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Evaluation of adaptogenic (antistress) activity of *Piper longum* fruits

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Abstract

The present research work was carried out to evaluate adaptogenic potential of methanolic extract of *Piper longum* fruits (MEPLF) at different dose levels (100, 250 and 500 mg/kg) using anoxia stress tolerance, chemical induced stress and cold restraint stress models in rats. In anoxia stress tolerance test, the mean time of appearing first convulsion in mice was taken as end point to determine the time of anoxia tolerance. The number of convulsions, severity of convulsions, rate of mortality and recovery of animals were recorded in chemical induced stress. In cold restraint stress model, the hematological parameters, biochemical parameters, organs weight, glutathione and lipid peroxidation levels and neurotransmitters such as dopamine, nor adrenaline and serotonin levels in rat brain were recorded. Treatment with standard and test extract significantly reversed the stress induced altered hematological parameters, biochemical parameters, organs weight, glutathione and lipid peroxidation levels and neurotransmitters such as dopamine, nor adrenaline and serotonin levels in rat brain. The findings from the present study suggest that methanolic extract of *Piper longum* fruits demonstrated significant adaptogenic effect.

Keywords: Adaptogenic, anoxia, convulsion, adrenal glands, serum cortisol..

Introduction

Adaptability is the most distinct characteristic of life. Dr. Hans Seyle defined stress is the sum of all non specific responses of the body to any external stimuli acting up on it. Basically, 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 forms (1).

In the present days, stress has become an integral part of human life (2). Stress is considered to be any condition which results in alteration of the body's homeostasis (3). If the level of stress is more, the body's homeostatic mechanisms of the organism become deficit and the survival of the organism is threatened (4). Stress has been postulated to be involved in the etiopathogenesis of a variety of disease states, namely; diabetes, hypertension, immunosuppression, peptic ulcer, reproductive dysfunctions and behavioural disorders like anxiety due to involvement of the central nervous system, endocrine system, and metabolic system (5). Drugs having antistress properties induce a state of non-specific resistance against stressful conditions. Drugs like benzodiazepines, certain CNS stimulants such as amphetamines and caffeine as well as some anabolic steroids are routinely prescribed to combat stress. The incidence of toxicity and dependence has limited the therapeutic usefulness of these drugs (6).

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* (7), *Emblica 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 energy (7).

Several poly herbal formulations namely Siotone (13), AVM (14) and Geriforte have been reported to possess significant antistress properties. AVM is a poly herbal formulation reported for adaptogenic activity consists of various ingredients namely Root of *Withania somnifera*, Fruit of *Emblica officinalis*, Root of *Asparagus racemosus*, Tuber of *Diascoria bulbifera*, Powder of Trikatu, Leaves of *Ocimum sanctum*, Powder of Shilajit, Areal parts of *Tribulus terrestris* and Fruit of *Piper longum*. AVM a ploy herbal formulation, some of its components namely *Withania somnifera* (7), *Emblica 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 an adaptogenic (antistress) property of *Piper longum* fruits has not been scientifically investigated so far. Hence, the present study was undertaken to evaluate adaptogenic activity.

Materials and Methods

Plant material

For this study, the fruits of *Piper longum* were purchased from the local market. The sample was 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.

Preparation of extract

Fruits of *Piper longum* were cleaned, shade dried and coarse powdered. Then the powdered material was defated with pet ether for the removal fatty material and then extracted with methanol using Soxhlet extraction method. Thereafter, the methanolic extract *Piper longum* (MEPLF) was concentrated using rotary flash evaporator resulted to yield 16.5% of crude extract. The extract was stored in airtight container in refrigerator below 10 ^{o}C for further studies.

The extract of *Piper longum* fruits was subjected to following studies

1. Preliminary phytochemical screening
2. Acute toxicity study (LD_{50})
3. Evaluation of adaptogenic activity

Preliminary phytochemical screening

Preliminary phytochemical screening was performed on test extract for the detection of various phytoconstituents. Tests for the presence of common phytochemicals were performed by following literature reported methods (15).

