EVALUATION OF POTENTIAL CENTRAL PROTECTIVE ROLE OF ETHANOL EXTRACT OF PEDALIUM MUREX LINN. IN ACUTE AND CHRONIC UNPREDICTABLE STRESS INDUCED MODELS IN SD RATS

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ABSTRACT

Objectives: An attempt is made to understand the protective role of ethanol extract of Pedalium murex Linn. herb (EEPM), using behavioral models, changes in the feeding patterns (food and water intake, and body weight) and biochemical studies by employing acute stress (AS) and chronic unpredictable stress (CUS). Methods: Animals were divided into five groups each for AS and CUS models. The animals were subjected to AS (immobility-induced stress) and CUS paradigms (immobility + forced-swimming + dark phase) for 10 days, followed by a battery of behavioral and biochemical analyses. The changes in food and water intake, body weight and general behavior were measured for 4 weeks. In addition, the effect of EEPM on the antioxidant enzyme systems [superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH)] in whole brain of animals, in vitro antioxidant and free radical scavenging activities were also screened.

Results: AS and CUS induced anxiety, depression and impairment of cognition and memory. Pretreatment with EEPM (200mg/kg and 400mg/kg; p.o.) for 30 days, significantly reduced stress-induced anxiety and related mood disorders. The normalization of SOD, CAT and GSH levels, further substantiates the protective role of EEPM. The herb was found to be effective in preventing CUS than the AS model. Conclusions: EEPM attenuated anxiety, depression and impairment of cognition and memory, more significantly in CUS rats, substantiating the hypothesis that EEPM may exert part of its neuroprotective effect through the brain antioxidant system. In addition, the protective ability could also be attributed to the antioxidant potential of EEPM, as indicated through diverse in vitro studies.
Keywords: Pedalium murex, acute stress, chronic unpredictable stress, behavioral studies, in vitro, brain antioxidants.

INTRODUCTION

Stress can be defined generally as responses to demands upon the body.$^{[1]}$ It is the body’s reaction to a change that requires a physical, mental or emotional adjustment or response.$^{[2]}$ It can come from any situation or thought that makes one feel frustrated, angry, nervous, or anxious. Conceptually, stress can be any threat, either real or perceived, to the well being of an organism and it can be of two types such as psychological stress, physical stress.

Sustained stress can have numerous pathophysiological effects such as activation of neuroendocrine system (limbic-hypothalamic-pituitary adrenal,$^{[3]}$ and hormonal functions (corticosterone release$^{[4]}$). Sustained and persistent stressful conditions can precipitate anxiety and affective disorders such as depression, which further leads to the excessive production of free radicals and oxidative burden.$^{[5]}$

Stressor is a stimulus, either internal or external, that activates the hypothalamic pituitary adrenal (HPA) axis and the sympathetic nervous system resulting in physiological change.$^{[6]}$ Long-term exposure to stressors can cause depression,$^{[7]}$ post-traumatic stress disorder, and anxiety disorders. Stressful events can activate the hypothalamo-pituitary-adrenal (HPA) axis$^{[8]}$ and increase the release of corticotrophin releasing hormone (CRH) from the hypothalamic paraventricular nucleus, causing the secretion of adrenocorticotropin (ACTH) from anterior pituitary, which in turn stimulates the secretion of glucocorticoids from the adrenal cortex.$^{[9, 10]}$ Glucocorticoids possess broad spectrum of actions affecting expression and regulation of genes throughout the body readying the organism for changes in energy and metabolism required for coping.$^{[11, 12]}$ Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions.$^{[13]}$ The brain tissue is made up of large amounts of polyunsaturated fatty acid, thus making it vulnerable to free radical attacks.$^{[14]}$

