



Screening of Antistress and Anxiolytic Activities of *Piper longum* Fruits Extract

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Abstract

The aim of the study was to investigate adaptogenic and anxiolytic activities of methanol extract of *Piper longum* fruits at different dose levels (100, 250 and 500 mg/kg) using different experimental animal models. In the present research work antistress activity was assessed by swimming endurance and immobilization stress models, and anxiolytic activity were assessed by elevated plus maze behavior of mice, light dark exploration test in mice and open field apparatus test in rats. In swimming endurance test, the mean time of swimming performance and swimming stress induced biochemical parameters such as serum cortisol, the weights of adrenal glands, ascorbic acid and cortisol levels were recorded in the adrenal gland. There was dose dependent significant increase in swimming performance time observed in mice pretreated with graded doses of the test extracts. Animals pretreated with test extracts at different dose levels showed significant and dose dependent fall in all the biochemical parameters, as compared to the stress control animals. Treatment with standard and test extracts significantly reversed the stress induced altered hematological parameters, biochemical parameters, organs weight, GSH and LPO levels and neurotransmitters such as dopamine, nor adrenaline and serotonin levels in rat brain. In immobilization stress. The test extracts demonstrated the significant increase in time spent and number of entries into open arm in elevated plus maze apparatus test and also increase in time spent and number of entries into lit box was observed in light box exploration test and reduction in time spent and number crossing into the dark compartment observed in animals pretreated with test extract. Pretreatment with test extracts demonstrated dose dependent significant increase in ambulation, rearing and self-grooming and significant decrease in fecal dropping in case of open field test.

1 Introduction

Adaptability is probably the most distinct characteristic of life. Dr. Hans Selye defined stress as the sum of all non-specific responses of the body to any external stimuli acting up on it. Fundamentally, it is a physiological response towards external stimuli and the primary objective of such a response is to restore the normal process of life. Perhaps the single most important property of an adaptogen is its proven ability to combat stress in all forms¹.

In the present days, stress has become an integral part of human life². Stress is considered to be any condition, which

results in perturbation of the body's homeostasis^{3, 4}. Stress is involved in the pathogenesis of peptic ulcer, reproductive dysfunctions and behavioural disorders like anxiety due to involvement of the central nervous system, endocrine system, and metabolic system⁵. Drugs having antistress properties induce a state of non-specific resistance against stressful conditions. Medicines like benzodiazepines, amphetamine and caffeine are regularly prescribed to contest the stress. The incidence of toxicity and dependence has limited the therapeutic usefulness of these drugs⁶.

The potential efficacy of risk less and cheaper medicinal plants as anxiolytic agents had been documented as they can endure stress without changing the physiological mechanisms of the body. Various herbs like *Withania somnifera*⁷, *Emblca officinalis*⁸, *Asparagus racemosus*⁹, *Ocimum sanctum*¹⁰, *Tribulus terrestris*¹¹ and *Trigonella foenum-graecum*¹² are claimed to have adaptogenic effect and the ability to improve vital energy⁷.

Several poly herbal formulations namely, Siotone¹³, AVM¹⁴ and Geriforte have been reported to possess significant antistress properties. AVM is a poly herbal formulation possessing adaptogenic activity consists of various ingredients namely Root of *Withania somnifera*, Fruit of *Emblca officinalis*, Root of *Asparagus racemosus*, Tuber of *Dioscorea bulbifera*, Powder of Trikatu, Leaves of *Ocimum sanctum*, Powder of Shilajit, Areal parts of *Tribulus terrestris* and Fruit of *Piper longum*.

AVM a poly herbal formulation, some of whose constituents like *Withania somnifera*⁷, *Emblca officinalis*⁸, *Asparagus racemosus*⁹, *Ocimum sanctum*¹⁰ and *Tribulus terrestris*¹¹ have earlier been reported to exhibit significant adaptogenic activity. However, the literature review reveals that adaptogenic and anxiolytic properties of *Piper longum* have not been scientifically validated so far.

Hence, the present study was undertaken to evaluate the *Piper longum* fruits for adaptogenic and anxiolytic activities and to establish the mechanism of action underlying these effects.

2 Materials and Methods

2.1 Plant material

For this study, the fruits of *Piper longum* were purchased from the local market of Bijapur, Karnataka. The samples were identified and authenticated by Dr. M. B. Mulimani, Professor of Botany, S.B Arts and K.C.P. Science College, Bijapur, Karnataka. The specimen was preserved in the herbarium of the HSK College of Pharmacy, Bagalkot- 587101.

