EVALUATION OF POTENTIAL CENTRAL PROTECTIVE ROLE OF ETHANOL EXTRACT OF P*edalium murex* Linn. IN ACUTE AND CHRONIC UNPREDICTABLE STRESS (CUS) INDUCED MODELS IN SD RATS

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai-32.

In partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

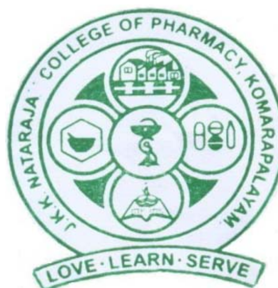
PHARMACOLOGY

Submitted by

Reg.No: 26113093

Under the Guidance of

Dr. R. SHANMUGA SUNDARAM, M.Pharm., Ph.D.,



DEPARTMENT OF PHARMACOLOGY

J.K.K. NATTRAJA COLLEGE OF PHARMACY

Komarapalayam – 638183.

Tamil Nadu.

SEPTEMBER – 2013

Certificates

CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"Evaluation of Potential Central Protective Role of Ethanol Extract of Pedalium murex Linn. in Acute and Chronic Unpredictable Stress (CUS) Induced Models in SD Rats",,** submitted to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment to the requirement for the award of Degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide work carried out by **Miss. SUDHA.M [Reg.No.26113093]** during the academic year 2012-2013, under the guidance and supervision of **Dr. R. SHANMUGA SUNDARAM, M.Pharm.,Ph.D.,** Vice Principal, Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

Place: Komarapalayam

Date:

Dr. R. Sambathkumar, M.Pharm., Ph.D.,

Principal & Professor

Department of Pharmaceutics,

J.K.K. Nattraja College of Pharmacy,

CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"Evaluation of Potential Central Protective Role of Ethanol Extract of *Pedalium murex* Linn. in Acute and Chronic Unpredictable Stress (CUS) Induced Models in SD Rats",** submitted to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment to the requirement for the award of Degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide work carried out by **Miss. SUDHA .M [Reg.No.26113093]**, during the academic year 2012-2013, under my guidance and direct supervision in the department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

Place: Komarapalayam

Date:

Dr. R. Shanmuga Sundaram, M.Pharm., Ph.D.,
Vice Principal and Professor
Department of Pharmacology,
J.K.K. Nattraja College of Pharmacy.

EVALUATION CERTIFICATE

This is to certify that the work embodied in this dissertation entitled **"Evaluation of Potential Central Protective Role of Ethanol Extract of Pedalium murex Linn. in Acute and Chronic Unpredictable Stress (CUS) Induced Models in SD Rats",** submitted to "The Tamil Nadu Dr. M.G.R. Medical University", Chennai, in partial fulfillment to the requirement for the award of Degree of **Master of Pharmacy** in **Pharmacology**, is a bonafide work carried out by **Miss. SUDHA.M [Reg.No.26113093]**, during the academic year 2012-2013, under my guidance and direct supervision in the Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam.

Internal Examiner **External Examiner**

DECLARATION

I hereby declare that the dissertation entitled **“Evaluation of Potential Central Protective Role of Ethanol Extract of Pedalium murex Linn. in Acute and Chronic Unpredictable Stress (CUS) Induced Models in SD Rats”,** has been carried out under the guidance and supervision of **Dr. R. Shanmuga Sundaram, M.Pharm., Ph.D.,** Vice Principal, Department of Pharmacology, J.K.K. Nattraja College of Pharmacy, Komarapalayam, in partial fulfillment of the requirements for the award of degree of **Master of Pharmacy** in **Pharmacology** during the academic year 2012-2013.

I further declare that, this work is original and this dissertation has not been submitted previously for the award of any other degree, diploma associateship and fellowship or any other similar title.

Place: Komarapalayam

Date:

SUDHA.M

Reg.No: 26113093

Department of Pharmacology,

J.K.K. Nattraja College of Pharmacy.

भारत सरकार / GOVERNMENT OF INDIA
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टी.एन.ए.यू. कैम्पस / T.N.A.U. Campus
लाउली रोड / Lawley Road
कोयंबत्तूर / Coimbatore - 641 003

टेलीफोन / Phone: 0422-2432788, 2432123, 2432487
टेलीफक्स / Telefax: 0422- 2432835
ई-मैल / E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No. BSI/SRC/5/23/2012-13/Tech. /1931

दिनांक/Date: 1 March 2013

सेवा में / To

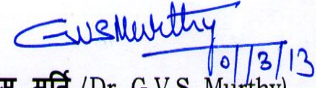
Ms. M. Sudha
II Year M. Pharm.
Department of Pharmacology
J.K.K. Nattraja College of Pharmacy
Komarapalayam – 638 183
Namakkal Dist., Tamil Nadu

महोदया/Madam,

The plant specimen brought by you for identification is identified as *Pedalium murex* L. - PEDALIACEAE

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,



(डॉ. जी.वी.एस. मूर्ति /Dr. G.V.S. Murthy)
वैज्ञानिक 'एफ' एवं कार्यालय अध्यक्ष /
Scientist 'F' & Head of Office

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Scientist 'F' & Head of Office
भारतीय वनस्पति सर्वेक्षण
Botanical Survey of India
दक्षिणी क्षेत्रीय केन्द्र
Southern Regional Centre
कोयंबत्तूर / Coimbatore - 641 003

Animal Ethical Committee Clearance Certificate

We, the Undersigned Chairman/Members of the Animal Ethical Committee, functioning in JKK Nattraja College of Pharmacy have studied the proposed research Subject/Project of **M. Sudha** titled **"Evaluation of potential central protective role of ethanol extract of *Pedalium murex* Linn. In acute and chronic unpredictable stress (CUS) induced model in SD Rats"** applying for permission for animal usage and hereby give the certificate of clearance of approval by this Ethical Committee.

Signature of the Chairman/ Members of the
Animal Ethical Committee

Name of the Institution:

Station :

Date :

Seal :

Dedicated to

Almighty,

My beloved family,

Teachers and Friends.

Acknowledgement

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I am proud to dedicate my deep sense of gratitude to the founder, (Late) Thiru **J.K.K. Nattaraja Chettiar,** providing us the historical institution to study.

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M. SUDHA

Reg.No:26113093

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Introduction

1.STRESS

Stress is a common experience of daily life and all organisms have developed mechanisms to cope with it. Sustained stress can have numerous pathophysiological effects such as activation of neuro−endocrine [limbic−hypothalamic−pituitary adrenal system] (*Bonfiglio et al., 2011*) and hormonal (corticosterone release) functions (*Fuchs & Flugge, 1998*). 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 (*Maes et al., 2011*).

Stressful events can activate the Hypothalamo-Pituitary-Adrenal (HPA) axis (*Kvetnansky et al., 2002*) 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 (*Pacak et al., 1993; Venihaki et al., 1997*). 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 (*Akil & Morano, 1995; Levine, 2005*). Stress has been postulated to be involved in the etiopathogenesis of a variety of disease state including hypertension, coronary heart disease (*Roy et al., 2001*), gastric ulcers (*Yadin & Thomas, 1996*), diabetes (*Fitzpatrick et al., 1992*), immuno-suppression (*Purret, 2001*), mental depression, memory loss (*Gareri et al., 2000*), and host of other diseases. The resultant disturbances may vary depending upon type, intensity, and the duration of a particular stressor and the strain\sex differentiation of the subjects (*Kioukia-Fougia et al., 2002*). Different animal models for stress have been developed and used

frequently to evaluate the anti-stress activity of compounds of both natural and synthetic origin.

In an organism, diverse stressors activate a wide spectrum of interacting hormonal and neuronal systems resulting in behavioral (anxiety disorders, decrease in food intake, decrease in sexual behavior, and loss of cognitive function) and physiological responses [activation of pituitary adrenal axis and release of glucocorticoids into the blood stream] (*Henry & Stephens, 1977*). These stressors are stimulators of arousal and lead to autonomic (changes in body temperature and tachycardia) and behavioral changes; however, when arousal increases to stress-like levels, it results in psychiatric and physical disorders (*Hennessy et al., 1979*) [Figure 1]. Different animal models have been developed for chronic stress induced neurological disorders such as the olfactory bulbectomy model, and the chronic unpredictable stress model. These animal models are used to screen various new chemical entities and to develop a better understanding of the underlying molecular pathway in chronic stress pathology. Stress responses are variable and there are individual differences both physiologically and behaviorally in how an organism perceives a perturbation and in the resulting adaptational/maladaptational processes (*Weiner, 1992*).

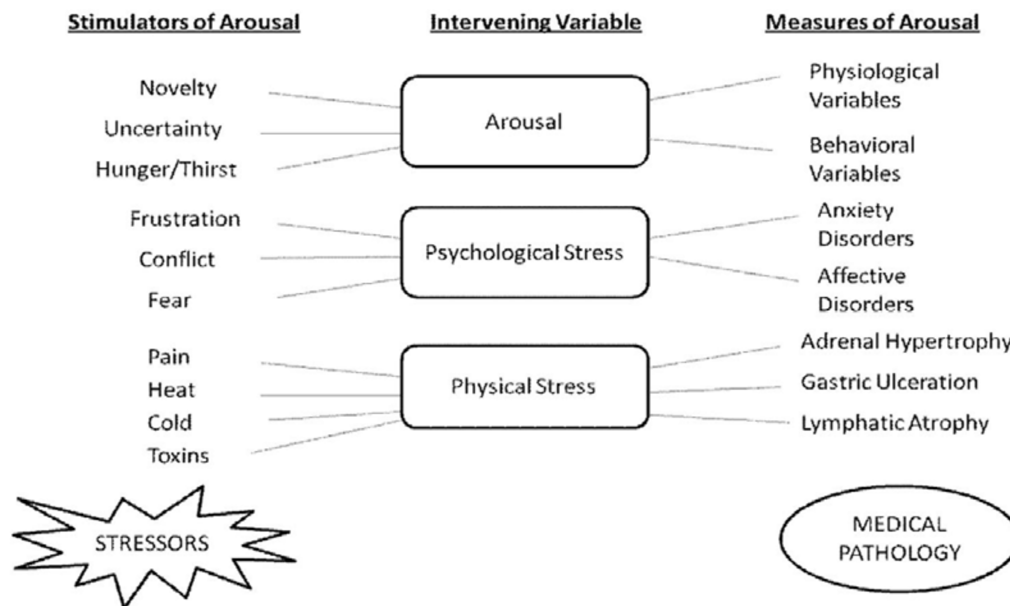
Stress and Stressors

Stress can be defined generally as responses to demands upon the body (*Koob, 1999*). It is the body's reaction to a change that requires a physical, mental, or emotional adjustment or response (*Selye, 1936*). 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.

Kumar, et al.: Stress

Figure 1: Relationship between arousal, psychological stress, physical stress and pathology



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 (*Maier & Watkins, 1998*). Long-term exposure to stressors can cause depression, (*Nirmal et al., 2008*) post-traumatic stress disorder, and anxiety disorders. The degree of behavioral control that an individual has over a stressor often determines the consequence of that stressor and plays a key role in the development of pathological behaviors after a traumatic event (*Christianson et al., 2009*). The potency to cope with the stressors is a fundamental requirement for survival. Brain is the target for different stressors because of its high sensitivity to

stress-induced degenerative conditions (*Sahin & Gumuşlu, 2007*). The brain tissue is made up of large amounts of polyunsaturated fatty acid, thus making it vulnerable to free radical attacks (*Gutteridge, 1995*).

Consequences of Stress

Normal development and preservation of life and species depend on a normally functioning stress system. Maladaptive neuroendocrine responses, i.e., dysregulation of the stress system, may lead to disturbances in growth and development, and cause psychiatric, endocrine/metabolic, and/or autoimmune diseases or vulnerability to such diseases.

Stress and anxiety

According to previous reports stress induces anxiety-like behavior in both humans and animals (*Liezmann et al., 2011*). In response to stress, there is an increase in CRF levels. The CRF level decreases when the stressor is no longer present. Lee, et al. reported that chronic stress increases the length and volume of expression of CRF in areas of the brain associated with fear and emotion, including the amygdale (*Lee et al., 2008*) [Figure 2]. Such chronic stress changes the body's response, and the resulting increased expression of CRF is believed to be the cause of health-related stress problems such as anxiety, depression, and infertility (*Kimura et al., 2010*). Exposure to stress represents an important factor for a number of neuropsychiatric disorders such as depression, post-traumatic stress disorder, and other anxiety disorders (*Horstmann& Binder, 2011*). There are earlier reports of enhanced noradrenergic or HPA axis activity in many psychopathological states such as depression and anxiety disorders (*Boyer, 2000; Kendler, 1996*).

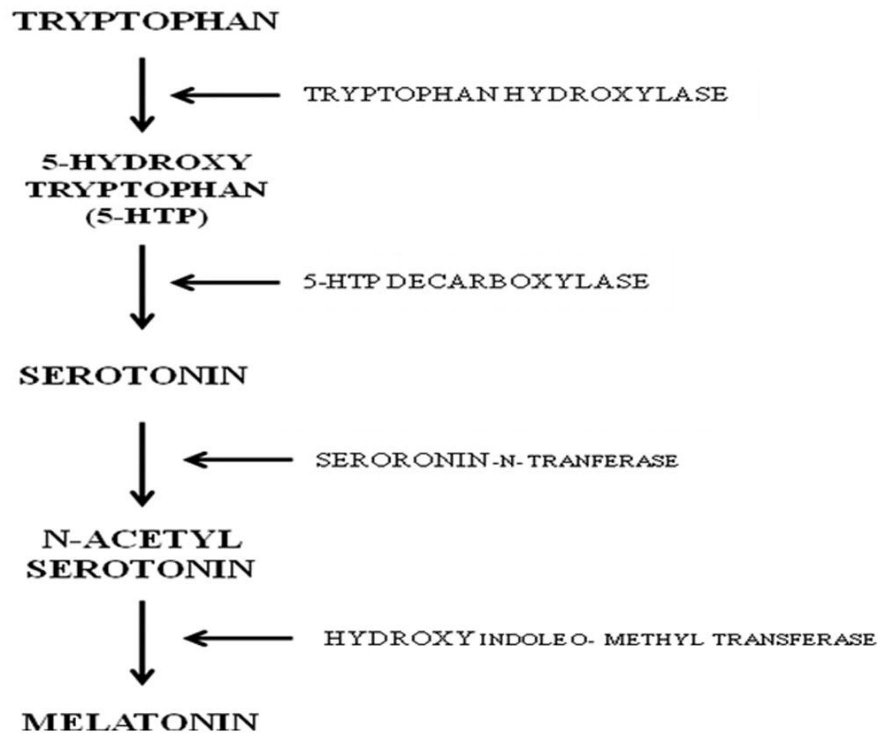


Figure 2: *De novo* synthesis pathway of melatonin.

Oxidative stress contributes toward neuronal degeneration in the central nervous system in the process of aging as well as neurodegenerative diseases (*Hovatta et al., 2010*). The production of reactive oxygen species (ROS) is greatly increased under many conditions of toxic stress (*Liu & Schubert, 2009*). One of the reasons for stress-induced enhancement of free radicals may be the elevation of nitric oxide (NO) production (*Matsumoto, 1999*). This is further supported by the present determination of nitrite levels, which revealed significant increase in brain NO levels in stressed mice. The reactive nitrogen species along with ROS, working in concert with an inflammatory process, may play a substantial role in the pathogenesis of depression (*Matsumoto, 1999*). Stress has been shown to be responsible for the depletion of several free radical detoxifying enzymes such as glutathione peroxidase, catalase, and superoxide dismutase (*Zaidi & Banu, 2004*).

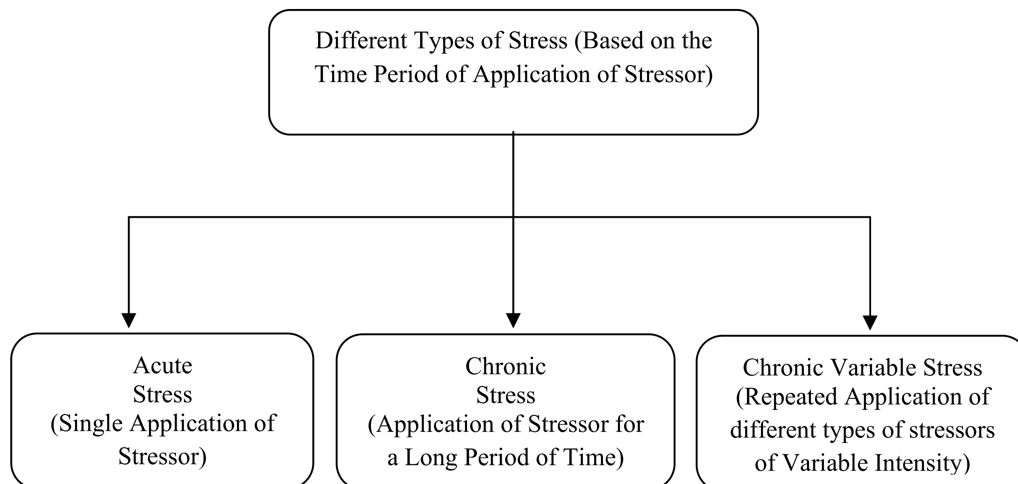
This results in oxidative burden, which has been implicated in stress as well as in the pathogenesis of several disease states. Since brain tissues consist of a high content of polyunsaturated fatty acids and one of the important consequences of oxidative stress is peroxidation of membrane lipids, this reaction produces marked damage to the structure and function of cell membranes in these tissues (*Jain et al., 1991*). Therefore, lipid peroxidation was supposed as the major biochemical alteration and consequence of oxidant-induced cell injury. Thus, the important consequences of stress could be attributed to stress-induced lipid peroxidation.

Stress and immunological changes

Stress has been associated with impaired immune function and increased susceptibility to infectious diseases (*Connor & Leonard, 1998*). It is now believed that the nervous, endocrine, and immune systems are so intimately connected that they should be regarded as a single network rather than as three separate systems (*Connor & Leonard, 1998*). It is widely accepted that psychological stress and psychiatric illness can compromise immune function (*Leonard, 1995*) and soluble mediators released by immune cells can affect the central nervous system, thus producing alterations in behavior. Exposure to stressful life events such as academic examinations and divorce was reported to cause impairments in various aspects of cellular immune function (*Bartrop et al., 1977*). There are also reports of immune activation, (*Bartrop et al., 1977*) in addition to immunosuppression in both the depressed and subjects exposed to stressful life events.

A requirement of all studies on stress is an adequate and appropriate animal model of stress. An ideal animal model should be able to reproduce each of the aspects of stress response and should be able to mimic the natural progression of the disease.

However, none of the models available is able to entirely reproduce stress response. Some models reproduce physical stress and associated neuroendocrine changes (*Kvetnansky & Mikulai, 1970*), whereas others better reproduce the psychological stress and associated behavioral changes (*Marcelo et al., 2007*). Acute models do not reproduce the neuroendocrine dysfunction whereas a chronic model might be able to do so. Therefore, a correct model should be used to evaluate specific aspects of the stress response. Each model has inherent limitations including lack of stability, lack of predictability of tissue damage, and lack of adjustability. And hence a literature survey of more than 35 years (1970-2007) was conducted based on the description of the models, potential utilization of the models, and value of the models for testing of new medical interventions for the management of stress. The purpose of this review was to assess different models of stress.



ANIMAL MODELS COMMONLY USED

- **Physical stress models**

  Animal models of stress that use physical stress can be subdivided into:

- Temperature fluctuation induced stress:

  1. Immersion in cold water with no escape
  2. Cold environment isolation

  - Immobilization induced stress
  - Electric foot shock induced stress
  - Forced swimming induced stress

- **Psychological stress models**

  Animal models of stress that use psychological stress can be subdivided into:

  - Neonatal isolation induced stress
  - Predatory stress
  - Day-night light change induced stress
  - Noise induced stress.

- **Chronic unpredictable stress**

Different stressors of mild to moderate intensity are applied on variable basis so as to prevent the emergence of adaptation or resistance to one particular type of

stressor. It involves the use of both physical and psychological stress models in a random way.

PHYSICAL STRESS MODELS

Temperature fluctuation induced stress

Acute change in temperature leads to stressful conditions by activation of temperature regulatory centre in the hypothalamus and subsequently HPA axis. It leads to acute release of adrenocortical hormones in the blood stream responsible for acute stressful response (*Sapolsky et al., 1986*). A sharp decrease in temperature using either cold water or freezer has been used frequently to induce acute stress.

1. Immersion in Cold Water (ICW)

In this method, the rats are placed individually in a tank of cold water (depth = 15.5 cm; temperature = 15-20°C) where they either swim or remain in an upright position, keeping their heads above water level (*Retana-Marquez et al., 2003; Iwona et al., 2003; Fernandez-Landiera, 2004; Yun et al., 2003*). This situation lasts for 15 minutes unless the rats sink. In that event, rats are removed before the cutoff time and are not included in the experiments. For acute stress, rats are sacrificed 30 minutes after the stress exposure. For chronic stress, animals are exposed to this stressor for 7-10 days. Rats are sacrificed 1 h after the last stress session. The major advantage of this type of stressor is that acute stress can be achieved in a relatively short period of time. However the major drawback of this model is that the body adapts to change in temperature on chronic exposure to low temperature and hence stress response gets highly diminished (*Pitman et al., 1988; Blustein et al., 1998*).

2. Cold environment isolation

In this method, rats are individually kept in a freezer with a temperature maintained at 4°C. The rats are kept for 15 minutes once for acute stress and for 7-10 days to develop chronic stress (*Kvetnansky et al., 1971*). This sharp fall in temperature leads to a sharp increase in the level of adrenocorticoids as explained above culminating in the development of stress response (*Kvetnansky et al., 2002; Staratakis & Chrousos, 1995*). Unlike the ICW model, rats are prevented from drowning in cold water hence it is relatively safe model however it also suffers from same drawback of development of resistance/adaptation on chronic exposure.

Immobilization induced stress

Immobilization has been used extensively as a stressor for the study of stress-related biological, biochemical and physiological responses in animals (*Kvetnansky & Mikulai, 1970; Kasuga et al., 1999; Marty et al., 1997*). Immobilization can be produced in two different ways. Animal can be either kept immobilized in a semi cylindrical acrylic tube (4.5 cm diameter and 12 cm long) with proper holes in it for air to pass (*Das et al., 2000*). Another way is to keep the animal with its limbs stretched on a board and its limbs are immobilized with adhesive tape. Movement of head is restricted by keeping the head in a metal loop coiled around the neck. The rats are kept immobilized in either of the above two ways for 150 minutes once to produce acute stress and for 7-10 days to produce chronic stress (*Dronjak & Gavrilovic, 2006*). The major advantage of using immobilization as a model of stress is that it produces an inescapable physical and mental stress to which adaptation is seldom exhibited (*Kasuga et al., 1999*).

