particular emphasis on behavioral and oxidative stress parameters.*

Methods: *CFS was induced in Wistar rats by subjecting them to a forced swimming protocol—continuous swimming for 15 minutes daily for 28 consecutive days. Animals were divided into five groups (n = 6): Group I (naïve control), Group II (stress control), Group III (standard, treated with Imipramine HCl), Group IV (EESCB 250 mg/kg), and Group V (EESCB 500 mg/kg). Fatigue severity was assessed by recording immobility duration on days 1, 7, 14, 21, and 28. Twenty-four hours after the final swimming session, behavioral assessments were conducted, including anxiety evaluation using the Elevated Plus Maze, muscle strength via the rota-rod test, and locomotor activity using an actophotometer. Oxidative stress markers, including catalase activity and malondialdehyde (MDA) levels, were measured to assess antioxidant status.*

Results: *Imipramine- and EESCB-treated groups (both doses) exhibited a significant reduction in immobility duration and anxiety-like behaviour compared with the stress control group ($p < 0.05$). These groups also demonstrated a significant improvement in locomotor activity and muscle grip strength. Biochemically, EESCB administration led to a marked decrease in MDA levels and a significant elevation in catalase activity, indicating attenuation of oxidative stress. The higher dose (500 mg/kg) showed a more pronounced effect, approaching the efficacy of the standard drug.*

Conclusion: *Therefore, the results highlighted that, EESCB has a prominent protection against experimentally induced CFS which is considered as beneficial for alleviating exercise- induced fatigue. The dose-dependent activity was observed with EESCB due to presence of enormous phytoconstituents.*

Keywords*: Chronic fatigue syndrome, Forced swim test, Oxidative stress, Imipramine Hcl, Anti-oxidant.*

Introduction

Fatigue is one of the most common symptoms encountered in both clinical and community settings. In most individuals, fatigue is transient, self-limiting, and attributable to identifiable circumstances such as physical exertion, emotional stress, or acute illness. However, a subset of individuals experience persistent and debilitating fatigue that is not relieved by rest and cannot be explained by an underlying medical condition such as anemia, hypothyroidism, or chronic infections. This condition is referred to as Chronic Fatigue Syndrome (CFS), also known as myalgic encephalomyelitis.

CFS is a complex, multisystem disorder characterized by severe fatigue lasting for at least six months, accompanied by additional symptoms such as headaches, unrefreshing sleep, impaired concentration, memory disturbances, muscle pain, and post-exertional malaise [1]. Immunological abnormalities have been reported in approximately two-thirds of CFS patients, and the prevalence of depression is higher compared with other chronic illnesses. Oxidative stress and dysregulated nitric oxide (NO) production are implicated in the pathophysiology of CFS, alongside proposed immune system disturbances [4]. While psychological and physical stress are not considered primary causes, they are known to act as triggering or aggravating factors in susceptible individuals [5].

Phytochemicals such as condensed tannins, flavonoids, gallotannins, and other antioxidants have shown potential in alleviating CFS symptoms and associated neuropsychiatric conditions like anxiety and depression [4,6]. Both in vitro and in vivo models have been used to identify natural extracts with significant anti-fatigue and neuroprotective properties. Many herbs and plant-derived products have been traditionally utilized to enhance endurance, boost vitality, and improve resilience against stress. These natural agents often contain bioactive compounds with antioxidant, adaptogenic, and neuromodulatory effects that may help mitigate fatigue and restore physiological balance.

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Caesalpinia bonduc L. Roxb. (CBLR), belonging to the family Caesalpiniaceae, is widely distributed throughout tropical and subtropical regions, with notable prevalence in India. Various parts of the plant have been documented for their antioxidant, anti-inflammatory, immunomodulatory, anti-tumor, and anxiolytic activities [6]. The seeds contain a potent glycoside known as bonducin, along with saponins, terpenoids, fatty oils, starch, sucrose, phytosterols, and fatty acids such as stearic, palmitic, oleic, linoceric, and lindenic acids, as well as unsaturated low molecular weight acids. The protein content of the seeds varies between 7.43% and 25.35% [7,8]. Additionally, the plant is rich in flavonoids, alkaloids, and saponins, which are traditionally used as pain relievers and in decoctions by tribal communities to reduce mental tension and induce sleep [9].

