



Protection of stress induced behavioural and physiological alteration by *Marsilea quadrifolia* in rodents

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ABSTRACT

The present study was designed to evaluate the antistress effect of *Marsilea quadrifolia* ethanolic extract in stress induced behavioural and physiological alteration in the mice. Anti-stress activity was evaluated using physical stress models viz. swimming endurance and post swimming motor function test, anoxic tolerance test and restraint stress test. Swiss albino mice (18-25 g) divided into four groups of six animals each were used for the study. Control group received CMC as vehicle and standard group received *Withania somnifera* (100 mg/kg) while *Marsilea quadrifolia* ethanolic extract (200 and 400 mg/kg) were administered orally for seven days. Change in immobility time in swim endurance and first clonic convulsion in anoxic tolerance test and stress-induced behavioral and biochemical alterations in immobilization stress was recorded as parameters. *Marsilea quadrifolia* ethanolic extracts significantly reduces the immobility timing along with increases the swimming endurance time, post motor function and clonic convulsion timing in anoxic tolerance test as compared to control group and significantly reversed the behavioral and biochemical alterations in restraint stress.

Keywords: Stress, *Marsilea quadrifolia*, Swim endurance test, Anoxic tolerance test, Restraint stress test.

INTRODUCTION

Stress can be described as “ a physical or physiological stimulus that can produce mental tension or physiological reactions that may lead to illness, it is also considered as to be any conditions which results in perturbation of body's homeostasis [1].

Stress and stress-related disorders are a significant cause of disease in modern times, contributing to perhaps 75% of all illnesses. Stress has been postulated to be involved in the etiopathogenesis of a diverse variety of diseases ranging from psychiatric disorder such as anxiety and depression, immunosuppression, endocrine disorders including diabetes mellitus, male sexual dysfunction, cognitive dysfunctions, peptic ulcer, hypertension and ulcerative colitis. Modern medical science has developed multiple approaches to coping with stress, including pharmacological approach, exercise, and relaxation techniques like meditation [2].

Herbal formulations have been in use for many years not only in Asian countries but also globally for human well-being. The herbal formulations claimed to enhance physical endurance; mental functions and non-specific resistance of the body have been termed as adaptogens. The potential utility of safer and cheaper herbal medicines as antistress agents have been reported as they can withstand stress without altering the physiological functions of the body. Various herbs like *Withania somnifera*, *Embllica officinalis*, *Asparagus racemosus*, *Ocimum sanctum*, *Tribulus*

terrestris and *Piper longum* are claimed to have immunomodulatory, adaptogenic, anabolic effects and the ability to improve vital energy [3].

Marsilea quadrifolia Linn, a member of Marsileaceae is a creeping herbaceous perennial plant. In Hindi it is commonly known as caupatiya, sunsunia. It is widely distributed in tropical and temperate regions of world and found throughout the India, in marshy places and along the banks of canals and rivers. In folk medicine; the herb is used as a vegetable for inducing sleep. As per the traditional claims the plant has been used for astringent, hypnotic, diuretic, expectorant, aphrodisiac, anodyne, ophthalmic, constipating, psychopathy, leprosy, haemorrhoids, skin diseases, fever, insomnia and febrifuge. The phytochemicals like marsilin (1-triacontanol-cerotate), 3-hydroxy-triacontan-11-one, hentriacontan-6-ol, methylamine, beta-sitosterol, marsileagenin A, flavonol-O-mono-and-diglycoside, C-glucosylflavones and C-glucosylxanthones have been isolated from the plant. The plant is traditionally used to reduce mental tension and to induce sleep, reducing anxiety and stress in emotional conditions. Therefore, with the reference to traditional and reported uses, the present study was undertaken to investigate the CNS activity of the psychopharmacological activity of the plant and provided a scientific rationale for its use [4].

EXPERIMENTAL SECTION

Plant Material

The whole plant *Marsilea quadrifolia* were collected from Raebareli, during the month of July. The plant material was authenticated by NISCAIR, Delhi and voucher specimens were deposited for future reference (Reference letter no.NISCAIR/RHMD/Consult/2013/2305/85).

Animals

Swiss albino mice weighing 18-25 gm of either sex were used in the study. The animals were maintained under 12 hr light and dark cycle. They were kept on standard pellet diet and water *ad libitum*. They were initially acclimatized to the laboratory environment for seven days prior to their use. The animal caring and handling were done according to CPCSEA guideline. Institutional Animal Ethics Committee (BBDNIIT/IAEC/028/2014)

Preparation of Ethanolic Extract

The collected plant material was washed with tap water to remove dirt particles then shade dried and pulverised in a mechanical grinder. The powdered plant was extracted with ethanol water (20:80) by maceration in a closed vessel for 72h. The dark brown coloured sticky residue was collected after complete removal of the solvent under reduced pressure [5].