Electric foot shock induced stress:

Electric foot shock (EFS) of mild intensity has also been used as a stressor. Rodents are very susceptible even to mild shock and exhibit rapid stress response. Researchers have used electric foot shock of varying degree to produce stressful conditions and hence to evaluate adaptogenic activity of various compounds. Stress by electric foot shock is given by placing the rats individually in a chamber with an electrified floor. Rats receive unavoidable electric foot shocks with an intensity of 3 mA, 200 ms of duration and a frequency of 1 per second over a 5-min period. For acute stress response, the rats are exposed once and sacrificed after 15 minutes of stress. Chronic stress is also produced by repeating the same treatment for 7-10 days and rats are sacrificed 1 h after the last stress session (*Retana-Marquez et al., 2003*). Some researchers have modified the method in which rats are subjected to inescapable electric foot shock for 60 minutes (0.15 mA shock, on a variable interval schedule with a mean inter shock interval of 60 seconds) (*Taysse et al., 2005*). The biggest advantage of this model is that it effectively produces high degree of stress in the animal. The major disadvantage of this model is the hazard of electric shock causing death of the animal and special caution that is required to perform this methodology.

Forced swimming induced stress

It is the tendency of the living being to escape or avoid a noxious stimuli/condition. If the animal is not able to escape the stressful stimuli or it feels threatened, the animal will show stress response. This principle is used for developing forced swimming model for inducing stress in laboratory animals. In order to produce swimming induced stress, rats are made to swim in a cylinder (30 cm diameter and

filled to a height of 20 cm with 15 cm of space above the head of the rat) for a single session of 2 h duration for acute stress, or for one 2 h session a day for five consecutive days for chronic stress (*Ferry et al., 1991*). Some authors have used forced swimming in warm (20°C) water for 3 minutes with the total session lasting for 1 h (*Kitchen & Pinker, 1990*). Although forced swimming induced stress is a highly safe model, adaptation to chronic swimming induced stress has been reported and inter-strain differences between rats to forced swimming behavior have also been documented (*Armario et al., 1995*).

PSYCOLOGICAL STRESS MODELS

Neonatal isolation stress

Early life events have profound consequences on subsequent quality of life. It has been shown that the early life stress of neonatal isolation in rats has immediate and enduring neural and behavioral effects (*Kuhn et al., 1990*). Such effects may reflect, in part, stress-induced morphological changes in hippocampus and other brain regions (*Kosten et al., 2005b*). In fact, the hippocampus provides negative feedback regulation of the hypothalamic-pituitary-adrenal (HPA) axis (*Herman & Cullinan, 1997*) and hence neonatal isolation induced stress can represent the stress response that may lead to neuro-degeneration at an early stage of life. This stress procedure is also useful in evaluating the effect of stress on cognition and memory development. In the neonatal isolation procedure, the litter of the inbred strain is removed from the cage on second day after the birth, weighed and placed individually in an opaque plastic container (9 cm diameter and 8 cm deep) with no bedding for 1 h (between 09:00 and 12:00) in a heated (30°C), humidity controlled chamber with white noise to mask other pups calls. The chamber has to be located in a room separate from

animal colony facility. Containers are placed 20-30 cm apart. After 1 h period the litters are placed back with their dams in home cage (*Kosten et al., 2000; Kosten et al., 2004*). This isolation procedure continues up to 8 days and hence it is used to induce chronic stress only. Neonatal isolation stress model has be used extensively to demonstrate the effect of early lifetime stress on vulnerability to addiction (*Kosten et al., 2005a*), and response to psychostimulants by impairment of hippocampal-dependent context induced fear in adult male rats.

Predatory stress

Direct encounter of an animal with its natural predator is one of the most stressful and anxiogenic event it can face and it leads to rapid development of „flight or fight response (*Lupien et al., 2006*). Exposure of rodents to natural predators or to their odors may induce stress like states (*Adamec & Shallow, 1993*). Under such circumstances, there is rapid sympathetic activation leading to rise in the levels of adrenocorticoids in blood causing acute stress response to develop. Direct encounter with a predator has been effectively used to evaluate the biochemical and physiological changes produced during such stressful conditions (*Marilia et al., 2007*). Predatory stress in mice is induced by series of short exposures to natural predator like cat (*Blanchard & Blanchard, 1989*) or to any substance having the odor of cat like the fecal pellets of cat (*Berton et al., 1998*). In one of the methods, mice are placed individually in different cages and after four initial 20-min cage habituation sessions each subject is submitted to two randomly-assigned 20-min predator confrontation sessions. Change in behavioral pattern such as locomotion, shrieking like voices and endocrinological changes after the stress exposure are observed (*Blanchard et al., 1998*). Another free-exploration test (*Griebel et al.,*

1993) was used consists of a PVC box (30x20x20 cm) covered with Plexiglas and subdivided into six equal square exploratory units, which are all interconnected by small entries. It could be divided in half lengthwise by closing three temporary partitions. Approximately 20 h before cat exposure, each subject is placed in one half of the apparatus with the temporary partitions in place, in order to be familiarized with it. The floor of this half was covered with fresh sawdust and the animal is given unlimited access to food and water. On the test day, mice of each strain are randomly allocated to the following four groups.

(a) Naive clay: animals are exposed to both familiar and novel compartments by removal of the temporary partitions. The novel compartment contains three modeling odor-free clay pellets.

(b) Naive feces: animals are exposed to both familiar and novel compartments. The novel compartment contains three cat feces pellets.

(c) Exposed clay: subjects are removed from the free-exploration box and confronted individually with a cat during a 5 min. session. The cat cage consists of a PVC box (82x56x62 cm) subdivided into two compartments, one containing the cat, the other the mouse. Separation consists of a transparent PVC wall with holes allowing the cat to reach the other side with its paws. The mouse is then put back in the free-exploration apparatus and is exposed 1 h later to both familiar and novel compartments. The novel compartment contains three modeling odor-free clay pellets.

(d) Exposed feces: same as previous group, but the novel compartment contains three pellets of feces from the cat used during exposure. The behavior of the mouse

is observed under red light for 5 min via a closed circuit TV camera by an observer located in an adjacent room.

The following parameters are recorded:

(a) Time spent in the novel compartment; (b) total unit entries and (c) total number of rearings.

The results are expressed as mean percentage of time spent in the novel compartment, mean total number of novel unit changes, and mean total number of rearings. Marmosets (*Callithrixpenicillata*) have also been employed for induction of predatory stress in a test battery known as Marmoset Predator Confrontation Test [MPCT] (*Cilia & Piper, 1997*). This model compares the behavioral response of experienced versus naïve adult black tufted-ear marmosets in confrontation with a taxidermized wild-cat predator stimulus. After four initial 20-min cage habituation sessions, each subject is submitted to two randomly-assigned 20-min predator confrontation sessions. Confrontation with the predator induces significant behavioral changes; i.e., proximic avoidance and *tsik-tsik* alarm call. Anti-stress drug administration, concomitant to predator exposure, reverses the behavioral changes observed (*Barros et al., 2004*). Predator induced stress is an established model to induce short term acute stress response but its major disadvantage is development of habituation to predator exposure hence the use of this model for inducing stress is justified for developing only acute stress.

Day-night light change induced stress

Changes in the circadian rhythm have profound effect on physical and psychological well being of an individual (*Atcheson & Tyler, 1975*). Laboratory animals, when

subjected to abrupt changes in day-night light pattern, exhibit acute stress response (*Kosten et al., 2005b*). Changes in circadian rhythms are regulated by pineal gland through the secretion of melatonin (*Nicholsonet al., 1985*). Melatonin is released from the pineal gland in response to dark or dim light where as its functional antagonist serotonin is secreted in response to bright light. It is this serotonin-melatonin cycle that is responsible for regulation of sleep-awake state of the body (*Bermudez et al., 1983; Hamm et al., 1983)*. To induce stress, cages of rat or mice are kept under bright light from 19:00 h over night (in the dark phase) and cages are kept in dark room with no light from 12:00 h in the light phase for 180 minutes for 7-10 days (*Marcelo et al., 2007*). This method is suitable for inducing short term stress response. Generation of stress can be evaluated by measuring the biochemical parameters associated with chronic stress response (*Rai et al., 2003*). The major disadvantage of this model is that it can be effectively used to generate short term stress response as on repeated exposure to this type of stressor, the animal adapts to the changed day-night light pattern. This major drawback can be minimized by using this model as a part of chronic unpredictable stress protocol.

Noise induced stress

Noise as a stressful stimulus is a widely accepted fact. A large number of people are exposed to potentially hazardous levels of noise levels in daily modern life. Experimental studies have demonstrated ultra structural modifications in rat cardiomyocytes mainly in mitochondria due to noise stress. These subcellular alterations are related to an imbalance in calcium homeostasis, which is supposed to be sustained by increased catecholamine innervations (*Paparelli et al., 1992*). When noise exposure of any kind exceeds 90 dB, noise becomes a stressor (*Ramsey, 1982*).

Noise stress has a depletory effect on free radical scavenging enzymes in the brain leading to moderate to severe oxidative stress (*Samson et al., 2005*) which can be a potential basis for hearing loss (*Fechter, 2005*). Noise stress in laboratory rats can be produced by loudspeakers (15 W), driven by a white noise generator (0-26 kHz), installed 30 cm above the cage. Thus a noise level can be set at 100 dB or above uniformly throughout the cage and can be monitored by a sound level meter. Each animal to be treated is exposed to noise stress for 4 h/day for 15 days. Control group rats are also kept in the above described cage during the corresponding period of time, without noise stimulation to avoid the influence of handling stress on evaluation of effects due to noise exposure (*Ravindran et al., 2005; Manikandan & Devi, 2005*). The effect of noise stress exposure can be determined by estimating the brain biogenic amine level.

CHRONIC VARIABLE (UNPREDICTABLE) STRESS MODELS

The major disadvantage of both physical stress models and psychological stress models is the development of adaptation / resistance on chronic exposure. The changes in physiological and behavioral responses to chronic stress can be related to the adaptation of the HPA axis. When the same stressor is repeated, the HPA response undergoes desensitization or become stable as it has been reported that rodents repeatedly exposed to restraint stress exhibited a habituated corticosterone response, when they were subsequently challenged with an acute exposure to restraint (*Magarinos & Evans, 1995; Gadek-Michalska & Bugajski, 2003*). On the other hand, the exposure to a multiple stress paradigm produced continued elevation in corticosterone levels, when the animals were subsequently subjected to acute restraint stress (*Magarinos & Evans, 1995*). It has also been suggested that the

adaptations of HPA axis depend on type, duration and severity of the stress regime (*Blanchard et al., 1998; Gadek-Michalska & Bugajski, 2003*). To prevent the development of resistance, Chronic Unpredictable Stress (CUS) models have been developed which involve the use of various physical and psychological stressors in a predetermined manner so that the animal is not able to adapt to the stressor. Adaptation to one type of stressor has been effectively prevented by employing various stressors such as immobilization stressor for 15 minutes followed by overnight sleep deprivation and rotation of the cage at a predetermined speed (horizontal shakes at high speed) for 50 minutes followed by swim stress in water (20ºC) of 4 minutes (*Ortiz et al., 1996*). Wetting the saw dust bedding of the animal all day to restrict movement followed by electric foot shock (ten shocks of one second duration each, in an unpredictable manner, at the intensity level of 0.4-1.8 mA) and stroboscopic light (for 13 h, 10 Hz) has also been used as a part of CUS protocol (*Margus et al., 2007*). Some researchers have used exposure to predator odor induced stress as a part of CUS protocol, in which mice are placed in a novel cage containing cat litter soiled with cat feces and urine (*Anisman et al., 2007*). Various authors have modified the stress models in order to accommodate them in their respective CUS protocol. Other additional stressors that have been applied as a part of CUS protocol are tail pinch with a clothes-pin placed 1 cm distal from the base of the tail for 5 min, strong illumination during predicted dark phase for 12 h, movement restriction in a small cage (11 cm x16 cm x 7 cm) for 2 h (*Ortiz et al., 1996*), ether anaesthesia until loss of reflex (*Renard et al., 2005*), and subcutaneous 0.9% saline injection (*Ladd et al., 2004*). Chronic variable stress models have been proven to be more useful as they are devoid of the problem of resistance in the animal species towards the commonly used stressors and also have the advantage of

the development of effective and long-term stress response. Thus CUS models are nowadays the preferred models for generation of a stress response.

Stress has been postulated to be involved in the etiopathogenesis of a variety of disease state including hypertension, coronary heart disease (Roy et al., 2001), gastric ulcers (*Yadin & Thomas, 1996*), diabetes (*Fitzpatrick et al., 1992*), immunosuppression (*Purret, 2001*), mental depression, memory loss (*Gareri et al., 2000*), and host of other diseases.

NEURODEGENERATIVE DISEASE

Neurodegenerative diseases comprise a wide range of diseases that share the common characteristic of progressive loss of structure or function of neurons and glial cells in the brain and spinal cord. Many neurodegenerative diseases are a result of neuronal loss, although glial cells are also involved (*Glass et al., 2010*).

Neurodegenerative diseases present a chronic and slowly progressive process. Neurons in neurodegenerative diseases are affected by neuronal dysfunction at the level of synaptic transmission, synaptic contacts, and axonal and dendritic degeneration. In different neurodegenerative diseases, neurite degeneration and cell loss of neurons are present within specific neurotransmitter populations. In addition, numbers of functional neurons in neurogenic regions, and adult neurogenesis are altered or decreased. Adult neurogenesis increases after several acute pathologic stimuli, including stroke, seizure and acute trauma (*Arvidsson et al., 2002; Rice et al., 2003; Parent, 2007*). Brain regions differ in their vulnerability to aging; some regions that are very sensitive to age-related neurodegenerative changes are the dentate gyrus (DG) of the hippocampus, subiculum (*Small, 2003*) and subventricular

zone (SVZ)⁄olfactory bulb (OB) *(Braak et al., 2003*). The generation and cell death of newly generated cells have critical roles in brain development and maintenance in the embryonic and adult brain, and alterations in these processes are seen in neurodegenerative diseases.

DIFFERENT NEURODEGENERATIVE DISORDERS:

Although neuronal degeneration predominantly affects or starts with specific neuronal populations [including DAergic neurons in PD, striatal medium spiny neurons in HD, motor neurons in amyotrophic lateral sclerosis, and cortical and hippocampal neurons in Alzheimer's disease (AD)], there are many similarities between different neurodegenerative disorders. These include atypical protein assemblies and oligomerization as well as induced cell death. At a late disease stage, protein aggregation is no longer restricted to specific brain regions.

Interestingly, in Parkinson's disease (PD) and Huntington's disease (HD), the specific alterations in neurogenic areas such as the DG and SVZ ⁄ OB system parallel the early or premotor symptoms that are seen in the early stages of neurodegenerative disease, such as depression, anxiety or olfactory dysfunction (*Simuni & Sethi, 2008*). Therefore, it is intriguing that the mechanisms of neurodegenerative diseases are closely linked to brain plasticity. Brain plasticity in the adult, originally conceived of as changes at the level of synaptic transmission, synaptic contacts and gene expression (reviewed in *Buonomano & Merzenich, 1998*), became a more complicated concept.

Genes that are key players in neurodegenerative diseases [α-synuclein, presenilin(PSEN)1, tau, huntington] are also physiologically involved in modulating

brain plasticity in the embryonic brain, specifically as membrane proteins and when concentrated in synapses. These proteins commonly show high conservation between species and are located close to membranes or are involved in microtubule transport. α-synuclein is a protein that is physiologically enriched in presynaptic termini (*Abeliovich et al., 2000*). Initially shown to be upregulated in a discrete population of presynaptic terminals of the song bird brain during a period of acquisition-related synaptic rearrangement (*George et al., 1995*), α-synuclein can interact with tubulin (*Alim et al., 2002*). In addition, it is involved in dopamine (DA) synthesis, metabolism and release, and slight changes in concentration can have vast effects on neurotransmitter release (*Nemani et al., 2010*).

NEURODEGENERATION IN DIFFERENT DISORDERS:

In non-pathological aging, cognitive impairment would seem easiest to attribute to the effects of oxidative stress, inflammatory reactions and changes in the cerebral microvasculature (Riddle et al., 2003). However, the aging brain in the absence of dementia is also affected to varying degrees by the neuropathological features. Many neurodegenerative diseases occur as a result of neurodegenerative processes including

- Parkinson's disease (PD)
- Alzheimer's disease (AD)
- Huntington's disease (HD)
- Amyotrophic lateral sclerosis (ALS) and
- Multiple sclerosis (MS)

Alzheimer's disease (AD)

Alzheimer’s disease (AD) was first described by Alois Alzheimer more than a century ago in Germany, and it constitutes one of the most common causes of senile dementia.AD refers to a clinical syndrome that occurs in the elderly and is severe enough to interfere with social and occupational activities. At least two clinical abnormalities are essential for diagnosis of the disease, namely, memory loss in an alert person and impairment of one or more of the following functions: language, attention, perception, judgment or problem solving (*Forstl & Kurz, 1999*).

The pathology of AD includes neuronal and synaptic loss, neurofibrillary tangles due to hyperphosphorylated tau proteins and deposition of amyloid-β (Ab) protein in senile plaques in the basal forebrain cholinergic neurons as well as in the cortex, hippocampus and amygdala (*Hardy & Selkoe, 2002*). Ab is the product of proteolysis of amyloid precursor protein (APP) by b- and c-secretase enzymess.

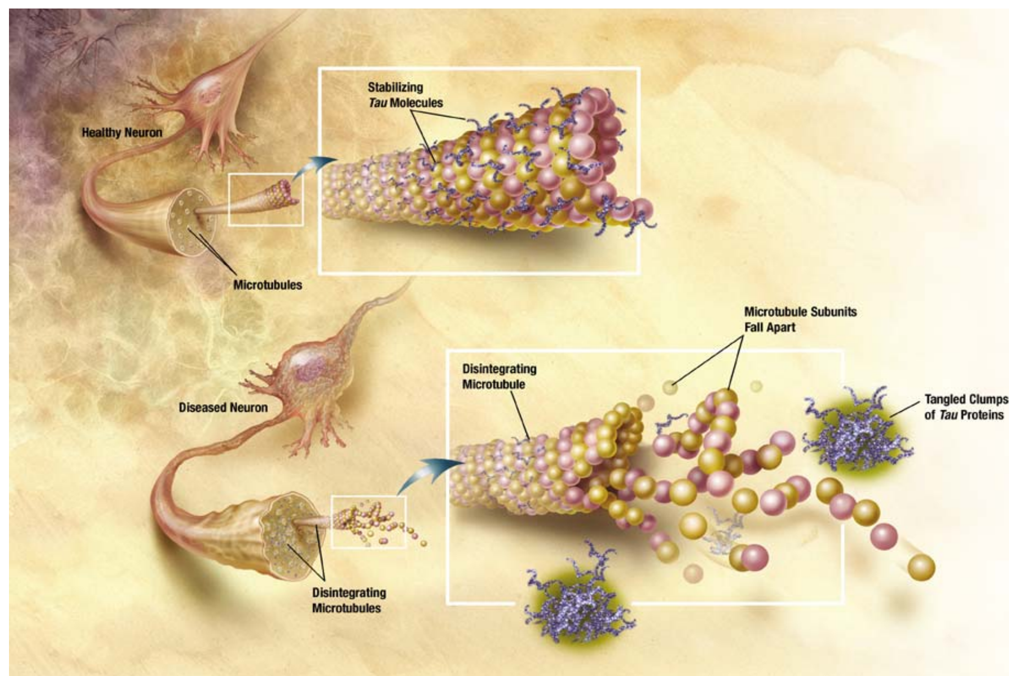


Fig.3 Changes in Tau protein lead to microtubule disintegration

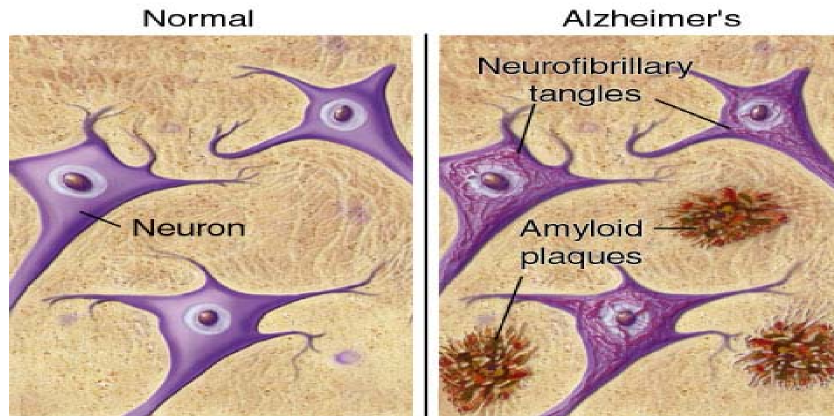
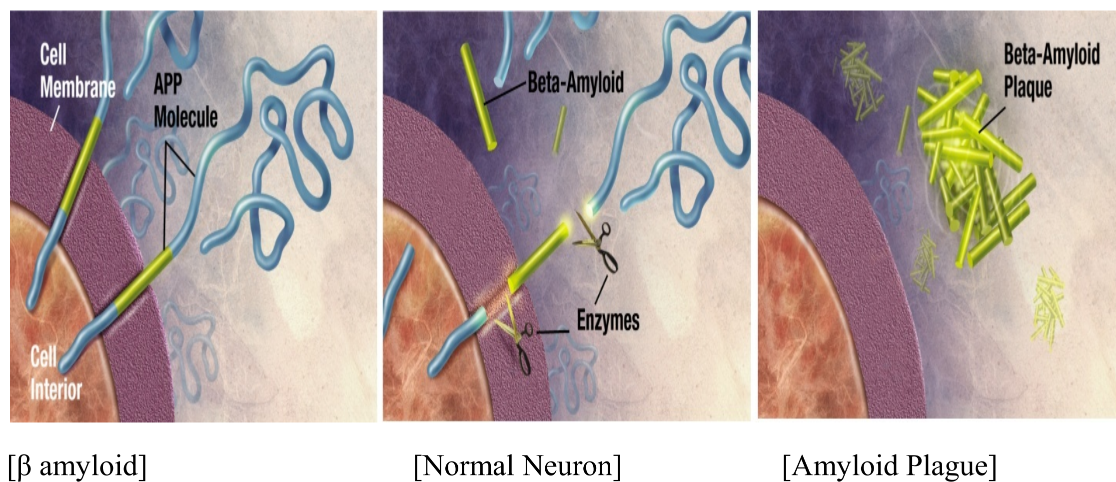


Fig.4 "Tangles" of a protein called "tau" occur in Alzheimer's patients' brains-causing neurons to lose their function and increasing memory loss. Illustration: National Institute on Aging/U.S. National Institutes of Health.



