ADAPTOGENIC ANTI-STRESS ACTIVITY OF STANDARDISED EXTRACT OF *MARSELIA MINUTA* L.

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Summary

Marsilea minuta (Mm) was investigated on a 14-day mild, unpredictable and inescapable foot shock stress (FSS) induced perturbations in behaviour (depression), suppressed male sexual behaviour and cognitive dysfunction in albino rats. Gastric ulceration, and adrenal gland and spleen weights were also used as the stress indices. *Panax ginseng* (PG) was used as the standard adaptogenic agent for comparison. FSS induced marked gastric ulceration, significant increase in adrenal gland weight with concomitant decrease in spleen weight. Chronic stress also suppressed male sexual behaviour, induced behavioural depression (Porsolt's swim despair test and learned helplessness test) and cognitive dysfunction (attenuated retention of learning in active and passive avoidance tests). All these FSS induced perturbations were attenuated dose dependently by Mm (100, 200 and 400 mg/kg, p.o.) and PG (100 mg/kg, p.o.). The results indicate that Mm has significant anti-stress activity, qualitatively comparable to PG, against a variety of behavioural and physiological perturbations induced by chronic stress, which has been proposed to be a better indicator of clinical stress than acute stress, and may indicate adaptogenic activity.

Keywords: *Marsilea minuta*, *Panax ginseng*, cognitive dysfunction, chronic stress, anti-stress, adaptogen.

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Introduction

Marsilea minuta Linn. (Marsileaceae) a common Indian species of water fern is found widely in wet and flooded low land [1, 2]. The plant as a whole is used as sweet, astringent, cooling, digestive, diuretic, hypnotic and expectorant. Treatment for psycopathy, diarrhea, cough, bronchitis, skin diseases and fever has also been reported in Ayurveda [2, 3]. The decoction of this plant was feed to the patients suffering from mental disorders along with their meal as a routine procedure in some mental clinics [4]. Marsiline (macrocyclic ketone) was isolated from chloroform extracts of leaves (yield- 0.1 to 0.05%) and from whole plant (yield 0.03 to 0.04 %) and reported for its sedative and convulsant properties [4]. Other reported activities include antifertility activity [5, 6], hypocholesterolemic activity [7], tranquilizing activity [8], antibacterial and antifungal activity [9, 10].

Stress is a global menace fortified by the advancement of industrialization and elicited by a variety of factors, viz., environmental, social or pathological phenomenons of life. Stress has been postulated to be involved in the etiopathogenesis of a variety of disease states, including hypertension, peptic ulcer, diabetes, immune-supression, reproductive function [11], and behavioural disorders like anxiety [12] due to involvement of the central nervous system (CNS), endocrine system, and metabolic system. Since the introduction of adaptogens [13], researchers have studied several plants that were once used as tonics due to their adaptogenic and rejuvenating properties in traditional medicine [14]. The drugs of plant origin are gaining increasing popularity and are being investigated for remedies of a number of disorders including anti-stress adaptogenic activity [15]. Earlier we have successfully demonstrated that standardised extract of *Marsilea minuta* (Mm) possess anti-amnestic [16], anxiolytic [17] and antidepressant activities [18]. The present work was undertaken to investigate the effects of standardised ethanolic extracts of Mm for its putative anti-stress adaptogenic activity in view of its aforementioned reported psychopharmacological activities.

Materials and Methods

Materials: Panax ginseng (PG) was obtained from Biological Evans Ltd., India. All other reagents used were of analytical grade. Whole plants of *Marsilea minuta* were collected during the month of July 2004 from Berhampur, Orissa, India. *Marsilea minuta* Linn. (Marsileaceae) was authenticated by Prof. N.K. Dubey, Incharge herbarium, Department of Botany, Banaras Hindu University, Varanasi. A specimen copy (Sept-2004-1) was deposited in the herbarium, Department of Botany, Banaras Hindu University.

Preparation of extract: Whole plant of *Marsilea minuta* was dried under shade in a drying room with a relative humidity of 40%. The room temperature was maintained in between 37 and 40°C. Drying process was carried out for 5–7 days. The shade-dried plant was reduced to coarse powder in roller grinder and further it was finely powdered. The fine powder was then passed through sieve (no. 40). About 500 g of plant powder was thoroughly extracted with 2.5 litre of 90% ethanol in soxhlet apparatus for 48 h. The extract was concentrated *in vacuum* at 50°C and then lyophilized (yield 16.3%, w/w), which was then stored at −20°C until required. Presence of steroids, flavonoids, alkaloids, and saponins were confirmed by preliminary phytochemical investigation of ethanolic extract of *Marsilea minuta* [19]. Marsiline was isolated as described previously [4] and characterised.