Given its rich phytochemical profile and traditional medicinal applications, *C. bonduc* presents a promising candidate for combating fatigue and associated neuropsychiatric symptoms. The present study was therefore undertaken to evaluate the therapeutic efficacy of the ethanolic extract of the seed coat of *Caesalpinia bonduc* L. Roxb. (EESCB) in a rat model of forced swimming-induced CFS, with an emphasis on behavioral and biochemical assessments to elucidate its potential mechanisms of action.

Materials and Methods

Collection and Identification of Plant Material

The seeds of *Caesalpinia bonduc* L. Roxb. were collected from the local herbal garden of Chitradurga, Karnataka, India. The seeds were washed thoroughly, shade-dried, and manually separated into seed kernels and seed coats. The seed coat material was identified and authenticated by a qualified botanist, and a voucher specimen was deposited for future reference.

Preparation of Plant Extract

The dried seed coats were coarsely powdered and subjected to extraction using 95% ethanol as the solvent in a Soxhlet apparatus [10]. The extract was filtered and concentrated to dryness under reduced pressure at 45 °C using a rotary flash evaporator to yield the Ethanolic Extract of Seed Coat of *Caesalpinia bonduc* L. Roxb. (EESCB). The percentage yield obtained was approximately 12.5% w/w. The dried crude extract was stored in an airtight glass container at refrigerated temperature until further use. For administration, a stock suspension of EESCB was prepared using 0.1% carboxymethyl cellulose (CMC) as the vehicle.

Experimental Animals

Thirty healthy adult Wistar albino rats of either sex, weighing 150–200 g, were procured from Biogen Laboratory Animal Facility, Bangalore (PIN: 562107). The animals were housed in standard polypropylene cages under controlled environmental conditions (12-hour light/dark cycle, 22 ± 2 °C, and 50–60% relative humidity) with free access to a standard pellet diet and water *ad libitum*. All experimental procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and approved by the Institutional Animal Ethics Committee (IAEC) (Approval Ref. No.: 02 C SJMCP/IAEC/2021-22).

Selection of Screening Dose

The doses were selected based on literature reports from previous pharmacological studies and acute toxicity data [11], following the OECD guidelines.

Two dose levels were selected for evaluation:

- **Low dose:** 250 mg/kg body weight
- **High dose:** 500 mg/kg body weight

Preliminary Phytochemical Analysis

Qualitative phytochemical screening of the EESCB was carried out to determine the presence of major classes of phytoconstituents, including alkaloids, flavonoids, tannins, saponins, glycosides, terpenoids, and phenolic compounds, following standard phytochemical procedures [12,13].

Experimental Design

Grouping of Animals

Weight-matched Wistar rats were randomly allocated into five groups, each containing six animals (n = 6):

- **Group A – Naïve Control:** No exposure to stress and no treatment administered.
- **Group B – Stress Control:** Subjected to forced swimming to induce Chronic Fatigue Syndrome (CFS) for 28 days without treatment.
- **Group C – Standard Drug:** Subjected to forced swimming + treated with Imipramine HCl (20 mg/kg) [14] for 28 days.
- **Group D – EESCB Low Dose:** Subjected to forced swimming + treated with EESCB (250 mg/kg) for 28 days.
- **Group E – EESCB High Dose:** Subjected to forced swimming + treated with EESCB (500 mg/kg) for 28 days.

Imipramine HCl and EESCB were administered orally 30 minutes before the daily forced swimming session throughout the 28-day study period.

Induction of Chronic Fatigue Syndrome

CFS was induced by the Forced Swim Test (FST) following the protocol of Dawson *et al.* [15] with slight modifications. Rats were placed individually in a transparent glass tank (height: 45 cm, diameter: 30 cm) filled with water to a depth of 30 cm, maintained at 22 ± 3 °C. Each animal underwent a 15-minute swimming session once daily for 28 consecutive days.