[β amyloid] [Normal Neuron] [Amyloid Plague]

Fig.5 Enzymes act on the APP (amyloid precursor protein) and cut it into fragments. The beta-amyloid fragment is crucial in the formation of senile plaques in AD.

Ab accumulates intracellularly in the neuronal endoplasmic reticulum but also extracellularly (*Trojanowski & Lee, 2000; Cuello, 2005*). Although Ab plaques are the neuropathological hallmarks of AD, the small Aboligomeric species rather than its amyloid counterpart is thought to be the toxic culprit in the disease. Evidence for this assumption includes a correlation between oligomerization and memory deficit

in both transgenic mice and humans, the presence of oligomers in the brains of transgenic mice, the toxicity of Ab dimer and trimer measured by long-term potentiation, and lack of a good correlation between plaque amount and AD (at least in the early phase of the disease) (*Walsh et al., 2002; Walsh & Selkoe, 2007*). Patient deficits include olfactory deficits, memory impairment, cognitive and functional decline, and death. These symptoms can be partly related to regions and functions of adult neurogenesis.

Parkinson's disease (PD)

Parkinson disease (PD) is the second most common neurodegenerative disease, affecting about 1% of the population over 65 years of age. PD is characterized clinically by resting tremor, rigidity and bradykinesia, resulting from the progressive and selective loss of dopamine (DA) neurons in the substantianigra (SN) pars compacta, and histopathologically by the eosinophilic proteinaceous intracytoplasmic inclusion known as Lewy bodies (LBs) in surviving dopaminergic cells (*Forno, 1996*). The etiopathogenesis of PD is probably multifactorial, including both environmental and genetic factors (*Di Monte, 2003; Hardy et al., 2003*). Asmentioned above, α-synuclein (α-syn) is the principal component of LBs (*Spillantini et al., 1997*). This was seen in molecular genetic investigations that identified autosomal dominant missense mutations of α-syn (A53T) in heritable cases of PD (*Polymeropoulos et al., 1997*). Another missense mutation on the α-syn gene (A30P) was associated with familial PD (*Kruger et al., 1998*) and a new mutation (A46K) was found recently in a Spanish family, associated with PD, dementia of Lewy bodies (*Zarranz et al., 2004*).

It was suggested that α-syn aggregation is the key event that triggers neuronal damage and death (*Forloni et al., 2000; Volles & Lansbury, 2003*). In this context, α-syn mutations would speed up the protein aggregation (*Conway et al., 2000*). It is likely that the α-syn concentration is crucial to switch from a physiological to a pathological condition, and only when a threshold concentration is reached do the deleterious effects of aggregation become evident. Misregulation in the homeostasis of α-syn (caused by mitochondrial complex I inhibition, environmental toxins, oxidative stress or proteasome impairment) would be sufficient to trigger α-syn chemical modifications and aggregation in sporadic PD too (*Sherer et al., 2003; Norris et al., 2003*).

Loss of Dopaminergic neurons in the substantianigra of the midbrain and loss of other neurotransmitter phenotype neurons in other brain regions are characteristic neuropathological hallmarks (reviewed in *Goedert, 2001*). Prominent clinical features of PD are motor symptoms (bradykinesia, tremor, rigidity and postural instability) and non-motorrelated PD symptoms (olfactory deficits, autonomic dysfunction, depression, cognitive deficits and sleep disorders). Non-DA brain regions that are affected in PD have recently attracted increasing interest because the onsets of the non-motor symptoms linked to these neuropathological alterations are observed early in the course of the disease. They include rapid eye movement (REM) sleep behavior disorder, subtle cognitive deficits, depression, olfactory dysfunction and constipation (for review see *Tolosa&Poewe, 2009*). As described above, a subset of these functions is connected to the stem and progenitor cell populations in the hippocampus and SVZ / OB system. Interestingly, several

monogenetic forms of PD show a decreased gray matter volume in the hippocampal region (*Reetz et al., 2010*).

Chaperone-like activity of α-synuclein:

Chaperones are proteins that prevent irreversible protein aggregation and facilitate the correct folding of non-native proteins through regulated binding and release in vivo (*Slavotinek & Biesecker, 2003*). α–Syn (α–Synuclein) has been suggested to function as a chaperone protein in vivo because, besides lipids (*Eliezer et al., 2001; Li et al., 2001; Davidson et al., 1998*), it appears capable of interacting with a variety of ligands and cellular proteins (*Ostrerova et al., 1999; Xu et al., 2002*), thus modifying their activities. It has recently been reported that the amino-terminal portion of α–syn shares 40% a homology with molecular chaperone 14-3-3 (*Ostrerova et al., 1999*), suggesting that the two proteins could sub serve the same function. The molecular chaperone 14-3-3 is particularly abundant in brain, where it comprises ~1% of total soluble proteins (*Boston et al., 1982*). Chaperone 14-3-3 accumulates in LBs, participates in neuronal development and cell growth control (*Fu & Masters, 2000*), and prevents apoptosis by antagonizing BAD, a proapoptotic member of the Bcl-2 family (*Yuan & Yanker, 2000*; Fig. 6). α-Syn binds to many of the same proteins as 14-3-3 (*Ostrerova et al., 1999*), including three proteins known to affect cell viability, protein kinase C (PKC), BAD, and extracellular regulated kinase (ERK). α-Syn interacts with 14-3-3 (*Ostrerova et al., 1999*), and the interaction between the two proteins produces a 54 to 83 kDa protein complex in PD brain (*Xu et al., 2002*). This complex is selectively increased in *substantianigra* but not in *cerebellum* or cortex. Thus α-syn may sequester 14-3-3, leading to a reduction in the amount of 14-3-3 protein available to inhibit apoptosis and rendering the cells

more susceptible to cellular stresses (*Xu et al., 2002*; see also Fig. 6). Both 14-3-3 and α-syn bind to tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, with divergent consequences: activity is stimulated by 14-3-3 but inhibited by α-syn (*Jenco et al., 1998*). The physical and functional homology between α-syn and 14-3-3 suggests that α-syn may normally act as a protein chaperone to help the cell deal with the effects of increased stress as part of an initial effort by the cell to protect itself against the accumulation of damaged proteins (*Ostrerova et al., 1999*). However, overexpression of wild-type α-syn is toxic to dividing cells and overexpression of its mutant forms A53T or A30P exhibits even greater toxicity (*Ostrerova et al., 1999*), which may be due to inhibition of PKC and interaction of α-syn with BAD, ERK, or other proteins involved in signal transduction (*Ostrerova et al., 1999*).

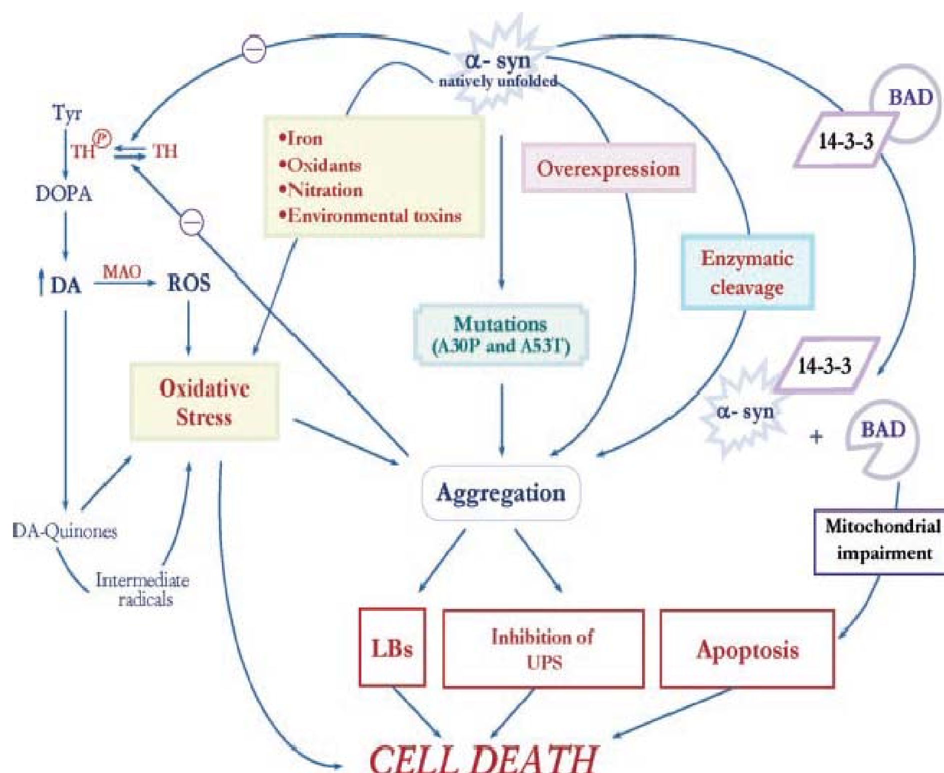


Figure 6. α-Syn aggregation and toxic effects in dopaminergic neurons. A hypothetical scheme depicts various pathways that, leading to aggregation of natively unfolded α-syn, oxidative stress, or mitochondrial impairment, cause cell death. For further details, refer to text. DA-dopamine; DOPA-dihydroxyphenylalanine; LBs-Lewy bodies; MAO-monoamine oxidase; ROS-reactive oxygen species; TH-tyrosine hydroxylase; THP-phosphorylated tyrosine hydroxylase; Tyr-tyrosine; UPS-ubiquitin proteasome system.

Huntington's disease (HD)

Hungtington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by motor dysfunction, cognitive impairment and psychosis (*Sharp et al., 1995*). The disease is caused by an *IT15* gene mutation on chromosome 4 (*Sharp et al., 1995*). This mutation produces a CAG / polyglutamate repeat expansion in the gene's protein product, huntington (*Sharp et al., 1995*). The DNA sequence CAG encodes the protein glutamate, an amino acid known primarily for its roles in metabolism and as a neurotransmitter (*Cotman & Monaghan 1986; Kandel, 2001*). Normal huntington protein contains 9-35 CAG repeats; however, its mutant form may contain up to 250 of these repeats (*Cattaneo et al., 2002*). Both forms of the protein are known to undergo caspase cleavage to generate smaller, truncated fragments; however, the mutant protein's fragments are distinct in their correlation with neurodegeneration (*Wellington et al., 2002*). Researchers are unsure why the mutant protein's CAG repeats cause HD; however, two theories exist to explain disease onset. One theory, the loss of function hypothesis, suggests that the CAG expansion disables huntington from carrying out its normal function (*Catteneo et al., 2002*). A second theory, the gain of function hypothesis, suggests that the *IT15*

mutation produces a toxic huntington protein with a distinct conformation that enables it to stick to both itself and normal huntington (*Catteneo et al., 2002*).

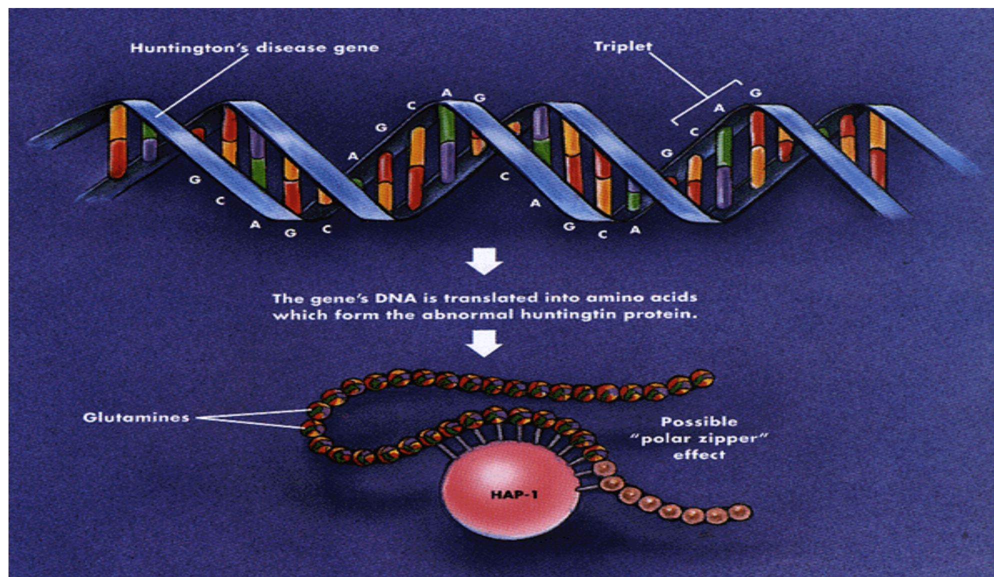


Figure 7. This conformation allows mutant huntington fragments to clump in aggregates and simultaneously inhibit the normal protein's proper function (*Catteneo et al., 2002*). Despite these opposing hypotheses regarding the exact role of CAG expansion, one idea is accepted across the board: mutant huntington forms inclusions in cell nuclei depending on the length of its CAG repeat, and longer repeats correlate with an increased presence of huntington inclusions (Senut et *al., 2000*). Moreover, these nuclear inclusions are associated with premature neuronal cell death, especially in the striatum and globuspallidus (*Hickey & Chesselet, 2003*).

Amyotrophic lateral sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS) is a progressive, degenerative disorder characterized by the selective loss of motor neurons in the brain and spinal cord leading to paralysis, muscle atrophy and eventually, death (*Pasinelli & Brown, 2006*). Two missense mutations in the gene encoding the human Vesicle-associated

membrane protein (VAMP)-Associated Protein B (hVAPB) causes a range of dominantly inherited motor neuron diseases including ALS8 (*Nishimura et al., 2004; Chen et al., 2010*). VAP family proteins are characterized by an N-terminal major sperm protein (MSP) domain, a coiled-coil (CC) motif and a transmembrane (TM)-spanning region. They are implicated in several biological processes, including regulation of lipid transport, endoplasmic reticulum (ER) morphology and membrane trafficking (*Lev et al., 2008*). Drosophila Vap-33-1 (DVAP) hereafter, regulates synaptic structure, synaptic microtubule (MT) stability and the composition of postsynaptic glutamate receptors (*Pennetta et al., 2002; Chai et al., 2008*). MSP domains in DVAP are cleaved and secreted into the extracellular space where they bind Ephrin receptors (*Tsuda et al., 2008*). MSPs also bind postsynaptic Roundabout and Lar-like receptors to control muscle mitochondria morphology, localization and function (Han et al., 2012). Transgenic expression of the disease-linked alleles (DVAP-P58S and DVAP-T48I) in the larval motor system recapitulates major hallmarks of the human disease, including aggregate formation, locomotion defects and chaperone upregulation (*Chen et al., 2010; Chai et al., 2008; Ratnaparkhi et al., 2008*). Several studies have also implicated the ALS mutant allele in abnormal unfolded protein response (UPR) (*Chen et al., 2010; Kanekura et al., 2006; Langou et al., 2010; Suzuki et al., 2009; Gkogkas et al., 2008*) and in the disruption of the anterograde axonal transport of mitochondria (*Mo´rotz et al., 2012*). However, it is unclear how these diverse VAP functions are achieved and which mechanisms underlie the disease pathogenesis in humans. One way to address these questions is to search for DVAP-interacting. We identified Sac1 (Suppressor of Actin 1), an evolutionarily conserved phosphoinositide phosphatase, as a DVAP-binding protein. Phosphoinositides are low-abundance

lipids that localize to the membrane–cytoplasm interface and function by binding various effector proteins. The inositol group can be reversibly phosphorylated at the 3′, 4′ and 5′ positions to generate seven possible phosphoinositide derivatives, each with a specific intracellular dynamic distribution (*Di Paolo & De Camilli, 2006*). Interestingly, SAC3 (also known as FIG4), another member of the Sac phosphatase family, is mutated in familial and sporadic cases of ALS (*Chow et al., 2009*). Inactivation of SAC3 in mice also results in extensive degeneration and neuronal vacuolization in the brain, most relevantly in the motor cortex (*Chow et al., 2007*). We identified Sac1 and DVAP as binding partners and show that DVAP is required to maintain normal levels of PtdIns4P. Loss of either Sac1 or DVAP function disrupts axonal transport, MT stability, synaptic growth and the localization of a number of postsynaptic markers. We also show that the disease causing mutation (DVAP-P58S) induces neurodegeneration and displays synaptic phenotypes similar to those of either Sac1 or DVAP loss-of-function, including an increase in PtdIns4P levels. Importantly, reducing PtdIns4P levels rescues the neurodegeneration associated with DVAP-P58S and suppresses the synaptic phenotypes associated with DVAP-P58S and DVAP loss-of-function alleles.

Multiple sclerosis (MS)

Multiple sclerosis is a chronic idiopathic demyelinating and neurodegenerative disease of the central nervous system. As such, both the onset and exacerbation of MS are thought to be influenced by multiple factors, including infectious agents, genetic composition and environment (*Hauser et al, 2006*).

DEFINITION:

MS is a chronic disease of the CNS, characterized by discrete areas of demyelination and axon injury associated with inflammatory activity as shown in figure 8. A key defining feature of MS is that lesions are disseminated in both space and time, i.e., they occur at more than one site and develop on more than one occasion. Additional information on the pathology of MS is provided below. Clinically, MS symptoms emerge Sbetween the ages of 20 and 40 years in approximately 70% of patients (*Weinshenker et al., 1989; Confavreux et al., 1980*) although changes visible on MRI are much more common than clinical activity and may well precede the latter (*O'Riordan et al., 1998; Sailer et al., 1999; Brex et al., 2002*).

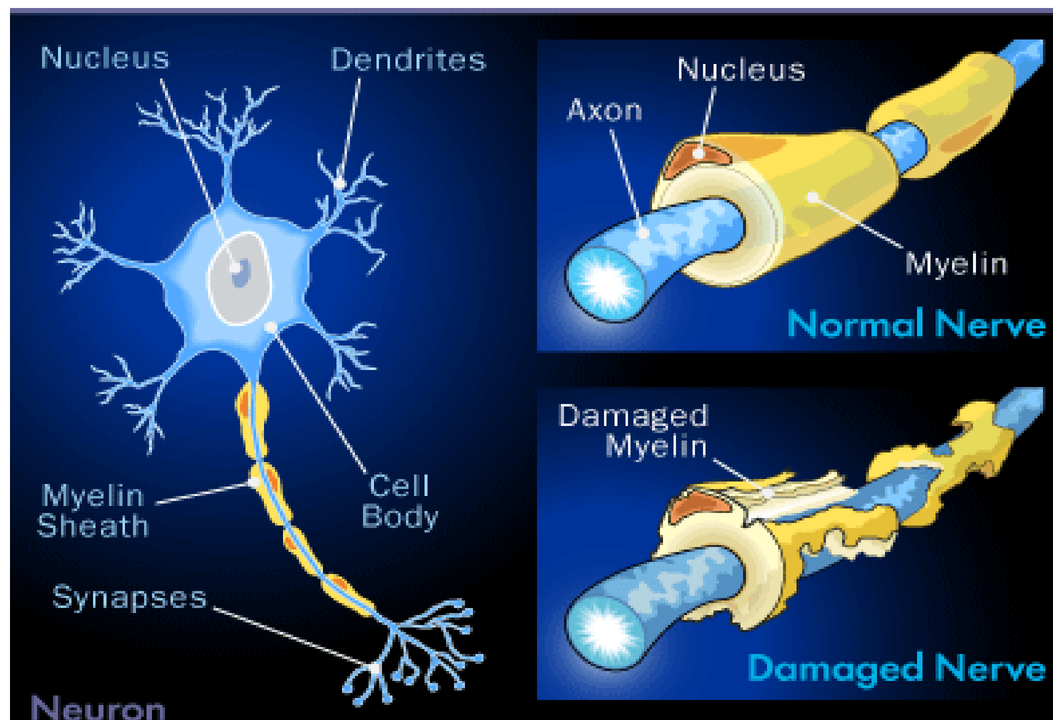


Figure 8. Demyelination of Myelin Sheath produces multiple sclerosis.

SYMPTOMS AND SIGNS:

Because MS lesions can occur in many different parts of the CNS, they can cause a wide variety of symptoms and signs. An exhaustive list of clinical findings seen in MS clinics at the Universities of British Columbia and Western Ontario, Canada (*Paty & Ebers, 1997*) together with estimates of the frequencies of each finding at onset and at any time. According to this list, initial neurologic symptoms and signs seen in 10% or more of patients include fatigue (20%, probably more common than this in many populations), optic neuritis (16%), internuclear ophthalmoplegia (17%), nystagmus (20%), vertigo (4–14%), gait disturbances (18%), sensory loss (30–50%, most commonly in the legs and implicating the posterior columns), increased deep tendon reflexes (20%), weakness in the legs (10%), spasticity (10%) and bladder disturbance (3– 10%). Symptoms and signs seen in 50% or more of patients at any time include cognitive changes (70%), euphoria (10–60%), depression (25–54%), fatigue (80%, probably nearer 90% in many populations), optic neuritis (65%), optic atrophy (77%), retinal nerve fiber loss (80%), nystagmus (85%), vertigo (5–50%), dysarthria (50%), limb ataxia (50%), ataxia of the gait and trunk (50–80%), sensory loss (90%, again, most commonly in the legs and implicating the posterior columns), increased deep tendon reflexes (90%), weakness in the legs (90%), spasticity (90%), extensor or flexor spasms (50%), cramps (50%), amyotrophy (50%), bladder disturbance (80%), and sexual disturbance (50% in women, 75% in men).

COMMON MOTIFS IN NEURO DEGENERATION

Neurodegenerative disorders such as Alzheimer's and Parkinson's disease account for a significant and increasing proportion of morbidity and mortality in the developed world (*Hebert et al., 2001; Hebert et al., 2003*). Largely as a result of

increased life expectancy and changing population demographics (i.e., the aging of baby boomers), neurodegenerative dementias and neurodegenerative movement disorders are becoming more common (*Brookmeyer et al., 1998; Samii et al., 2004*). As our population ages, an improved understanding of these diseases will be vital to developing more effective therapies and combating the staggering personal, social, and economic costs of these diseases (*Ernst et al., 1997*). Unifying theories of pathogenesis in neurodegenerative disease provide an avenue for developing therapeutic strategies with broad applicability for disease prevention and an opportunity for decreasing morbidity and mortality from these disorders in the elderly population (*Forman et al., 2004*). Converging lines of investigation have revealed a potential single common pathogenic mechanism underlying many diverse neurodegenerative disorders.