In traditional practices of medicine, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases such as Alzheimer’s disease (AD), Parkinson’s disease (PD) and other memory related disorders. The past decade has also witnessed an intense interest in herbal medicines in which phytochemical constituents can have long-term health promoting or medicinal qualities. Phytochemicals present in vegetables
and fruits are believed to reduce the risk of several major diseases including cardiovascular diseases, cancers as well as neurodegenerative disorders. Therefore, people who consume higher vegetables and fruits may be at reduced risk for some of diseases caused by neuronal dysfunction.\cite{15, 16} In Indian system of medicine, the plant Pedalium murex (P. murex) is being used to treat many illnesses, successfully, for centuries. A number of studies have attributed the curative effect of P. murex to its high content of flavonoids. But there is lack of studies related with protective effect of P. murex in human and experimental animal models too. Hence, in order to contribute further to the knowledge of Indian traditional medicine and its sacred and rich history, leaves of P. murex were subjected to evaluate the possible protective effect against acute stress (AS) and chronic unpredictable stress (CUS) in rats. After subjecting the animals to AS and CUS, changes in food and water intake, and body weight were observed from the time of induction of either AS or CUS. Thereafter, the animals were subjected to a battery of behavioral studies, viz., anxiolytic tests using elevated plus maze (EPM), depressive test using forced swim test (FST) and cognitive and memory impairment test using 8-arm radial maze (RAM). In addition, the perturbations in the levels of brain antioxidant system like superoxide dismutase (SOD), catalase (CAT) and the activities of glutathione (GSH) were also measured.

MATERIALS AND METHODS

Test Drugs and Chemicals
Corticosterone was purchased from Sigma Aldrich Chemicals Ltd., Bangalore, India. All other biochemical and chemicals used for the experiments were of analytical grade obtained from SD Fine Chemicals, Mumbai, India.

Collection of plant materials and plant extraction
The aerial parts of the herb P. murex Linn. were collected as fresh plants from Bhavani, Erode district, Tamilnadu, India. The whole plant was washed and leaves were separated from other aerial parts of the plant, freed from earthy material and shade dried with occasional sifting at room temperature, powdered, and was subjected to extraction by cold maceration with 90% ethanol (8.34 % yield) at room temperature with continuous stirring (300 rpm). The solvents were evaporated using rotary vacuum and stored in desiccator. The ethanol extract of P. murex (EEPM) was then subjected to the following tests.

Animals
Animals used in this study were male Swiss albino mice (20-25 g) and female Sprague-
Dawley rats (150-210 g). The animals were housed for at least one week in the laboratory animal room prior to testing in standard polypropylene cages at room temperature of 22 ± 30°C and at 60-65% relative humidity. Food (fed with commercially pellet diet supplied by M/s Hindustan Lever Ltd., Mumbai, India) and water were given ad libitum unless otherwise specified. All experimental protocols were approved by the Institutional Animal Ethics committee of J.K.K. Nattraja College of Pharmacy, Komarapalayam, Tamilnadu, India.

1. **In Vitro Antioxidant Activity**
   a) Free radical scavenging activities of EEPM
   i) DPPH radical scavenging activity
   The antioxidant activity of EEPM was assessed using DPPH (1,1-diphenyl-2-picrylhydrazyl) assay according to the method of Blois.\[17\] Absorbance at 517 nm was determined after 30 min. at room temperature and the scavenging activity were calculated as a percentage of the radical reduction. Each experiment was performed in triplicate. Vitamin C was used as a reference compound.

   ii) Nitric oxide radical scavenging activity
   The interaction of EEPM with nitric oxide (NO) was assessed by the nitrite detection method.\[18\] NO interacted with oxygen to produce stable products, leading to the production of nitrites. After incubation for 60 min. at 37°C, Griess reagent (α-napthyl-ethylenediamine 0.1% in water and sulphanilic acid 1% in H₃PO₄ 5%) was added. The same reaction mixture without the extract of sample but with equivalent amount of distilled water served as control. Quercetin was used as positive control.

   iii) LPO assay
   The mixture (egg phosphatidylcholine in 5 mL saline) was sonicated to get a homogeneous suspension of liposome. Lipid peroxidation was initiated by adding 0.05 mM ascorbic acid to a mixture containing liposome (0.1 mL). The pink chromogen was extracted with a constant volume of n-butanol and absorbance of the upper organic layer was measured at 532 nm. The experiment was performed in triplicate.\[19\]

   iv) CUPRAC assay
   1 mL CuCl₂, 1 mL neocuproine, and 1mL NH₄Ac buffer and mixed; 0.5 mL of dilute plant extract (previously diluted with MeOH at a volume ratio of 1:10) followed by 0.6 mL of
water (total volume = 4.1 mL) were added together and mixed. Absorbance against a reagent blank was measured at 450 nm after 30 min.\textsuperscript{[20]}