2.2 Preparation of plant extract

The fruits were graded, cleaned, shade dried and coarse powdered. Then the powdered material was extracted with pet ether for the removal fatty material followed by methanol using soxhlet extraction method. Thereafter, the methanol extract of *Piper longum* fruits (MEPLF) was concentrated using rotary flash evaporator and resulted to yield 16.5%. The obtained crude extracts was stored in the airtight container in a refrigerator below 10 °C for further studies.

2.3 Preliminary phytochemical screening

The crude extract was then subjected to preliminary phytochemical screening following the standard procedures described in the literature¹⁵.

2.4 Experimental animals

The Albino mice 20 - 30 g and Wistar rats 150 - 200 g of either sex were used in the experimentation. The animals were procured from Sri Venkateshwara Enterprises, 4304, 13th main 2nd cross, Subramanyanagar, Bangalore-21 (237/CPCSEA). After randomization into various groups, animals were acclimatized for the period of 10 days under standard husbandry condition as follows.

Room temperature: 27 ± 3 °C

Relative humidity: 65 ± 10%

12 hr. light/dark cycle

All the animals were fed with rodent pellet diet (VRK Nutritionals Industries, Pune, India) and water *ad libitum* under strict hygienic condition. Study protocol was approved from Institutional Animal Ethics Committee (IAEC) before initiation of the experiment. (Ref. No. BLDEA's COP/IAEC/51 dated 29/07/2013)

2.5 Preparation of stock solution of methanol extract of *Piper longum* fruits

Appropriate concentration of stock solution of the test extracts was prepared by suspending in 2% w/v of tween 80 in distilled water. This stock solution was administered orally at 1 ml/100 g body wt. of mice and 0.5 ml/100 g body wt. of rats.

2.6 Acute toxicity study (LD₅₀)¹⁶

The acute toxicity of methanol extracts of *Piper longum* fruits was determined in female Albino mice (20-30 g). The animals were fasted overnight prior to the experiment. Fixed dose (OECD Guideline No. 423) method was employed for toxicity study. Based on the result of the study, 1/40th, 1/20th and 1/10th of LD₅₀ cut off value, the screening doses of extract was selected for anti-stress and anxiolytic activities.

2.7 Evaluation of antistress activity of methanol extract of *Piper longum* fruits

2.7.1 Swimming endurance test^{17,18}

Albino mice of either sex weighing 20 -30 g divided into six groups of six animals each

- | | | |
|-----------|---|--|
| Group I | - | Normal control |
| Group II | - | Stress control, received vehicle only 1 ml/kg p.o. |
| Group III | - | Std. (<i>Withania somnifera</i> 100 mg/kg, p.o.) |
| Group IV | - | MEPLF 125 mg/kg p.o. |
| Group V | - | MEPLF 250 mg/kg p.o. |
| Group VI | - | MEPLF 500 mg/kg p.o. |

Treatment was given to mice for 7 days. On seventh day 1 hr. after treatment, all the mice except normal control were subjected to swimming endurance test. The mouse was allowed

to swim individually in swimming tank (30 cm height with 20 cm diameter) containing water of 25 cm height maintained at 26 ± 1 °C temperature. The mouse was allowed to swim till exhausted and moment when animals drowned is considered as the endpoint. The mean swimming time for each group is calculated.

2.7.1.1 Post swimming antifatigue and motor coordination test

The animals were immediately taken out, dried with tissue paper and subsequently all the animals placed on digital rota rod (15 rpm) to monitor antifatigue and motor coordination effects.

2.7.1.2 Biochemical estimations

The blood was collected (orbital sinus) from all the animals subjected to post swimming antifatigue effect for estimation of serum cortisol level. Then the animals were sacrifice for the removal of adrenal glands to record their weight. Then the two glands were used for the estimation of ascorbic acid and cortisol levels using standard procedures reported in the literature¹⁸.

2.7.2 Immobilization stress¹⁷

Adult albino rats of either sex weighing 150 – 200 g were selected and divided into six groups of six animals each.

- Group I - Normal control (Unstressed, untreated)
- Group II - Stress control (Stressed, received vehicle)
- Group III - Standard (*W.somnifera*, 100 mg/kg p.o.)
- Group IV - MEPLF125 mg/kg p.o.
- Group V - MEPLF 250 mg/kg p.o.
- Group VI - MEPLF 500 mg/kg p.o.

The treatment was made as stated above for 10 days 1hr. before the exposure of stress. Stress was induced by immobilizing rats with head down, supine position by fixing the forelimbs and hind limbs to a wooden board inclined at an angle of 60°, daily 2 hrs. for a period of ten days.