Dose Selection

Two doses (200 and 400 mg/kg) [6] of *Marsilea quadrifolia* ethanolic extract and *Withania somnifera* (100 mg/kg) [7] was selected for this study.

Swim Endurance and post swimming motor function test:

Animals were randomly divided into four groups of six animals in each.

Group I received only CMC as vehicle.

Group II animals were treated with Ashwagandha 100 mg/kg orally.

Group III and IV animals were provided with *Marsilea quadrifolia* at doses of 200 and 400 mg/kg orally for 7 days. On 8th day, the animals were allowed to swim in a propylene tank of dimension 40x18cm, filled with water to a height of 15 cm. The total duration of immobility was measured in a time period for 30 minutes [8]. The animals were considered to be immobile whenever they remained floating passively in water with their head above the water. After each swim session the mice were removed from water dried with towel and placed in warm enclosure for 10 min and then return to their homecage. The container was implied and washed thoroughly after testing of each animal. The animals were subsequently tested for muscle coordination on a rota rod apparatus rotating at 15 rpm and the duration of stay on the rod was recorded as parameter [9].

Anoxic Tolerance Test:

Animals were randomly divided into four groups of six animals each.

Group I treated with CMC as vehicle.

Group II animals were received water-soluble powder of Ashwagandha 100 mg/kg orally.

Group III and IV animals were provided with *Marsilea quadrifolia* at doses of 200 and 400 mg/kg orally, for 7 days [10]. On 8 day, animal was subjected to anoxic stress by keeping them in a confined airtight 500 ml conical flask. Conical flasks of 500 ml capacity was used for the test. These flasks was made airtight using rubber cork before beginning the experiment. The time taken for the mice to exhibit the first clonic convulsion was taken as the end point. The animal was removed immediately from the vessel for recovery and resuscitated if needed. The time duration between animal entry into the vessel and the appearance of the first convulsion was taken as a parameter for the time of anoxia tolerance [11].

Restraint Stress Test:

Animals were randomly divided into five groups of six animals each.

Group I was treated with CMC as vehicle.

Group II received CMC.

Group III received Ashwagandha for seven days.

Group IV and V received 200 and 400 mg/kg orally doses of *Marsilea quadrifolia* for 12 days. On the day 12, one hour after last treatment, the forelimbs and hind limbs of the mice in Groups 2nd, 3rd, and 4th,5th were tied with adhesive tape thereby immobilizing them for 2 hr. After the induction of stress for 2 hr, the adhesive tape was removed and the blood was collected from retroorbital plexus. The blood obtained from retroorbital plexus was centrifuged and the serum obtained was used for the estimation of blood glucose, triglyceride, cholesterol as biochemical parameter and stress-induced behavioural changes were assessed using actophotometer, rota rod and light and dark model [12].

RESULTS**Effect of *Marsilea quadrifolia* swimming endurance and post swimming motor function test:**

Seven days pretreatment with *Marsilea quadrifolia* ethanolic extract at the doses 200 and 400 mg/kg, p.o. significantly reduced the immobility time of mice (318.00±1.25 and 297.83±1.49) as compared to control group (482.233±1.82) as shown in [Table no. 1] and Figure 1. The duration of stay on rota rod was significantly increased in both the doses of *Marsilea quadrifolia* ethanolic extract (9.23±0.16 and 12.75±0.16) as compared to control group (5.90±0.22) as shown in [Table no. 1] and Figure 2.

Effect of *Marsilea quadrifolia* on anoxia stress tolerance in mice:

The time taken for the mice to exhibit clonic convulsions was taken as the end point in the anoxic tolerance test. Seven days pretreatment with *Marsilea quadrifolia* ethanolic extract 200 and 400 mg/kg, p.o. significantly increased the time taken for clonic convulsions (56.04±1.07 and 66.17±1.49) as compared to control animals (41.40±0.79) as shown in [Table no.2] and Figure 3.

Effect of *Marsilea quadrifolia* in restraint stress test:**Biochemical Parameter**

All the biochemical parameter were significantly increased by restraint stress, while *Marsilea quadrifolia* ethanolic extract 200 and 400 mg/kg, p.o. reduced glucose (170.33±1.27,158.17±1.56), cholesterol (136.67±1.54, 121.85±0.83) and triglyceride level (70.33±1.65,76.07±0.99) as compared to stressed groups (186.17±1.28, 154.00±1.25, 90.69±1.50) as shown in [Table no.3] and Figure 4,5,6.

Table no.1 Effect of *Marsilea quadrifolia* on immobility time and rota rod falling time

S.N.	Treatment	Duration of immobility in sec	Falling time on rota rod in sec
1.	CMC	482.233±1.82	5.90±0.22
2.	Ashwagandha, 100 mg/kg.p.o.	247.17±1.61 ***	14.21±0.16 ***
3.	MQEE 1, 200 mg/kg.p.o.	318.00±1.25 ***	9.23±0.16 ***
4.	MQEE 2, 400 mg/kg.p.o.	297.83±1.49 ***	12.75±0.16 ***

Results expressed as Mean ±SEM significant at ***P < 0.001 vs control group by one way ANOVA followed by Tukey test.