COMMON NEUROPATHOLOGICAL HALLMARKS:

Neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases and amyotrophic lateral sclerosis make up a group of pathologies characterized by a separated etiology with distinct morphological and pathophysiological features. These disorders are defined by a multifactorial nature and have common neuropathological hallmarks such as (a) abnormal protein dynamics with defective protein degradation and aggregation; (b) oxidative stress and free radical formation; (c) impaired bioenergetics and mitochondrial dysfunctions; (d) neuro inflammatory processes (*Jellinker, 2003; Jellinger, 2010*). It is difficult to establish the correct sequence of these events, but it has been shown that the oxidative damage to the brains of affected individuals is one of the earliest pathological markers. Oxidative and nitrosative stresses arise from the imbalance

between the increased production of both the reactive oxygen species (ROS) and the reactive nitrogen species (RNS) and the cellular antioxidant defense systems (*Valko et al., 2007*). At low levels, ROS function as signaling intermediates for the modulation of cellular activities but, at higher concentrations, they contribute to neuronal membrane damage.

ROS is a collective term, which includes not only the oxygen radicals (O_2 •−, and •OH) but also some non-radical derivatives of oxygen. These include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O_3) (*Bandhopadhyay et al., 1999*).

Over about 100 disorders like rheumatoid arthritis, hemorrhagic shock, cardiovascular disorders, cystic fibrosis, metabolic disorders, neurodegenerative diseases, gastrointestinal ulcerogenesis and AIDS have been reported as ROS mediated. Some specific examples of ROS mediated diseases include Alzheimer's disease, Parkinson's disease, Atherosclerosis, Cancer, Down's syndrome and ischemic reperfusion injury in different tissues including heart, liver, brain, kidney and gastro intestinal tract. The role played by ROS in stress induced gastric ulcer and inflammatory bowel diseases have been well established, as well as their involvement in the process of ageing. The role of radicals in various diseases is dealt in detail.

Aging biology

In the biological process of aging, the following cause–effect relationships are demonstrated: formation of intra and intermolecular cross-linkings, as in the case of muscle cells aging. Modifications of immunological reactions, usually resulting in

decrease of their activities. Telomere shortening with decrease or interruption of cell proliferation, which can mean an unbalance between cells lost and reposition rates, culmination with organ and system failure and death of the organism. Cell damages provoked by free radicals are common during aging; once in this life step cells produce less concentrations of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), etc. Gene activation with inevitable aging-related physiological changes (*Harman, 1998; Ferrari, 2001*). In healthy human centenarians, although both plasmatic and red blood cell-SOD were decreased (in concentration with the increasing levels since <60 years until 99 years), the remarkable increase of plasmatic Vitamins A and E contribute the first indication that these vitamins are favorable to longevity (*Mecocci et al., 2000*).

Inflammation

An inflammatory response implicates macrophages and neutrophils, which secrete a number of mediators (eicosinoids, oxidants, cytokine and lytic enzymes) responsible for initiation, progression and persistence of acute or chronic state of inflammation (*Lefkowitz et al., 1999*). NO along with superoxide (O_2 •−) and the products of their interaction initiates a wide range of toxic oxidative reactions causing tissue injury (*Hogg, 1998*). Likewise, the neutrophils too produce oxidants and release granular constituents comprising of lytic enzymes performing important role in inflammatory injury (*Yoshikawa & Naito, 2000*). Reactive oxygen intermediates (ROI) are believed to be mediators of inflammation and responsible for the pathogenesis of tissue destruction in rheumatoid arthritis (*Valentao et al., 2002*). The role of ROS/RNS in inflammation is clearly demonstrated by the anti-inflammatory effects of the antioxidants. Nitric oxide synthase inhibitors are also effective as anti-

inflammatory agents in carrageenan-induced rat paw edema method as SOD. These may be due to the removal of O_2 •− by SOD, so preventing O_2 •− dependent formation of a factor chemotactic for nutrophils (*Miller et al., 1992*).

REACTIVE OXYGEN SPECIES

There have been several reports on the role of ROS / RNS in neurodegenerative diseases. Parkinson's disease usually appears in the middle to old age often as a rhythmic tremor in a foot or hand especially when the limb is at rest. Comparison of the brains of Parkinson's disease with that of the neurologically normal brains shows several parameters consistent with increased oxidative stress and defective mitochondrial function. Damaged mitochondria may generate more ROS than usual and ROS / RNS (includingO_2 •−, •OH, ONOO−) can inactivate complex I. Hence it is possible that oxidative stress and mitochondrial defects form a vicious cycle (*Halliwell & Gutteridge, 1999*).

The ROS mainly involved in neurodegeneration are the superoxide anion (O_2 −), the hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO•). RNS, such as nitric oxide (NO), can react with O_2 to produce peroxynitrite (ONOO−), a powerful oxidant that may decompose itself to form HO (*Spillantini et al., 2011*). Cells normally employ a number of defense mechanisms against free radical such as enzymes (Cu/Zn- and Mn, superoxide dismutase, GSH peroxidase, GSH reductase, catalase, and methionine sulfoxide reductase) and low molecular weight antioxidants (vitamin E, ascorbate, and GSH) (*Markesbery, 1999*). Macromolecules such as lipids, proteins, and DNA undergo damage and subsequently cell death mainlyby apoptosis when the antioxidant defense network is not sufficient (*Sayre et al., 2001*).

Antioxidant defense

It is evident through the reactions of oxygen, that it is toxic; still only the aerobes survive its presence, primarily because they have evolved an inbuilt antioxidant defense. Antioxidant defenses comprise:

• Agents that catalytically remove free radicals and other reactive species like SOD, CAT, peroxidase and thio specific antioxidants.

• Proteins that minimize the availability of peroxidase such as iron ions, copper ions and haem.

• Proteins that protect biomolecules against oxidative damage example heat shock proteins.

• Low molecular mass agents that scavenge ROS and RNS, example GSH, ascorbic acid, tocopherol. The antioxidants may be defined as "any substance, when present at low concentrations compared with that of an oxidizable substrate that significantly delays or prevents oxidations of that substrate". The term oxidizable substrate includes every type of molecule found in vivo. Antioxidant defense include the antioxidant enzymes like SOD, CAT, GSH-px, etc, low molecular agents and dietary antioxidants (*Halliwell & Gutteridge, 1999*).

As stated earlier, Alzheimer's disease is a process characterized by oxidative properties. Therefore, antioxidants, in general, should have positive effects in both the prevention and treatment of Alzheimer's. A study found that antioxidants such as vitamin A, vitamin D, lycopene, and beta-carotene were all significantly lower in Alzheimer's disease patients than controls (*Foy et al., 1999*).The best studied of the remaining antioxidants is vitamin C. A study found that plasma vitamin C levels are

lower in patients with Alzheimer's disease and that these levels are associated with the degree of cognitive impairment (*Riviere et al., 1998*).A prospective study of 633 patients aged 65 years and older found that high-dose supplementation with vitamin C decreased the risk of developing Alzheimer's disease (*Morris et al., 1998*).None of the 23 high-dose vitamin C users in this study developed Alzheimer's when, statistically speaking, 3.3 would have been expected to develop the disease. A case-control study found that beta carotene levels are lower in Alzheimer's disease patients than in healthy controls (*Zaman et al., 1992*).A study of 38 Alzheimer's patients and 42 healthy control subjects found that beta carotene levels are lower in patients than in healthy individuals (*Jimenez-Jimenez et al., 1999*).Other antioxidants such as selenium, glutamine, taurine, coenzyme Q10, pantethine, and magnesium would likely be beneficial to Alzheimer's patients but have not yet been thoroughly studied. Magnesium, for instance, is a particularly good agent to study because of its demonstrated to block the absorption of aluminum in the intestines as well as across the blood-brain barrier.

CURRENT SCENARIO

Neurodegeneration is a process involved in both neuropathological conditions and brain ageing. It is known that brain pathology in the form of cerebrovascular and neurodegenerative disease is a leading cause of death all over the world, with an incidence of about 2/1000 and an 8% total death rate (*KolominskyRabas et al., 1998*). Cognitive dysfunction, is a major health problem in the 21st century, and many neuropsychiatric disorders and neurodegenerative disorders, such as schizophrenia, depression, Alzheimer's Disease (AD) dementia, cerebrovascular

impairment, seizure disorders, head injury, Parkinsonism can be severely functionally debilitating in nature (*Commenges et al., 2000*).

A recent study (*Andlin-Sobocki et al., 2005*) has evaluated the total cost of brain diseases per year, including direct and indirect costs, in 28 countries in Europe at about 386 billion Euros for the year 2004. This represented 35% of the total burden of diseases affecting about 27% of the 465 million people who are suffering brain diseases. If mental disorders are excluded from the calculation the total cost of neurological diseases including dementia could be about 146 billion Euros per year and the total specific cost of the neurodegenerative diseases could be as much as 72 billion Euros (Table 1). These diseases are found in about 5% of the total number of patients suffering brain diseases. They are characterized by more or less selective neuronal degenerations inducing neurological syndromes, and affect both sensory-motor areas and cognitive functions.

Table 1.Number of cases and cost per case and per year of neurodegenerative diseases in 28 selected European countries (2004)*

	Number of cases	**Cost per case/year**	**Total cost/year**
Parkinson's disease	**1 160 000**	**7 500 Euros**	**8.70 billion Euros**
Dementia	**4 890 000**	**11 000 Euros**	**53.80 billion Euros**
Multiple sclerosis	**380 000**	**24 000 Euros**	**9.12 billion Euros**

* from *Andlin-Sobocki et al. (2005)*

In industrial countries, PD has a prevalence of approximately 0.3% in the general population and affects about 1% of those older than 60 (*de Lau & Breteler, 2006*).

This disease rarely occurs before the age of 50, and men are at higher risk than women. In Europe, PD affected 1.2 million people in 2010, resulting in costs per patient of EUR 5,626 for direct health care and EUR 4,417 for non-medical care. In 30 European countries, the total cost of all care for patients with PD in 2010 was EUR 13.9 billion (*de Lau & Breteler, 2006*).

According to a recent estimation, it is possible that almost 80% of individuals with dementia suffer from AD (*Jellinger & Attems, 2010*). AD is a severe progressive neurodegenerative brain disorder that affects approximately 5% of the population older than 65 years (*Shah et al., 2008*). According to the US Centers for Disease Control and Prevention (2003), the number of people in the world who are over the age of 65 will increase to around 1 billion by 2030. It has also been projected that by 2050 the number of dementia cases will reach around 14 million in Europe (*Mura et al., 2010*) and 13.2 million in the United States (*Hebert et al., 2001*). Furthermore, it has been estimated that the annual incidence of AD in the United States will increase from the 337,000 cases recorded in 1995 to 959,000 cases in 2050 (*Hebert et al., 2001*). At the level of individuals, AD decreases the quality of life and shortens life expectancy. At the societal level, the long-term care of AD patients in nursing homes is an economic challenge in Western countries, as illustrated by a report in which Olesen and colleagues (2012) showed that in Europe the annual cost for patients with dementia was EUR 105.2 billion in 2010. The mentioned date certainly indicate the tremendous impact of AD in terms of the enormous number of patients with this disease, the pressure on their relatives, and the negative socioeconomic consequences. In short, it can be said that AD is one of the major public health problems in the world.

CURRENT THERAPEUTIC APPROACHES IN NEURODEGENERATION

Drugs to improve memory generally work by altering the balance of particular chemicals (neurotransmitters) in the brain that are involved in the initial

Table 2. List of cognition enhancing drugs acting at neurotransmitter level

SN	Category	Name	Mechanism	Comment	Reference
1	Cholinergic agents	Donepezil Galantamine Rivastigmine	Acetylocholinesterase inhibitor. Acetylcholinesterase inhibitor; also possible cholinergic agonist Acetyl cholinesterase andbutyryl cholinesterase inhibitor.	Symptomatic treatment of AD, Vascular dementia and dementia associated with PD.	(Narahash-i et al., 2004)
2	Glutaminergic agents	D-cycloserine Ampakine Memantine	Partial NMDA agonist enhances glutamate. Signaling	Significant broad benefits in moderate-to-severe AD, vascular dementia and combined of non-specified dementia	(Lanni et al., 2008; Tullyet al., 2003; Lynch, 2004)
3	Nicotinic agonist	Nicotine	Acetylcholine agonist and Releaser	Facilitates learning/memory performance	(Ahijevyc-h et al., 2002)
4	Monoamines and agents acting on them	Methyl -penidate Modafinil	Effectson catecholamine, serotonin, glutamate, gamma amino-butyric acid,orexin, and histamine systems	Improve cognition in children and adults with ADHD	(Turner et al., 2004)
5	Adenosine and phosphodiesterase	Rolipram	Selective type-4 phosphodiesterase inhibitor	Improve LTP	(Gong et al., 2004)

learning of a memory or its subsequent reinforcement. Some of them along with their mechanism are listed in table 2. Some acts by selective enhancement of cerebral blood flow and metabolism, including enhanced glucose uptake, which may protect against the effects of hypoxia and ischemia. Reports from literature reveal that some medications currently available to patients with memory disorders mayalso increase performances in healthy people. Drugs designed for psychiatric disorders can also be used to enhance certain mental functions. However, the long-term effects of these drugs are unknown. Drugs which act as cognition enhancer increase synaptic plasticity by, regulating release of neurotransmitter from the pre-synaptic terminal and increasing sensitivity and specificity of receptors and ion channels in the membranes of synapse to neurotransmitter signaling. Some of the agents also modulate the process at transcriptional and translational level.

HERBAL MEDICINES

In traditional practices of medicine, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases such as Alzheimer's disease (AD) and other memory related disorders. Various studies have been undergone to identifying potential new drugs from plant sources, including those for memory disorders. There are numerous drugs available in market that have been isolated from plants, e.g. alkaloids from plant sources have been investigated for their potential in AD therapy, and are now in clinical use. Usually herbal preparations are well tolerated but they may have harmful side-effects, including interactions with pharmaceuticals (*Howes et al., 2003*). Herbal medicines, such as, Ginkgo Biloba, Bacopamonier (Bramhi) (*Das et al., 2002*), Shankhpushpi etc. has been found to increase memory power. Some of the herbal medicinal plants with

potential cognitive enhancement activity are listed in table 3, (*Howes & Houghton, 2003; Kennedy et al., 2003*).

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. In contrast, many medicinal plants exert specific medicinal actions without serving a nutritional role in the human diet and may be used in response to specific health problems over short- or long-term intervals. 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 (*Selvam, 2008; Lobo et al., 2010*).

Table 3. Some putative cognitive enhancing plants

Acoruscalamas	*Embeliaribes*	*Nicotianatabacum*
Angelica archangelica	*Emblicaofficinalis*	*Paeoniaemodi*
Asparagus racemosus	*Eugenia caryophyllus*	*Panax ginseng*
Bacopamonniera	*Evodiarutaecarpa*	*Piper longum*
Biota orientalis	*Galanthusnivalis*	*Polygonummultiflorum*
Boerhaviadiffusa	*Ginkgo biloba*	*Polygala tenuifolia*
Celastruspaniculatus	*Glycyrrhizaglabra*	*Pongamiapinnata*
Centellaasiatica	*Huperziaserrata*	*Rosmarinusofficinalis*
Clitoriaternatea	*Hydrocotylasiatica*	*Salvia lavandulifolia*
Codonopsispilosula	*Lawsoniainermis*	*Salvia miltiorrhiza*
Convolvulus pluricaulis	*Lycoris radiate*	*Schizandrachinensis*
Coptischinensis	*Magnolia officinalis*	*Terminaliachebula*
Crocus sativus	*Melissa cordifolia*	*Tinosporacordifolia*
Curcuma longa	*Nardostachysjatamansi*	*Withaniasomnifera*

Herbal medicine has long been used to treat neural symptoms. Although the precise mechanisms of action of herbal drugs have yet to be determined, some of them have been shown to exert anti-inflammatory and/or antioxidant effects in a variety of peripheral systems. Now, as increasing evidence indicates that neuroglia-derived chronic inflammatory responses play a pathological role in the central nervous system, anti-inflammatory herbal medicine and its constituents are being proved to be a potent neuroprotector against various brain pathologies. Structural diversity of medicinal herbs makes them a valuable source of novel lead compounds against therapeutic targets that are newly discovered by genomics, proteomics, and high-throughput screening. This review will highlight the importance of phytochemicals on neuroprotective function and other related disorders, in particular their mechanism of action and therapeutic potential (*Pueyo & Calvo, 2009*).

Phytochemicals in neuroprotection

There has been considerable public and scientific interest in the use of phytoconstituents for neuroprotection or to prevent neurodegenerative diseases. Many phytochemicals have been shown to exert neuroprotective actions in animal and cell culture models of neurological disorders. For example, a chalcone (safflor yellow B) can protect neurons against ischemic brain injury and piceatannol can protect cultured neurons against Aβ-induced death. Epidemiological studies of human populations, and experiments in animal models of neurodegenerative disorders, have provided evidence that phytochemicals in fruits and vegetables can protect the nervous system against disease (*Liu, 2003; Joseph et al., 2005*). The vast majority of studies on health benefits of phytochemicals have focused on the fact that many of the active chemicals possess antioxidant activity. Neuroprotective

effects of various phytochemicals are associated with reduced levels of oxidative stress. For example, resveratrol, quercetin and catechins reduced oxidative stress and protected cultured hippocampal neurons against nitric oxide-mediated cell death (*Larson, 1988*). Some of the neuroprotective herbs with their major bioactive compound and mode of action were shown in table 4. Hundreds of articles have been published reporting neuroprotective effects of compounds in natural products, including α-tocopherol, lycopene, resveratrol, ginkgo biloba and ginsenosides (*Ikeda et al., 2003*).

Flavonoids

Recently, there has been intense interest in the potential of flavonoids to modulate neuronal function and prevent age-related neurodegeneration. Dietary intervention studies in several mammalian species, including humans, using flavonoid rich plant or food extracts have indicated an ability to improve memory and learning, by protecting vulnerable neurons, enhancing existing neuronal function or by stimulating neuronal regeneration.

Table 4: Nootropic herbs with their active constituents' that help in neuroprotection

Plant name	Active constituents	Phytochemical group	Activity
Acorus calamus	Asarone	Monoterpene	Sedaative and important medhya drug, capable of improving memory power and intellect
All cruciferous plants	Sulforaphane	Isothiocyanate	Decreased brain edema
Centella asiatica	Asiaticoside, centelloside, madecassoside, and asiatic acid	Triterpenoid saponins	Brain tonic, cognition and anti-anxiety
Corydalis ternata	Protropine	Alkaloid	Anti-cholinesterase and anti-amnesic properties
Curcuma longa	Curcumin	Phenols	Protects against synaptic dysfunction
Emblica officinalis	Vit-C, phyllemblin	Vitamin polyphenol	Anti cholinesterase activity
Evolvulus alsinoides	Betaine, sankhapushpine and evolvine	Alkaloids	Memory enhancing agent used in treating dementia
Ginkgo biloba	Bilobolide, ginkgolides, kaemferal, quercetin, isorhamneting sitosterol and stigmasterol	Terpenoids flavanoids steroids	Action an corticosterone produce neuronal atrophy and cell death in the hippocampus
Glycyrrhiza glabra	Glycyrrhizin	Triterpenoid saponins	Improved learning and memory on scopolamine induced nuerodisorders
Huperzia serrata	Huperzine A and B	Alkaloids	Action on neuromuscular systems related to cholinesterase activity.
Ilex paraguariensis	Chlorogenic acid caffeine, theophylline, and theobromine quercetin, kaemferol	Polyphenols xanthenes flavanoids	Memory enhancing activity on dementia
Magnolia officinalis	4-0-methyl honokiol, honokiol and magnolol	Phenols	For the treatment of neurosis, anxiety, stroke, dementia
Uncaria rhynchophylla	Rhynchophylline, corynoxeine, isorhynchophylline and isocorynoxeine	Alkaloids	Neuroprotective in particular against ischemia-induced neuronal loss
Zingiber officinale	Gingerol, shogaol and zingerone	Phenols	Brain acetyl cholinesterase inhibition activity
Ziziphus spinosa	Jujuboside A, and jujuboside B	Saponins	Anti-calmodulin action

Individual flavonoids such as the citrus flavanone tangeretin, have been observed to maintain nigro-striatal integrity and functionality following lesioning with 6-hydroxydopamine, suggesting that it may serve as a potential neuroprotective agent against the underlying pathology associated with Parkinson's disease (*Youdim et al., 2004*). In order for flavonoids to access the brain, they must first cross the blood

brain barrier (BBB), which controls entry of xenobiotics into the brain (*Ehrnhoefer et al., 2006*). Flavanones such as hesperetin, naringenin and their *in vivo* metabolites, along with some dietary anthocyanins, cyanidin-3-rutinoside and pelargonidin-3-glucoside, have been shown to traverse the BBB in relevant *in vitro* and *in situ* models (*Youdim et al., 2004; Youdim et al., 2002*). Anthocyanins can possibly cross the monolayer in blood-brain barrier models *in vitro*.

Flavonoids and tannins are phenolic compounds that are a major group of compounds act as primary antioxidants or free radical scavengers (*Polterait, 1997*).

THE ROLE OF PLANT FLAVONOIDS IN NEURODEGENERATION

There has been a recent explosion of interest by research scientists in the flavonoid compounds, with a multitude of medically useful properties having been demonstrated in experimental, as well as, clinical studies of flavonoids. For instance, flavonoids have been shown to act as powerful free radical scavengers for a multitude of free radical species, even the powerful peroxynitrite radical (*Jovanovic et al., 1998*). In addition, several flavonoids have shown powerful metal-chelating properties, especially for iron and copper, two of the most potent-free radical catalysts (*Morel et al., 1998*). Of equal importance are several studies that have shown that flavonoids interact with cell membranes, improving their fluidity, thereby protecting them from lipid peroxidation (*Saija et al., 1995; Ratty & Das, 1988*). Along these same lines is the protection of micro vessels in the nervous system by specific flavonoids from free radical damage (*Kuttan et al., 1981*). This not only prevents leakage of such vessels, but has been shown to preserve the blood–brain barrier as well (*Robert et al., 1977*). There is also evidence that several of the flavonoids can inhibit platelet adhesiveness, thereby preventing strokes (*Tzeng et al.,*

1991). Finally, some of the flavonoids have the unique ability to inhibit certain enzymes, such as the COX-2 enzyme (*Kim et al., 1998*).

FLAVONOIDS AS FREE RADICAL SCAVENGERS

The flavonoid compounds have two properties that make them especially useful as antioxidants. First, many are powerful, primary free radical scavengers against a wide variety of radicals, including singlet oxygen, superoxide, peroxyl, hydroxyl, and the peroxynitrite radicals (*Saija et al., 1995*). Second, several are known to be very effective metal chelators (*Duthie et al., 1997*). Most flavonoids are present in plants as glycosides. In the intestines, this moiety is cleaved off, leaving the aglycone form of the flavonoids (*Griffiths, 1982*). It is the aglycone form that is thought to have the highest antioxidant activity in biological systems. There is experimental evidence that hydrogen peroxide accumulation occurs during the process of catecholamine catabolism, making it especially important in PD (*Li et al., 1995*). Recent evidence also indicates that H_2O_2 plays an important role in the toxicity of Alzheimer's plaques. As we have seen, iron accumulation within neurons is characteristic of ageing of the nervous system, but is especially high in the case of neurodegeneration. A multitude of phytochemicals have specific properties that make them especially useful in combating neurodegeneration, and a list of nutrients that stimulate energy generation, primarily through the mitochondrial system.