The extract was standardised for marsiline (purity, 94.32%) using a PerkinElmer's HPLC with diode array detector. The method was standardised and validated with an initial sampling of 5 µg/ml. Eight replicates of this concentration (5 µg/ml) was prepared and analysed. Limit of detection and limit of quantification obtained was 1.53 and 5.11 µg/ml, respectively. Average percentage recovery and coefficient of variation was found to be 91.75 and 1.11%, respectively. Standard curve was prepared using five standards at 10, 20, 50, 100 and 200 µg/ml. The curve showed good linearity with r^2 value of 0.942. The standardised ethanolic extract (1.15%, w/w of marsiline) was used for the pharmacological evaluations.

Animals: Swiss albino mice (20±2 gm) and Wistar rats (200-250 gm) of either sex were obtained from the Central Animal House, Institute of Medical Sciences, Banaras Hindu University (Regd. no. 542/02/ab/CPCSEA). Animals were randomly housed in groups of six in polypropylene cages at an ambient temperature of 25±1^{0}C and 45-55% relative humidity, with a 12 h light/dark cycle (lights on at 7 am). The animals had free access to standard pellet (Hindustan Lever, India) and water *ad libitum*. Experiments were conducted between 8:00 and 14:00 h. The experiments were conducted according to the norms of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Prior permission was obtained from Institutional Animal Ethics Committee (IAEC) to carry out the experiments.

Drug treatments: Acute toxicity study and LD_{50} determination of alcoholic extract of Mm was studied by many researchers [3, 5, 7] and was found safe up to a maximum dose of 1 gm/kg by both oral and intraperitoneal route in rodents. Based on our earlier studies, standardised ethanolic extract (1.15% w/w of marsiline, HPLC) of Mm was administered orally, as polyethylene glycol (PEG) suspension in the doses of 100, 200 and 400 mg per kg once daily for 14 consecutive days, 1 hr before the induction of stress. Experiments were conducted on day 14, 1 h after the last stress procedure and 2 h after drug or vehicle administration. *Panax ginseng* (Biological E. Ltd., India) was used as the standard adaptogenic agent for comparison. Control animals were treated with the vehicle (10%, v/v PEG suspension, 10ml/kg/day, p.o.).

Induction of chronic stress: The method of Armando *et al*. [20] was used. The rats were randomly assigned to the unstressed control, stress and drug treated stress groups. Those assigned to the vehicle or drug treated groups were subjected daily (including Sundays) to 1 h of foot shock through a grid floor in a standard conditioning chamber with the escape route closed. The duration of each shock (2 mA) and the interval between the shocks were randomly programmed in between 3 and 5 sec and 10 and 110 sec, respectively in order to make them unpredictable [21]. Animals were tested on day 14, 1 h after the last shock procedure on completion of the test procedure involved.

Techniques used for assessment of stress intensity: The following parameters were used to assess the intensity of stress-induced effects:

(a) ***Gastric ulceration:*** The stomach was removed and split open along the greater curvature. The numbers of discrete ulcers were noted by the help of magnifying glass. The severity of the ulcers was scored after histological confirmation as, 0=no ulcers, 1=changes limited to superficial layers of the mucosa with no congestion, 2-half the mucosal thickness showing necrotic changes and congestion, 3= more than two-third of mucosal thickness showing necrotic changes and congestion, and 4=complete destruction of the mucosa with marked haemorrhage. Thereafter, the period ulcer severity score was calculated after adding up individual scores [22].

(b) ***Adrenal cortex and spleen weights:*** The adrenal gland and spleen were removed and weighed [23].

Methods used to assess stress-induced perturbations

(a) Stress-induced 'behavioural depression: The following methods were used to assess behavioural depression.

(i) ***Stress-induced 'behavioural despair test :*** Rats were forced to swim individually in a polypropylene vessel (45x40x30 cm) with a water level of 20 cm, which ensured that the rat's feet did not touch the floor of the vessel and that it could not climb out of it. The rat was allowed to swim for 10 min. Thereafter, during the next 5 min, the total period of immobility, characterised by complete cessation of swimming with the head floating above water level, was noted. This immobility period, after initial frenzied attempts to escape, is postulated to represent 'behavioural despair' as an experimental model of endogenous depression [24].

