A STUDY ON ADAPTOGENIC ACTIVITY OF STEM EXTRACTS OF *TINOSPORA MALABARICA* (LAMK)

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Summary

The objective of the study was to investigate adaptogenic activity of petroleum ether, alcohol and aqueous extracts of stem of *Tinospora malabarica* (Lamk.). All the three extracts were subjected to preliminary phytochemical investigation and also for acute oral toxicity study. Adaptogenic activity was evaluated in various animal models like anoxia stress tolerance test in mice, forced swimming and cold induced stress in rats. The preliminary phytochemical screening revealed the presence of flavonoids, carbohydrates and amino acids in aqueous extract, flavonoids, alkaloids and carbohydrates in alcoholic extract, while petroleum ether extract contains only steroids. Aqueous and alcohol extracts found to be non toxic upto a dose of 5000 mg/kg while petroleum ether extract was non toxic up to a dose of 2000 mg/kg. Forced swimming and cold resistant stress altered various serum biochemical parameters like glucose, cholesterol, triglycerides BUN, cortisol, blood cell count (RBC,WBC,DLC) and weight of organs like liver, spleen and adrenal glands . The aqueous and alcohol extracts had reduced stress induced elevated levels of serum biochemical parameters, blood cell count, prevented alterations in the weight of the liver, adrenal gland and increased the weight of the spleen following the treatment with extracts. The present investigation revealed that *Tinospora malabarica* exhibited adaptogenic activity by preventing stress induced elevated levels of biochemical, hematological changes and preventing the alteration in organ weights.

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Introduction

Stress is a psycho-physiological response to a change in the environment [1]. Stress has been postulated to be involved in the etiopathogenesis of variety of disease states, ranging from psychiatric disorder, indigestion, gastritis, bowel disturbance, muscle pain, immunosupression, endocrine disorder, male impotency, cognitive dysfunction, peptic ulcer, hypertension and ulcerative colitis [2].

Recently some plants have been reported to possess anti stress or adaptogenic activity in animals and man used to overcome stress related disorder and diseases. In recent days, there has been huge volume of work aimed at scientific validation of efficacy of herbal drugs used in the traditional system. Mechanism of T. malabarica as antistress in restraint stress was explored [3] wherein the stress period was of short duration and in the present study it was selected to study in various stress induced animal models like anoxia stress tolerance in mice, swimming endurance and cold resistance stress in rats.

Materials And Methods

Drugs and Chemicals

Withania somnifera root powder (Standard drug) obtained from sami labs, Bangalore. Absolute alcohol, petroleum ether, EDTA solution, anesthetic ether, chloroform, Erba kits for estimation of glucose, cholesterol, triglyceride and BUN.

Animals

Adult swiss albino mice (20-25g) and wistar rats (160-200g) procured from Sri Venkateshwara enterprises, Bangalore, were used to study antistress activity. All animals were maintained under standard husbandry conditions (Light / dark period of 12 hrs day / night and temperature 24^0 C ± 2^0C) with free access to food and water ad *libitum*. The experimental protocols were approved by Institutional Animal Ethics Committee and all experiments were performed between 10:00 -17:00hrs.

Preparation of plant extracts

T. malabarica (Lamk.) was purchased from Alwa Pharmacy, Moodbidre, Mangalore, Dakshin Karnataka and was authenticated by Prof. Laxmayya, botanist, V.L college of pharmacy, Raichur. The dried stems were reduced to coarse powder and were successively extracted in soxhlet apparatus using petroleum ether and absolute alcohol for 18 hrs. The same marc was extracted by maceration process with water for 24 hrs to get aqueous extract and percentage yield for all the three extracts were 2.43. 4.53 and 6.5 g respectively.

Preliminary Phytochemical screening

All the three extracts were subjected to preliminary phytochemical investigation for the presence of various phytochemical constituents as described by Khandewal [4].

Acute oral toxicity studies (LD_{50}):

The acute toxicity of petroleum ether, alcoholic and aqueous extracts of *Tinospora malabarica (Lamk.)* were determined in 3 hrs fasted female albino mice by OECD guide lines No. 425. The

LD_{50} of the test extracts were calculated using AOT 425 software provided by Environmental Protection Agency, USA.