2. PLANT PROFILE

Scientific classification

Kingdom	:	Plantae
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Lamidae
Order	:	Caryophyllales
Family	:	Pedaliaceae
Genus	:	Pedalium
Species	:	murex
Binomial name	:	***Pedalium murex Linn.***

Vernacular Names

Tamil	:	Yanai Nerunjil
English name	:	Land Caltrops
Hindi name	:	Bada Gokshur
Sanskrit name	:	Brihat Gokshur
Kannada	:	Ane Neggilu
Malayalam	:	Ananerinnil
Oriya	:	Gokara
Marathi	:	Gokharu
Gujarati	:	Kadva Gokhru

Habitat:

It is distributed in tropical Africa, Ceylon, India, Mexico and Pakistan. It is a common herb grows throughout India but it is found commonly along the western and corommandal coasts as a weed of waste places. It also occurs in Delhi, Rajasthan and Punjab, Tamil Nadu and Gujarat and Deccan peninsula (Sukla & Thakur, 1983; Bhakuni et al., 1992).

Description:

Large Caltrops is a shrubby, stiff-stemmed herb, native to India, grown for reputed medicinal and other uses. It is diffuse annual, much branched, spreading, succulent, and glandular, up to 60 cm tall. Roots similar to turmeric in colour. Leaves are simple, opposite, ovate or oblong-obovate, 1-4.5 cm long, irregularly and coarsely crenate-serrate. Yellow flowers 1.5-2 cm across, stalk 1-2 mm long, increasing up to

4 mm in fruit. Sepals 2 mm long; Teeth linear, scaly outside, persistent. Petals fused into a broad tube, 1-3 cm long; lobes obtuse. Stamens 0.5-1 cm long; anthers kidney shaped. The four angled seed is with 5 extremely sharp spines. It is an important famine food - leaves eaten as vegetable.

Chemical Constituents:

Fruit : Alkaloids 3.5%–5%, stable oil, aromatic oil, resins, glycosides, carbohydrates, saponins and triterpenoids.

Stem : Saponins, phytosterols, tannins and carbohydrates.

Root : Reducing sugars, phenolic compounds, saponins, xanthoproteins, alkaloids, triterpenoids and flavonoids.

Leaves : Flavonoids, alkaloids, steroids, resins, saponins and proteins.

Medicinal Uses:

- Nervine weakness, Pains, Inflammation, Indigestion, Piles, Constipation, Heart related problems, Cough, Asthma, Epitasis, Frigidity, Impotence, Renal calculi, Dysurea, Infections.
- Leaves are antibilious. Seeds are demulcent, diuretic, tonic, mucilaginous and aphrodisiac. Used in male impotence, gonorrhoea, and incontinence.

REVIEW OF LITERATURE

Pedalium murex has demonstrated antioxidant potential in animal studies.

Jalaram H et al., (2011) had elucidated ***IN VITRO* ANTIOXIDANT ACTIVITY OF AQUEOUS FRUIT EXTRACT OF *Pedalium murex.*** Free radicals are involved in more than 80 diseases including Diabetes mellitus, arthritis, cancer, ageing, etc. in addition the free radicals are also play role in the pathogenesis of liver toxicity, peptic ulcer, diabetic nephropathy etc. In treatment of these diseases, antioxidant therapy play key role. Current research is now directed towards finding naturally occurring antioxidant of plant origin. In Indian system of medicine *Pedalium murex* is an important medicinal plant and it has been used traditionally in various disorders and as a health tonic. To understand the mechanisms of pharmacological actions, the in vitro antioxidant activity of aqueous extract of fruits of *Pedalium murex* was investigated for DPPH scavenging activity and superoxide scavenging activity. Percentage inhibition of free radicals was measured. The antioxidant property may be related to the phenolic acids and micronutrients present in the extract. Results clearly indicate that Pedalium murex is effective free radical scavenger.

Srinivas P et al., (2011) had evaluated **ANTIOXIDANT ACTIVITY OF *Pedalium murex* FRUITS IN CARBON TETRACHLORIDE INDUCED HEPATOPATHY IN RATS.** The decreased activity of antioxidant enzymes, such as superoxide dismutase (SOD), Catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GRD) in CCl_4 –intoxicated rats, and its retrieval towards near normalcy in CCl_4 + MEC administered rats revealed the efficacy of methanol extract of fruits of

Pedalium murex (MEC) in combating oxidative stress due to hepatic damage. Elevated level of glutathione transferase(GTS) observed in hepatotoxic rats too showed signs of returning towards normalcy in MEC co-administered animals, thus corroborating the antioxidant efficacy of MEC.

Patel DK et al., (2011) had estimated ***Pedalium murex L.*** **(PEDALIACEAE) FRUITS: A COMPARATIVE ANTIOXIDANT ACTIVITY OF ITS DIFFERENT FRACTIONS.** Here the antioxidant activities of *Pedalium murex* were evaluated using six *in-vitro* assays, namely total antioxidant assay, DPPH assay, reducing power, nitric oxide scavenging, hydrogen peroxide scavenging and deoxyribose scavenging assays, and total phenol contents were also investigated.

Bhakuni RS et al., (1992) had computed **FLAVONOIDS AND OTHER CONSTITUENTS FROM *Pedalium murex*.** Two new compounds isolated from the fruits of *Pedalium murex* were characterized as 2′,4′,5′-trihydroxy-5,7-dimethoxyflavone and triacontanyl dotriacontanoate by physico-chemical methods. Luteolin, rubusic acid, nonacosane, tritriacontane, triacontanoic acid, tritriacontanoic acid and sitosterol-β-d-glucoside have also been isolated and identified.

Siva V et al., (2012) had determined **EVALUATION OF ANTIPYRETIC ACTIVITY OF *Pedalium murex* AGAINST BREWER'S YEAST-INDUCED PYREXIA IN RATS.** The aqueous and ethanolic extracts of *Pedalium murex* (Pedaliaceae) was investigated for antipyretic activity in rats using Brewer's yeast-induced pyrexia models. Brewer's yeast (15%) was used to induce pyrexia in rats. Both the extract (200 and 400 mg/kg body weight p.o

produced a significant ($p<0.05$) dose dependent inhibition of temperature elevation compared with the standard drug Paracetamol (150mg/kg body weight). At doses of 200mg/kg b.w, the aqueous extract significantly ($P<0.001$) decreased yeast induced pyrexia in rats. These results indicate that leaf extracts of *Pedalium murex* possesses potent antipyretic effects and thus pharmacologically justifying its folkloric use in the management of fever.

Banji D et al., (2010) had composed **SCRUTINIZING THE AQUEOUS EXTRACT OF LEAVES OF *Pedalium murex* FOR THE ANTIULCER ACTIVITY IN RATS.** Peptic ulcer is manifested largely due to an alteration in lifestyle and diet. The antiulcer efficacy of the aqueous extract of leaves of *Pedalium murex* on ethanol induced gastric lesions was investigated in our studies. This has been substantiated by ascertaining the content of total acid, acid volume, total protein, ulcer index and glutathione. Ulceration was induced in 36 hours fasted rats by the administration of 80% ethanol (1 ml/kg) orally. The reference standard (famotidine, 3 mg/kg) and aqueous extract of leaves of *Pedalium murex* in doses of 50, 100, 200 mg/kg was given to different groups, one hour before the administration of ethanol. Marked gastric mucosal lesions were observed with ethanol. A perceptible elevation in ulcer index, total acidity, acid volume, total protein and diminution of glutathione was observed. Pretreatment with aqueous extract of leaves of *Pedalium murex* particularly at a dose of 200 mg/kg in a single schedule and 100 mg/kg for 15 and 30 days treatment annihilated these alterations and elevated the level of glutathione. Therefore the aqueous extract of leaves of *Pedalium murex* could be regarded

as a favorable antiulcerogen which could be attributed to its content of flavonoids and mucilage.

Mohana lakshmi S et al., (2012) had presented **A REVIEW ON MEDICINAL PLANTS FOR NEPHROPROTECTIVE ACTIVITY.** Medicinal plants may serve as a vital source of potentially useful new compounds for the development of effective therapy to combat a variety of kidney problems. Many herbs have been proven to be effectual as nephroprotective agents while many more are claimed to be nephroprotective but there is lack of any such scientific evidence to support such claims. Developing a satisfactory herbal therapy to treat severe renal disorders requires systematic investigation of properties like acute renal failure, nephritic syndrome and chronic interstitial nephritis. Herbal medicines possess curative properties due to the presence of their chemical components. The present review is aimed to elucidate the list of nephroprotective medicinal plants, which are scientifically proved in treating renal disorders.

Methekar Chandrika et al., (2012) had scrutinized **A COMPARATIVE ANTI-TUSSIVE ACTIVITY OF *LAGHU GOKSHURA* [*Tribulus terrestris* Linn.] AND *BRIHAT GOKSHURA* [*Pedalium murex* Linn.] *PANCHANGA* IN SWISS ALBINO MICE.** As it is used in the treatment of *Kasa*, in the present study a comparative anti-tussive activity of whole plant (*Panchanga*) of *Laghu*and *Brihat Gokshura* was evaluated against sulphur dioxide induced cough in mice. The mice were used as experimental animals and were randomly divided in to three groups of 6 animals each. The test drugs were administered orally at a dose of 780 mg/kg. Recodex, which contains

codeine phosphate (2 mg/ml) and chlorpheniramine maleate (0.8 mg/ml), was used as standard anti-tussive drug for comparison. The *Panchanga* of *Laghu Gokshura* and *Brihat Gokshura* have shown moderate anti-tussive activity, among them *Brihat Gokshura* was found to be better. Hence in non-availability of root samples of these plants and also to prevent destructive harvesting, whole plants can be used in the treatment of *Kasa*.

Mukundh N et al., (2008) had assessed **ANTI HYPERLIPIDEMIC ACTIVITY OF *Pedalium murex* (LINN.) FRUITS ON HIGH FAT DIET FED RATS.** The main objective of the study was to investigate about the anti-hyperlipidemic potential of the ethanolic extract from the fruits of *Pedalium murex* at doses of 200 and 400mg/kg/p.o. in high fat diet fed rats. Biochemical parameters like serum total cholesterol (TC), High Density Lipoprotein (HDL), Low Density Lipoprotein (LDL), very low density lipoprotein (VLDL) and triglycerides (TG) levels were compared with animals concurrently treated with reference standards Gemfibrozil and Atorvastatin. The ethanolic extract showed a significant decrease in triglycerides ($p<0.01$), LDL ($p<0.001$), VLDL ($p<0.01$), cholesterol ($p<0.001$) and a significant increase in HDL ($P<0.05$) Levels at the tested doses.

Sharma V et al., (2012) had analyzed **A COMPARATIVE STUDY OF ETHANOLIC EXTRACTS OF *Pedalium murex* LINN. FRUITS AND SILDENAFIL CITRATE ON SEXUAL BEHAVIORS AND SERUM TESTOSTERONE LEVEL IN MALE RATS DURING AND AFTER TREATMENT.** Present findings provide experimental *in-vivo* and *in-vitro* evidence that the ethanolic extract of *Pedalium murex* fruits possesses

aphrodisiac property. Study lends growing support for the traditional use of *Pedalium murex* as a sexual stimulating agent and offers a significant potential for studying the effect on male sexual response and its dysfunctions. The findings justify the concept of Rasayana as rejuvenative tonics and support their role in prevention or delay of the aging process.

DK Patel et al., (2012) had reported **APHRODISIAC ACTIVITY OF ETHANOLIC EXTRACT OF *Pedalium murex* LINN FRUIT.** The study represents an interesting case report for a very good aphrodisiac activity observed during an oral glucose tolerance test performed while evaluating the antidiabetic potential of *Pedalium murex* Linn. fruit. Pregnancy was observed in the treated groups after 20-25 days of treatment in females which resulted in birth of pubs ranging upto ten in some females (more significant in case of 500 mg/kg p.o.). The observation also showed a significant increase in weights of pubs along with a normal behavior pattern. The increased pregnancy rate in the drug treated groups may be due to the healthy viable sperm and enhancement of sexual desire of the rats.

Patel K et al., (2012) had enumerated **ALDOSE REDUCTASE INHIBITORY ACTIVITY OF ALCOHOLIC EXTRACT OF *Pedalium murex* LINN FRUIT.** Physico-chemical investigation of ethanolic extract of fruit of the *Pedalium murex* (EPM) was carried out. EPM was screened for aldose reductase inhibitory activity using rat lens AR enzyme. HPTLC fingerprinting analysis of EPM was performed in chloroform: methanol (8:2) solvent system.

Rajashekar V et al., (2012) has studied **BIOLOGICAL ACTIVITIES AND MEDICINAL PROPERTIES OF GOKHRU (*Pedalium murex* L.)** Bada Gokhru (*Pedalium murex L.*) is perhaps the most useful traditional medicinal plant in India. This plant is now considered as a valuable source of unique natural products for development of medicines against various diseases and also for the development of industrial products. This review gives a bird's eye view mainly on the biological activities of some of this compounds isolated, pharmacological actions of the extracts, clinical studies and plausible medicinal applications of gokhru along with their safety evaluation.

Muruganantham Sermakkani (2011) had explored **EVALUATION OF PHYTOCHEMICAL AND ANTIBACTERIAL ACTIVITY OF *Pedalium murex Linn*. ROOT.** In the present study, the Petroleum ether, Chloroform, Acetone and Methanolic extract of *Pedalium murex* L. root was subjected to preliminary phytochemical compounds and antibacterial activity of certain human pathogenic microorganisms. The extracts indicated the presence of flavonoids, glycosides, steroids, phenols, alkaloids and tannins. Maximum antibacterial activity was observed in methanolic extract against gram positive bacteria, Streptococcus pyogene*s* and Enterococcus faecalis than the gram negative bacteria.

Kevalia J et al., (2011) had observed **IDENTIFICATION OF FRUITS OF TRIBULUS TERRESTRIS LINN. AND *Pedalium murex Linn.*: A PHARMACOGNOSTICAL APPROACH.** *Gokshura* is a well-known Ayurvedic drug that is used in many preparations. Botonically it is identified as *Tribulus terrestris* Linn., especially the roots and fruits of the plant. But

instead the fruits of another plant *Pedalium murex* Linn. are commonly used and the drug is frequently substituted. Pharmacognostical study has been carried out to identify the distinguishing features, both morphological and microscopic, of the fruits of *Tribulus terrestris* Linn. and *Pedalium murex* Linn. This knowledge should help reduce the problem of substitution of the genuine drug.

Sahayaraj K et al., (2008) had pronounced **INSECTICIDAL AND ANTIFEEDANT EFFECT OF *Pedalium murex Linn.* ROOT AND ON *SPODOPTERA LITURA* (FAB.) (LEPIDOPTERA: NOCTUIDAE).** *Pedalium murex* reduced the food consumption index, growth rate, approximate digestability, eficiency of conversion of ingested food, efficiency of conversion of digested food of *S. litura* indicating the antifeedant activity of this plant. Qualitative analysis of *Pedalium murex* root extract revealed that it contains phytochemical such as, steroids, terpenoids, phenolics, saponines, tannins and flavanoids. Phenol, 2-(5,6-dimethyl pyrazinyl) methyl (molecular weight 214); O-Terphenyl-13C (molecular weight 230) and 3,3A, 4,9B-Tetrahydro- 2H-Furo(3,2-C)(1) Benzopyran (molecular weight 206) were identified from the ethanol root extract of *Pedalium murex* by using GC-MS. *Pedalium murex* impact was more than the neem based biopesticide neem gold. Hence this plant can be explored as biopesticidal plant in the near future.

Patel DK et al., (2011) had outlined ***Pedalium murex Linn*.: AN OVERVIEW OF ITS PHYTOPHARMACOLOGICAL ASPECTS.** Different parts of the plant are used to treat various ailments like, cough, cold and as an antiseptic. Phytochemically the plant is popular for the presence of a considerable amount

of diosgenin and vanillin which are regarded as an important source and useful starting materials for synthesizing steroidal contraceptive drugs and isatin alkaloids. Other phytochemicals reported in the plant includes quercetin, ursolic acid, caffeic acid, amino acids (glycine, histidine, tyrosine, threonine, aspartic acid and glutamic acid) and various classes of fatty acids (triacontanoic acid, nonacosane, tritriacontane, tetratriacontanyl and heptatriacontan-4-one). Pharmacologically, the plant have been investigated for antiulcerogenic, nephroprotective, hypolipidemic, aphrodisiac, antioxidant, antimicrobial and insecticidal activities. From all these reports it can be concluded that the plant were found to have a better profile with potential natural source for the treatment of various range of either acute or chronic disease.

Thamizh Mozhi M et al., (2011) had summarized **PHYTOCHEMICAL AND PHARMACOGNOSTICAL STUDIES ON *Pedalium murex Linn*.** The entire plant of *Pedalium Murex* Linn. was subjected to various Pharmacognostical evaluations like Morphological, Microscopical and Powder analysis. Results have revealed clearly that the entire plant is genuine. The Phytochemical constituents of Leaves of *Pedalium Murex* Linn. have been worked out. The dry powder of the entire plant was successfully extracted with Total Petroleum ether extract, Alcohol Extract, Chloroform extract, and Aqueous Extract. All the extracts were subjected to Preliminary Phytochemical screening. It showed the presence of Carbohydrates, Glycosides, Alkaloids, Steroids and Flavonoids. As per Materia medica (Krithikar and Basu), studies are required for the screening of various

Pharmacological activities like plant pacifies vitiated vata, pitta, urinary retention, kidney stone, seminal weakness, amenorrhea, inflammation, flatulence and fever.

Shukla YN et al., (1983) had worked on **HEPTATRIACONTAN&4&ONE, TETRATRIACONTANYL OCTACOSANOATE AND OTHER CONSTITUENTS FROM *Pedalium murex* Linn.** Two new compounds isolated from the fruits of *Pedalium murex* are characterized as heptatriacontan-4-one and tetratriacontanyl octacosanoate by spectral studies. Pentatriacontane, sitosterol, hexatriacontanoic acid, hentriacontanoic acid, ursolic acid and vanillin have also been isolated and identified.

Thangadurai Chitra et al., (2013) had revealed **LABORATORY AND FIELD EFFICACY OF *Pedalium murex* AND PREDATORY COPEPOD, *Mesocyclops longisetus* ON RURAL MALARIA VECTOR, *Anopheles culicifacies***, To test the potentiality of the leaf extract of *Pedalium murex* and predatory copepod *Mesocyclops longisetus* (*M. longisetus*) in individual and combination in controlling the rural malarial vector, *Anopheles culicifacies* (*An. culicifacies*) in laboratory and field studies. Predator survival test showed that the methanolic extract of *Pedalium murex* is non-toxic to the predatory copepod, *M. longisetus*. Experiments were also conducted to evaluate the efficacy of methanolic extract of *Pedalium murex* and *M. longisetus* in the direct breeding sites (paddy fields) of *An. culicifacies*. Reduction in larval density was very high and sustained for a long time in combined treatment of *Pedalium murex* and *M. longisetus*.

3. AIM AND OBJECTIVE

In this study, we had investigated whether pretreatment and subsequent exposure to an acute and chronic unpredictable stress protect the animals from the corresponding effects on behavior, cognition, brain antioxidant system and other parameters.

Stress plays a major role and has a cascading effect on the functioning of the defense mechanisms, neuroendocrine, neurotransmitter, behavior, cognition, etc.,

There are, indeed, a multitude of paradigms assessing various aspects of the behavioral performance and cognitive abilities. Till now, some of the paradigms will be not used at all in the evaluation of *Pedalium murex* leaf extract against behavioral consequences of adult rats in stress.

Exposure to stressful events often results in long-lasting changes in the responsivity of a variety of systems. For example, repeated exposure to the same stressor (homotypic stress) often results in habituation of the hypothalamic–pituitary–adrenal (HPA) axis and brain stem catecholaminergic activity. Conversely, exposure to a test stressor that is different (heterotypic stress) from that used during the initial repeated exposure results in sensitization of the HPA axis and brain stem catecholaminergic activity (Sakellaris & Vernikos-Danellis, 1975; Vernikos et al., 1982; Konarska et al., 1989; Lachuer et al., 1994). In addition, a single exposure to a stressor has been shown to sensitize central pathways involved in drug reward (Pi- azza & Le Moal, 1998), fear and anxiety (Agid et al., 2000; Goenjian et al.,2000), and neuroendocrine responses (van Dijken et al., 1993; Schmidt et al., 1996).

As human life expectancy has increased, so too has the incidence of stress related neurodegenerative disorders such as Alzheimer’s disease, Parkinson’s disease and

Huntington's disease. Plant extracts have a wide range of medicinal actions, and throughout history, they have been used to treat many different types of diseases. In the treatment of many diseases, antioxidant therapy plays a key role, so current research is now directed towards finding naturally occurring antioxidant of plant origin.

In Indian system of medicine, the plant *Pedalium murex* is being used to treat many illnesses, successfully, for centuries. A number of studies have attributed the curative effect of *Pedalium murex* to its high content of flavonoids. But there is lack of studies related with protective effect of *Pedalium murex* in human and experimental animal models too.

Although *Pedalium murex* shares many medicinal properties with '*rasayans'* in Ayurvetha, its neuroprotective effect in different experimental neurodegenerative models are still debatable. Actually, little is known about the feeding pattern and feeding rhythms that underly the hypophagia of the stress treated rats. The general behavior, anxiolytic, antidepressant and cognitive, antioxidant enzyme examination of the rat whole brain, pattern of food and water intake are used for the first time, to assess the protective effect of *Pedalium murex* against stress induced neurodegenerative injury. We anticipate that the behavioral tests used in the present study could contribute to the evaluation of potential drugs effective in the prevention of functional deficits induced by neurotoxic agent and may shed an insight into the mechanism of action. Hence, a special attention is focused to understand the treatment of neurodegenerative diseases by natural phenolic antioxidants from this plant.

Hence, in order to contribute further to the knowledge of Indian traditional medicine, and its sacred and rich history, the objective of the present study is to subject the traditionally well-known leaves of *Pedalium murex* to extraction using suitable solvents and the dried extract will be used to evaluate the possible neuroprotective effect of *Pedalium murex* against stress induced rats.

SPECIFIC OBJECTIVE:

- To elucidate the possible mechanism of action of *Pedalium murex* by employing stress.
- To screen preliminary phytochemicals of ethanol extract of *Pedalium murex.*
- To study the *in vitro* antioxidant and radical scavenging and reducing power of *Pedalium murex.*
- To observe behavioral parameters (anxiety, depression and cognition) following stress induced neurodegeneration.
- To estimate and study the perturbations in the levels of antioxidant defense systems – SOD, CAT, GSH, LPO and corticosterone in the whole brain of the rat.