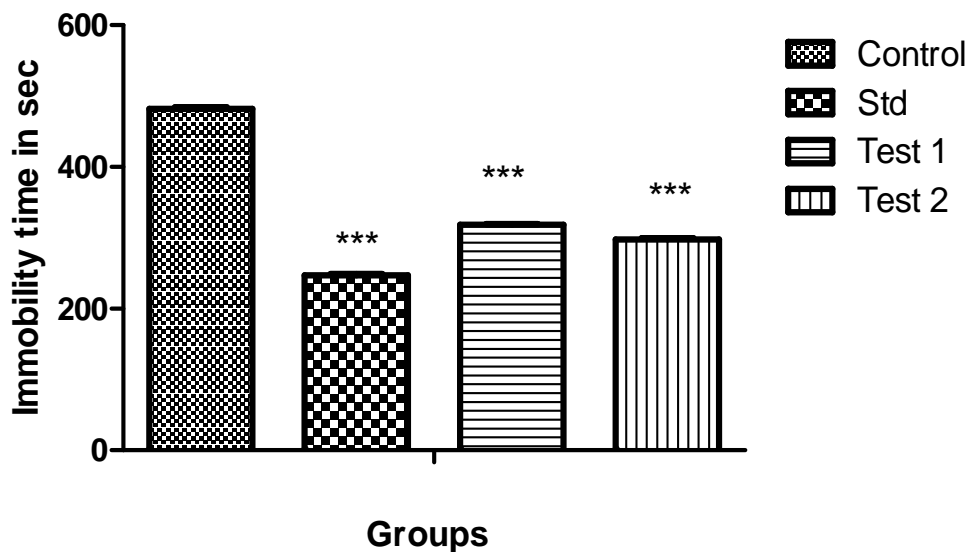
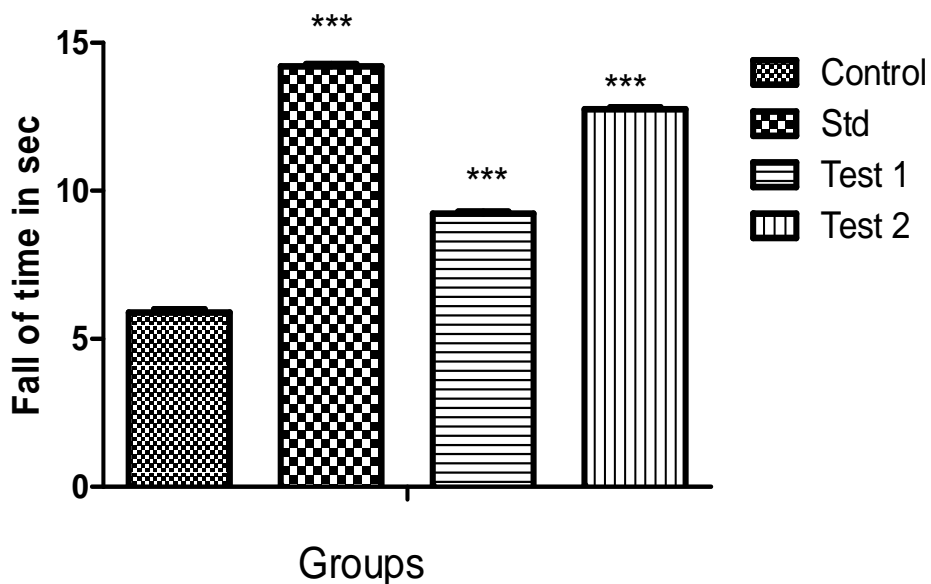
Fig.1 Effect of *Marsilea quadrifolia* on duration of immobility in swimming endurance testFig. 2 Effect of *Marsilea quadrifolia* on Rota rod test with swim endurance test