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 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 libitium* 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)

Preparation of stock solution of methanolic extract of *Piper longum* fruits

Appropriate concentration of stock solution of the test extract 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.

Acute toxicity study (LD_{50}) (16)

The acute toxicity of methanolic extract 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 LD_{50} cut off value, the screening doses of extract selected for adaptogenic activity.

Evaluation models for antistress activity of methanolic extract of *Piper longum* fruits

Anoxia stress tolerance test (11,12)

Albino mice of either sex weighing 20 - 30 g were selected and divided into five groups of six each.

Group I	-	Control, received vehicle only
Group II	-	Std. (*Withania somnifera*, 100 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.

Animals were treated as shown above for three weeks. At the end of 1st, 2nd and 3rd week i.e. on 7th, 14th, and 21st day 1 hr. after the treatment, stress was induced in all the groups of animals by placing each mouse individually in the hermetic vessel of 300 ml capacity to record anoxia stress tolerance time. The moment when the animal showed the first convulsions removed immediately from the vessel. The time duration of animal entry into the hermetic vessel and the appearance of the first convulsion was recorded as anoxic stress tolerance time. Appearance of convulsion is very sharp end point, as delay by minute of removal of the animal from the vessel may lead to death.

Chemical induced stress (17)

PTZ (Pentylenetetrazol) induced convulsions

The mice of either sex weighing between 20 - 30g were randomly divided into five groups of six animals each.

Group I	-	Control, received vehicle only
Group II	-	Std. 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 considered as normal control received only vehicle. The group II animals were administered with Diazepam (2 mg/kg, p.o.). The group III, IV and V animals were treated with test extract of different doses for a period of seven days. At the end of the seventh day, all the animals were injected with pentylenetetrazol (80 mg / kg, i.p.), one hour after oral administration of the drug. The onset of convulsions, severity of convulsions, rate of mortality and recovery of animals were recorded.

Cold restraint stress (12,17, 18)

In the present study, albino rats of either sex weighing 150 – 200 g were divided into six groups of six animals each.

Group I	-	Control, untreated
Group II	-	Stress control, received vehicle only
Group III	-	Std. (*Withania somnifera* 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.

Grouping and treatment of animals were done as shown above. The hind and fore limbs of rats were tied and subjected to cold stress by exposing them to cold environment 4 ± 1 ºC for 2 hr. daily in refrigerator 1 hr. after the treatment, except control group. The extract was given orally daily for 10 days.

Hematological and biochemical estimations

At the end of specified period the blood was collected from retro orbital plexus of stressed and unstressed rats for estimation of hemoglobin (Hb), RBC, WBC, differential leucocytes count (DLC) and platelets using digital cell counter. The biochemical parameters such as serum glucose (GOD-POD method), cholesterol (CHOD-PAP method), triglycerides (GPO-Triender method) and BUN (Blood Urea Nitrogen, GLDH-UREASE method) were also measured using Erba Chem semi auto analyser.

The rats then scarified and their organs such brain, stomach, liver, spleen and adrenal glands were removed. The weight (expressed 100 g body weight of animal) of

liver, spleen and adrenal gland were recorded after washing with alcohol.

Brain was isolated rapidly from all the animals and kept in ice cold normal saline. Half portion of brain was used for determination of lipid peroxidation (LPO) and reduced glutathione (GSH) levels and rest of the portion was used for the estimation of neurotransmitters such as Noradrenalin (NA), Dopamine (DA) and Serotonin (5HT).

Assessment of oxidative stress (19,20)

Part of the rat brain was processed to get homogenate in cold buffer using homogenizer to estimate lipid peroxidation (LPO) and reduced glutathione (GSH) levels.

Estimation of brain neurotransmitters (21)

Noradrenalin, dopamine and serotonin levels were estimated using of rest of the portion of brains of all the animals exposed to cold restraint stress using high performance liquid chromatographic (HPLC) technique coupled with photodiode array (PDA) detection.