ADAPTOGENIC ACTIVITIES

Anoxia stress tolerance

Albino mice of either sex weighing between 18-22 g were divided into 4 groups of six in each. Hermetic vessel of 500 ml air capacity was used for this test. Each animal was kept in the hermetic vessel and the time to show the first sign of convulsion was noted, it was immediately removed from the vessel and resuscitated if needed. After one week of drug treatment the animals were once again exposed to the anoxia stress. Similarly the animals were also observed at the end of 2^{nd} and 3^{rd} week with the same treatment and the time duration for anoxia stress tolerance was noted [5].

Swimming endurance test in rats

Rats of either sex (160-200 g) were divided into five groups of six in each were used for the study. Stress was exerted in rats by keeping them in cylindrical vessels (length 48 cm and width 30 cm) filled with water to a height of 25 cm and the total swimming time for individual rat was noted, the rats were allowed to swim daily till exhausted . Extracts were given to rats once daily for a period of 7 days and on 8^{th} day mean swimming time for each group was calculated, blood was collected through retro orbital plexus under light ether anesthesia to estimate biochemical parameters like blood glucose, triglycerides, cholesterol, BUN and blood cell count (RBC, WBC, DLC). Animals were sacrificed by cervical dislocation and the weight of organs such as liver, adrenals, spleen were recorded after washing with alcohol [6, 7].

Cold resistant stress:

Rats were divided into 5 groups of 6 animals in each were subjected to cold stress by exposing them to 4 ± 1^0 C daily for 2 hours [8]. The extracts were administered daily once to respective groups. This procedure was repeated for a period of 10 days. Blood was collected through orbital plexus under light ether anesthesia as mentioned above for estimation of biochemical parameters as mentioned above [9, 10]. The animals were sacrificed and the weight of organs such as liver, spleen and adrenal gland were recorded after washing them with alcohol [11].

Statistical Analysis

All the values are expressed as mean ± SEM. Statistical differences between means were determined by one-way ANOVA followed by Dunnett's post hoc test. $P < 0.05$ was considered as significant. The statistical analysis was performed using Instat ® software (Graph pad Inc., Santabarba, CA)

Results

Preliminary phytochemical investigation

The preliminary phytochemical screening with stem extracts of *Tinospora malabarica (Ham.)* revealed the presence of flavonoids, carbohydrates and amino acids in aqueous extract, flavonoids, alkaloids and carbohydrates in alcoholic extract and only steroids in petroleum ether extract.

Acute toxicity study:

Aqueous and alcohol extracts up to a dose of 5000 mg/kg and petroleum ether extract up to a dose of 2000 mg/kg were found to be non toxic.

Evaluation of Adaptogenic activity

Effect of stem extracts of *Tinospora malabarica* on anoxia stress tolerance test in mice

Pretreatment with aqueous and alcoholic extracts had significantly increased the anoxia stress tolerance at the end of 1st, 2 nd and 3 rd weeks of treatment with both doses i.e., 500 mg/kg ($p<0.05$) and 1000 mg/kg ($p<0.01$) while a significant ($p<0.01$) increase in anoxia stress tolerance time was observed with petroleum ether extract with higher dose (1000 mg/kg) only (Table 1) when compared to stress control

Table 1 Effect of *T.malabarica* on anoxia stress tolerance test in mice and swimming induced ulcer in rats

Groups	Dose	Mean duration of anoxia tolerance time in mice (min)			Swimming endurance test in rats		
		1st Week	2nd Week	3rd Week	Mean Ulcer index	% Protection	Swimming Survival time (min)
Normal control	2ml /kg	--	--	--	0.00	100	------
Stress control	2 ml /kg	47.00±1.592	48.167±1.662	49.167±1.778	3.5±0.846	0.00	138.83±11.25
Withania somnifera	100 mg/kg	59.16**±1.66	62.667**±1.706	66.33±2.290**	0.833**±0.21	76.2	182.33±11.57
Aqueous extract	500 mg/kg	56.50*±2.553	57.33*±2.092	58.33*±2.076	1.16*±0.401	66.65	177.17±5.115
	1000 mg/kg	59.5**±1.945	66.00**±1.84	70.33**±2.629	--		--
Alcoholic extract	500 mg/kg	55.50*±2.045	56.33*±2.044	57.83*±2.242	2.16±0.872	38.08	155.67±10.42
	1000 mg/kg	57.83**±1.74	61.167**±1.6	65.333**±1.820	--		--
Petroleum ether	500 mg/k	51.333±3.211	54.333±2.565	56.500±2.156	1.417±0.238	59.51	152.0±11.41
	1000 mg/kg	56.16**±1.58	58.00**±1.88	60.167**±2.056	--		--

Results are expressed as Mean ±SEM; *Significance at* $P<0.05$*, $P<0.01$** as compared to stress control.