v) Metal chelating assay
The reaction mixture containing 1 mL O-phenanthroline, 2 mL ferric chloride, and 2 mL extract at various concentrations ranging from 2 to 1000 µg/mL in a final volume of 5 mL was incubated for 10 min. at ambient temperature. The absorbance at 510 nm was recorded. Ascorbic acid was added instead of extract and absorbance obtained was taken as equivalent to 100% reduction of all ferric ions. Blank was carried out without drug. Experiment was performed in triplicate.\textsuperscript{[21-23]}

vi) Reducing power
The reducing power of extract was determined according to the method of Yen and Duh, 1993.\textsuperscript{[24]} Different concentrations of extracts (0.5-2.5 µg/mL) were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% K\textsubscript{3}Fe(CN)\textsubscript{6}. The mixtures were incubated at 50°C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid were added to the mixtures, followed by centrifugation for 10 min. The upper layer (5 mL) was mixed with 5 mL of distilled water and 1 mL of 0.1% ferric chloride and the absorbance of the resultant solution were measured at 700 nm.

b) Bioactive compounds
i) Total phenol
Total phenolic content was determined according to the method described by Singleton and Rossi.\textsuperscript{[25]} 1.0 mL of sample was mixed with 1.0 mL of Folin and Ciocalteu's phenol reagent. After 3 min. 1.0 mL of saturated Na\textsubscript{2}CO\textsubscript{3} (~35 %) was added to the mixture and made up to 10 mL by adding distilled water. The reaction was kept in the dark for 90 min. after which its absorbance was read at 725 nm. A calibration curve was constructed with different concentrations of catechol (0.01- 0.1 mM) as standard. The results were expressed as mg of catechol equivalents/g of extract.

ii) Total flavonoids
This was assayed as described by Jia et al.\textsuperscript{[26]} 0.5 mL of the sample was added into a test tube containing 1.25 mL of distilled water. 0.075 mL of 5 % NaNO\textsubscript{2} solution was added and allowed to stand for 5 min. Then 0.15 mL of 10% AlCl\textsubscript{3} was added after 6 min. 0.5 mL of 1.0 M NaOH was added and the mixture were diluted with another 0.275 mL of distilled water.
The absorbance of the mixture at 510 nm was measured immediately. The flavonoid content was expressed as mg catechin equivalents/g sample.

iii) Total tannins

The quantitative tannin content in samples was estimated as described by Price and Butler[27] with some modifications. 0.1 g of dry plant sample was transferred to 100 mL flask; 50 mL water was added and boiled for 30 min. After filtration with cotton filter the solution was further transferred to a 500 mL flask and water was added to 500 mL mark. 0.5 mL aliquots were finally transferred to vials, 1 mL of 1% K₃Fe(CN)₆ and 1 mL of 1% FeCl₃ were added, and was made upto 10 mL with distilled water. After 5 min. the solutions were measured spectrophotometrically at 720 nm.

2. Pharmacological Studies

a) Acute Oral Toxicity Study

Acute oral toxicity study for EEPM leaves was carried out according to the OECD guideline 423. Adult female SD rats were used in the present study. All the rats were kept at room temperature (22 ± 30°C). They were housed and treated as per the internationally accepted ethical guidelines for the care of laboratory animals. EEPM leaves were suspended in 0.5% CMC, and was administered once daily to the animals by oral route for 30 days. Diazepam (2mg/kg, i.p.) was used as a standard drug.

b) Stress Induction

Animals remained in their home cages as controls (HCC) or were exposed to stress immediately after the extract was given. After stressor stimulation, rats were returned to their home cages. We preferred two different types of stress to produce different patterns of effects such as,

i) Acute stress (Immobilization induced stress)

Immobilization has been used extensively as a stressor for the study of stress-related biological, biochemical and physiological responses in animals using the model described by Das et al.[28]

ii) Chronic Unpredictable Stress

The animals were subjected to 3 varieties of chronic stressors such as, forced swimming induced stress,[29] immobilization induced stress,[28] and dark phase induced stress[30] daily for
10 days from 21st day to 30th day.