2.7.2.1 Hematological and biochemical estimations¹⁹⁻²¹

At the end of 10th day one hour after drug treatment the blood was collected from retro orbital plexus in sodium citrated tubes for estimation of hemoglobin (Hb), RBC, WBC, differential leucocytes count (DLC) and platelets carried out using digital cell counter and for the estimation of biochemical parameters such as, serum glucose (GOD-POD method), cholesterol (CHOD-PAP method), triglycerides (GPO-Trinder method), BUN (Blood Urea Nitrogen, GLDH-UREASE method) were measured using semi auto analyzer.

The rats then scarified and their organs such brain, liver, spleen and adrenal glands was removed. The weight of liver, spleen

and adrenal glands were recorded after washing with alcohol per 100 g body weight of animal.

2.7.2.2 Estimation of brain neurotransmitters²²

Noradrenalin (NA), dopamine (DA) and serotonin (5HT) levels were estimated after isolation of brains of all animals exposed to immobilization stress using high-performance liquid chromatographic (HPLC) technique coupled with photodiode array (PDA) detection.

2.8 Evaluation of anxiolytic activity of methanolic extract of *Piper longum* fruits

2.8.1 Elevated plus test²³

Albino mice of either sex weighing between 20-30 g were divided into five groups of six mice in each were fasted overnight prior to the test but water was supplied *ad libitum*.

- Group I - Control, received vehicle only
- Group II - Diazepam (2 mg/kg, p.o.)
- Group III - MEPLF125 mg/kg p.o.
- Group IV - MEPLF 250 mg/kg p.o.
- Group V - MEPLF 500 mg/kg p.o.

All the groups were received vehicle, standard and different test doses respectively once daily for 10 days. On 10th day one hour after the treatments, each mouse was individually placed on the center of the elevated plus maze with its head facing the open arm. During the entire experiment, mouse was allowed to socialize. Every precaution was taken out to ensure that no external stimuli, other than the height of the plus-maze could invoke maze anxiety. During the 5 min experiment, following behaviors of the mouse were recorded.

- Number of entries into the open arm
- Number of entries into the closed arm
- Time spent in the open arm and
- Time spent in the closed arm

Every time before placing each animal, the arena was washed with 5% alcohol to eliminate the possible bias due the odor left by the previous animal.

2.8.2 Light/dark exploration test²³

Albino mice (20-30 g) of either sex were divided into five groups of six mice in each fasted overnight prior to the test but water was supplied *ad libitum*.

- Group I - Control, received vehicle only
- Group II - Diazepam (2 mg/kg, p.o.)
- Group III - MEPLF125 mg/kg p.o.
- Group IV - MEPLF 250 mg/kg p.o.

Group V - MEPLF 500 mg/kg p.o.

The treatment was given once daily for 7 days. On 7th day 60 min after administration of the vehicle, standard drug and test extracts to different groups, the mouse were placed in the center of light box and the following parameters were recorded during a test session of 5 min.

- Latency to the first crossing into the dark compartment
- Number of crossings between the light and dark areas

Total time spent in the illuminated part of the cage

2.8.3 Open- Field apparatus test²³

Wistar rats (150-200 g) of either sex were divided into 05 groups of 06 in each were fasted overnight prior to the test but water was supplied ad libitum.

Group I - Control, received vehicle only

Group II - Diazepam (2 mg/kg, p.o.)

Group III - MEPLF 125 mg/kg p.o.

Group IV - MEPLF 250 mg/kg p.o.

Group V - MEPLF 500 mg/kg p.o.

Group I is maintained as normal control was received vehicle only once daily for 7 days, group II received diazepam (2 mg/kg, p.o.), Groups III, IV and V were treated with different doses of test extracts p.o. respectively once daily for 7 days. On 7th day 60 min after administration of the vehicle, standard and test extracts each rat was placed in the center of open field arena, and the following parameters were recorded during a test session of 5 min.

Ambulation: Measured in terms of the number of squares crossed by the animal.

Rearing: Number of times the animal stood on its hind limbs.

Self grooming: Number of times the animal groomed facial region, and licked /washed / scratched various parts of its body.

Fecal droppings: Number of fecal droppings excreted during the period.

2.9 Statistical analysis

The data obtained from the above findings were subjected to statistical analysis following one-way ANOVA followed by Tukey's Kramer Multiple Comparison Test to assess the statistical significance of the results using Graph pad prism software.

3 Results

3.1 Percentage yield of the test extract

The methanol extract of the *Piper longum* fruits resulted yield 16.5%.

3.2 Preliminary phytochemical screening

Results of the preliminary phytochemical investigation on methanol extract of *Piper longum* fruits reveal the presence of alkaloids, carbohydrates, flavonoids and tannins.