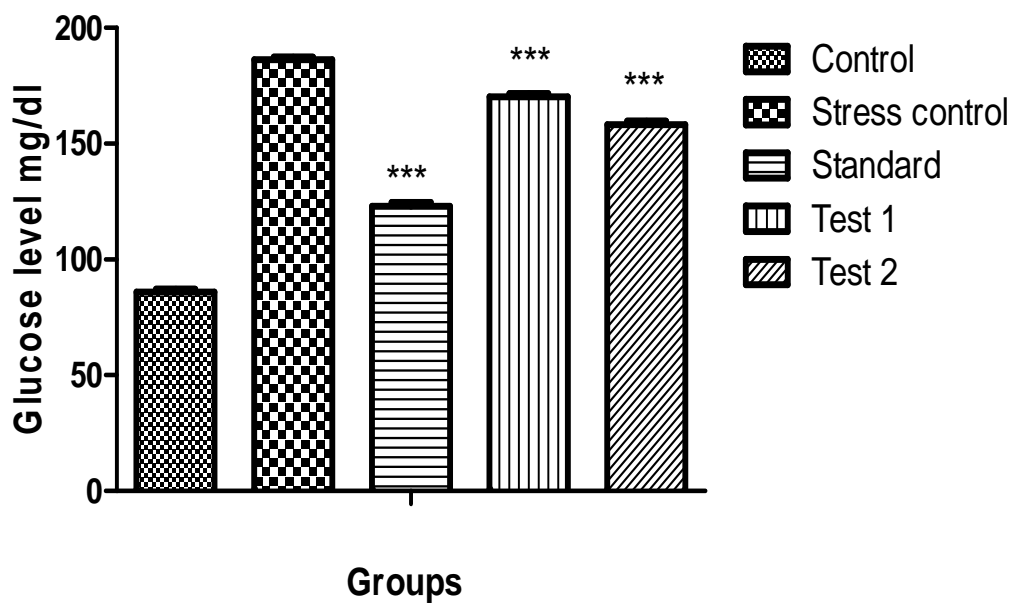
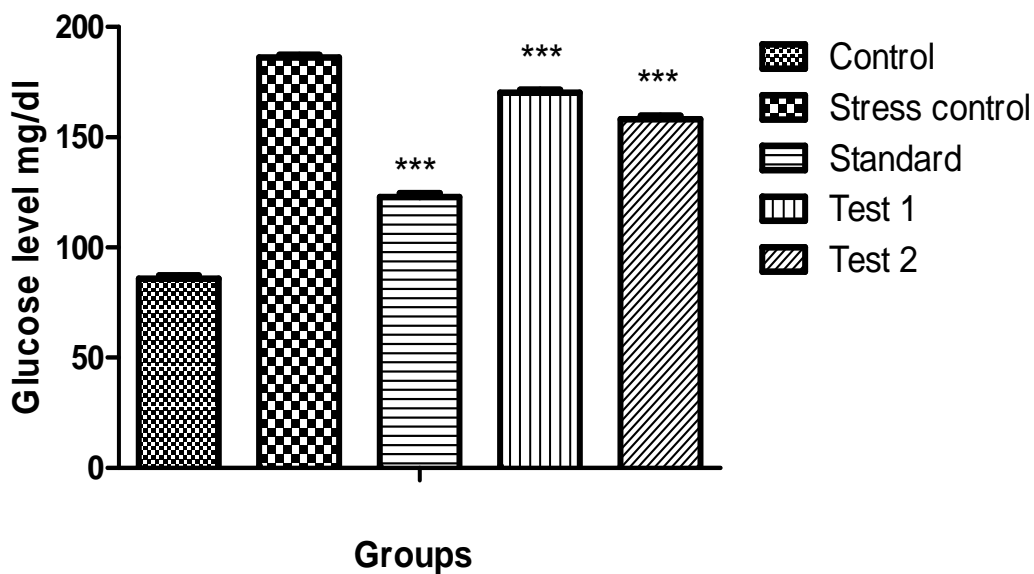
Fig. 3 Effect of *Marsilea quadrifolia* on the latency of convulsion in the anoxic tolerance testFig. 4 Effect of *Marsilea quadrifolia* on glucose level in restraint stress test