(ii) ***Learned helplessness test:*** On day 12 of the investigation, rats were subjected to footshock (60 scrambled shocks, 15 sec duration, 0.8 mA, every min) in a two compartment jumping box (Techno) with the escape door to the adjoining unelectrified compartment closed. The exercise continued for 1 hr. On day 14, 48 hr later, the rats were subjected to avoidance training, using the same apparatus but keeping the escape route to the unelectrified chamber open. During this avoidance training the rats were placed in the electrified chamber and allowed to acclimatize for 5 min before being subjected to 30 avoidance trials, with an inter-trial interval of 30 sec. During the first 3 sec of the trial a buzzer stimulus (conditioned stimulus, CS) was present followed by electroshock (unconditioned stimulus, UCS) (0.8 mA) delivered via the grid floor for the next 3 sec. The avoidance response was characterised by escape to the adjoining 'safe' chamber during CS. Failure to escape during UCS within 15 sec assessed as 'escape' failure which is postulated to indicate despair or depression [25].

(iii) ***Stress-induced inhibition of male sexual behaviour:*** A male rat was placed in a cage in a dimly room for 10 min with 2 oestrinised (sequentially treated with oestradiol valerate 5 μg/rat, followed 48 hr later by hydroxyprogesterone 1.5 mg/rat, sc) female rats. The total numbers of mounts were counted [26, 27].

(b) Stress-induced cognitive dysfunction: The following parameters were used to assess the effect of stress on retention of a learned task as memory:

(i) ***Active avoidance test:*** Rats were trained for an active avoidance task before subjecting them to stress. During training, the rat was placed in the right electrified compartment of a shuttle box (Techno) and allowed to acclimatize for 5 min. Thereafter, the animal was subjected to 15 sec of a buzzer stimulus (CS) which was followed by electric shock (1 mA, 50 Hz) given through the grid floor (UCS). The rats were given at least 10 trails, with an inter-trial interval of 60 min, until they reached the criterion of 100% avoidance response of jumping to the unelectrified left chamber of the shuttle box during CS. The test was repeated on day 14 in order to assess the retention of the active avoidance learning [28].

(ii) ***Passive avoidance test:*** The test apparatus was a rectangular box (45x30x40) with an electrified grid floor. An 8 cm high platform (17x12 cm) was fixed to the centre of the floor. A rat was placed on the platform and allowed to step down. 24 hr later, on day 1 of the experiment, the rat was again placed on the platform and on stepping down, received foot shock (0.75 mA, 2 sec) through the grid floor. The rat was given 3 more trials until the latency of step down had stabilized. The test was repeated on day 14 and retention of learning as memory, for each rat was recorded [27, 29].

Statistical analysis: The values are expressed as mean ±SD. Statistical significance of the difference between control and treated groups was calculated using Kruskal Wallis one way analysis of variance (ANOVA) followed by Mann-Whitney U-Test (two tailed), $P<0.01$ was considered to be significant.

Results

(1) ***Gastric ulceration:*** Chronic stress markedly increased the incidence, number and severity of gastric ulcers. Mm (100, 200 and 400 mg/kg, p.o.) and PG (100 mg/kg, p.o.) significantly reduced these stress-induced gastric indices (Table 1).

Table 1: Effect of *Marsilea minuta* on chronic stress induced gastric ulcerations in rats

Treatment	Dose (mg/kg)	Ulcer incidence (%)	Number of ulcers	Severity of ulcers
Vehicle+Stress (VS) (10)	-	100	8.5±1.5	16.6±3.1
Mm+VS (6)	100	67	4.2±2.1†	8.5±4.2†
Mm+VS (6)	200	33	2.1±1.5†	4.6±3.2†
Mm+VS (6)	400	33	2.1±1.5†	4.5±1.8†
Panax ginseng + VS (6)	100	67	2.1±1.0†	4.6±1.8†

Values in parentheses indicate number of animals. † indicates difference with VS (ANOVA followed by Mann-Whitney *U*-test).

(2) ***Adrenal cortex and spleen weights:*** Chronic stress significantly increased adrenal gland weight and reduced that of spleen. These stress-induced changes were significantly attenuated by Mm (100, 200 and 400 mg/kg, p.o.) and PG (100 mg/kg, p.o.) (Table 2).

Table 2: Effect of *Marsilea minuta* on chronic stress induced changes in adrenal gland and spleen weights in rats

Treatment	Dose (mg/kg)	Adrenal gland wt (mg/100g)	Spleen wt (mg/100g)
Vehicle (10)	-	24.10±2.5	195.65±10.50
Vehicle+Stress (VS) (10)	-	41.5±3.70^{a}	128.60±8.50^{a}
Mm+VS (6)	100	30.70±2.80^{b}	157.65±7.00^{b}
Mm+VS (6)	200	27.00±2.48^{b}	178.40±6.05^{b}
Mm+VS (6)	400	26.90±2.40^{b}	180.40±6.05^{b}
Panax ginseng + VS (6)	100	28.40±2.65^{b}	170.05±9.10^{b}

Values in parentheses indicate number of animals. ^{a} indicates difference with vehicle treated group. ^{b} indicates difference with VS (ANOVA followed by Mann-Whitney *U*-test).