3.3 Acute toxicity study

In an acute toxicity studies, the test extract of title plant did not cause any mortality (0/3 mice died) of the animals at dose of 2000 mg/kg, even at repeated dosing using 3 new mice. Hence, 5000 mg/kg was taken as LD₅₀ cutoff value as per fixed dose method of OECD guideline number 423.

The doses selected for the evaluation of anti-stress and anxiolytic activities of the test extract was:

125 mg/kg - 1/40th dose of LD 50 cut off value, 5000 mg/kg b.w.

250 mg/kg - 1/20th dose of LD 50 cut off value, 5000 mg/kg b.w.

500 mg/kg - 1/10th dose of LD 50 cut off value, 5000 mg/kg b.w.

3.4 Effect of MEPLF on swimming endurance test in mice

Mice pretreated with graded doses (125, 250, 500 mg/kg) of the test extract for seven days, significantly increased the swimming performance time compared to the normal control group. The effect seen was dose-dependent and statistically significant. The percentage increase in the swimming time was found to be ranging from 18 to 43. The effect of MEPLF on swimming performance time at dose of 500 mg/kg was found closer to that of reference standard drug, *Withania somnifera*. The results are tabulated in table 1.

3.5 Effect of MEPLF on post swimming antifatigue and motor coordination effect

Mice subjected to the motor coordination test followed by swimming stress exhibited significant fatigue and motor incoordination effect compared to control group. Animals pretreated for seven days with test extract demonstrated significant antifatigue and motor coordination effect in dose dependent manner when compared to stress control group. The results are tabulated in table 1.

3.6 Effect of MEPLF on swimming stress induced biochemical parameters in mice

Swimming stress resulted in an increase in adrenal glands weight in association with depletion of adrenal contents namely ascorbic acid and cortisol, there was an elevated serum cortisol level also seen in swimming control as compared to normal control animals. Treatment with test extracts of different doses prevented the adrenal hypertrophy. Increased serum cortisol level was reversed with treatment of MEPLF significantly. Furthermore, a significant increase in adrenal ascorbic acid and cortisol contents were observed in animals treated with different doses MEPLF. The results are presented in table 2.

3.7 Effect of MEPLF on hematological parameters in Immobilization stress

Immobilization stress in rats resulted in decrease in Hb level, percentage of lymphocytes and eosinophils whereas an increase in RBC, WBC, platelets count and increase in percentage of neutrophils and monocytes when compared to

normal control. Treatment with standard and test extracts at different doses significantly attenuated stress induced altered hematological parameters. The results of the test extract at a dose of 500 mg/kg found almost similar to that of standard drug *Withania somnifera* (100 mg/kg). The results are tabulated in table 3.

Table 1: Effect of MEPLF on swimming endurance time and post swimming antifatigue effect in mice

Groups	Treatment	Dose mg/kg	Swimming endurance time (min)	% increase in swimming time	Fall of time from rotarod (sec)	% antifatigue effect
I	Normal control		---	---	110 ± 4.25	--
II	Stress control		222.2 ± 10.93	---	28.32±2.5	---
III	Std. (W S)	100	411.2 ± 6.71***	45.95	58.24±2.3***	51.72
IV	MEPLF	125	274.4 ± 2.15***	18.97	33.26±2.1 ^{ns}	15.15
V	MEPLF	250	338 ± 18.86***	34.31	39.15±1.8**	28.20
VI	MEPLF	500	396.8 ± 4.14***	43.93	46.25±1.2***	39.13

The values are expressed as Mean ± SEM, (n=6); * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

Table 2: Effect of MEPLF on swimming stress induced biochemical parameters in mice

Groups	Treatment	Dose mg/kg	Adrenal gland weight (mg/100 g b.w)	Serum cortisol (µg/dl)	Ascorbic acid (mg/100 g of adrenal wt.)	Cortisol (mg/100 g of adrenal wt.)
I	Normal control		7.25±0.62	23.56±0.58	280.2±0.25	3.73±0.45
II	Stress control		15.65 ± 0.12	45.95±0.2	128.2 ± 2.3	1.05 ± 0.06
III	Std. (W S)	100	10.20 ± 0.71***	25.25±0.6***	258.5 ± 2.3***	3.10 ± 0.03***
IV	MEPLF	125	14.4 ± 0.15***	35.97±0.4***	150.2±2.1***	1.35 ± 0.05***
V	MEPLF	250	13.2 ± 0.86***	30.31±0.8***	193.1±1.8***	1.89 ± 0.07***
VI	MEPLF	500	11.8 ± 0.14***	27.93±0.1***	226.2±1.2***	2.58 ± 0.01***

3.8 Effect of MEPLF on biochemical parameters in Immobilization stress

Immobilization stress caused a significant increase in the levels of serum glucose, total cholesterol, triglycerides and BUN when compared to normal control. Pre-treatment with graded doses of test extracts and standard drug significantly reversed the elevated levels of these biochemicals. The results are presented in table 4.