c) Stress Protocol
The rats were divided into nine groups of 6 animals each. Except the control and AS and CUS groups, all other animals were pretreated with EEPM for 30 days in four groups. The animals were grouped as mentioned below

i) Acute Stress (AS) Protocol
**Group- I** served as control and received 0.9% normal saline; **Group- II** was subjected to AS only; **Group-III** served as positive control (diazepam 2 mg/kg); **Group- IV** pretreated with EEPM (200 mg/kg) and subjected to AS for one day; **Group- V** pretreated with EEPM (400 mg/kg) and subjected to AS for one day.

ii) Chronic Unpredictable Stress (CUS) Protocol
**Group- I** served as control and received 0.9% normal saline; **Group- II**, was subjected to CUS only; **Group-III**, served as positive control (diazepam 2 mg/kg); **Group- IV** pretreated with EEPM (200 mg/kg) and subjected to CUS; **Group- V** pretreated with EEPM (400 mg/kg) and subjected to CUS.

d) Behavioral studies in AS and CUS rats

i) Elevated plus maze test
The maze consists of 2 open arms (50cm × 10cm) crossed with 2 enclosed arms of the same dimensions with walls 40 cm height. The arms connected with central squares (10cm×10cm) to give the apparatus plus sign appearance. The maze was elevated 70cm above the floor in a room.[31] Rodents have a natural aversion for high and open spaces and prefer enclosed arms, which have burrow like ambience and therefore spend greater amount of time in the enclosed arm. When exposed to the novel maze allay the animal experience an approach–avoidance conflict, which is stronger in the open arms as compared to enclosed arms. The parameters noted were (i) percent preference for open arm as first entry and (ii) number of entries in the open arm and closed arm.

ii) Forced swim test
In experimental room animals were placed in Plexiglas non-transparent cylinders (40×20cm) filled with fresh water (25°C) up to a depth of 15cm. On day 1, at least 60 min before the beginning of the habituation session, mark the animals and randomly assign them to a drug
treatment. All animals within a cage receive the same treatment.\cite{32,33} Weigh two animals individually, then place one rat in each of the two cylinders for 15 min (habituation session). No scoring of immobility is performed during the habituation session.

On the test day, administered the test substance 30 min (for intra peritoneal) prior to the session. Tested animals by placing in cylinder containing fresh water and observed their behavior for 6 min. Scored the duration of immobility by summing the total time spent immobile\cite{34,35} (i.e., the time not spent actively exploring the cylinder or trying to escape from it. Included within the time spend immobile are the short periods of slight activity where the animals just make those movements necessary to maintain their heads above water).

**iii) Radial arm maze test**

RAM consists of eight horizontal arms (57x11cm) placed radially around a central platform above the floor. Experimental subjects are placed on a central platform from which they have to collect hidden baits placed at the end of the arms. One piece of reinforce (bait) is placed at the end of each arm in a well that hides the food from sight, and the animal is allowed to freely explore the maze. Each session lasts until (a) all eight arms have been entered (consider enter an arm when the whole body, except the tail, is inside the arm), (b) 10 min passed since the start of the test, or (c) 2 min passed since the animal’s last arm entrance.\cite{36,35}

Arm entries are recorded for later analysis. To prevent odor cues, the maze must be wiped clean between animals. The variables commonly used for the analysis of the performance are (a) the number of errors in each session (entering an arm that has been visited previously counted as an error) and the total number of errors across eight sessions, (b) the number of correct choices in the first eight arm entries of each session, (c) the location of the first error in each session, (d) the number of adjacent arm entries in each session (trials) and (e) the number of sessions to reach the criterion of one error or less, averaged over four consecutive days of training.\cite{36-38}

e) **General behavioral alterations in CUS rats only**

During the entire period of study the animals were observed for any changes in behavior and suitably noted.

i) **Measurement of food intake**

The measurement of food intake was studied\cite{39,40} by presenting pre-weighed food to the
animals in all the groups’ immediately following stress and drug treatment. The amount of food (Brook Bond, Lipton, India) consumed by the animals (food intake in g/g weight of rat) was evaluated daily.