Effect of stem extract of *T.malabarica* on swimming endurance survival time in rats

On 8th day animals in stress control group swam for 139 ± 11.2 minutes and with standard and aqueous extracts treated but not alcohol and petroleum ether extracts treated groups showed a significant ($p<0.05$) increase in swimming endurance time **(**table 2).

Table-2 Effect of stem extracts of *T. malabarica* on biochemicals and organ weights in swimming endurance test

Groups	Biochemical parameters					Weight of organ /100gm body wt.			
	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	BUN (mg/dl)	Cortico sterone (ng/ml)	Liver (g)	Spleen (g)	Adrenal Gland (mg)	Kidney (g)
Group 1	98.35± 5.102	60.072± 2.853	54.347± 1.219	71.667± 5.772	15.20± 4.22	4.085± 0.0581	0.567± 0.0212	13.85± 0.312	0.819± 0.0562
Group 2	135.84± 5.991	74.612± 1.439	68.400± 1.279	97.167± 3.156	37.5± 3.73	5.055± 0.0881	0.378± 0.0179	20.98± 0.424	0.907± 0.0554
Group 3	110.83** ±3.960	64.395** ±1.282	61.104** ±1.613	79.333* *±2.445	24.8** ±1.24	4.217** ±0.1298	0.535** ±0.0192	14.89** ±0.462	0.778± 0.0204
Group 4	111.68** ±5.946	70.248 ±1.959	65.008 ±1.417	81.00** ±2.633	25.66** ±2.076	4.313* ±0.2056	0.497** ±0.0254	15.49** ±0.643	0.904 ±0.0270
Group 5	115.06* ±3.28	70.343 ±2.676	65.202 ±1.409	81.50** ±1.765	26.83* ±2.151	4.334* ±0.1671	0.485* ±0.0234	17.06 ±0.674	0.901 ±0.0975

Results are expressed as mean ± SEM, Significance at $P<0.05$*, $P<0.01$.** as compared to stress control Group 1– Normal Control , Group 2-Stress control , Group 3-*Withania somnifera* (Standard 100mg/kg), Group 4-Aqueous extract (500mg/kg), Group 5-Alcohol extract (500mg/kg)

Effect of extracts of *T. malabarica* on ulcer index in swimming endurance test.

Swimming stress induced ulcers in stress control group and pretreatment with standard and aqueous but not alcoholic and petroleum ether extracts of *T. malabarica* had significantly prevented the swimming stress induced ulceration. (Table1)

Effect of stem extracts of *T. malabarica* on biochemical parameters

Biochemical parameters like glucose, cholesterol, triglycerides, BUN and cortisol were found to be increased in forced swimming and cold induced stress models in rats which were reduced on pretreatment with standard drug (*Withania somnifera*), while aqueous and alcoholic extracts reduced glucose, cholesterol and cortisol only.(Table 2).

Effect of stem extracts of *T. malabarica* on organ weights

Weight of the organs like liver, spleen increased, while spleen was reduced in both stress models. Pretreatment with standard drug, aqueous and alcoholic extracts but not petroleum ether extract significantly reduced the weight of the liver, adrenal gland and increased weight of the spleen however alcoholic extract did not reduced weight of the adrenal gland significantly in forced swimming stress model. While no significant change was observed with the weight of the kidney in any of the stress models.(Table 3 and 4).

Table-3 Effect of *T. malabarica* on Blood cell count in swimming endurance test in rats

Treatment	RBC millions /cumm	WBC Cells/cumm	Differential Leucocytes count (cells/cumm.)			
			Neutrophils	Lymphocytes	Eosinophils	Monocytes
Group 1	3.05± 0.143	9000± 187.9	2119.5± 44.2	6750.0± 140.9	130.1± 2.57	9±0.258
Group 2	3.78± 0.060	12250± 517.5	2884.8± 121.8	9187.5± 388.1	177.5± 7.42	12± 0.730
Group 3	3.10** ±0.068	10017** ±340.0	2358.9** ±80.0	7514.0** ±255.5	140.1* ±3.31	10± 0.731
Group 4	3.28** ±0.124	10600* ±366.9	2496.3* ±86.4	7966.7* ±274.8	136.6* ±17.73	11± 0.730
Group 5	3.30** ±0.085	11333 ±342.2	2669.0 ±80.5	8500.0* ±256.6	164.0 ±5.01	12± 0.8165