4. PLAN OF WORK

PHASE I:

1. **Phytochemical Studies:**

   - Collection and extraction of leaves of *Pedalium murex* Linn.
   - Phytochemical screening of leaves of *Pedalium murex* Linn.

2. ***In vitro* antioxidant studies**

   a) DPPH radical scavenging activity

   b) Nitric oxide radical scavenging activity

   c) LPO assay

   d) CUPRAC assay

   e) Metal chelating assay

   f) Reducing power

3. **Bioactive compounds**

   a) Total phenol

   b) Total flavonoids

   c) Total tannins

PHASE II:

1) Pharmacological Studies:

a) Acute oral toxicity study:

It will be carried according to the OECD guideline 423.

b) Stress induction:

i) Acute stress:

- Immobilisation induced stress

ii) Chronic unpredictable stress:

- Forced swimming induced stress
- Immobilisation induced stress
- Dark phase induced stress

2) Evaluation of general behavioral alterations in Stress induced rats:

a) General parameters

- Body weight
- Food intake
- Water intake

b) Behavioural studies

i) Test for anxiety studies

- Elevated plus maze

ii) Test for depression

- Forced swim test

iii) Test for learning and memory

- Radial arm maze

PHASE III:

Biochemical Analysis

- *In vivo* antioxidant studies
  - Superoxide dismutase (SOD)
  - Catalase (CAT)
  - Total glutathione (GSH)

5. MATERIALS AND METHODS

Collection of Plant

The leaves of *Pedalium murex* was collected from surrounding areas of Komarapalayam and vatamalai, Namakkal District, Tamilnadu, India.

Authentication of Plant

The Plant was authenticated by Dr. G.V.S. Murthy, scientist F, Botanical survey of India, Coimbatore, Tamilnadu (No.BSI/SRC/5/23/2012-13/Tech/1931).

Extraction Procedure

The leaves of *Pedalium murex* were carefully washed with tap water, and dried under shade, in room temperature for one week. Then they were ground into powder and stored in room temperature. The grind materials were passed though sieve no. 44 and 72, and the powdered materials of identical size present in-between those two sieves were collected and stored in an airtight container for further use. About 400gm of powdered materials were taken in 1000ml round bottom flask and extracted with ethanol for 24 h using cold maceration process with continuous stirring. The extract was decanted into pre-weighed glass vials. The solvent present in the extract was removed by evaporating at room temperature, to obtain a syrupy greenish mass. The extracted residues were weighed and were preserved in a vacuum desiccator for subsequent use in the study.

PRELIMINARY PHYTOCHEMICAL ANALYSIS

The ethanolic extract of *Pedalium murex* (EEPM) was subjected to qualitative tests for the identification of various plant constituents (Harborne et al., 2005; Krishnaswamy, 2003; Gurudeep et al., 2003; Kasture et al., 2003).

1) TEST FOR ALKALOIDS

(a) Dragondorff's Test: - 1 ml of the extract was added with 1 ml of dragondorff's reagent (potassium bismuth iodide solution). An orange red precipitate indicates the presence of alkaloids.

(b) Mayer's Test: - 1 ml of the extract was added with 1 ml of Mayer's reagent (potassium mercuric iodide solution). Whitish yellow coloured precipitate indicates the presence of alkaloids.

(c) Hager's Test: - 1 ml of the extract was added with 3 ml of Hager's reagent (saturated aqueous solution of picric acid), yellow coloured precipitate indicates the presence of alkaloids.

(d) Wagner's Test: - 1 ml of the extract was added with 2 ml of Wagner's reagent (Iodide in potassium Iodide), formation of reddish brown precipitate indicates the presence of alkaloids.

(e) Tannic acid Test: - 1 ml of the extract was added with 1 ml of 10% tannic acid solution, buff coloured indicates the presence of alkaloids.

2) TEST FOR SAPONINS

(a) Foam Test: - The extract was diluted with 20 ml of distilled water and shaken in a graduated cylinder for 15 min. lengthwise. A 1 cm layer of foam indicates the presence of Saponins.

(b) Lead acetate Test: - 1 ml of sample solution was treated with 1% lead acetate solution, formation of white precipitate indicate the presence of saponins.

(c) Hemolytic Test: - The extract or dry powder was added one drops of blood placed on glass slide. If hemolytic zone appears shows the presence of saponins.

3) TEST FOR GLYCOSIDES

(a) Legal's Test: - Dissolved the extract in pyridine and added sodium nitroprusside solution to make it alkaline. The formation of pink red to red colour shows the presence of glycosides.

(b) Baljet Test: - 1 ml of the test extract was added with 1 ml of sodium picrate solution and the yellow to orange colour shows the presence of glycosides.

(c) Keller- killiani Test: - The ethanolic extract 0.5 ml of strong solution of lead acetate was added and filtered. The filtrate is shaken with 5 ml of chloroform. The chloroform layer is separated in a porcelain dish and removes the solvent by gentle evaporation. Dissolve the cool residue in 3

ml of glacial acetic acid containing 2 drops of ferric chloride solution. Carefully transferred this solution to the surface of 2 ml of concentrated sulphuric acid. A reddish brown layer forms at the junction of the two liquids and the upper layer slowly becomes bluish green, darkening with standing.

(D) Borntrager's Test: - Added a few ml of dilute sulphuric acid to 1 ml of the extract solution. Boiled, filtered and extracted the filtrate with chloroform the chloroform layer was treated with 1 ml of ammonia. The formation of red colour of the ammonical layer shows the presence of anthraquinone glycosides.

4) TEST FOR CARBOHYDRATES AND SUGARS

(a) Molisch's Test: - 2 ml of the extract was added with 1 ml of α- napthol solution was added and also added concentrated sulphuric acid though the side of the test tube. Reddish violet colour at the junction of the two liquids indicates the presence of carbohydrates.

(b) Fehling's Test:- 1 ml of the extract was added with equal quantities of Fehling solution A and B were added, upon heating formation of a brick red precipitate indicates the presence of reducing sugars.

(c) Benedict's test: - 1 ml of extract was added with 5 ml of Benedict's reagent, was added and boiled for 2 min. and cool. Formation of red precipitate shows presence of sugars.

(d) **Tollen's Test: -** 1 ml of extract was added with 2 ml of tollen's reagent was added and boiled. A silver mirror is obtained inside the wall of the tube which indicates the presence of aldose sugar.

(e) **Seliwanoff's Test: -** The extract was treated with hydrochloric acid and resorcinol and heated. Formation of red colour shows presence of glucose.

(f) **Bromine water Test: -** The little quantity of test extract, bromine water was added. Bromine water decolorization indicates the presence of aldose sugar.

5) TEST FOR TANNINS

(a) **Gelatin Test: -** 1 ml of extract was added with 1% gelatin solution containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannins.

(b) **Ferric chloride Test:-**1 ml of extract was added with 1 ml ferric chloride solution, formation of dark blue or greenish black product shows the presence of tannins.

(c) **Vanillin hydrochloride Test:-**1 ml of extract was added with vanillin hydrochloride. Formation of purplish red colour indicates the presence of tannins.

(d) **Lead acetate Test: -** Taken a little quantity of test solution was taken and mixed with basic lead acetate solution. Formation of white precipitate indicates the presence of tannins.

(e) A little quantity of test extract was treated with potassium ferric cyanide and ammonia solution. A deep red colour indicates the presence of tannins.

(f) P**otassium dichromate Test**:- The sample solution was treated with 1 ml of 10% Potassium dichromate solution gives yellowish brown precipitate indicate the presence of tannins.

6) TEST FOR FLAVONOIDS

(a) **Shinoda's Test:-** The extract solution, few fragments of magnesium ribbon was added and add concentrated HCL drop wise gives cherry red colour appears after few min., shows the presence of Flavonoids.

(b) **Alkaline reagent Test: -** The extract was treated with sodium hydroxide; formation of yellow colour indicates the presence of Flavonoids.

(c) Little quantity of extract was treated with lead acetate, a yellow colour solution formed, disappears on addition of an acid indicates the presence of Flavonoids.

(d) The extract was treated with concentrated sulphuric acid, formation of yellow or orange colour indicates the presence of flavonoids.

7) TEST FOR STEROIDS

(a) **Libermann- Burchard's Test: -** 2 ml of extract was added with chloroform solution, 1-2 ml of acetic anhydride and 2 drops of concentrated sulphuric acid was added along the sides of the test tube. Appearance of bluish-green colour shows the presence of steroids.

(b) Salkowsky's Test:-Dissolve the extract in chloroform solution, 2 ml conc. sulphuric acid was added. If chloroform layer appear red colour indicate the presence of steroids.

8) TEST FOR PROTEINS AND AMIN.O ACIDS

(a) Biuret Test: - 1 ml of the extract was treated with 4% NaOH and few drops of $CuSO_4$ solution, Formation of purple violet colour indicate the presence of proteins.

(b) Ninhydrin Test:- 1 ml of the extract was treated with 3 drops of 5% Ninhydrin solution in boiling water bath for 10 min.; formation of purplish or bluish colour appearance indicate the presence of proteins, peptides or amino acid.

(c) Xanthoproteic Test: - 1 ml of the extract was treated with 1 ml of concentrated nitric acid. A white precipitate formed, it was boiled and cooled. Then 20% of sodium hydroxide or ammonia is added. Orange colour indicates the presence of amino acids.

(d) Millon's Test: - 1 ml of the extract was treated with millon's reagent (mercuric nitrate in HNO_3) white precipitate turns to brick red indicates the presence of proteins.

9) TEST FOR TRITERPENOIDS

(a) Knoller's Test: - Dissolved 2 or 3 granules of tin metal in 2 ml thionyl chloride solution. Then added 1 ml of the extract into the test tube and warm, the formation of pink colour indicates the presence of Triterpenoids.

10) TEST FOR FIXED OILS AND FATS

(a) Spot Test: - Pressed a small quantity of extract between two filter papers, the stain on the filter paper indicates the presence of fixed oils.

(b) **Saponification Test: -** Added a few drops of 0.5 N of alcoholic potassium hydroxide to small quantity of various extract along with a drop of phenolphthalein separately and heat on water bath for 1 to 2 h. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

11) TEST FOR GUMS AND MUCILAGE

10 ml of ethanolic extract was slowly added to 25 ml of absolute alcohol with constant stirring, the precipitate was filtered and dried in air. The precipitate for its swelling property indicates the presence of carbohydrates.

***IN VITRO* ANTIOXIDANT ESTIMATION**

A) FREE RADICAL SCAVENGING ACTIVITY

♦ ***DPPH radical scavenging activity:***

The free-radical scavenging activities of these compounds were tested by

their ability to bleach the stable radical DPPH. The antioxidant activity using the DPPH (1, 1-diphenyl-2- picrylhydrazyl) assay was assessed by the method of Blois. The reaction mixture contained 100 µM DPPH in methanol and different concentrations (0.5-2.5 µg/ml) of compounds. 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. BHT was used as a reference compound.

- ***Nitric oxide radical scavenging activity:***

The interaction of ethanol extract of *Pedalium murex* with nitric oxide (NO) was assessed by the nitrite detection method. The chemical source of NO was sodium nitroprusside (10 mm) in 0.5 m phosphate buffer, pH 7.4, which spontaneously produced NO in an aqueous solution. 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 H_3PO_4 5%) was added. The same reaction mixture without the extract of sample but with equivalent amount of distilled water served as control. Ascorbic acid was used as positive control.

- ***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 (Sudheerkumar, 2003).

- ***CUPRAC assay:***

To a test tube, the following solutions were added: 1 mL $CuCl_2$, 1 mL neocuproine, and 1mL NH_4Ac 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 were added (total volume = 4.1mL), and mixed. Absorbance against a reagent blank was measured at 450 nm after 30 min. (the tested plant extracts were checked to reach steady state absorbance within this period). The trolox equivalent molar concentration of the plant extract sample in final solution may be found by dividing the observed absorbance to the molar absorptivity (ε) for trolox (optical cuvette thickness = 1 cm).

- ***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 (Sreejayan, 1996; John, 1984; Benzie, 1996).

♦ ***Reducing power:***

The reducing power of extract was determined by the method of Yen and Duh. 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% potassium ferricyanide. 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

♦ ***Total phenol:***

Total phenolic content was determined by the method described by Singleton and Rossi. 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_2CO_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.

♦ ***Total flavonoids:***

This was assayed as described by Jia et al. 0.5 ml of the sample is added into a test tube containing 1.25 ml of distilled water. Then added 0.075 ml of 5 %

sodium nitrite solution and allowed to stand for 5 min. Added 0.15 ml of 10% aluminium chloride, after 6 min. 0.5 ml of 1.0 M sodium hydroxide were 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.

- ***Total tannins:***

The quantitative tannin content in samples was estimated by the method of Price and Butler 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 1% $K_3Fe(CN)_6$ and 1 ml 1% $FeCl_3$ were added, and was made up to 10 ml with distilled water. After 5 min. the solutions were measured spectrophotometrically at 720 nm.

ACUTE ORAL TOXICITY STUDIES

Animals

Swiss albino mice weighing 20-25 gms were used for the study. The animals were housed in propylene cages and were maintained under standard laboratory conditions (25° ± 2°c). They were fed with standard diet and water *ad libitum.* Ethical clearance (for handling of animals and the procedures used in study) was obtained from the Institutional Animal Ethical Committee before performing the study on animals.

Acute toxicity test:

Acute oral toxicity study for ethanolic extract of *Pedalium murex* leaves was carried out as per OECD guideline 423. The test procedure minimizes the number of animals required to estimate the acute oral toxicity study. The test allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

PHARMACOLOGICAL EVALUATION

Animal study:

Adult female Sprague Dawley rats were used in the present study.

Housing and feeding condition:

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. Prior to the experiments, rats were fed with standard food, water *ad libitum* and were acclimatized to the standard laboratory conditions of temperature (22 ± 30°c) and maintained a 12:12 h light: dark cycle. All the experimental procedures were performed on the animals after approval from the ethics committee and in accordance with the recommendations for the proper care and use of laboratory animals.

Extract:

Ethanolic extract of *Pedalium murex* leaves were suspended in 0.5% Carboxy Methyl Cellulose, and was administered once daily to the animals by oral route for 30 days.

Standard drug used:

Diazepam (2mg/kg, i.p.,)

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. Different animal models for stress have been developed and used frequently to evaluate the anti-stress activity of compounds of natural origin.

Acute models do not reproduce the neuro endocrine models dysfunction whereas chronic model might be able to do so, but chronic unpredictable stress prevents the emergence of adaptation or resistance to one particular type of stress. Thus we preferred two different types of stress to produce different patterns of effects such as,

- **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 (Kvetnansky & Mikulai, 1970; Kasuga et al., 1999; Marty et al., 1997). Animals were kept immobilized in a semi cylindrical acrylic tube (4.5 cm diameter and 12 cm long) with proper holes in it for air to pass (Das et al., 2000) The rats were kept immobilized for 120 min. (2 h) once to produce acute stress (Dronjak & Gavrilovic, 2006).

- **Chronic Unpredictable Stress**

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

- **Forced swimming induced stress:**

In order to produce swimming induced stress, rats were made to swim in a cylinder (30cm diameter and filled to a height of 20 cm with 15 cm of space above the head of the rat) for 30 min. session in a day for ten consecutive days (Ferry et al., 1991).

- **Immobilization induced stress:**

Animals were kept immobilized in a semi cylindrical acrylic tube (4.5 cm diameter and 12 cm long) with proper holes in it for air to pass (Das et al., 2000) The rats were kept immobilized for 15 min. session upto 10 days to produce chronic stress (Dronjak & Gavrilovic, 2006).

- **Dark phase induced stress:**

To induce stress, cages of rats were kept in dark room with no light upto 30 min. per day for 10 days (Marcelo et al., 2007).

To avoid habituation to stressors, unpredictability was maintained by changing the time and sequence of stressors daily during the 10 days of the stress paradigm.

Experimental Protocol:

The rats were divided into nine groups of 6 animals each. The treatment period comprised of 30 days in four groups, and stress period for chronic unpredictable stress (CUS) was 10 days and for acute stress was 2 h. They were grouped under the following regimen:

Group- I, served as control and it received 0.9% normal saline

Group-II, served as positive control and it received acute stress, Diazepam (2 mg/kg)

Group- III, served as positive control and it received CUS, Diazepam (2 mg/kg)

Group- IV, received acute stress only

Group- V, received CUS only

Group- VI, pretreated with extract (200 mg/kg) and it received acute stress

Group- VII, pretreated with extract (400 mg/kg) and it received acute stress

Group-VIII, pretreated with extract (200 mg/kg) and it received CUS

Group- IX, pretreated with extract (400 mg/kg) and it received CUS.

(A) Evaluation of general behavioral alterations in Stress induced rats:

The animals were subjected to the following behavioral procedures initially and after induction of stressors. During the entire period of study the animals were observed for any changes in behavior and suitably noted.

♦ ***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.

♦ ***Measurement of food intake***

The measurement of food intake was studied 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 gram /gram weight of rat) was evaluated by weighing the remaining amount of food, 24 h after food presentation with accuracy to ± 0.1 g. Spillage of food pellets was rare, but any obvious spillage was noted and those data excluded from the analysis. The food pellets were placed at a height accessible to the experimental animals (5 cm from the floor of the cage), so they did not need to rear up to reach water and food. Water and food intake evaluation started immediately after stress administration.

♦ ***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 / gram body weight of rat) consumed over a 24 h period, following induction of stress, in all the groups. Clean water was provided in

graduated burettes with drinking spouts allowing direct volumetric measurements of intake to the nearest 0.1 ml. The drinking spouts were placed at a height accessible to the experimental animals (5 cm from the floor of the cage), so that they did not need to rear up to reach water.

(B) Evaluation of behavioural studies

- **Test for anxiety studies**

  - **Elevated plus maze:**

The elevated plus maze (EPM) was performed on rats as a standard test of fear and anxiety, where anxiety-related behavior is measured by the degree to which the rodent avoids elevated, unenclosed arms of the maze and exhibits defense behaviors such as head dips and scanning posture. The maze was elevated 50 cm above ground and consisted of four arms 48 cm in length. Two opposing arms were open with no walls, while 48 cm high walls enclosed the other two opposing arms. There was a 10 x 10 cm open area at the confluence of the four arms. Testing procedures followed the Behavioral Neuroscience protocol for elevated plus maze (Current Protocols in Neuroscience 2001 John Wiley and Sons: Supplement 10 section 8.3.6 Basic Protocol 4).

One h before animals were put in the maze, they were placed in a darkened holding room followed by testing in a dimly lit room. Animals were placed individually in the center facing an open arm, and the rodent's behavior recorded by video camera. Tapes were scored by a researcher blind to

treatment for 1) first arm preference, 2) entries into closed arms and 3) entries into open arms.

- **Test for depression**

  - **Forced swim test:**

A modified forced swim test procedure consisting of an increase in water depth was used to enhance sensitivity for detecting putative antidepressant activity of drugs (Porsolt et al., 1991). Rats were placed into plastic buckets (19 cm diameter, 23 cm deep, filled with 23–25°C water) and videotaped for the entire session. As described previously by Porsolt, only the last 4 min. were scored for mobility duration.

- **Test for learning and memory**

  - **Radial arm maze:**

The 8-arm radial maze testing was performed to evaluate spatial memory and stimulus-response (S–R). Maze arms were 55 x 10 x 10 cm (L x W x H) and the center arena was 35 cm in diameter with 20 cm high walls. Feed rewards were recessed at the ends of each arm so that the feed was not visible unless the animal was adjacent to the edge of the arm. Testing procedures followed the Behavioral Neuroscience protocol assessing working vs. reference memory (Current Protocols in Neuroscience, 2001 John Wiley and Sons: Supplement 4 section 8.5A.1 Basic Alternate Protocol 1). Food bait was placed at the ends of four arms after which the animal was placed in the center of the maze. The test animal was removed only after 6 min. had elapsed. Animals were scored based on number of entries into baited arms

and sequence of arms visited. The number of arm entries per min. was also recorded.

(C) Evaluation of biochemical analysis:

At the end of the behavioural 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 alumin.ium foil for further use.

Test drug and chemicals:

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

Preparation of homogenate:

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.

- **Estimation of Superoxide Dismutase (SOD):**

  Reagents

  Carbonate buffer (100 mM, pH 10.2); Epinephrine (3 mM);

Procedure

The SOD activity in supernatant was measured by the method of Misra and Fridovich (1967). The supernatant (500 µl) was added to 0.800ml of carbonate buffer (100 mM, pH 10.2) and 100 µl of epinephrine (3 mM). The change in absorbance of each sample was then recorded at 480 nm in spectrophotometer for 2 min. at an interval of 15 sec. Parallel blank and standard were run for determination SOD activity.

One unit of SOD is defined as the amount of enzyme required to produce 50% inhibition of epinephrine auto oxidation.

Reagents	(Standard)	(Sample)	Blank
Carbonate buffer	0.900 ml	0.800 ml	1.0 ml
Supernatant	……	0.1 ml	……
Epinephrine	0.1 ml	0.1 ml	……

The reaction mixtures are diluted 1/10 just before taking the readings in spectrophotometer.

Calculation:

% Inhibition= [X A_{480nm}/min. Uninhibited – X A_{480nm} /min. inhibited X 100] /

[X A_{480nm} /min. Uninhibited – X A_{480nm} /min. Blank]

Units/ml enzyme = [% Inhibition X V_t] / [(50%) X V_s]

Units/mg protein = [Units/ml enzyme] / [mg protein/ml enzyme]

Estimation of Catalase (CAT):

Reagents

1. Phosphate buffer solution (50 mM)

A) Dissolving 6.81 gm of KH_2PO_4 in 1000 ml distilled water.

B) Dissolving 6.9 gm of Na_2HPO_4 in 1000ml distilled water.

390 ml from solution (A) are mixed with 610 ml from solution (B), the pH is adjusted to 7.

2. Hydrogen peroxide (H_2O_2) 30 mM

0.34 ml of 30% H_2O_2 is diluted with phosphate buffer to 100 ml.

Procedure

Catalase activity was measured by the method of Aebi. 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 H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein. A unit is defined as the velocity constant per second.

Reagents	**Sample**	**Blank**
Phosphate buffer solution	1.9 ml	2..9 ml
Supernatant	0.1 ml	0.1 ml
H_2O_2	1 ml	……

The reaction occurs immediately after the addition of H_2O_2.

Solutions are mixed well and the first absorbance (A_1) is read after 15 seconds (t_1) and the second absorbance (A_2) after 30 seconds (t_2).The absorbance is read at wave length 240 nm.