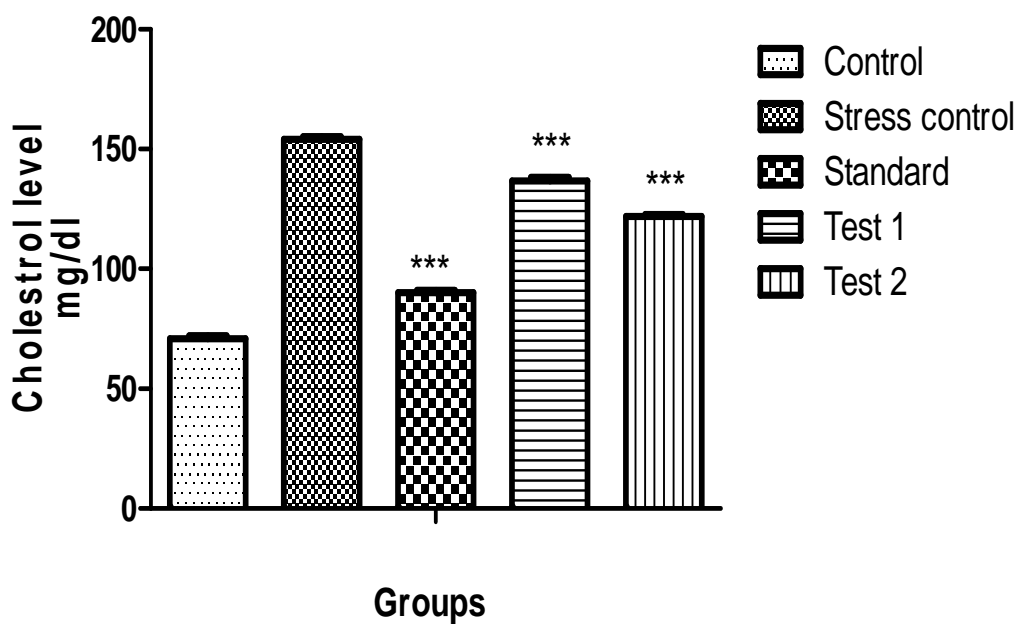
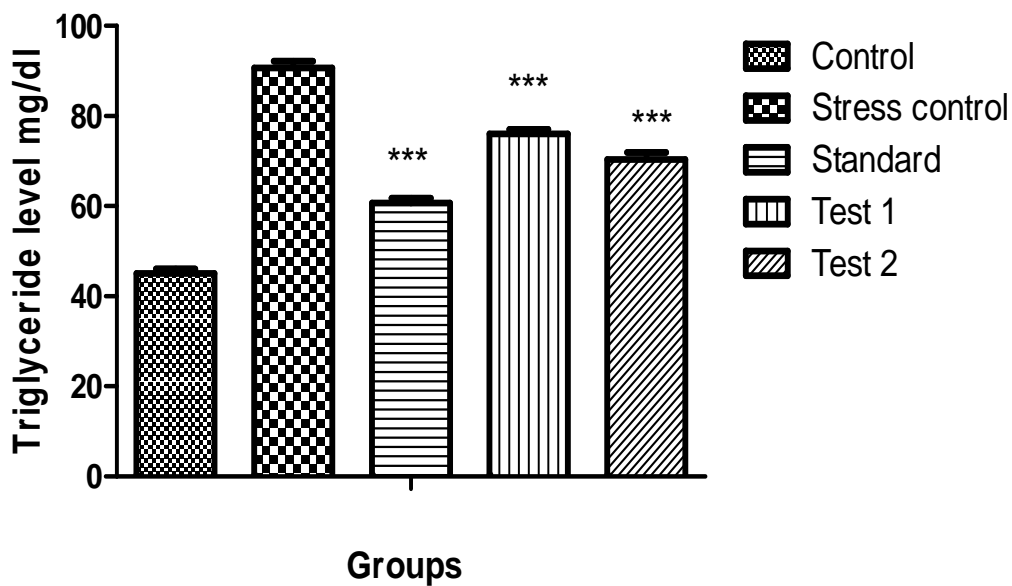
Fig. 5 Effect of *Marsilea quadrifolia* on cholesterol level in restraint stress testFig. 6 Effect of *Marsilea quadrifolia* on triglyceride level in restraint stress test

Table No. 2 Effect of *Marsilea quadrifolia* on anoxic tolerance

S.N.	Treatment	Time of convulsion in min
1.	CMC	41.40±0.79
2.	Ashwagandha	74.47±1.33 ***
3.	MQEE 1, 200 mg/kg, p.o.	56.04±1.07 ***
4.	MQEE 2, 400 mg/kg, p.o.	66.17±1.49 ***

Results expressed as Mean ±SEM significant at * $P < 0.05$ VS control group, *** $P < 0.001$ vs control group by one way ANOVA followed by Tukey test.

Table no. 3 Effect of *Marsilea quadrifolia* on Glucose, cholesterol and triglyceride in restraint stress

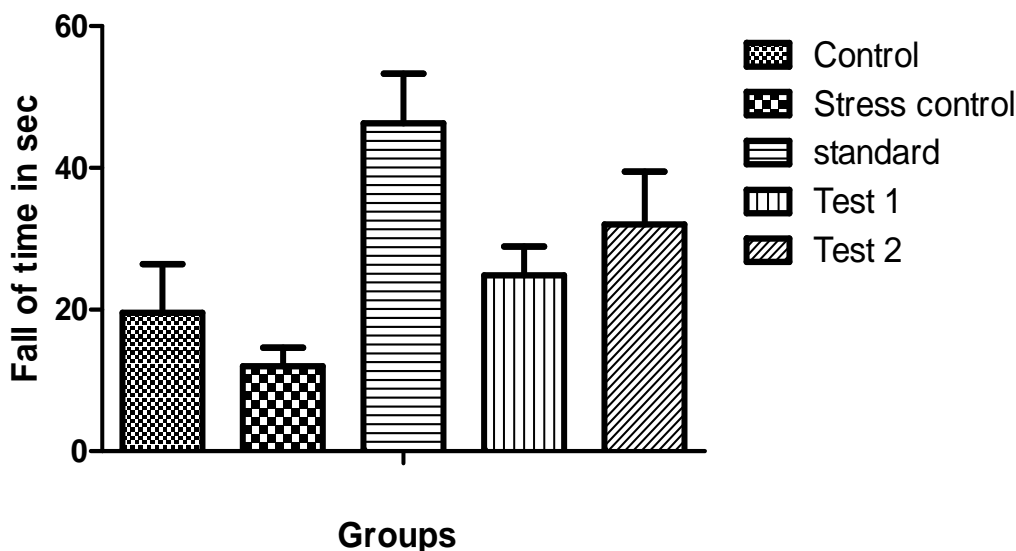
S.N.	Treatment	Glucose	Cholesterol	Triglyceride
1.	CMC	85.92±1.34	70.83±1.25	45.07±1.01
2.	CMC	186.17±1.28	154.00±1.25	90.69±1.50
3.	Ashwagandha, 100 mg/kg, p.o.	122.83±1.69***	90.04±1.07***	60.71±1.09***
4.	MQEE 1, 200 mg/kg, p.o.	170.33±1.27***	136.67±1.54***	76.07±0.99***
5.	MQEE 2, 400 mg/kg, p.o.	158.17±1.56***	121.85±0.83***	70.33±1.65***