Assessment of stress–induced gastric ulcer (22)

The stomach of each animal was cut longitudinally along the grater curvature and ulcer index was recorded.

Ulcer index:

The following arbitrary scoring system was used to grade the incidence and severity of lesion,

0 = Normal

1 = Red coloration

2 = Spot ulcers

3 = Haemorrhagic streaks

4 = Ulcers > 3 but < 5

5 = Ulcers > 5.

Mean ulcer score for each animal was expressed as Ulcer Index.

The percentage of ulcer protection was determined by

$$\% \text{ protection} = \frac{\text{Control mean ulcer index - test mean ulcer index}}{\text{Control mean ulcer index}} \times 100$$

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.

Results

Preliminary phytochemical screening

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

Acute toxicity study

In an acute toxicity studies, the MEPLF 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 LD_{50} cutoff value as per fixed dose method of OECD guideline number 423.

The doses selected for the evaluation of anti-stress activity of the test extract were:

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.

Preparation of stock solution of MEPLF

Appropriate concentration of stock solution of the test extract 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.

Anoxia stress tolerance time in mice

In this model, the test extract demonstrated dose and duration dependent significant adaptogenic activity. This was evident by observing subsequent increase in anoxic stress tolerance time at the end of 1st, 2nd and 3rd week of the study when compared to control group. Though, there was increase in anoxia stress tolerance time observed at dose of 125 mg/kg on 7th day but the results found statistically not significant. Antistress effect of 500 mg/kg dose of the extract was found to be less effective than that of standard drug, *Withania somnifera*. The results are tabulated in Table 01.

Chemical induced stress (PTZ Induced convulsions)

The MEPLF at graded doses significantly increased the onset of convulsions, percentage protection and decreased number of convulsions, rate of mortality in mice compared to control group, which was found to be dose dependent. The results are given in table 02.

Effect of MEPLF on hematological parameters

Cold restraint stress in rats resulted in decrease in Hb level, percentage of lymphocytes and eosinophils whereas increase in RBC, WBC, platelets count and increase in percentage of neutrophils and monocytes when compared to normal control. Treatment with standard and test extract at different doses significantly attenuated stress induced altered hematological parameters. The results of test extract at a dose of 500 mg/kg found almost similar to that of standard drug *Withania somnifere* (100 mg/kg). The results are tabulated in table 03.

Effect of MEPLF on biochemical parameters

Cold restraint stress caused a significant increase in the levels of serum glucose, total cholesterol, triglycerides and BUN when compared to normal control. Pre treatment with different doses of test extract and standard drug significantly reversed the elevated levels of these biochemicals. The results are tabulated in table 04.

Effect of MEPLF on organs weight

Exposure of rats to cold restraint stress resulted in significant increase in liver and spleen weight whereas decrease in the weight of adrenal glands when compared to normal control. Pre treatment with standard drug *Withania somnifera* (100 mg/kg) and graded doses of the test extract significantly reversed the altered organs weight. Though the results of the test extract at a dose of 125 mg/kg shows statistically not significant in spleen and adrenal glands weight. The results are presented in the table 05.

Effect of MEPLF on different oxidative stress parameters in brain

Significant increase in the lipid peroxidation (LPO) and reduced glutathione (GSH) levels in rats brain were observed in stress control over normal control. Pre treatment with standard and test extract at different doses reversed the lipid peroxidation and glutathione levels in a dose dependent manner. The results are given in table 06.

Effect of MEPLF on brain neurotransmitter level

Determination of brain neurotransmitters level by HPLC revealed that cold restraint stress caused a significant ($p < 0.001$) depletion of Noradrenalin (NA), Dopamine (DA) and Serotonin (5HT) leading to state of depression. Treatment with MEPLF 125, 250 and 500 mg/kg augmented the levels of NA, DA and 5HT significantly. The test extract at a dose of 125 mg/kg in NA found to be not significant. The results are shown in table 07.