(3) ***Stress-induced 'behavioural despair' and 'learned helplessness' test:*** Chronic stress increased the duration of immobility, while increasing escape failures with concomitant decrease in avoidance response in the learned helplessness test, features indicative of depression. Mm (100, 200 and 400 mg/kg, p.o.) and PG (100 mg/kg, p.o.) tended to significantly reverse the stress-induced behavioural changes (Table 3 and 4).

Table 3: Effect of *Marsilea minuta* on chronic stress-induced changes in swim stress immobility, suppression of sexual behaviour and memory deficit in active avoidance response in rats

Treatment	Dose (mg/kg)	Duration of immobility (sec)	Number of mountings (N)	Active avoidance response on day 14 (%)
Vehicle (10)	-	116.13±9.10	6.10±0.75	75
Vehicle+Stress (VS) (10)	-	258.89±7.30[a]	1.65±0.70[a]	20[a]
Mm+VS (6)	100	178.40±8.60[b]	3.20±0.75[b]	50
Mm+VS (6)	200	137.28±6.08[b]	3.80±0.90[b]	70[b]
Mm+VS (6)	400	131.58±5.89 [b]	3.95±0.85[b]	70[b]
Panax ginseng + VS (6)	100	141.40±6.55[b]	4.10±0.80[b]	70[b]

Values in parentheses indicate number of animals. [a] indicates difference with VS (ANOVA followed by Mann-Whitney *U*-test).

Table 4: Effect of *Marsilea minuta* on chronic stress-induced changes in learned helplessness test in rats

Treatment	Dose (mg/kg)	Escape failure (N)	Avoidance response (N)
Vehicle (10)	-	13.70±1.75	3.85±0.69
Vehicle+Stress (VS)(10)	-	25.04±1.90[a]	0.79±0.15[a]
Mm+VS (6)	100	19.30±1.50[b]	1.65±0.52[b]
Mm+VS (6)	200	16.65±1.25[b]	2.85±0.60[b]
Mm+VS (6)	400	15.45±1.20[b]	2.95±0.54[b]
Panax ginseng + VS (6)	100	15.50±1.15[b]	2.90±0.51[b]

Values in parentheses indicate number of animals. [a] indicates difference with vehicle treated group. [b]indicate difference with VS (ANOVA followed by Mann-Whitney *U*-test).

(4) ***Stress-induced inhibition of male sexual behaviour:*** Chronic stress significantly decreased the sexual behaviour of male rats, as indicated by decrease in the number mountings. This stress effect was significantly reversed by Mm (100, 200 and 400 mg/kg, p.o.) and PG (100 mg/kg, p.o.) (Table 3).

(5) ***Active and passive avoidance test:*** Chronic stress produced significant decrease in the retention of acquired active and passive learning. These stress induced memory deficits were significantly reduced by Mm (100, 200 and 400 mg/kg, p.o.) and PG (100 mg/kg, p.o.) (Table 3 and 5).

Table 5: Effect of *Marsilea minuta* on chronic stress-induced memory deficit in passive avoidance response in rats

Treatment	Dose (mg/kg)	Latency (Step-through) in sec	
		Day 1	Day 2
Vehicle (10)	-	13.45±2.05	19.85±2.40
Vehicle+Stress (VS)(10)	-	12.10±1.80	7.32±0.51[a]
Mm+VS (6)	100	11.95±1.85	17.10±2.00[b]
Mm+VS (6)	200	12.60±1.90	17.45±1.90[b]
Mm+VS (6)	400	12.65±1.86	18.65±1.80[b]
Panax ginseng + VS (6)	100	12.00±1.88	18.95±1.91[b]

Values in parentheses indicate number of animals. [a] indicates difference with vehicle treated group. [b]indicate difference with VS (ANOVA followed by Mann-Whitney *U*-test).