Initially, animals exhibited vigorous activity, followed by intermittent episodes of immobility, which is characterized by minimal movement required to keep the head above water. Endurance capacity and fatigue level were assessed by measuring the immobility time. This was recorded on days 1, 7, 14, 21, and 28 for all groups.

Behavioral Assessments

a) Elevated Plus Maze (EPM) Test

The EPM was used to evaluate anxiety-like behavior in rats [16,17]. The apparatus consisted of two opposite open arms (50 × 10 cm) and two opposite closed arms (50 × 10 × 40 cm) connected by a central platform (10 × 10 cm) and elevated 50 cm above the floor.

Each rat was placed at the center of the maze, facing an open arm, and observed for 5 minutes. The following parameters were recorded:

- Time spent in open and closed arms (seconds)
- Number of entries into open and closed arms

An entry was considered valid when all four paws of the rat were within the arm.

b) Assessment of Locomotor Activity by Actophotometer

Locomotor activity was evaluated using a digital actophotometer consisting of a square arena (30 × 30 cm) enclosed by four walls equipped with photocells [18].

Prior to testing, the photocells were checked to ensure proper functioning. Each rat was placed individually in the arena, and the number of beam breaks (crossings) was recorded automatically for a period of 5 minutes. All groups were assessed for locomotor activity 30 minutes after administration of the respective test or standard drug.

c) Assessment of Muscle Grip Strength by Rota-Rod

Neuromuscular coordination and muscle grip strength were assessed using a rota-rod apparatus set to a constant rotation speed of 25 revolutions per minute (RPM) [19]. Each rat was placed individually on the rotating rod, and the latency to fall (fall-off time) was recorded in seconds, with a maximum cut-off time of 300 seconds.

Biochemical Studies

a) Preparation of Brain Homogenate

Twenty-four hours after the final forced swimming session, animals were sacrificed by cervical dislocation. The brains were quickly excised, rinsed with ice-cold saline to remove blood, and weighed. A 10% (w/v) brain homogenate was prepared in 0.1 M phosphate buffer (pH 7.4) using a homogenizer under chilled conditions. The homogenate was centrifuged, and the supernatant was used for biochemical assays of lipid peroxidation and catalase activity.

b) Measurement of Oxidative Stress

Lipid Peroxidation (MDA Content)

Malondialdehyde (MDA) levels, an indicator of lipid peroxidation, were estimated by the thiobarbituric acid reactive substances (TBARS) method described by Wills [20]. TBARS were quantified spectrophotometrically using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, and results were expressed as nanomoles of MDA per milligram of protein. Tissue protein content was determined by the Biuret method.

Catalase Activity

Catalase activity was measured according to the method of Claiborne [21], and results were expressed as micromoles of hydrogen peroxide decomposed per minute per milligram of protein.

Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's–Kramer multiple comparison test to determine intergroup significance. A p-value < 0.05 was considered statistically significant.

Results

Preliminary Phytochemical Analysis

Qualitative phytochemical screening of the ethanolic extract of the seed coat of *Caesalpinia bonduc* L. Roxb. (EESCB) confirmed the presence of alkaloids, glycosides, flavonoids, tannins, steroids, saponins, triterpenoids, resins, proteins, and carbohydrates. These bioactive constituents are known to possess antioxidant, adaptogenic, and neuroprotective properties, which may contribute to the observed pharmacological effects.

Assessment of Immobility by the Forced Swim Test (FST)

In the stress control group (Group B), the immobility duration progressively and significantly increased on days 14, 21, and 28 compared to the naïve control group (Group A), indicating the successful induction of chronic fatigue syndrome.

Administration of EESCB at both 250 mg/kg (Group D) and 500 mg/kg (Group E) resulted in a significant and time-dependent reduction in immobility duration when compared to the stress control group. A similar reduction was observed in the standard drug-treated group (Group C, Imipramine HCl 20 mg/kg).