3.9 Effect of MEPLF on organs weight in Immobilization stress

Exposure of rats to Immobilization stress resulted in the significant increase in liver and adrenal glands weight whereas decrease in the spleen weight of when compared to normal control. Pre-treatment with standard drug *Withania somnifera* (100 mg/kg) and graded doses of the test extract MEPLF

significantly reversed the altered organs weight. The results are displayed in the table 5.

3.10 Effect of MEPLF on brain neurotransmitter level in Immobilization stress

Determination of a brain neurotransmitters level by HPLC method revealed that Immobilization stress caused a significant (p < 0.001) depletion of Noradrenalin (NA), Dopamine (DA) and Serotonin (5HT) leading to a state of depression. Treatment with MEPLF at 125, 250 and 500 mg/kg significantly augmented the levels of these neurotransmitters. The results are shown in table 6.

3.11 Effect of MEPLF on elevated plus maze behaviors of mice

The graded doses of the MEPLF (125, 250 and 500 mg/kg) and diazepam (2 mg/kg) exhibited a significant increase in number

of entries into the open arm and time spent in the open arm and time spent in a closed arm compared to control group. The results are tabulated in table 7.

Table 3: Effect of MEPLF on hematological parameters in Immobilization stressed rats

Gp.	Treat.	Dose mg/kg	Hb g %	RBC millio./cmm	Platelets lakh/cmm	WBC thou./cmm	Differential leucocytes count (%)			
							N	L	M	E
I	Normal control	--	13.03±0.05	5.38±0.3	8.50±0.5	10.06±0.4	40.0±0.4	38.0±0.8	4.0±0.4	2.9±0.3
II	Stress control	Vehicle	10.05±0.01 [@]	9.89±0.6 [@]	13.30±0.3 [@]	16.03±0.1 [@]	70.5±0.8 [@]	14.0±0.6 [@]	8.5±0.7 [@]	1.0±0.1 [@]
III	Std.	100	12.01±0.02 ^{***}	5.02±0.1 ^{***}	8.90±0.2 ^{***}	10.98±0.8 ^{***}	45.3±0.5 ^{***}	35.7±0.7 ^{***}	4.5±0.1 ^{***}	3.7±0.02 ^{***}
IV	MEPLF	125	10.25±0.05 ^{**}	8.50±0.2 [*]	11.60±0.2 ^{**}	13.50±0.5 ^{**}	62.2±0.3 ^{***}	20.2±0.2 ^{***}	6.7±0.3 [*]	1.7±0.01 ^{***}
V	MEPLF	250	11.60±0.02 ^{***}	7.05±0.3 ^{***}	10.68±0.2 ^{***}	12.75±0.2 ^{***}	54.4±0.2 ^{***}	26.1±0.5 ^{***}	6.2±0.6 ^{**}	2.0±0.02 ^{***}
VI	MEPLF	500	12.50±0.03 ^{***}	5.50±0.2 ^{***}	09.30±0.1 ^{***}	11.15±0.6 ^{***}	47.3±0.3 ^{***}	31.3±0.6 ^{***}	5.5±0.4 ^{***}	2.5±0.01 ^{***}

Values are expressed as Mean ± SEM, (n=6), where [@] p < 0.001 compared to normal control.; * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

Table 4: Effect of MEPLF on serum biochemical changes in Immobilization stress in rats

Groups	Treatment	Dose mg/kg	Biochemical estimations			
			Glucose mg/dl	Total Cholesterol mg/dl	Triglyceride mg/dl	BUN mg/dl
I	Control untreated	--	95.6±5.2	49.4±2.5	56.9±3.2	20.1±1.0
II	Stress control	Vehicle	145.5±6.1 [@]	135.9±2.9 [@]	97.3±2.9 [@]	35.5±0.9 [@]
III	Std. (W S)	100	105.1±4.3 ^{***}	55.6±3.0 ^{***}	58.3±2.0 ^{***}	22.5±0.9 ^{***}
IV	MEPLF	125	125.0±2.5 [*]	122.4±2.3 [*]	82.2±2.0 ^{***}	31.1±1.0 [*]
V	MEPLF	250	115.1±3.2 ^{***}	95.3±3.2 ^{***}	73.1±1.9 ^{***}	27.2±0.9 ^{***}
VI	MEPLF	500	109.0±2.6 ^{***}	62.4±2.1 ^{***}	62.4±1.5 ^{***}	23.3±0.8 ^{***}