ii) Measurements of water intake

The animals in all groups had free access to water during the entire duration of the study. The intake of water was studied by measuring the volume of water (water intake in mL/g body weight of rat) consumed per day on particular time interval.\textsuperscript{[41]}

iii) Measurement of body weight

The body weight of the animals was monitored daily by weighing on an electrical balance with accuracy to ± 0.1 g. All measurements were made every day between 8.30 and 9.15 h, immediately before administration of stress. Changes in body weight were calculated by subtracting the weight of the animal obtained on last day of stress from that of the animal weight immediately before the first stressor.\textsuperscript{[40, 42]}

f) Biochemical analysis in AS and CUS rats

At the end of the behavioral studies, the animal models were anesthetized with mild chloroform and sacrificed by cervical dislocation; the whole brain were quickly removed, rinsed in ice-cold isotonic saline and packed in an aluminum foil for further use. The brain tissue were weighed and 10% tissue homogenate was prepared with 0.025M Tris-Hcl buffer, pH 7.5. After centrifugation at 10,000 x g for 10 min. the resulting supernatant was used for enzyme assays for the estimation of non-enzymatic and enzymatic antioxidants.

i) Estimation of SOD

SOD activity was measured according to the method of Misra and Fridovich (1976).\textsuperscript{[43]} The supernatant (500 µl) was added to 0.800 mL of carbonate buffer (100mM, pH 10.2) and 100 µL of epinephrine (3mM). The changes in absorbance of each sample were then recorded at 480 nm in spectrophotometer for 2 min. at an interval of 15 sec.

ii) Estimation of CAT

CAT activity was measured according to the method of Aebi et al (1974).\textsuperscript{[44]} 0.1 mL of supernatant was added to cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H₂O₂. The rate of
decomposition of H$_2$O$_2$ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of CAT was expressed as units/mg protein.

**iii) Estimation of GSH**

Reduced glutathione (GSH) was measured according to the method of Ellman et al (1959). The PMS of rat brain (720 µl) and 5% TCA were mixed to precipitate the protein content of the supernatant. After centrifugation at 10,000 x g for 5 min, the supernatant was taken. DTNB (5,5’-dithio-bis(2-nitrobenzoic acid)) and Ellman’s reagent were added and the absorbance was measured at 412 nm.

**Statistical analysis**

All data are presented as mean ± SEM. The behavioral and biochemical data was analyzed using one way analysis of variance (ANOVA) followed by Dunnett’s test, to analyze differences between the groups. A probability level (p) of value of less than 0.001 was considered to be statistically significant. The statistical analysis was performed using GraphPad Prism for Windows (Graph Pad Prism Software (version 6.01), San Diego, California, USA).

**RESULTS**

**In vitro antioxidant activity**

Free radicals are chemical entities that can exist separately with one or more unpaired electron. The propagation of free radicals can bring about thousands of reactions and thus may cause extensive tissue damage. Antioxidants may offer resistance against oxidative stress by scavenging the free radicals, inhibiting the lipid peroxidation (Fig. 1), etc.,

**i) Inhibition of DPPH radical**

EEPM had significant scavenging effects on the DPPH radical and the effect was found to be enhanced with an increase in the concentration between 1.95 and 125 µg/mL of the extract (Fig. 1a). Compared with that of Vitamin C, the scavenging effect of EEPM was significantly less. The IC$_{50}$ of EEPM was found to be 6.87µg/mL. The DPPH radical is considered to be a model for a lipophilic radical chain reaction initiated by the lipid auto oxidation and has been widely used to evaluate the free radical scavenging capacity of antioxidants. DPPH is relatively stable free radical and the assay determines the ability of EEPM to reduce DPPH radical to the corresponding hydrazine by converting the unpaired electrons to paired ones. In addition, antioxidants are known to interrupt the free radical chain of oxidation and to donate
hydrogen from phenolic hydroxyl groups, thereby, forming stable free radicals, which do not initiate or propagate further oxidation of lipids.\cite{49} The determination of scavenging stable DPPH was Blois method to evaluate the antioxidant activity of the extracts. With this method it was possible to determine the antiradical power of an antioxidant activity by measurement of the decrease in the absorbance of DPPH at 517 nm. The dose dependent inhibition of DPPH radical indicates that EEPM caused reduction of DPPH radical in a stoichiometric manner.\cite{50}