Results are expressed as Mean ±SEM; *Significance at P*<0.05*, *P*<0.01** as compared to stress control. Group 1– Normal Control , Group 2-Stress control , Group 3-*Withania somnifera* (Standard 100mg/kg), Group 4-Aqueous extract (500mg/kg), Group 5-Alcohol extract (500mg/kg)

Table-4 Effect of stem extracts of *T. malabarica* on biochemicals and organ weights in cold stress in rats

Groups	Biochemical parameters				Weight of organs /100gm body wt.			
	Glucose (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	BUN (mg/dl)	Liver (g)	Spleen (g)	Adrenal glands (mg)	Kidney (g)
Group 1	98.35 ±5.102	60.07 ±2.853	54.34 ±1.219	71.76 ±5.766	4.085 ±0.058	0.567 ±0.021	13.85 ±0.312	0.819 ±0.056
Group 2	132.66 ±2.315	73.46 ±1.637	65.46 ±1.923	94.92 ±1.658	5.527 ±0.188	0.418 ±0.022	20.13 ±0.578	1.023 ±0.082
Group 3	105.57** ±2.833	64.15** ±1.661	56.98* ±1.676	78.80** ±1.766	4.470** ±0.193	0.532** ±0.019	15.55** ±0.407	0.848 ±0.079
Group 4	110.85** ±3.069	65.07** ±1.718	59.41 ±2.028	81.83** ±1.533	4.650* ±0.201	0.519** ±0.019	16.71** ±0.557	0.850 ±0.089
Group 5	115.27** ±2.906	69.20 ±1.398	61.40 ±2.188	88.73 ±2.719	4.693* ±0.208	0.503* ±0.020	17.71* ±0.685	0.925 ±0.092

Results are expressed as Mean ±SEM; *Significance at P*<0.05*, *P*<0.01** as compared to stress control.
Group 1– Normal Control , Group 2-Stress control , Group 3-*Withania somnifera* (Standard 100mg/kg), Group 4-Aqueous extract (500mg/kg), Group 5-Alcohol extract (500mg/kg)

Effect of T. malabarica on Blood cell counts

Forced swimming as well as cold stress significantly altered the hematological parameters i.e.,increased RBC, WBC and DLC counts. Pretreatment with standard drug, aqueous and alcoholic but not petroleum ether extracts significantly inhibited the stress induced changes in these parameters (Table 3 and 5) .

Table-5 Effect of *T. malabarica* on Blood cell count in cold stress in rats

Treatment	RBC millions /cumm	WBC cells/cumm	Differential Leucocytes count (cells/cumm.)			
			Neutrophils	Lymphocytes	Eosinophils	Monocytes
Group 1	3.05 ±0.143	9000 ±187.97	2118.83 ±44.244	6750 ±140.98	130.16 ±2.57	9.00 ±0.258
Group 2	3.90 ±0.096	12500±200.07	4689.3 ±99.19	7499.2 ±98.16	293.17 ±4.67	18.33 ±0.843
Group 3	3.28** ±0.094	10073** ±194.02	2758.8** ±113.90	7148.5 ±84.02	150.83** ±2.91	15.16** ±0.307
Group 4	3.38** ±0.079	10433** ±215.51	3263.5** ±105.61	6996.5** ±113.5	158.0** ±3.26	15.33** ±0.421
Group 5	3.45** ±0.076	11133** ±278.89	3920.3** ±142.30	7013.5** ±139.7	185.0** ±5.17	16.16* ±0.477

Results are expressed as Mean ±SEM; *Significance at* $P<0.05$*, $P<0.01$** as compared to stress control. Group 1– Normal Control , Group 2-Stress control , Group 3-*Withania somnifera* (Standard 100mg/kg), Group 4-Aqueous extract (500mg/kg), Group 5-Alcohol extract (500mg/kg)

Discussion

All the body functions, including cellular respiration depends on the oxygen supply .Lack of any vital element will play havoc on all body mechanisms. Increase in adaptation due to the depletion of any vital elements during stress by any drug that increases the tolerance can acts as adaptogenic agent. Adaptogens produce beneficial effects in stress which are believed to act by increasing the non specific resistance. In the present study depletion of oxygen in hermetic vessel leads to convulsions in animals and treatment with *T. malabarica* extracts had increased the stress tolerance indicating their adaptogenic activity [12, 13].