Results expressed as Mean ±SEM Significant at, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs stress control group, **** $P < 0.0001$ vs stress control group by one way ANOVA followed by Tukey test.

Table no. 4 Effect of *Marsilea quadrifolia* on Rota rod test, locomotion and light and dark in restraint stress

S.N.	Treatment	Rota rod falling time in sec	Number of count in photoactometer	Time spent in light side(sec)
1.	CMC	48.33±1.93	129.33±1.09	32.33±0.85
2.	Stress control	12.00±0.43	29.67±1.03	19.83±0.90
3.	Ashwagandha, 100 mg/kg, p.o.	49.67±0.74***	71.50±0.98***	39.83±0.62***
4.	MQEE 1, 200 mg/kg, p.o.	24.33±0.79***	45.33±1.44***	30.67±1.05***
5.	MQEE 2, 400 mg/kg, p.o.	39.00±0.86***	53.00±1.50***	34.00±1.09***

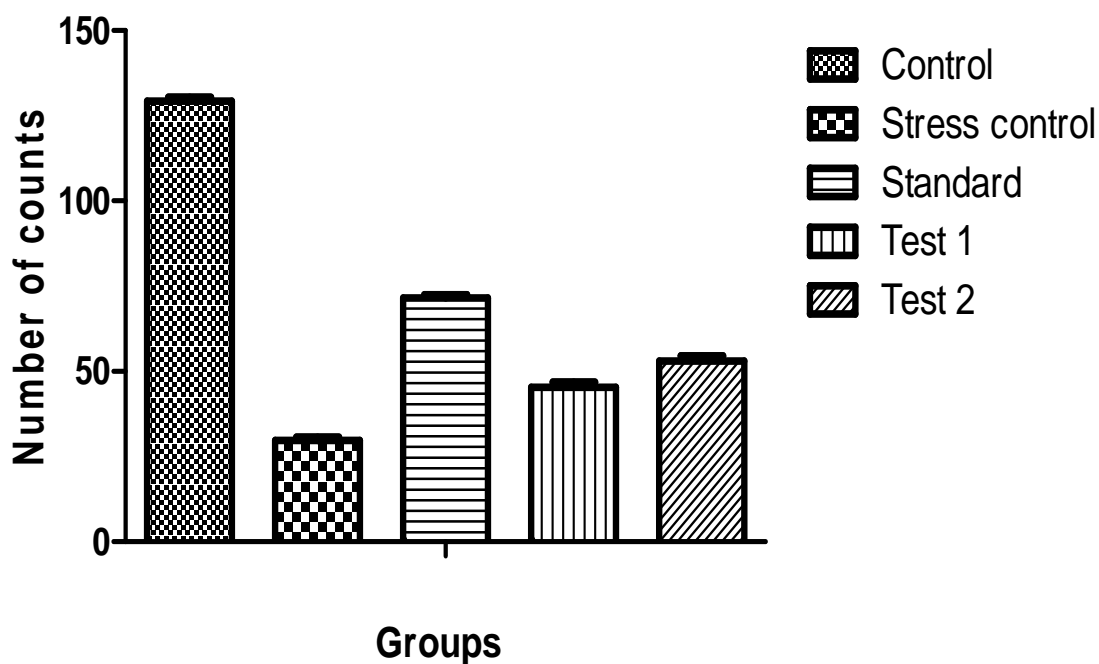
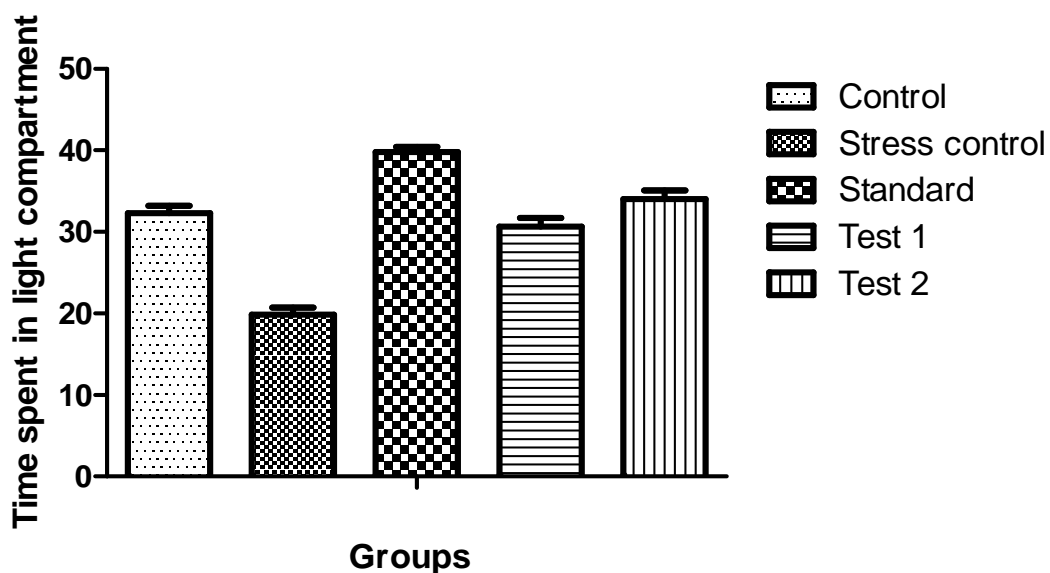
Results expressed as Mean ± SEM Significant at * $P < 0.05$, ** $P < 0.01$ vs stress control group, *** $P < 0.001$ vs stress control group by one way ANOVA followed by Tukey test.