Discussion

Adaptogens are the plant derived biologically active substances, which appear to induce a state of non-specific increase of resistance of the organism to diverse aversive assaults which threaten internal homeostasis and which improve physical endurance for doing work even in adverse circumstances and in difficult environmental conditions [23, 30]. These agents are basically preventive rather than curative in action and appear to function best when the resistance of the body is diminished, as seen in the case of prolonged illness, chronic stress and old age. They increase tolerance to change in environmental conditions and resistance to noxious stimuli such as exposure to cold, heat, pain, general stress and infectious organisms. Such agents have been claimed to arrest ageing process and age induced deterioration in physical and mental performance. Stress research in laboratory animals has assumed an important role in understanding the biological and behavioural consequences of external or internal stressors, which threaten to perturb homeostasis, and may induce a number of clinical diseases when the body fails to counter the stress situation [31]. A variety of stressful situations have been employed and the lack of consistency of the stress protocols is astounding [31]. Likewise, there is wide variation in the physiological consequences of the stressors utilized in animal research [32]. However, it is now widely accepted that chronic inescapable intermittent stress, particularly of an unpredictable pattern, is more likely to induce neural, endocrine and biochemical perturbations than either acute or chronic stress of a predictable nature [31]. The validity of the method used in the present study is demonstrated by the biological effects induced by it which include gastric ulcerations, increase in adrenal gland weight and decrease in the weight of the spleen. All these parameters have been conclusively shown to be stress-induced effects [28, 33].

The prevention and management of stress disorders remains a major clinical problem. Benzodiazepines (BDZs) appear to be effective against acute stress but fail to prevent the consequence of chronic stress [31]. In addition, the problems of the tolerance and physical dependence exhibited by BDZs, on prolonged use, limit their utility [31]. An answer to this vexing problem was first provided when it was reported that some plant-derived agents could induce a state of non-specific increase of resistance to affect internal homeostasis [34]. These agents, named adaptogens, appeared to be effective only when the physiological perturbations were discernible following prolonged illness, old age and exposure to chronic

stress [34]. Adaptogen *Panax ginseng* (PG) was shown to be effective in attenuating stress induced adverse effects in astronauts, soliders and athletes in the USSR [34]. PG, the first clinically used adaptogen, has been extensively investigated experimentally and clinically for its stress-attenuating activity [35].

Both Mm and PG prevented chronic stress-induced gastric ulcerations, in the term of the incidence and severity of the ulcers. Involution of the spleen and increase in adrenal gland weight, are also consequences of chronic stress [31], both responses being significantly reversed by Mm and PG.

There is considerable experimental and clinical evidence to suggest that chronic stress induces endogenous depression [36]. A number of animal models of depression and based on the use of uncontrollable stress and the biochemical correlates of such tests are consonant with those seen in chronic stress, including monoamine deficiency and increased activity of the corticotrophin-releasing factor [36]. Both Mm and PG were able to significantly reverse chronic stress-induced indices validated as animal models of depression. Chronic stress is known to affect other endocrine responses as well, which can induce sexual debility in males [37] and perturb glucose metabolism [37]. Maturity-onset diabetes mellitus may represent a state of stress-induced disturbance in glucose homeostasis [38]. Mm and PG significantly reversed chronic stress-induced inhibition of male sexual behaviour.

Stress is known to interfere with cognitive functions, tending to retard the memory engram rather than the acquisition of learning [39]. The mechanisms involved in the memory-attenuating effect of stress remains conjectural but a similar neurochemical basis operating in the induction of stress-induced depression, may be responsible [39]. Mm and PG significantly attenuated the stress-induced deficit to retention of learned tasks, both in the active and passive avoidance parameters, thus facilitating memory and its recall.

The findings indicate that, like the standard adaptogen PG, Mm can attenuate chronic stress-induced biochemical, behavioural and physiological perturbations in rats. PG has earlier been reported to reverse chronic stress induced defects in humans [34]. Japanese traditional medicinal plant formulations, like *Gosya-jinki-gan, Kyushin* and *Reiousan,* essentially based on *Ginkgo biloba,* have been reported to reduce the adverse effects of chronic hanging stress on sexual and learning behaviours in mice [40].

Increased generation of oxidative free radicals (OFR), or impaired antioxidant defence mechanisms, have been implicated in chronic stress induced perturbed homeostasis including immunosuppression, inflammation, diabetes mellitus, peptic ulceration and other stress-related diseases [41]. Thus, the observed adaptogenic anti-stress effect of Mm may be at least partly due to its antioxidant activity.

The present investigation indicates that Mm has significant adaptogenic anti-stress activity as shown by its mitigating effects on several chronic stress induced physiological and behaviour perturbations, comparable to that induced by the well accepted adaptogenic agent, *Panax ginseng.*

Conclusion

We report for the first time that adaptogenic anti-stress activity of standardised extract of *Marsilea minuta* L. which may be potentially valuable for the treatment of stress and stress related disorders.

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