Calculation

$$K = [(V_t / V_s) \times (2.3 \times \Delta_t) \times (\text{Log } A_1 / A_2) \times 60]$$

Where,

K = Rate constant of the reaction; $\Delta_t = (t_2 - t_1)$ =15 seconds; A_1= absorbance after 15 seconds; A_2= absorbance after 30 seconds; V_t = total volume (3 ml); V_s = volume of the sample (0.1ml);

- **Estimation of reduced glutathione (GSH)**

Reduced glutathione (GSH) was measured by 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) Ellman's reagent was added to it and the absorbance was measured at 412 nm. A standard graph was drawn using different concentration of standard GSH solution.GSH contents were calculated in the rat brain.

6. RESULTS

Phase I: Phytochemical studies

5.1.1 Extractive value and percentage yield of EEPM

The nature of the extract obtained following cold maceration with 90% ethanol and percentage yield of leaves of EEPM is shown in Table 5.

Table 5. Percentage yield and nature of EEPM

Plant / Extract	Nature of the extract	Extraction Yield (% w/w)
Ethanol extract of leaves of *Pedalium murex*	Dark green semisolid	8.34 %

5.1.2 Preliminary phytochemical screening of plant extract

The ethanolic extract of *Pedalium murex* leaves was analyzed for the presence of flavonoids, amino acids, tannins, steroids, glycosides and reducing sugars, etc., according to standard methods of Harborne et al., (2005); Kasture et al., (2003) and Gurudeep et al., (2003).

Table 6. Preliminary phytochemical analysis of *Pedalium murex* leaf extract

Phytoconstituents	Ethanol Extract
Reducing sugars	+
Glycosides	+
Alkaloids	-
Steroids	+
Flavonoids	+
Proteins	-
Amino Acids	+
Tannins	+
Fixed oils & fats	-
Gum & mucilage	-
Saponins	-

+ Present; **-** Absent

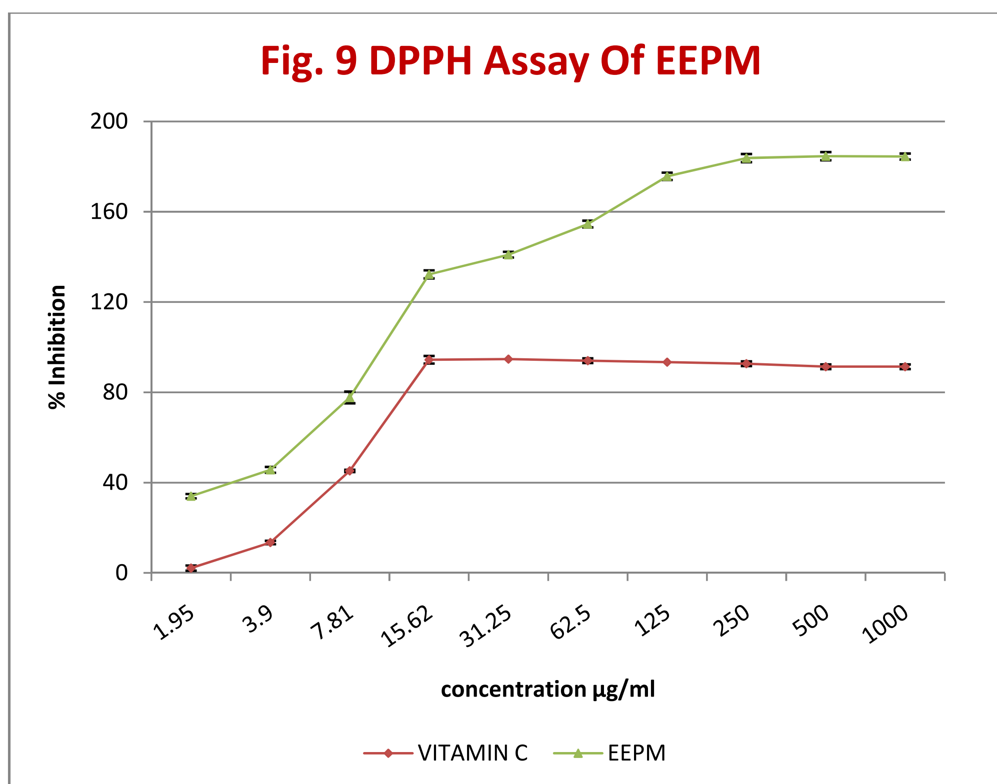
5.1.3 In vitro antioxidant studies

Free radicals are implicated in many disease conditions. Antioxidants may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by other mechanisms to prevent diseases.

5.1.3a. Inhibition of DPPH radical

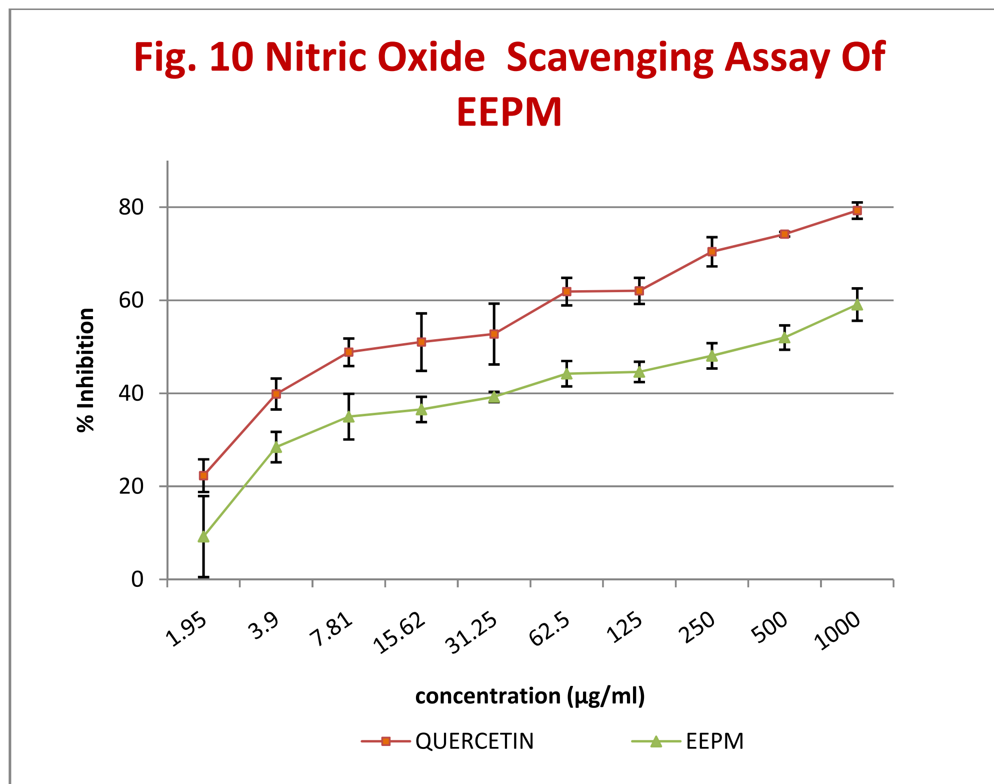
EEPM had significant scavenging effects on the DPPH radical and the effect was found to be enhanced with an increase in the concentration between 10µg/ml and 50µg/ml of the extract (Figure.9). Compared with that of butylated hydroxyl anisole (BHA), the scavenging effect of EEPM was significantly less. The IC_{50} of EEPM on

DPPH radical scavenging assay 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.



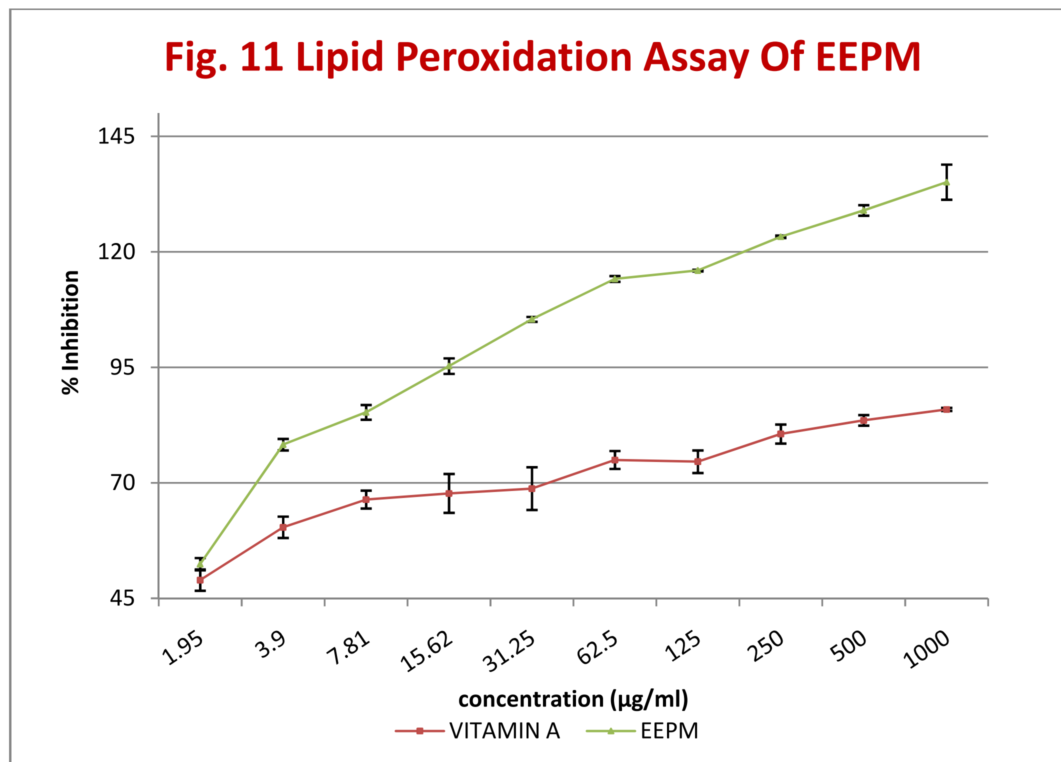
5.1.3b. Inhibition of Nitric oxide radical

It is known that nitric oxide has an important role in various types of inflammatory processes. The concentration of EEPM needed to produce 50% inhibition of nitric oxide release was found to be 7.81µg/ml, whereas 15.62µg/ml was needed for quercetin, used as a reference compound. The results were shown in Figure.10, which illustrates the percentage inhibition of NO generation by EEPM. It is well known that nitric oxide has an important role in various types of inflammatory processes.



5.1.3c. Lipid peroxidation

Figure. 11 illustrates the antioxidative activities of various concentrations (µg/ml) of EEPM. The IC_{50} of EEPM was found to between 7.81 and 15.62 µg/ml. The results indicate that, EEPM significantly inhibits the linoleic acid peroxidation. The antioxidative activity of the leaf extract of EEPM was measured using the ammonium thiocyanate method. This method was used to measure the level of peroxides, during the initial stages of lipid oxidation. The antioxidant activity of EEPM might be due to the reduction of hydrogen peroxide, inactivation of free radicals or both. This antioxidant activity of EEPM might be attributed to the presence of flavonoids and biflavones.



5.1.3d. Effect of 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 and are shown in table7. (Apak et al., 2007; Apak et al., 2006; Lee et al., 2011)

SAMPLE	Copper Reducing Equivalents	R^2
EEPM	675.3109	0.9922

5.1.3e. Effect of EEPM on metal chelating activity

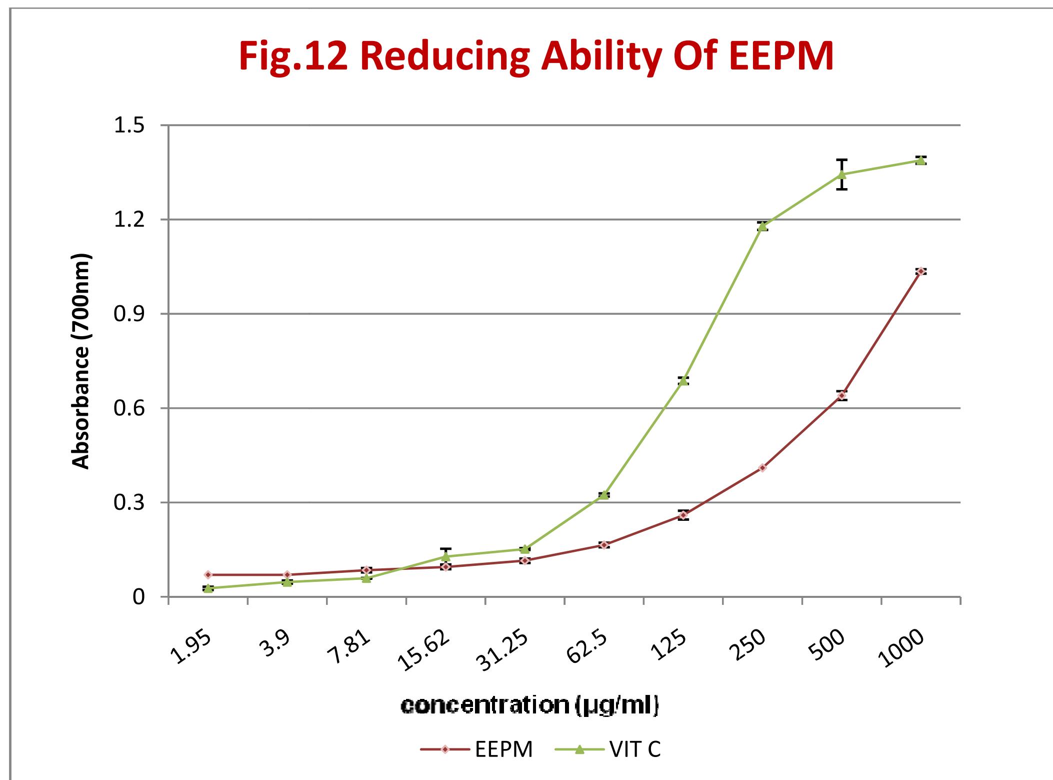
Transition metals have been proposed as the catalysts for the initial formation of radicals. Chelating agents may stabilize transition metals in living systems and inhibit the generation of radicals, consequently reducing free radical-induced damage. To better estimate the antioxidant potential of the 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 ethylenediamine tetraacetic acid (EDTA) was found to be 59.43± 0.7808 (at 30µg/ml) shown in table 8.

Conc. (µg/ml)	EDTA	A
	MEAN	MEAN
30	59.43± 0.7808	9.49± 1.543

5.1.3f. Reductive ability

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 700nm. Figure.11 shows the reductive capabilities of EEPM and Vitamin C (VIT.C). The reducing power of EEPM and Vitamin C rose with an increase in concentration and it was found to be 62.5 and 500µg/ml, respectively.

The antioxidant activity of the herbal preparations or phenolic compounds may be attributed to concomitant reducing power (Yen & Duh, 1993) and may serve as a significant indicator of its potential antioxidant activity.



***5.1.4* Bioactive compounds**

5.1.4a. Estimation of total phenolic compounds

The estimation of total phenolic content in EEPM has revealed that 100 µg/ml of EEPM contains 0.164 gallic acid equivalent of phenolics. The result indicates a strong association between antioxidative activities of phenolic compounds (r^2 = 0.9264), suggesting that phenolic compounds are probably responsible for the antioxidative activities of PM.

Phenols exhibit significant antioxidant and free radical scavenging ability (Shahidi & Naczk, 1995) 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.

5.1.4b. Estimation of total flavonoids

Total flavonoid content (expressed as µg quercetin equivalent/ml) were derived from a quercetin standard in the range of 20 -100 µg/ml (R^2= 0.9805).As was observed for total polyphenols, total flavonoid contents of the ethanolic extract of *Pedalium murex* (EEPM)was 0.156 µg/ml.

5.1.4c. Estimation of total tannins

Quantitative analysis of EEPM for tannic acid content is shown in the table 9.

SAMPLE	TANNIC ACID EQUIVALENT	Y value at 100µg/ml	R^2
EEPM	4.3948	0.167	0.9409

5.2 Phase II: Pharmacological studies

5.2.1 Effect of EEPM on general behavior parameters in acute stress treated rats

Results of behavior studies on acute stress-induced model

5.2.1. General behavior

After induction of stress, the animals were observed for general behavior upto 5 hours. Stress may result in psychological and physiological changes in behavior,

attributable to excitotoxicity. The observations made on general behavior for a period of 45 minutes following administration of EEPM, diazepam is described below:

Rats treated with stress exhibited a prolonged suppressive behavior in comparison to the control rats. Pretreatment with EEPM 1 hour before stress, resulted in profound calmness. Diazepam-treated animals exhibited little or no obviously observable behavior effects.

5.2.2 Effect of *Pedalium murex* and diazepam on elevated plus maze behavior in acute stress-treated rats on day 30.

Anxiety levels were determined with a plus-maze apparatus and the results are shown in figure.13 and table 10. When tested on an elevated plus-maze, a significant increase in anxiety levels (time spent in open arms: 20.97±2.9; time spent in closed arms: 159.2±9.2) were seen with acute stress-treated rats on Day 1 and a significant change in anxiety levels were observed on Day 30 with acute stress rats (time spent in open arms: 36.34±7.3; $p<0.01$; time spent in closed arms: 143.6±13.0), when compared to control.

The rats treated with EEPM in doses employed, produced a significant decrease in anxiety levels on Day 1 (time spent in open arms: 55.38±7.625; time spent in closed arms: 114.2±11.13) and was found to be even more marked on Day 30 (time spent in open arms: 4.172±1.217; time spent in closed arms: 138.5±12.68) with 200 mg/kg dose level in comparison to acute stress treated rats.

Analysis of the results of the first part of the experiment, which examined the effects of acute stress on Day 1 in EPM, revealed that the acute stress-treated rats showed

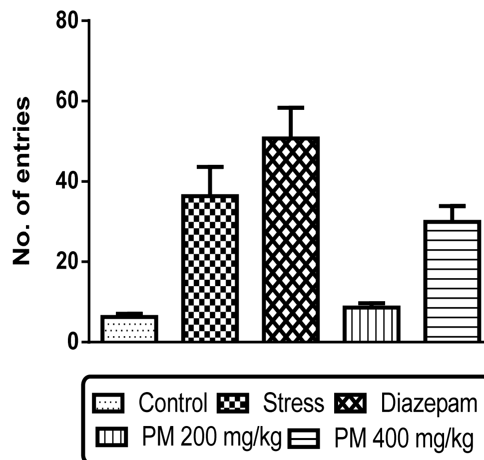
significant levels of anxiety as is evident from the time spent in open and closed arms.

Treatment with EEPM in the doses employed exhibited a dose dependent and significant decrement in the anxiety parameters studied and was most effective at the dose level of 200 mg/kg.

Table .10 Summary of elevated plus maze in acute stress treated rats

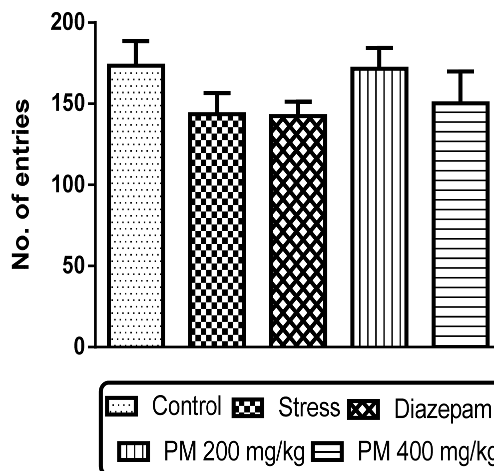
GROUPS	**TIME SPENT IN ARMS (sec)**	
	OP ENTRY (DAY30)	**CL ENTRY (DAY30)**
Control	6.283±0.7884	173.4±15.19
Stress	36.34±7.330**	143.6±13.00 ns
Diazepam	50.67±7.719***	142.4±8.842 ns
EEPM 200	8.608±1.117 ns	171.6±12.75 ns
EEPM 400	29.98±3.889*	150.3±19.65 ns

Fig.13a Effect Of EEPM, Diazepam On The Time Spent In Open Arm In EPM, In Acute stress Treated Rats



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001

Fig.13b Effect Of EEPM, Diazepam On The Time Spent in Closed Arm in EPM, In Acute stress Treated Rats



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001

5.2.3 Effect of *Pedalium murex* and diazepam on forced swim test in acute stress-treated rats

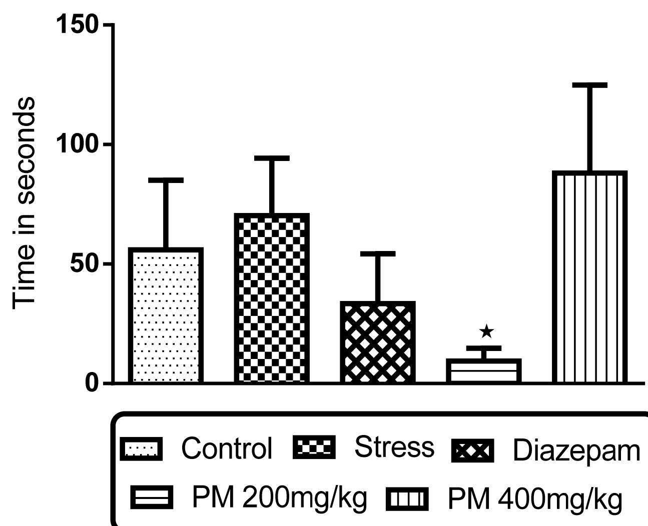
The effects of acute stress, EEPM and diazepam on the duration of immobility of rats in the forced swimming test are shown in figure.14 and table 11. Pretreatment with EEPM was found to attenuate the period of immobility, significantly (EEPM 200: 8.608±1.1; EEPM 400: 171.6± 12.7).

Analysis of the results of the experiment, which examined the effect of acute stress (70.27±9.8), following pretreatment for Day 30 by FST, revealed that changes in the freezing time displayed by the rats were less apparent when compared with stressed group. The duration of immobility was found to be 33.48±8.4 for rats treated with diazepam displaying antidepressant activity.

Table. 11 Summary of forced swim test in acute stress treated rats

GROUPS	IMMOBILITY TIME ON DAY 30 (in sec)
Control	55.50±10.30
Stress	46.00±8.333 ^{ns}
Diazepam	27.15±5.994 ^{ns}
EEPM 200	101.1±12.43**
EEPM 400	81.24±8.503 ^{ns}

Fig.14 Effect Of EEPM, Diazepam On Forced Swim Test In Acute stress Treated Rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

5.2.9 Effect of *Pedalium murex* and diazepam on radial arm maze activity in acute stress-treated rats

Analysis of the results of the experiment, which examined the effects of acute stress on day 30 on an RAM, showed an increased number of trials, and committed more errors in comparison to control rats. EEPM and diazepam treated rats took less trials, committed less errors to learn the radial arm-maze in comparison to acute stress-treated rats. However the number of days to learn the task remained unchanged in comparison to vehicle treated groups. Further, the drug treated rats differed in their learning pattern in comparison to the control group indicating altered learning.