Values are expressed as Mean ± SEM, (n=6), where p < 0.001 compared to normal control.; * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

Table 5: Effect of MEPLF on organs weight in Immobilization stress in rats

Groups	Treatment	Dose mg/kg	Organs weight (g/100 g b.w.)		
			Liver	Spleen	Adrenal glands
I	Normal control	--	3.00 ± 0.22	0.242 ± 0.01	0.150 ± 0.02
II	Stress control	Vehicle	6.89± 0.80 [@]	0.058± 0.03 [@]	0.490 ± 0.01 [@]
III	Std. (W S)	100	3.48 ± 0.13 ^{***}	0.218 ± 0.01 ^{***}	0.271 ± 0.04 ^{***}
IV	MEPLF	125	5.00 ± 0.12 ^{**}	0.075 ± 0.01 ^{***}	0.427 ± 0.05 [*]
V	MEPLF	250	4.00 ± 0.10 ^{***}	0.105 ± 0.02 ^{***}	0.378 ± 0.03 ^{***}
VI	MEPLF	500	3.61 ± 0.13 ^{***}	0.135± 0.01 ^{***}	0.310± 0.01 ^{***}

Values are expressed as Mean ± SEM, (n=6), where [@] p < 0.001 compared to normal control.; * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

3.12 Effect of MEPLF on light/dark exploration test

In this model, the mice pre dosed with MEPLF and diazepam significantly delayed the latency of animal towards dark compartment and also significantly increase the time spent by animal in a light area. Further the test extracts exhibited a significant increase in number of entries to light area than the dark area as compared control group. The anxiolytic effect of

the test extracts was observed to be dose dependent manner. Though there was increase in latency to dark compartment at a dose of 125 mg/kg in both the test extracts but the results found to be statistically not significant. The anxiolytic activity of the test extracts at a dose of 500 mg/kg was found closer to the reference standard drug, diazepam. The results are presented in table 8.

Table 6: Effect of MEPLF & MEDBT on rat brain neurotransmitter level in Immobilization stress

Groups	Treatment	Dose mg/kg	Noradrenalin µg/g	Dopamine µg/g	Serotonin (5HT) µg/g
I	Normal control	--	0.19 ± 0.001	16.61 ± 0.23	2.04 ± 0.21
II	Stress control	Vehicle	0.06 ± 0.002 [®]	9.23 ± 0.17 [®]	0.86 ± 0.02 [®]
III	Std. (W S)	100	0.14 ± 0.002 ^{***}	14.53 ± 0.24 ^{***}	1.94 ± 0.01 ^{***}
IV	MEPLF	125	0.09 ± 0.002 ^{***}	10.90 ± 0.15 ^{***}	1.25 ± 0.01 [*]
V	MEPLF	250	0.10 ± 0.001 ^{***}	12.00 ± 0.13 ^{***}	1.39 ± 0.02 ^{***}
VI	MEPLF	500	0.12 ± 0.003 ^{***}	13.50 ± 0.17 ^{***}	1.48 ± 0.03 ^{***}

Values are expressed as Mean ± SEM, (n=6), where [®] p < 0.001 compared to normal control.; * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

Table 7: Effect of MEPLF on elevated plus maze behavior of mice

Groups	Treatment	Dose mg/kg	Number of entries (5 min)		Time spent in (Sec)	
			Open arm	Closed arm	Open arm	Closed arm
I	Control	--	4.22 ± 0.32	12.39 ± 0.39	180 ± 2.8	320 ± 1.9
II	Std. Diazepam	2	13.2 ± 0.73 ^{***}	3.99 ± 0.28 ^{***}	390 ± 1.5 ^{***}	110 ± 2.0 ^{***}
III	MEPLF	125	6.98 ± 0.89 [*]	07.18 ± 0.19 ^{***}	210 ± 2.5 ^{***}	298 ± 1.8 ^{***}
IV	MEPLF	250	8.38 ± 0.29 ^{***}	06.23 ± 0.87 ^{***}	270 ± 2.1 ^{***}	230 ± 1.3 ^{***}
V	MEPLF	500	11.40±0.56 ^{***}	4.4 ± 0.60 ^{***}	320 ± 1.98 ^{***}	170 ± 2.5 ^{***}

Values are expressed as Mean ± SEM, (n=6).; * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