Fig. 7 Effect of *Marsilea quadrifolia* on falling time in rota rod in restraint stress test

Behavioural Parameter

In Rota rod test:

Marsilea quadrifolia ethanolic extract at the doses 200 and 400 mg/kg, p.o. it was found significantly increases the falling time of mice (39.00±6.16 and 24.33±5.28) on the rota rod as compared to stressed control group (12.00±0.43) shown in [Table no. 4] and Figure 7.

Fig. 8 Effect of *Marsilea quadrifolia* on locomotion in restraint stress testFig. 9 Effect of *Marsilea quadrifolia* in light and dark model in restraint stress test**In Actophotometer test:**

Locomotor activity was significantly increases by *Marsilea quadrifolia* ethanolic extract (45.33 ± 1.44 and 53.00 ± 1.50) as compared to the stressed group (29.67 ± 1.03) in actophotometer as shown in [Table no.4] and Figure 8.

In Light and Dark model:

Marsilea quadrifolia ethanolic extract at the doses 200 and 400 mg/kg, p.o, time spent in light chamber were significantly increased (30.67 ± 1.05 and 34.00 ± 1.09) as compared to stressed control group (19.83 ± 0.90) as shown in Table no.4 and Figure 9.

DISCUSSION

Stress is a global menace fortified by the advancement of industrialization and elicited by a variety of factors, viz., and environmental, social or pathological phenomenon of life. Considerable evidence published in the last decade has focused on a constellation of neurochemical, biochemical and molecular effects caused by stress in the CNS, endocrine system, and immune system. Normally stress induced changes are self-limiting and adaptive until events override "threshold" limits, becoming irreversible and pathological. Advancements in the understanding of processes leading to the pathogenesis of stress-induced disorders cannot obscure the simple fact that the exhaustion of energy supply is still the basis for triggering the disorders and collapse of energy metabolism following glucose deprivation in the circulation. The desire to augment the coping mechanism has led to the emergence of the science of adaptation that focuses on elucidating mechanisms that may help to counteract excessive and unnecessary responses to stress [13].

In the present study, the antistress activity of the ethanolic extract of *Marsilea quadrifolia* whole plant was evaluated by using various acute stress experimental models. The swimming endurance test, anoxic tolerance test and restraint stress test are stress model used for the evaluation of antistress activity [14].

The swimming endurance test is one of the most suitable paradigms, widely used for the routine evaluation of anti-stress and antidepressant property of novel compounds [15].

Central neurotransmitter are functionally involved in the regulation of stress responses. These chemical substances are release in response to stress are meant to strengthen the organism by providing resistance against the stressful events a process known as adaptation. However prolonged severe stress creates ineffective adaptation, which results in reduced stamina or mood [16].

This test is based on the observation that animals swim in water, eventually assumed a characteristic immobile posture, devoid of any activity. The appearance of immobility therefore, reflects a state of tiredness, fatigue, reduced stamina or a lowered mood (hopelessness or despair). These designs represent some of the core symptoms observed in individuals under intense stress [17].

The swim endurance test results indicate clearly that the MQEE has the properties whereby it increases the physical endurance as well as post swimming motor function as compared to control group in mice and possess significant anti-stress activity. It may be possibly normalizing the plasma level of catecholamine and MAO. It may be due to increased utilization of the adenosine tri phosphate pathway, increased levels of muscle glycogen (a storage form of glucose that can provide energy for more prolonged activities), or decreased concentrations of muscle lactic acid and ammonia (two toxic by-products of muscular effort). It can be attributed to the anti-oxidant effect of plant extract which prevent the free radical-induced damage of the vital organs [18].