The anti-fatigue effect was more pronounced in the higher dose EESCB group (500 mg/kg), with values approaching those of the standard group by day 28. Both test groups demonstrated a consistent attenuation of immobility over the study period, suggesting improved endurance and reduced fatigue-like behavior.

The detailed values and statistical significance are presented in Table 1.

Table 1: Effect of EESCB on Immobility by Force Swim Test

Sl. No.	Treatment	Immobility time in (Sec)				
		Day 1	Day 7	Day 14	Day 21	Day28
I	Naive	123.5 ± 5.39	128.0 ± 5.87	132.7 ± 5.81	135.7 ± 5.81	139.5 ± 5.99
II	Stress induced	159.0 ± 4.83	162.8 ± 4.70	166.2 ± 47	169.2 ± 4.77	173.0 ± 5.00
III	Standard Imipramine Hcl	105.2 ± 16.65***	101.1 ± 16.57***	97.50 ± 16.65***	95.00 ± 16.00***	94.50 ± 16.69***
IV	Low Dose of EESCB	131.7 ± 6.339	130.8 ± 3.198	128.3 ± 3.528*	127.8 ± 4.750*	112.5 ± 3.557*
V	High Dose of EESCB	123 ± 4.465*	119.3 ± 7.223*	118.2 ± 4.262***	117.5 ± 6.908**	105.3 ± 3.739**

Values were expressed as Mean ± SEM (n=6); Significance values are:
**P < 0.05, **P < 0.01 and ***P < 0.001. Stress group vs all groups.*

Assessment of Cognitive Behaviour by the Elevated Plus Maze (EPM) Apparatus

Chronic exposure to forced swimming in the stress control group (Group B) produced a pronounced anxiety-like state, as evidenced by a significant increase in the number of entries into the closed arms and increased time spent in the closed arms, along with a marked reduction in both the number of entries and the time spent in the open arms and central platform when compared to the naïve control group (Group A). In contrast, treatment with Imipramine HCl (Group C) and EESCB at both 250 mg/kg (Group D) and 500 mg/kg (Group E) produced a significant anxiolytic effect. These groups showed a higher number of entries and longer duration in the open arms and center, accompanied by a reduction in closed arm entries and time spent in closed arms when compared to the stress control group ($p < 0.05$).

The effect was dose-dependent, with EESCB 500 mg/kg producing results comparable to the standard drug.

Detailed results are presented in Table 2.

Table No 2: Effect of EESCB on cognitive behaviour by Elevated plus maze

Sl. No.	Treatment	Number of entries (counts/5min)			Time spent (sec)in 5mins		
		Open arm	Closed arm	Centre	Open arm	Closed arm	Centre
I	Naive	2.833 ± 0.30	4 ± 0.73	4.667 ± 0.61	38.17 ± 7.98	218.5 ± 10.14	43.67 ± 11.19
II	Stress control	1.5 ± 0.22	8.5 ± 0.76	2.6667 ± 0.33	20 ± 7.61	265.5 ± 10.74	14.5 ± 4.12
III	Standard (ImipramineHcl) 20 mg/kg	7 ± 0.5***	3.833 ± 0.30***	7.333 ± 0.55***	145.5 ± 8.32***	86.67 ± 5.34***	67.33 ± 9.45***
IV	Low Dose of EESCB (250mg/kg)	3 ± 0.36	5.5 ± 0.56*	5.167 ± 0.47*	85.33 ± 3.98**	188.2 ± 11.25**	34.33 ± 6.04
V	High Dose of EESCB (500 mg/kg)	3.5 ± 0.42*	5.333 ± 0.42**	6 ± 0.57**	111.5 ± 17.6***	132.5 ± 21.8***	55.67 ± 5.30**

*Values were expressed as Mean ± SEM (n=6); Significance values are: *P < 0.05, **P < 0.01 and ***P < 0.001. Stress group vs all groups.*

Assessment of Locomotor Activity by Actophotometer

Locomotor activity, expressed as the ambulatory score (beam crossings over a 5-minute observation period), was markedly reduced in the stress control group (Group B) compared to the naïve control (Group A), indicating a decrease in spontaneous movement and overall physical activity due to chronic forced swimming.