**ii) Inhibition of nitric oxide radical**

It is known that NO has an important role in various types of inflammatory processes. The concentration of EEPM needed to produce 50% inhibition of NO release and was found to be 7.81 µg/mL whereas 15.62 µg/mL was needed for quercetin, used as a reference compound. Fig. 3 illustrates the percentage inhibition of NO generation by EEPM. It is well known that NO has an important role in various types of inflammatory processes. NO is an important chemical mediator generated by endothelial cells, macrophages, neurons, toxins, stress, etc. and involved in the regulation of various physiological processes.\cite{51} Excess concentration of NO is associated with several diseases.\cite{52} Oxygen reacts with the excess NO, to generate nitrite and peroxynitrite anions. Moreover, NO is implicated in several neurological disorders.\cite{53} The extract competes with oxygen for NO and thus inhibits the generation of the anions and hence has an important protective role against neurological diseases.

**iii) LPO assay**

The IC\textsubscript{50} of EEPM was found to between 7.81 and 15.62 µg/mL. Initiation of the lipid peroxidation by FeSO\textsubscript{4} takes place either through ferrylperferryl complex or through OH radical by Fenton’s reaction. Figure 1d shows that EEPM inhibited lipid peroxidation in egg phosphatidylcholine as a dose dependent manner. The inhibition could be caused by absence of ferryl-perferryl complex or by scavenging the OH radical or by reducing the rate of conversion of ferrous to ferric or by chelating the iron itself. The free radical is highly reactive and can damage biological molecules, when it reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydro-peroxides is produced. Lipid hydroperoxide can be decomposed to produce alkoxy and peroxy radical which eventually yield numerous carbonyl products such as malondialdehyde (MDA). The carbonyl products are responsible for DNA damage, generation of cancer and aging related diseases. Thus the
The decrease in the MDA level in egg phosphatidylcholine with the increase in the concentration of the extract indicates the role of the extract as an antioxidant.

iv) CUPRAC assay
Polyphenols are a group of chemicals found in many plants. They possess antioxidant properties due to their phenolic –OH group. Polyphenol profiling can be done using this CUPRAC methods were various polyphenol groups which differs on number and position of –OH groups can be reduced/ oxidized by CUPRAC reagent which determines the antioxidant capacity of the respective polyphenolic groups. Polyphenols, flavonoid containing plant extracts can be measured easily.[54-56]

v) Metal chelating assay
Transition metals have been proposed as the catalysts for the initial formation of radicals. Chelating agents may stabilize transition metals in living systems and inhibit the generation of radicals, consequently reducing free radical-induced damage. To better estimate the antioxidant potential of the extract, its chelating activity was evaluated against Fe^{2+}. The scavenging activity of the sample was found to be 9.49 ± 1.543 at the concentration of 30µg/mL. However, the metal chelating activity of positive control ascorbic acid was found to be 59.43± 0.7808 (at 30µg/mL).

vi) Reducing power
It has been demonstrated that the power of certain antioxidant is associated with the reducing power of their atoms, which is associated with the presence of reductones. During the reducing power assay, the presence of reductants (antioxidants) in the tested samples would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The Fe^{2+} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm. The reducing power of EEPM and reference compound Vitamin C increased with an increase in the concentration and it was found to be 0.64 and 0.771, at 500 µg/mL, respectively (Fig. 1c). All doses of EEPM showed almost similar activity like the reference compound. The antioxidant activity of the herbal preparations or phenolic compounds may be attributed to concomitant reducing power[24,57] and may serve as a significant indicator of its potential antioxidant activity.[58] The reducing property of EEPM implies that it is capable of donating hydrogen atom in a dose dependent manner. A substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atom.[59] High content of phenolic compounds in
the extract may be a contributing factor towards antioxidant activity due to the presence of hydroxyl groups, which can function as hydrogen donor.[60, 61]

**Bioactive compounds estimation**

The result indicates a strong association between antioxidative activities of phenolic compounds, suggesting that phenols exhibit significant antioxidant and free radical scavenging ability[62] due to the presence of hydroxyl groups and effective hydrogen donating ability. Thus therapeutic properties of PM may possibly be attributed to the antioxidant property of phenolic compounds present in EEPM. Total flavonoid content (expressed as µg quercetin equivalent/mL) was derived from a quercetin standard in the range of 20 -100 µg/mL ($R^2 = 0.9805$). As was observed for total polyphenols, a total flavonoid content of the EEPM was 0.156 µg/mL.