Effect of MEPLF on stress–induced gastric ulcer

The administration of test extract at different doses (125, 250 and 500 mg/kg) showed significant reduction in the ulcer index as compared to that of stress control group in a dose dependent manner. Test extract at dose of 500 mg/kg showed more protection over ulcer index than 250 and 125 mg/kg. The percentage protection at doses of 500 mg/kg and 250 mg/kg was found to be 78.40 And 52.81 where std drug *Wihtania somnifera* 100 mg/kg showed the protection of 93.66. The results are placed in table 08.

Discussion

Since the introduction of adptogens, several plants that had once been used as tonics in the Ayurvedic medicine, have been investigated for their antistress property due to their adaptogenic and rejuvenating properties (17).

In the present study, MEPLF at different dose levels (125, 250 and 500 mg/kg) was investigated for adaptogenic activity of against anoxic tolerance test, chemical induced stress and cold restraint stress animal models.

Anoxia is a very severe stressor. All the body functions including cellular respiration depends on oxygen supply to them. Lack of this essential element causes destruction on all body mechanisms. Increase in adaptation during stress by any drug could be considered as its major antistress effect. The results of the anoxic tolerance test showed that MEPLF significantly delayed the latency of convulsions in experimental animals, which therefore confirm its antistress activity.

Pentylenetetrazol has an inhibitory function of the GABAergic system in the brain (23). Gamma amino butyric acid (GABA) plays a major role in the central integration of the hypothalamic-pituitary adrenocortical (HPA) stress responses. GABAergic neurons in the bed nucleus of the stria terminalis, preoptic area and hypothalamus can directly inhibit paraventricular nuclei outflow, and thereby, reduce adrenocorticotropic hormone secretion. Thus, GABA produces a marked inhibitory effect on HPA axis activity (24,25). Stress also causes a rapid decrease in GABA receptor binding in the central nervous system (26). The adaptogenic activity of MEPLF against

pentylenetetrazol is observed by increasing the latency for the onset of convulsions, mortality protection, and decreasing the duration of convulsions.

Cold stress typically increases total leukocyte (WBC) and erythrocyte (RBC) count. During stress heart rate, blood pressure, rate of blood flow and oxygen demand increases, to meet these extra demands RBC and WBC count increases (27).

Plant adaptogens are smooth pro-stressors which reduce the reactivity of host defense system and decrease the damaging effects of various stressors due to increased basal levels of mediators involved in the stress response (28). Pretreatment with test extract MEPLF significantly reduced the stress-induced elevated levels of hematological parameters in rats indicates its anti-stress activity.

Exposure of experimental rats to the cold restraint stress resulted in hyperglycemia, this is because during stressful condition adrenal cortex secrets excess cortisol (29). Excessive secretion of cortisol maintains the internal homeostasis through the process of gluconeogenesis and lipogenesis (30). The results of the present study revealed that the extract of the title plant exhibited promising effect in controlling hyperglycemia indicating the ability to prevent the alterations on adrenal cortex and helping in maintaining the homeostasis. Stress is known to be a triggering factor for hyperlipidemia (31) as such in our investigation it was proved that cold restraint 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 glucocorticoids (32). In the present study significant restoration of altered biochemical markers seen in treated group, thus indicating the ability 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 (29,30,33). Elevated serum cortisol resulted in increased liver mRNA levels there by causes liver hypertrophy (34,35). The hypotrophy of spleen in stressed animals is due to release of more RBC from spleen (36). Rats pretreated with *Withania somnifera* and MEPLF significantly reversed the altered organs weight of adrenal glands, liver and spleen.

In cold restraint stress, peroxidation causes damaging effect on nervous system due to enriched substrates susceptible to oxidation and poor antioxidant defense mechanism (37). Lipid peoxidation causes reduced glutathione content and leads to oxidative damage (38). In our study also the elevated LPO and decreased GSH content was an indicator of oxidative injury in rat brain exposed to stress which were significantly attenuated by pretreatment of test extract at different doses.