The ability of *Marsilea quadrifolia* to reduce the duration of immobility in this study suggests that it possesses phytochemically active compound with anti-stress property.

The exposure of hypobaric environment to mice for a specified period causes significant decrease in brain neurotransmitters, i.e. nor epinephrine (NE), dopamine (DA), serotonin (5-HT) and acetylcholine (ACh). The significant increase in anoxia tolerance time is an indication of either resistance to it or reduction in cerebral oxygen consumption. Both these effect are quite useful to protect neuronal cell against oxidative stress, a clinical condition increasing with the modernisation of life [19].

All the body functions, including cellular respiration depends on oxygen supply. Lack of any vital element will play havoc on all body mechanisms. Increased adaptation due to the depletion of any vital elements during stress by any drug that increases the tolerance can act as adaptogenic agent. Adaptogen producing beneficial effects in stress are believed to act by increasing non-specific resistance. In the present study depletion of oxygen in hermetic vessel

leads to convulsions in animals and pretreatment with ethanolic extract of *Marsilea quadrifolia* had increased the stress tolerance indicating their anti-stress activity [20].

Phytochemically active constituents i.e, flavonoid and glycoside, which could have increased the level of central neurotransmitter. Prolongation of mean time to convulsion could be as a result of its powerful antioxidant and free radical scavenging activities [21].

Stress response is sub-served by complex system with subsequent involvement of hypothalamus pituitary adrenal axis. Stresses, both physical and emotional, act via neural pathway to hypothalamus and leads to increase in corticotrophin releasing hormone and hence adrenocorticotropin hormone and cortisol secretion. Release of ACTH in stress stimulates adrenals to increase production of hormones epinephrine and corticosteroids. One of the best explored models of stress in mice is forced immobilization. As painful stimuli are not directly involved in restraint stress, this form of stress is probably more akin to physiological stress, combining emotional stress and physical stress resulting in both restricted mobility and aggression [22].

Stress is an aversive stimulus which perturbs the physiological homeostasis and its impact is reflected on a variety of biological systems. Complex mechanisms contribute to the breakdown in adaptational process resulting in various behavioural and endocrinological changes. Stress involved in the etiopathogenesis of variety of disease such as depression & anxiety, cognitive dysfunction, male impotency, hypertension and ulcerative colitis. The hypothalamic-pituitary-adrenal (HPA) axis and adrenal glands are crucial for the regulation of stress physiology. The activation of the HPA (Hypothalamic-pituitary-adrenal) system due to stress results in secretion of corticotrophin hormone, adrenocorticotropin hormone (ACTH), β -endorphin and glucocorticoids into the circulation. Release of ACTH in stress stimulates adrenals to increase production of hormones- epinephrine, nor epinephrine and corticosteroids. CRF (corticotrophin releasing factor) as a neurotransmitter or neuromodulator in brain, is known to act within the central nervous system to modulate a number of behavioral, neuroendocrine and autonomic responses to environmental stimulation through its action on the HPA-axis, resulting in increased level of serum cortisol. Increased cortisol level has been linked with anxiety like behavior and depressed motor response in humans. During stressful conditions, changes in monoamines (NA, DA & 5-HT) are well associated with transient behavioral aberrations in memory, learning and other mood disorders. Deregulated function of monoamines is one of the principle reasons for memory dysfunction during stressful conditions [23].

The mechanism by which stress raises serum cholesterol is likely to be related to the enhanced activity of hypothalamo- hypophyseal axis, resulting in increased liberation of catecholamines and corticosteroids which lead to elevated lipids from adipose tissues [24]. After treatment with *Marsilea quadrifolia* extract cholesterol was reduced in immobilization stress model. The effect of stress on serum triglycerides has been shown to be variable; probably catecholamines mobilize lipids from adipose tissue. In the present study immobilization stress model showed an increase in triglyceride levels [25].

In acute restraint stress model, pretreatment of mice with *Marsilea quadrifolia* at 200, 400 mg/kg body wt have been found to inhibit stress-induced rise in plasma glucose, cholesterol and triglyceride level compared to stress control group.

The result of present study it is concluded that *Marsilea quadrifolia* has potential protective effect against different paradigms of stress and the antistress property of *Marsilea quadrifolia* could be attributed to the presence of constituents like polyphenols, saponin and flavonoid.

CONCLUSION

The present study indicates that the ethanolic extract of *Marsilea quadrifolia* possessed a significant antistress activity in various acute stress models. The antistress activity of *Marsilea quadrifolia* ethanolic extract was probably due to its anti-oxidant property and the presence of flavonoid, glycosides, saponin, tannins and phenols. The results are encouraging to pursue further studies on the other bioactivity guided fractionation of these extracts to isolate and characterize probable bio active molecules.

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