**EEPM and Acute Stress**

**Effect on EEPM on AS induced alterations in Nootropic behaviors**

Application of AS for one day significantly reduced anxiety, depression and impairment of cognition and memory (nootropic behaviors) as compared to the control group. EEPM (200 and 400 mg/kg) treatment significantly improved and antagonized anxiety, depression and impairment of cognition and memory by increasing the time spent in open arms and reduced the time spent in closed arms in an EPM (Fig. 2a, 2b; P<0.01), decreased immobility time in FST (Fig. 3a; P<0.01), and reduced number of trials and errors (Fig. 4a; P<0.05) as compared to AS treated animals. EEPM (200 mg/kg) exhibited significant effect on time spent in closed arms in EPM and immobility time spent in FST and also number of trials and errors in RAM as compared to control group.

**Effect on EEPM on AS induced CAT, SOD and GSH enzyme levels**

A significant decrease in the CAT, SOD and GSH level was observed in whole brain of the AS treated groups as compared to the control group (Fig. 6a). On the other hand, EEPM (200 and 400 mg/kg) treatment significantly stimulated CAT, SOD and GSH levels in the whole brain (P<0.0001) in comparison to AS treated groups, whereas EEPM (400 mg/kg) treatment did not demonstrated any notable change in CAT level, as compared to control group.
Figure 1. *In Vitro* Antioxidant Assays of Ethanolic Extract of Pedalium murex.

Figure 2. Behavioral studies using elevated plus maze.
Figure 3. Behavioral studies using forced swim test

Figure 4. Behavioral studies using 8-arm radial maze

Figure 5. Behavioral studies using food intake and water intake.
**Figure 5. General behavioral studies in CUS treated rats**

**Figure 6. Biochemical Studies in AS, CUS Treated Rats**

EEPM and Chronic Stress

**Effect on EEPM on CUS induced changes in General behavioral tests**

The feed and water intake, body weight of the animals are shown in Fig. 5a, 5b and 5c respectively. Application of CUS displayed greater decrease in food intake (0.195 g/g) and body weights (-7 g) but a notable increase in water intake (0.7543 mL/g) of the rats as compared to control group. EEPM (200 and 400 mg/kg) treatment significantly improved the food intake and body weight and reduced water intake as compared to CUS treated group. But it did not demonstrate any statistical significance on the effect of the feed, water intake and body weight as compared to control group.
Effect on EEPM on CUS induced alterations in Nootropic behaviors

Administration of CUS for 10 days significantly reduced anxiety, depression and impairment of cognition and memory (nootropic behavior), as compared to the control group. EEPM (200 and 400 mg/kg) treatment significantly improved and reversed anxiety, depression and impairment of cognition and memory by increasing the time spent in open arms and reduced the time spent in closed arms in an EPM (Fig. 2c, 2d; P<0.01), decreased immobility time in FST (Fig. 3b; P<0.01), and reduced number of trials and errors in RAM (Fig. 4b; P<0.01) as compared to CUS treated animals. EEPM (200 mg/kg) had no significant effect on the time spent in open and closed arms in EPM, immobility time spent in FST and also number of trials and errors in RAM as compared to control group.

Effect on EEPM on CUS induced CAT, SOD and GSH enzyme levels

A significant decrease in the CAT, SOD and GSH level was observed in whole brain of the CUS treated groups as compared to the control group (Fig. 6b). On the other hand, EEPM (200 and 400 mg/kg) treatment significantly stimulated CAT, SOD and GSH levels in the whole brain (P<0.0001) in comparison to CUS treated groups, whereas EEPM (400 mg/kg) demonstrated had no statistically significant effect on the enzymes level, as compared to control group.