NA, DA and 5HT are the important biogenic amines distributed in brain and their functional role is ascertained well in stressful conditions (39,40). 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 (41,42). In the present investigation, cold restraint stress significantly decreased these monoamines in rats brain. The animals pretreated with test extract exhibited antistress potential by restoring the altered brain levels of NA, DA and 5HT.

Stress produces ulceration in the stomach because of involvement of HPA axis (43), which is highly responsive to stress. The hyper activation of paraventricular nucleus of the hypothalamus causes decrease in mucosal blood flow and hyper contractility through descending projections causes gastric ulcers (44,45). The cold restraint gastric ulcer model was used to evaluate agents inhibit the development of gastric ulcers by antiulcer or antisress effect (46). In the present investigation pretreatment of WS and MEPLF prevented ulcer index in dose dependent manner.

Conclusion

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

Conflict of interest

The authors report no conflicts of interest.

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Table 01. Effect of MEPLF on anoxia stress tolerance time in mice

Groups	Treatment	Dose (mg/kg)	Duration of anoxia stress tolerance time (min)		
			7^{th} Day	14^{th} Day	21^{st} Day
I	Normal control	--	25.6 ± 0.67	27.3 ± 1.78	31.6 ± 1.78
II	Std. (Withania somnifera)	100	50.2 ± 2.8***	54.0 ± 1.98***	57.6 ± 2.1***
III	MEPLF	125	33.4 ± 2.8^{ns}	38.5 ± 2.1**	41.2 ± 1.7*
IV	MEPLF	250	36.4 ± 2.4*	39.3 ± 1.9**	43.5 ± 2.3**
V	MEPLF	500	42.0 ± 2.7***	44.2 ± 2.3***	47.3 ± 2.4***

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

Table 02. Effect of MEPLF on chemical induced stress (PTZ induced convulsions)

Groups	Treatment	Dose mg/kg	Onset of convulsion (Sec)	No. of convulsions	Mortality (%)	Protection (%)
I	Normal control	---	08.23±0.50	06.60± 0.22	100	00
II	Std. (Diazepam)	2	22. 2±1.37***	00	00	100
III	MEPLF	125	14.58± 1.34*	06.02± 0.9**	85.66± 2.32**	28.18±2.32***
IV	MEPLF	250	16.16±1.89***	03.56±0.32**	53.23±3.32***	55.97±2.89***
V	MEPLF	500	19.63±1.02***	01.19±0.02***	37.39±2.57***	78.56±1.98***

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

Table 03. Effect of MEPLF on hematological parameters in cold restraint stressed rats

Groups	Treatment	Dose (mg/kg)	Hb g %	RBC (millions/cmm)	Platelets (lacks/cmm)	WBC (thousands/cmm)	Differential leucocytes count (%)			
							N	L	M	E
I	Normal control	--	12.03±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	Untreated Stress control	Vehicle	10.05±0.01$^{@}$	9.29±0.6$^{@}$	13.30±0.3$^{@}$	16.03±0.1$^{@}$	70.5±0.8$^{@}$	14.0±0.6$^{@}$	5.6±0.7$^{@}$	1.0±0.6$^{@}$
III	Std. (W S)	100	13.01±0.02***	5.02±0.1***	8.90±0.2***	10.98±0.8***	45.3±0.5***	35.7±0.7***	4.0±0.1***	2.7±0.1***
IV	MEPLF	125	11.09±0.05**	8.98±0.9*	12.40±0.7*	15.01±0.2*	63.0±0.3*	20.7±0.4***	5.1±0.4*	1.5±0.8*
V	MEPLF	250	11.72±0.02***	6.87±0.5***	10.29±0.3**	14.05±0.6*	55.4±0.2**	25.5±0.3**	4.9±0.7*	1.9±0.2*
VI	MEPLF	500	13.00±0.03***	5.00±0.2***	8.20±0.2***	10.02±0.8***	44.9±0.8**	32.9±0.1***	4.4±0.3**	2.1±0.3**