Administration of Imipramine HCl (Group C) significantly improved locomotor activity compared to the stress group ($p < 0.05$). Similarly, treatment with EESCB at both 250 mg/kg (Group D) and 500 mg/kg (Group E) produced a progressive, dose-dependent increase in ambulatory counts.

The EESCB high-dose group (500 mg/kg) demonstrated locomotor activity levels comparable to the standard drug group, suggesting a potent restorative effect on physical activity impaired by chronic fatigue.

Detailed numerical data are presented in Table 3.

Table 3: Effect of EESCB on Loco-Motor activity by Actophotometer

S. No.	Treatment	Ambulatory Score
I	Naive group	88.50±2.125
II	Stress group	60.17±2.242
III	Standard group (Imipramine Hcl) 20 mg/kg	104.3±3.303***
IV	Low dose of EESCB (250 mg/kg)	70.00±1.461*
V	High dose of EESCB (500 mg/kg)	74.50±2.012**

*Values were expressed as Mean ± SEM (n=6); Significance values are: *P < 0.05, **P < 0.01 and ***P < 0.001. Stress group vs all groups.*

Assessment of Muscle Grip Strength by Rota-Rod Apparatus

Muscle grip strength, evaluated by the mean fall-off time on the Rota-rod, served as an indicator of neuromuscular coordination and endurance. The stress control group (Group B) exhibited a marked reduction in fall-off time compared to the naïve control (Group A), indicating impaired muscle strength and coordination as a result of prolonged forced swimming.

Treatment with Imipramine HCl (Group C) significantly increased fall-off time compared to the stress group ($p < 0.05$). EESCB administration at both 250 mg/kg (Group D) and 500 mg/kg (Group E) also produced a dose-dependent improvement in grip strength, with the higher dose approaching the performance of the standard drug group.

These findings suggest that EESCB mitigates muscle weakness and incoordination associated with chronic fatigue.

Corresponding data are summarized in Table 4.

Table 4: Effect of EESCB on muscle grip strength by Rota rod apparatus

Sl. No.	Treatment	Fall of time (sec)
I	Naive group	151.3±2.917
II	Stress group	81.67±4.709
III	Standard group (Imipramine Hcl) 20 mg/kg	235±3.759***
IV	Low dose of EESCB (250 mg/kg)	126.5±15.15*
V	High dose of EESCB (500 mg/kg)	139.8±16.07**

*Values were expressed as Mean ± SEM (n=6); Significance values are: *P < 0.05, **P < 0.01 and ***P < 0.001. Stress group vs all groups.*

Effect of EESCB on Catalase Activity and Lipid Peroxidation (LPO)

Biochemical analysis of brain homogenates revealed that chronic stress (Group B) led to a significant reduction in catalase activity accompanied by an increase in lipid peroxidation (measured as MDA levels) compared with the naïve control (Group A), indicating heightened oxidative stress.

Treatment with Imipramine HCl (Group C) as well as EESCB at 250 mg/kg (Group D) and 500 mg/kg (Group E) significantly ($p < 0.05$) elevated catalase activity and reduced MDA levels relative to the stress control. The higher EESCB dose demonstrated antioxidant effects comparable to the standard

drug, suggesting its potential in mitigating oxidative damage associated with chronic fatigue syndrome.

Detailed values are presented in Table 5.