DISCUSSION

The development of anxiety, depression, and impairment of cognition and memory were evident in both the AS and CUS rats, as observed from decreased activity in EPM, FST and RAM respectively. In the EPM paradigm, an enormous shortening of the entries, and time spent in open arms, and prolongation of entries and time spent in closed arms, corresponds to the findings in mice.\[^{63}\] Compared to the control animals, prolonged start and transfer latencies in stress-treated rats indicated the inability to escape rapidly from the open arm into the closed arms is somewhat disturbed (Fig. 2). In spite of significant recovery with the EEPM treated groups, the scores in stress-treated animals exceeded substantially those measured in the controls. It is worth mentioning that when placed on the distal end of the open arm, the EEPM and stressed\[^{64}\] animal remained motionless. Thereafter, they displayed locomotion accompanied with typical arm- and air-sniffing. Thus, both initial immobility and freezing of stressed animals were replaced by a remarkable exploration. It was observed that EEPM-treated animals preferred to stay on the open arms. We suggest that the preferences of the open arms may relate to reduced anxiety, stress or frustration. Animals showing less
anxiety spent more time in the open arms of the maze whereas animals with higher anxiety level ducked in the closed arms.[64]

Nevertheless, shortening of transfer latency, seen with the EEPM-treated, can be interpreted as reflecting a definite ability to remember the configuration of the maze. Two different mechanisms, one involved in the emotionality and the other triggered by memory formation, may participate in the behavioral performance of animals subjected to the EPM task.[65]

A dissimilar portion of both factors could influence behavioral performance of EEPM-treated and intact animals. Even, it is possible to consider that an individual’s reaction towards an anxiogenic stimulus can be changed by cognitive processes.

EEPM treatment also attenuated the stress-induced depression in FST, which supports many previous findings.[66] Stress induced depression is evident from increased freezing time of rats in FST (Fig. 3). Treatment with EEPM, in a dose dependent manner, attenuated the depressive effects on feeding pattern, water consumption and suppression in body weight gain. Rats forced to swim in a restricted area assumed an immobile posture after initial attempts to escape. In subsequent immersions, the beginning of the immobility is faster and marked. Porsolt et al (1978)[66] named this phenomenon “behavior despair” and attributed the animals’ response to the development of a depression process.

The extract was also found to improve the disturbances in memory and cognitive abilities of rats caused by stress (Fig. 4). This was very much evident from the number of trials and errors committed by the rats. Memory impairment is a common and usual co-morbidity associated with exposure to chronic stress and has been found to induce cognitive dysfunction in psychiatric patients, which leads to the loss of synaptic connectivity and perhaps neuronal networks in limbic brain structures including the hippocampus and cortex. This further leads to loss of cholinergic neurons and results in a state of dementia. Activation of the stress system leads to behavioral and hormonal changes that improve the ability of the organism to adjust to homeostasis and increase its chances of survival.

Antidepressant drugs are clinically effective after chronic, but not in acute treatment. Similarly, in the present study, chronic EEPM pretreatment attenuated the effects of CUS-induced increase in food and water intake, body weight, anxiety, cognition and depressive behavior of the rats. These findings provide further support for the similarity between stress-
induced immune activation, anxiety and depression. The depletion in the level of SOD and CAT, and increased activity of GSH following a single and 10 days of AS and CUS respectively, are consistent with previously reported studies.

CONCLUSION

The AS and CUS paradigm appears to be very robust in inducing an anxiety and related mood disorders. The behavioral analyses revealed that the CUS paradigm successfully caused severe anxiety in rats, in comparison to acute model of stress. These findings suggested that the animals exhibited anxiety, depression and, impairment of memory and cognition following acute and CUS. EEPM was found to attenuate all the changes induces by stress.

The present data support the view that a series of tests and not a single test should be used to characterize animal behavior. The testing paradigms used here are reproducible in the evaluation of potential drugs effective in the consequent prevention of long-term behavioral functioning induced by stress treatment. These results clearly show that stress can lead to oxidative stress leading to neurodegeneration. Hence it may be concluded that the protective ability of P.murex, may be attributed to the presence of number of phenolic compounds, flavonoids, tannins, triterpenes, alkaloids, steroids, fixed and volatile oils.

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