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 stress control

Table 04. Effect of MEPLF on serum biochemical changes in cold restraint stress in rats

Groups	Treatment	Dose mg/kg	Biochemical estimations			
			Glucose mg/dl	Total Cholesterol mg/dl	Triglyceride mg/dl	BUN mg/dl
I	Normal 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	98.5±4.3***	55.6±3.0***	58.3±2.0***	22.5±0.9***
IV	MEPLF	125	130.0±3.5**	120.6±5.0**	85.4±3.1**	31.5±0.4*
V	MEPLF	250	120.0±4.2***	90.5±4.0**	73.3±2.1***	28.6±0.5**
VI	MEPLF	500	105.0±2.1***	60.5±3.8***	60.6±4.2***	25.1±0.4***

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 stress control

Table 05. Effect of MEPLF on organs weight in cold restraint stress in rats

Groups	Treatment	Dose mg/kg	Organs weight (g/100 g b.w.)		
			Liver	Spleen	Adrenal glands
I	Normal control Untreated	--	3.28 ± 0.22	0.058 ± 0.01	0.490 ±0.02
II	Stress control	Vehicle	6.59± 0.18$^{@}$	0.172 ± 0.03$^{@}$	0.150 ± 0.01$^{@}$
III	Std. (W S)	100	3.38 ± 0.13***	0.065 ± 0.01***	0.471 ± 0.04***
IV	MEPLF	125	4.90 ± 0.12***	0.132 ± 0.01^{ns}	0.274 ± 0.03^{ns}
V	MEPLF	250	4.80 ± 0.18***	0.098 ± 0.01*	0.353 ± 0.07*
VI	MEPLF	500	4.01 ± 0.27***	0.072 ± 0.01**	0.452 ± 0.06***

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 stress control

Table 06. Effect of MEPLF on oxidative measurements of brain in cold restraint stress

Groups	Treatment	Dose mg/kg	Oxidative parameters	
			Lipid peroxidation (LPO) (n moles of MDA/ml)	Glutathione (GSH) µg of GSH/g of wet tissue
I	Normal control Untreated	--	5.16 ± 0.78	0.53±0.003
II	Stress control	Vehicle	21.57 ± 0.8$^{@}$	0.10±0.005$^{@}$
III	Std. (W S)	100 mg/kg	7.38 ± 0.3***	0.45±0.002***
IV	MEPLF	125 mg/kg	17.74 ± 0.9**	0.12±0.006*
V	MEPLF	250 mg/kg	13.30 ± 0.4**	0.25±0.004**
VI	MEPLF	500 mg/kg	09.36 ± 0.6***	0.38±0.001***

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 stress control

Table 7. Effect of MEPLF on rat brain neurotransmitter level in cold restraint stress

Groups	Treatment	Dose mg/kg	Noradrenalin µg/g	Dopamine µg/g	Serotonin (5HT) µg/g
I	Normal control Untreated	--	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	0.05 ± 0.000^{ns}	10.44 ± 0.22**	1.79 ± 0.07***
V	MEPLF	250	0.08 ± 0.003**	11.87 ± 0.19***	1.57 ± 0.01***
VI	MEPLF	500	0.13 ± 0.006***	12.82 ± 0.09***	1.86 ± 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 stress control

Table 08. Effect of MEPLF on stomach ulcer index in cold restraint stress

Groups	Treatment	Dose mg/kg	Ulcer index	% Protection
I	Normal control Untreated	----	0.00 ± 0.0	-----
II	Stress control	Vehicle	3.26 ± 0.03$^{@}$	-----
III	Std. (W S)	100	0.96±0.02***	93.66
IV	MEPLF	125	3.00±0.09**	29.57
V	MEPLF	250	2.01±0.04***	52.81
VI	MEPLF	500	1.20±0.01***	78.40

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 stress control.