The present study investigated the ability of the extracts of *T. malabarica* to suppress stress induced changes in biochemical parameters and organ weights in forced swimming and cold induced stress models. Forced swimming stress involves physical exercise and psychological stress which results in increased serum corticosterone and protein levels, while cold stress is of psychogenic type only [14].

Response to stress is highly contradictory with regard to blood sugar levels. Studies related to stress in rats shows fluctuation in blood sugar levels ranging from initially a slight decrease followed by relative increase and further no changes in blood sugar levels[7].

In the present study, a significant hyperglycemia was observed with both swimming and cold stress models. Under stressful conditions cortisol in man and corticosterone in rats will be secreted by adrenal cortex [15]. Hyper secretion of cortisol helps in the maintenance of internal homeostasis through the process of gluconeogenesis and lipogenesis [16]. Treatment with *T. malabarica* extracts significantly reduced the forced swimming and cold stress related hyperglycemia by reducing the hyper activity of adrenal cortex and also by maintenance of homeostatic mechanism. This was further in agreement with plasma corticosterone levels observed after forced swimming test.

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[17]. In the present study a significant increase in plasma corticosterone was observed in cold stress as well as in forced swimming stress. After treatment with plant extracts it was reduced in forced swimming stress. Since forced swimming stress requires more exercise than cold stress and the cholesterol level might be increased due to the mobility of fat which could not be suppressed by extracts.

The effect of stress on serum triglycerides has been shown to be variable probably catecholamines mobilize lipids from adipose tissues. In the present study with forced swimming and cold stress models showed an increase in triglyceride levels. However *T. malabarica* extracts were not able to suppress the stress induced increase in triglycerides levels. These results suggest that aqueous and alcoholic extracts have weak adaptogenic activity in altering the lipid profiles during stress.

BUN levels were increased in forced swimming and cold stress as these are the end products of protein metabolism. In excess adrenocortical activity, due to increased metabolism of protein increases urea excretion, in the present study a similar effect was observed. However extracts of *T.malabarica* decreased the BUN levels as compare to stress control, indicating a diminished catabolism of protein under stressful conditions.

Adrenal glands and liver weights were significantly increased in forced swimming and cold stress models than control group. Stress induces adrenomedullary response in man to release adrenaline which in turn stimulates β_2 receptors on the pituitary gland. It leads to greater release of ACTH that can stimulate the adrenal medulla as well as cortex[16] resulting in further release of adrenaline and increase in weight of adrenal gland to greater extent. Cortisol increases m-RNA levels in liver cells, since the protein required for repair of wear and tear in swimming stress is more along with higher metabolic changes too that leads to show a significant increase in liver weight and it was well in correlation with the observed .increase in plasma corticosterone levels. Spleen contracts during stress and releases more amount of blood (RBC) into circulation hence its weight decreases. Pretreatment with aqueous and alcoholic extracts of *T malabarica* prevented the stress induced increase in weight of liver and adrenal glands and a decrease in spleen weight indicating their protective effect against stress. Stress causes alteration in hematological parameters like increase in RBC, WBC and DLC counts, neutrophils were increased more significantly in cold stress than forced swimming stress. Pretreatment with aqueous extract reduced the hematological parameters in both stresses, while alcohol and petroleum ether extracts reduced WBC, lymphocytes, eosinophils and monocyte counts in cold stress model. Aqueous extract of *T malabarica had* attenuated gastric ulcerogenesis due to forced swimming stress.

In conclusion the effect of alcoholic and aqueous extracts on biochemical, hematological parameters, organ weights indicated an adaptogenic activity of these against forced swimming

and cold stress in rats. However petroleum ether extract does not possess any adaptogenic activity. Phytochemical profiles indicated the presence of flavonoids, alkaloids and carbohydrates in alcoholic extract where as flavonoids, carbohydrates and amino acids in aqueous extract. The adaptogenic activity of aqueous and alcohol extracts of .*T malabarica* may be due to the presence of alkaloids or flavonoidal glycosides.

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