Table 8: Effect of MEPLF on light/dark exploration test in mice

Groups	Treatment	Dose mg/kg	Latency to first crossing into the dark compartment	Number of entries		Time spent in illuminated part (Light)
				Light	Dark	
I	Control	--	20.20±1.25	4.5 ± 2.5	10.3 ± 0.9	180.6 ± 2.4
II	Std. Diazepam	2	38.09±1.80 ^{***}	19.3±1.9 ^{***}	3.3±0.7 ^{***}	245.1 ± 2.8 ^{***}
III	MEPLF	125	24.10±2.00ns	12.7±2.3ns	5.4 ± 0.3 ^{**}	201.8 ± 1.9 ^{***}
IV	MEPLF	250	29.14±1.98 [*]	14.2±1.7 [*]	4.8±0.9 ^{***}	215.3 ± 3.4 ^{***}
V	MEPLF	500	33.18±1.73 ^{***}	17.5±1.8 ^{***}	3.8±1.0 ^{***}	223.7 ± 2.5 ^{***}

Values are expressed as Mean ± SEM, (n=6).; * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

3.13 Effect of MEPLF on open field apparatus test behaviors of rats

The results of this experimental model showed that the test extracts at graded doses significantly increased ambulation, rearing and self-grooming and significant decrease in fecal dropping in a dose dependent manner as compared to the

control group. The result of the test extract in MEPLF at a dose of 500 mg/kg was found nearer to the reference standard drug diazepam. Though there was the considerable protective effect of test extract at dose of 125 mg/kg in ambulation and self-grooming but the results are found statistically not significant. The results are presented in the table 9.

Table 9: Effect of MEPLF & MEDBT on behavioural changes of rats in open field test

Groups	Treatment	Dose (mg/kg)	Ambulation or No. of square crossed	Rearing	Self-grooming	Fecal drops
I	Control	--	35.1 ± 3.2	2.50 ± 0.4	2.1 ± 0.2	10.7 ± 1.6
II	Std. Diazepam	2	70.2 ± 1.2 ^{***}	15.23 ± 0.9 ^{***}	7.1 ± 0.2 ^{***}	0.5 ± 1.2 ^{***}
III	MEPLF	125	38.2 ± 2.8 ^{ns}	8.80 ± 1.8 ^{ns}	2.5 ± 0.1 ^{ns}	4.3 ± 0.9 [*]
IV	MEPLF	250	49.2 ± 1.9 ^{***}	9.24 ± 1.5 [*]	4.3 ± 0.2 ^{***}	3.3 ± 1.1 [*]
V	MEPLF	500	54.1 ± 0.7 ^{***}	12.10 ± 1.8 ^{***}	5.7 ± 0.6 ^{***}	1.2 ± 1.9 ^{**}

Values are expressed as Mean ± SEM, (n=6); * p < 0.05, ** p < 0.01, *** p < 0.001 as compared to control.

4 Discussions

Several plants that had been used as tonics in the Ayurvedic medicine, have been investigated for their antistress property due to their adaptogenic and rejuvenating properties¹⁹.

In the present study, MEPLF at different dose levels (125, 250 and 500 mg/kg) was investigated for antistress activity of against swimming endurance test and immobilization stress and anxiolytic activity by elevated plus maze, light/dark exploration test and open field apparatus test.

The swimming performance test is the broadly accepted physical stress model for the evaluation of antistress activity of new drug. The swimming performance test model is based on the observation that when animals forced to swim in water tank shows a immobile posture. The appearance of immobility, therefore, reflects a state of reduced stamina, fatigue and tiredness with the end point being the movement when the animal could not swim further and started drowning²⁴⁻²⁶. The results of the swimming test demonstrated the marked increase in swimming time in mice, pretreated for seven days with test extract with enhanced physical performance and thus confirming its antistress property.

Motor coordination test was used to find out the spontaneous motor activity. Post swimming muscle coordination (anti-fatigue) was carried out using rota rod test, which determines an animal's ability to support its own body weight by holding on to the rotating rod. The loss of muscle grip is an indication of relaxation, which is recorded by fall of time²⁷. The stress control animals showed an early fall from rota rod which interprets the reduced muscle coordination. The MEPLF showed increased the duration (sec) of stay on rota-rod in rats significantly as

compared to stress control animals in a dose dependent fashion.

Stress induces adreno-medullary response resulted in greater release of ACTH, which stimulates adrenal medulla and cortex which leads to increase in the weight of adrenal glands^{28,29}. Treatment with test extracts of different doses prevented the adrenal hypertrophy. Increased serum cortisol level was reversed with treatment of MEPLF significantly. Furthermore, a significant increase in adrenal ascorbic acid and cortisol contents was observed in animals treated with different doses of MEPLF.