Table 5: Effect of EESCB on Catalase assay and LPO assay

Sl. No.	Treatment	Catalase (µmol/min/mg of protein)	MDA (nmol /mg of protein)
I	Naive group	1.270±0.0340	4.465±0.125
II	Stress group	0.688±0.158	19.34±0.015
III	Standard group (Imipramine Hcl) 20 mg/kg	4.610±0.05***	4.309±1.641***
IV	Low dose of EESCB (250 mg/kg)	1.216±0.0955*	10.25±0.1500**
V	High dose of EESCB (500 mg/kg)	2.183±0.0675***	9.550±0.05000**

*Values were expressed as Mean ± SEM (n=6); Significance values are: *P < 0.05, **P < 0.01 and ***P < 0.001. Stress group vs all groups.*

Discussion

Chronic fatigue syndrome (CFS) is a complex global health problem with multifactorial etiology. Although the exact causes remain undetermined, several triggers have been proposed, including infectious diseases, nutritional deficiencies, food intolerance, extreme physical or mental stress, and oxidative damage [22]. Oxidative damage to mitochondrial membrane lipids is considered one of the most critical factors impairing mitochondrial function, and there is growing evidence linking mitochondrial dysfunction directly to fatigue in humans [23,24]. Oxidative stress-induced cytokines promote nitric oxide formation, which combines with superoxide to generate peroxynitrite—a highly potent oxidant. Elevated peroxynitrite disrupts mitochondrial function and damages essential mitochondrial lipids such as glycophospholipids and fatty acids. This lipid peroxidation has been linked to increased oxidative injury in the brain, as reported in mice subjected to chronic swim stress [25].

In the present study, chronic forced swimming for 28 days significantly increased immobility time in the stress control group, suggesting depressive-like behavior and fatigue. This was accompanied by reduced muscle grip strength (rota-rod) and decreased locomotor activity, potentially due to abnormalities in carnitine homeostasis—an important factor in muscle fatigue and CFS pathophysiology. Furthermore, animals in the stress control group exhibited increased anxiety-like behavior in the elevated plus maze, possibly linked to inactivation of GABAA_AA receptors, which mediate anti-anxiety responses.

Biochemical assays revealed decreased catalase activity and elevated malondialdehyde (MDA) levels in the stress control group, confirming heightened oxidative stress. Continuous forced swimming thus produced a CFS-like state in rats, characterized by behavioral, neuromuscular, and oxidative changes.

Treatment with Imipramine HCl and EESCB (250 and 500 mg/kg) significantly reversed these behavioral deficits and biochemical alterations. The protective effects of EESCB may be attributed to its flavonoid content, which can scavenge peroxynitrite and superoxide radicals, thereby reducing oxidative injury. In addition, flavonoids can restore tetrahydrobiopterin (BH4_44)—a cofactor essential for melatonin synthesis. Melatonin, with its potent antioxidant properties, may further contribute to the observed protection against fatigue.

Free radical generation occurs naturally during respiration and other cellular processes but can increase pathologically following chemical or radiation injury. Catalase, a heme-containing enzyme located in peroxisomes, catalyzes the breakdown of hydrogen peroxide into water and oxygen, preventing oxidative injury [14]. Lipid peroxidation, measured via MDA levels, reflects oxidative damage to cell membranes, proteins, and DNA. MDA is mutagenic and carcinogenic, capable of forming adducts with deoxyguanosine and deoxyadenosine [26,27].

The current findings demonstrate that EESCB treatment increased catalase activity and decreased MDA levels compared to stress controls, indicating a potent antioxidant effect. These results suggest that EESCB could mitigate oxidative stress and behavioral deficits associated with CFS, potentially offering a natural therapeutic approach for managing fatigue-related disorders.

Conclusion

The findings of this study indicate that the ethanolic extract of *Caesalpinia bonduc* L. Roxb. seed coats exerts significant protective effects against chronic fatigue syndrome in a rat model. This protection appears to be closely linked to its antioxidant properties, as evidenced by enhanced catalase activity and reduced lipid peroxidation. By improving behavioral, neuromuscular, and biochemical parameters, the extract demonstrates potential as a natural intervention for fatigue-related disorders. Future studies should focus on isolating and characterizing the bioactive constituents, elucidating the precise molecular mechanisms involved, and validating these effects in clinical settings to support its development as a therapeutic candidate for CFS.

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