Immobilization stress typically increases total leukocyte (WBC) and erythrocyte (RBC) count. During stress heart rate, blood pressure, rate of blood flow and oxygen demands increases, to meet these extra demands RBC and WBC count increases³⁰.

Plant adaptogens are smooth pro-stressors which reduce the reactivity of host defense system and reduce the harmful effects of various stressors due to increased basal levels of mediators involved in the stress response³¹. In the present study animals pretreated with MEPLF significantly reduced the stress-mediated elevated levels of hematological parameters indicates its anti-stress activity.

Exposure of experimental rats to the Immobilization stress resulted in hyperglycemia, this is because during stressful condition adrenal cortex secretes excess cortisol³². This excessive cortisol secretion maintains the internal homeostasis through the process of gluconeogenesis and lipogenesis³³. The results of the present study revealed that the extract of the title plants demonstrated promising effect in controlling hyperglycemia indicating the ability to prevent the alterations on adrenal cortex and helps in maintenance of the homeostasis.

Stress is known to be a triggering factor for hyperlipidemia³⁴ as such in our investigation, it was observed that immobilization stress leads to hyperlipidemia in experimental animals. The marked elevated levels of serum cholesterol, triglycerides and BUN in stress control animals is due to stimulating effect on hypothalamo-hypophyseal axis (HPA) leads to release of catecholamines and glucocorticoids³⁵ into a blood stream. In the present study significant restoration of altered biochemical markers seen in a treated group, is an indication of the ability of test extract to prevent the stimulation of HPA system and helping in normal functioning of the body.

Stress induces adreno-medullary response resulted in greater release of ACTH, which stimulates adrenal medulla and cortex which leads to increase in the weight of adrenal glands^{32,33,36}. Elevated serum cortisol resulted in increased liver mRNA levels there by causes liver hypertrophy^{37,38}. The hypotrophy of spleen in stressed animals was also seen due to release of more RBC from spleen²². Rats pretreated with *Withania somnifera*, MEPLF significantly reversed the altered organs weight of adrenal glands, liver and spleen.

NA, DA and 5HT are the important biogenic amines distributed in brain, and their functional role is ascertained well in stressful conditions^{31,32}. Exposure to sever stressful conditions results in significant decrease in these monoamine levels which is associated with central and peripheral ailments like depression, anxiety, hyperglycemia and declined immunity^{33,34}. In the present investigation, Immobilization stress significantly decreased these monoamines in rats brain. The animals pretreated with test extract MEPLF exhibited antistress potential by restoring the altered brain levels of NA, DA and 5HT. Anxiety is involved in the pathogenesis of psychiatric disorders, endocrine disorders, depression, ulcer, hypertension³⁹.

The most widely used behavioral test in mice for screening anxiolytic activity is elevated plus maze test. The decrease in the aversion to the open arms is the result of an anxiolytic effect, expressed by the enhanced time spent and number of entries in open arms and can be increased by antianxiety drug⁴⁰. In our studies, MEPLF significantly increases the time spent in open arms and number of entries in open arm while time spent in closed arms decreased significantly indicating that the plant showed antianxiety activity.

The light dark test may be useful to predict the anxiolytic like activity of the drugs. The administration of MEPLF showed dose dependent and significant increase in the time spent in light box, number of crossings, and the time of latency with decrease in time spent in the dark box.

The open field test is used to evaluate the animal emotional state. When the animals are placed in open field apparatus test exhibits anxiety and fear characterized by altered time spent in center arena and rearing, number of squares crossed and

number of fecal drops produced. The open field test showed that administration of MEPLF increased the time spent in the center of the arena, increased rearing, increased number of square crossed and a decreased number of fecal drops as compared with control animals. This observation supports the view that the crude extract imparts anxiolytic activity.

The literature reports indicated that extracts of medicinal plants containing flavonoids and tannins known to possess significant antistress activity⁴¹ and alkaloids are the most important secondary metabolites in many plants that are held responsible for their anxiolytic actions⁴². In our study also flavonoid, tannin and alkaloid contents of crude extract of the title plant may be responsible for observed antistress/ adaptogenic and anxiolytic activities.

5 Conclusion

In conclusion, the findings from the present study suggest that methanol extract of *Piper longum* fruits demonstrated adaptogenic and anxiolytic effects. However, the present study did not include the tests for establishing the exact mechanism of action.

6 Conflict of interests

The authors report no conflicts of interest.

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8 Author's contributions

NHM and SH are involved in conducting the research work, writing manuscript. PVP, MIS and CVM are involved in collection of data, literature review and draft the manuscript. All authors read and approved the final manuscript.

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