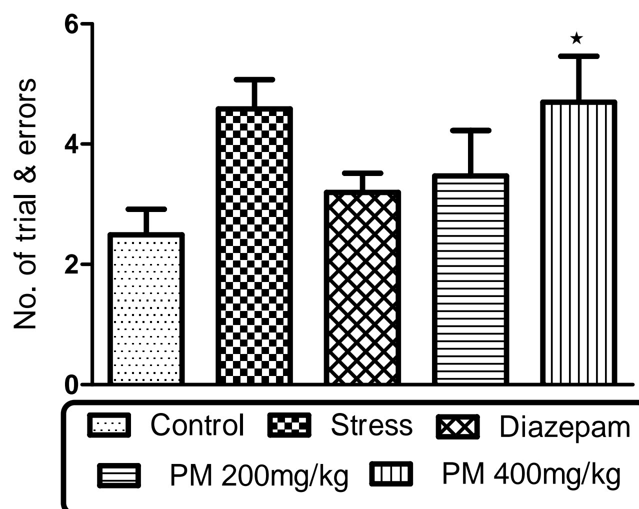
The results of 8-arm radial maze testing are shown in figure 15 table 12.A significant differences in the errors between the 4 groups (control, acute stress,

diazepam and EEPM-treated 200mg/kg and 400mg/kg was apparently observable 2.67±0.21, 6.17±0.16 and 3.66±0.21 and 5.12±). The CUS treated group had significantly increased errors, time and days (3.17±0.16, 175.58±6.69 and 06.87±0.33). In this study, treatment with diazepam completely prevented the acute stress-induced increase in number of trials, days, and errors largely but not completely.

Table. 12 Summary of radial arm maze studies on acute stress induced rats

GROUPS	NO.OF TRIALS AND ERRORS
Control	2.492±0.4242
Stress	4.587±0.4834 ns
Diazepam	3.199±0.3202 ns
EEPM 200	3.474±0.7560 ns
EEPM 400	4.697±0.7667*

Fig.15 Effect of EEPM, diazepam on 8-Arm Radial Maze In Acute Stress Treated Rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

In the present study, EEPM and diazepam completely prevented the errors, time and days and a full protection was observed in long-term reference memory, which was severely impaired by acute stress. These results indicate that EEPM and perhaps diazepam are potential candidates as therapeutic agents for stress-induced brain damage

Results of *Pedalium murex* and diazepam on behavior in CUS-treated rats

5.2.4 ***General behavior***

Induction of CUS for 10 days resulted in sickness, poverty in behavior and, invariably all the animals exhibited anxiety & depression after the administration of CUS. In comparison to CUS score (0.7±0.18), rats treated with EEPM 200mg/kg (1.78±0.32) and EEPM 400mg/kg (1.14± 0.17)scored more in motor activity, in similar to diazepam treatment (score 1.72±0.36) and was found to reverse the general behavior response on exposure to CUS. They exhibited significantly increased hyperactivity and antagonized the effect of these drugs with CUS.

5.2.5 Feed and water intake

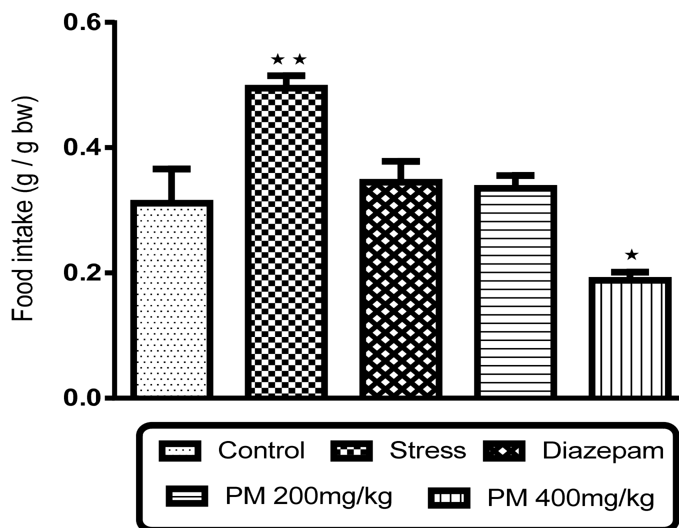
Administration of CUS markedly increased food intake by about 0.495±0.01 g/g body wt. ($p<0.01$) in comparison to control rats, which consumed 0.3113±0.05 g/g body wt.

The consumption of food with the EEPM treated groups are as follows: 0.335±0.02 g/g body weight for 200mg/kg and 0.1883±0.013 g/g body weight ($p<0.05$) for 400mg/kg respectively. The food intake was found to be markedly reduced with the EEPM treated rats. Treatment with diazepam (0.345±0.03 g/g body weight) significantly reduced the intake of food (figure 16 and table 13).

Induction of CUS notably increased water intake by about 0.7543±0.036 ml/g body weight, in comparison to all the groups (0.6357±0.035 ml/g bw. in control, 0.4243±0.030 ml/g bw. in EEPM 200, 0.3543±0.02487 ml/g bw. in EEPM 400 and 0.6671±0.04040 ml/g in diazepam), indicating that induction of CUS caused prompt but transient increase in water intake, which was antagonized by pretreatment with EEPM (figure 17 and table 14).

Table. 13 Summary of food intake in CUS treated rats.

GROUPS	FOOD INTAKE (in g/g body wt.)
Control	0.3113±0.054
Stress	0.495±0.019**
Diazepam	0.345±0.03344^{ns}
EEPM 200	0.335±0.02045 ^{ns}
EEPM 400	0.1883±0.01302*

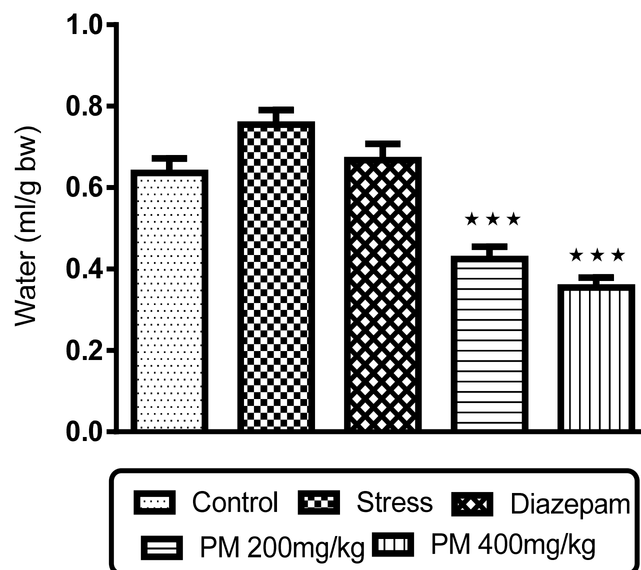


(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

Table. 14 Summary of water intake in CUS treated rats.

GROUPS	WATER INTAKE (in ml/g body wt.)
Control	0.6357±0.03565
Stress	0.7543±0.03631 ns
Diazepam	0.6671±0.04040 ns
EEPM 200	0.4243±0.03046***
EEPM 400	*0.3543±0.02487****

Fig.17 Effect Of EEPM, Diazepam On Water Intake In CUS Treated Rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

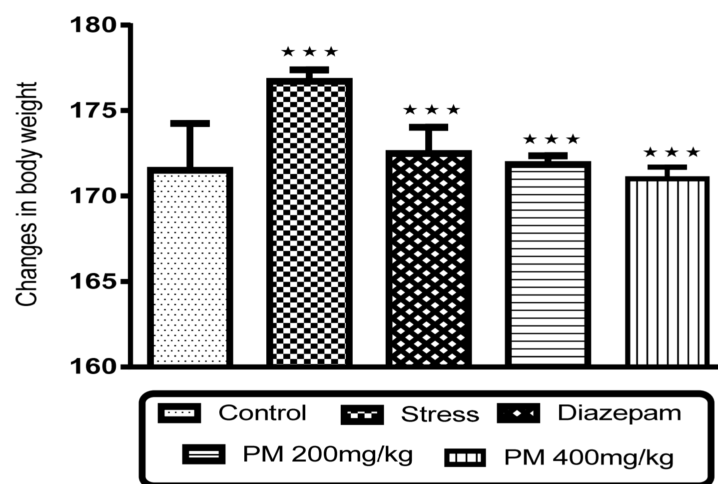
5.2.6 Body weight

Figure 18 and table 15, show body mass changes of all groups of rats investigated. Administration of CUS caused a significant increase in body weight (17.7± 0.67 g; p<0.001) in 10 days, from the day of induction of CUS, in comparison to the control rats (11.5± 1.5 g). The stress-induced increase of body mass was found to be antagonized by treatment with EEPM 200

Table. 15 Summary of body weight in CUS treated rats.

GROUPS	BODY WEIGHT (in gram)
Control	161.5±1.594
Stress	176.7±0.6703***
Diazepam	172.5±1.547***
EEPM 200	171.9±0.5132***
EEPM 400	171.0±0.6895***

Fig. 18 Effect of EEPM, Diazepam on Body Weight in CUS Treated Rats



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001

(11.9± 0.51 g; p<0.001), EEPM 400 (17.0± 0.68 g; p<0.001) and diazepam (12.5± 1.5 g; p<0.001). Pretreatment with EEPM for 30 days protected the rats from increase in body mass, in comparison to control rats (11.5± 1.5 g). The body weight of rats treated with diazepam was not significantly different from that of controls. Treatment with diazepam notably attenuated the increase in body mass induced by CUS.

5.2.7 Effect of *Pedalium murex* and diazepam on elevated plus maze behavior in CUS-treated rats on day 30.

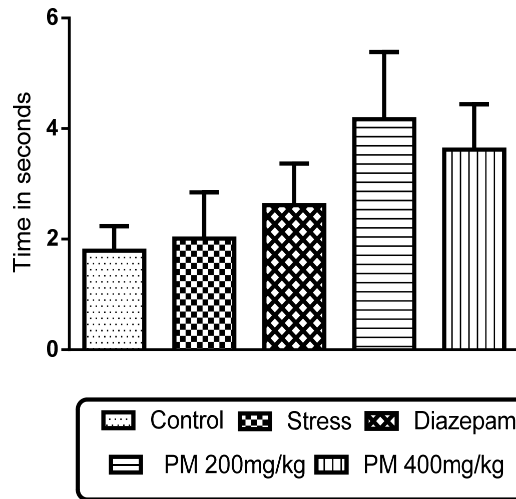
Tests on anxiety were determined using an elevated plus-maze apparatus and the results are shown in figure.19 and table 16. A notable change in anxiety levels were observed on day 30 (time spent in open arms: 2.014±0.83; time spent in closed arms: 136.7±10.42), following 10days of CUS in comparison to control groups (time spent in open arms: 1.792±0.44; time spent in closed arms: 136.7±12.28).

The rats treated with EEPM in the doses employed, produced a marked decrease in anxiety levels on day 30 (time spent in open arms: 4.172±1.2; time spent in closed arms: 138.5±12.6) with 200 mg/kg dose level in comparison to CUS treated rats. The entry into open & closed arm with diazepam treated groups was found to be 2.61± 0.75 and 154.4± 15.28, respectively.

Table. 16 Summary of elevated plus maze in CUS treated rats.

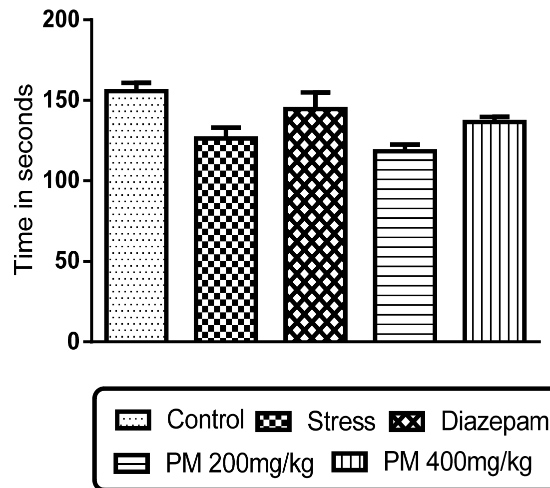
GROUPS	TIME SPENT IN ARMS (sec)	
	OP ENTRY (DAY30)	**CL ENTRY (DAY30)**
Control	1.792±0.4439	136.7±12.28
Stress	2.014±0.8348 ^{ns}	136.7±10.42 ^{ns}
Diazepam	2.618±0.7528 ^{ns}	154.4±15.28 ^{ns}
EEPM 200	4.172±1.217 ^{ns}	138.5±12.68 ^{ns}
EEPM 400	3.621±0.8218 ^{ns}	128.0±13.60 ^{ns}

Fig.19a Effect of EEPM, Diazepam On The Time Spent In Open Arm, In EPM In CUS Treated rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

Fig.19b Effect of EEPM, Diazepam On The Time Spent In Open Arm, In EPM In CUS Treated rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01: ★★★P<0.001

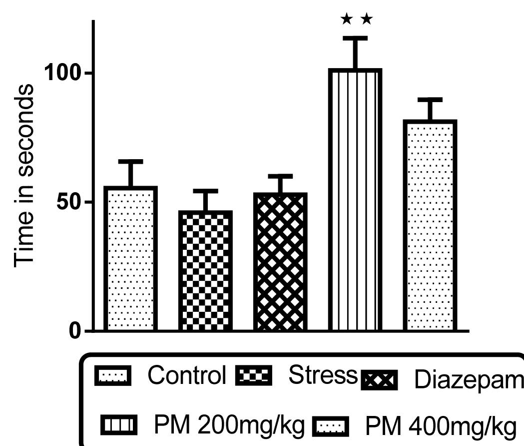
5.2.8 Effect of *Pedalium murex* and diazepam on forced swim test in CUS-treated rats

The effects of CUS, EEPM and diazepam on the duration of immobility of rats in the forced swimming test are shown in figure 20 and table 17. The onset of depression in CUS group was evident from increased period of immobility as against control (55.50± 10.3 seconds).

Table. 17 Summary of forced swim test in CUS treated rats.

GROUPS	IMMOBILITY TIME ON DAY 30 (in sec)
Control	55.50±10.30
Stress	46.00±8.333 ns
Diazepam	27.15±5.994 ns
EEPM 200	101.1±12.43**
EEPM 400	81.24±8.503 ns

Fig.20 Effect Of EEPM, Diazepam On Forced Swim Test In CUS Treated Rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

Analysis of the results of the experiment, which examined the effect of CUS (46.00±8.33) on Day 30 by FST, revealed that changes in the freezing time displayed by the rats were less apparent when compared with EEPM 200 mg/kg (immobility period: 101.1±12.43, $p<0.01$) and EEPM 400 mg/kg (immobility period: 81.24± 8.503). The immobility period for diazepam treated group was found to be significantly antagonized (57.15± 5.99).

5.2.9 Effect of *Pedalium murex* and diazepam on radial arm maze activity in CUS-treated rats

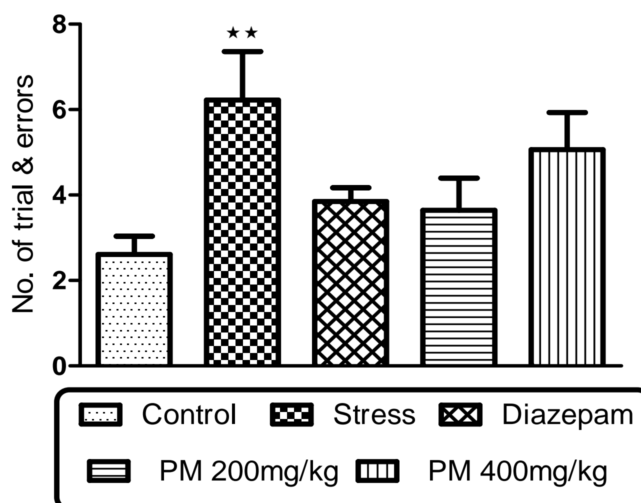
Analysis of the results of the experiment, which examined the effects of CUS on day 30 on an RAM, showed an increased number of trials, and committed more errors in comparison to control rats. EEPM and diazepam treated rats took less trials, committed less errors to learn the radial arm-maze in comparison to CUS-treated rats. However the number of days to learn the task remained unchanged in comparison to vehicle treated groups. Further, the drug treated rats differed in their learning pattern in comparison to the control group indicating altered learning.

The results of 8-arm radial maze testing are shown in Table 18. A significant differences in the errors between the 4 groups (control, CUS and EEPM-treated 200mg/kg and 400mg/kg was apparently observable 2.67±0.21, 6.17±0.16 and 3.66±0.21 and 5.12±). The CUS treated group had significantly increased errors, time and days (6.17±0.16, 257.62±6.69 and 07.67±0.33).

Table 18. Summary of radial arm maze in CUS treated rats.

GROUPS	NO. OF TRIAL AND ERRORS
Control	2.609±0.4256
Stress	6.225±1.132 **
Diazepam	3.850±0.3176 ns
EEPM 200	3.641±0.7557 ns
EEPM 400	5.065±0.7557 ns

Fig.21 Effect of EEPM, diazepam on 8-Arm Radial Maze In CUS Treated Rats



(Values are mean SEM observations from six animals in each group)
★P<0.05; ★★P<0.01; ★★★P<0.001

In this study, treatment with diazepam completely prevented the CUS induced increase in number of trials, days, and errors largely but not completely.

In the present study, EEPM and diazepam completely prevented the errors, time and days and a full protection was observed in long-term reference memory, which was

severely impaired by CUS. These results indicate that EEPM and perhaps diazepam are potential candidates as therapeutic agents for stress-induced brain damage.

5.3 Phase III: Biochemical analysis of brain antioxidants

5.3.1 Effect of EEPM on rat brain anti oxidant system in acute stress-treated rats

5.3.1a. Effect of EEPM on SOD (U/mg protein) levels in acute stress- treated rat brain

The SOD profile in the rat brain was depicted in Table 17.In comparison to control rats (143.7± 74.36), acute stress induction for 2 h resulted in significant reduction of SOD level of brain (70.3± 6.306). Pre-treatment with EEPM (200mg/kg: 97.57± 10.62; 400mg/kg: 147.6± 81.64) for 30 days significantly improve the SOD activity in comparison to stress-treated groups in the brain studied. Among all the groups, a marked increase in SOD status was observed with EEPM 400mg/kg treatment (147.6± 81.64).

The results suggest a significant dose dependent increase SOD status following EEPM pre-treatment for 30 days. Diazepam (115.49± 7.928) significantly attenuated the stress effect on SOD status and increased the antioxidant level in all the regions.

5.3.1b. Effect of EEPM on CAT (U/mg protein) levels in acute stress-treated rat brain

Table 19. showed the alteration of CAT levels in various brain regions studied. In comparison to control rats (88.89± 16.66), acute stress induction for 2 h resulted in significant reduction of CAT level of brain (15.095± 2.804). Pre-treatment with

EEPM (200mg/kg: 50.39± 5.196; 400mg/kg: 79.7± 42.99) for 30 days significantly improve the CAT activity in comparison to stress-treated groups in the brain studied.

Among all the groups, a marked increase in CAT status was observed with control groups (88.89± 16.66). The results suggest a significant dose dependent increase CAT status following EEPM pre-treatment for 30 days. Diazepam (15.095± 2.804) significantly elevated the stress effect on CAT status and increased the antioxidant level in all the regions studied.

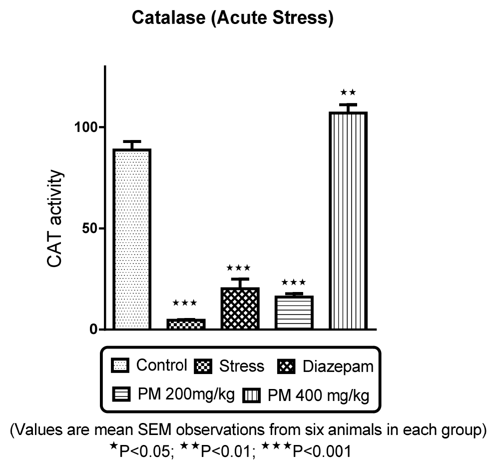
5.3.1c. Effect of EEPM on GSH (mg/g protein) levels in acute stress-treated rat brain

The effect of drugs employed on GSH levels are summarized in Table 19. In comparison to control rats (4.769± 2.61), acute stress induction for 2 h resulted in significant increase of GSH level of brain (86.129± 9.36). Pre-treatment with EEPM (200mg/kg: 36.692± 4.99; 400mg/kg:

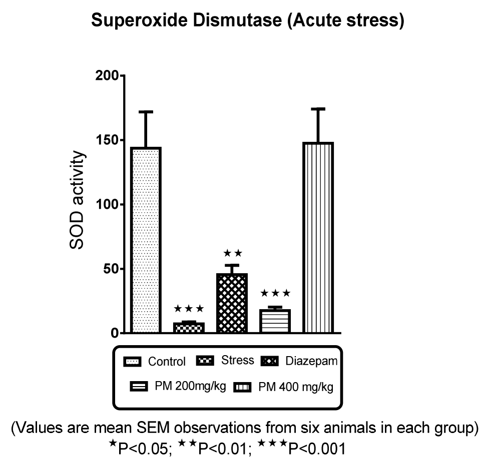
Table 19. Effect of EEPM, diazepam on different antioxidant (U/mg protein) levels in acute stress-treated rat brain

GROUPS	CAT	SOD	GSH
Control	88.72±4.200	143.7±28.18	19.57±1.162
Stress	4.672±0.3028***	7.337±1.405***	16.31±1.089 ^{ns}
Diazepam	20.21±4.773***	45.49±7.216**	6.370±0.9767*
EEPM 200	16.07±1.648***	17.57±2.692***	4.892±0.7670**
EEPM 400	107.0±4.111**	147.6±26.56 ^{ns}	80.63±6.372***

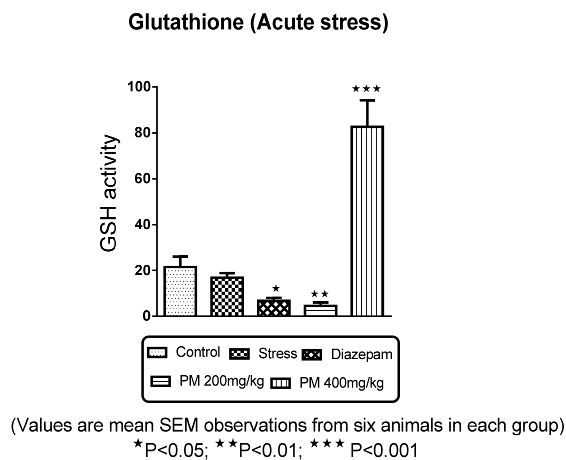
Fig.22 Effect Of EEPM, Diazepam On Different Antioxidant Levels In Acute Stress Treated Rats



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; *** P<0.001

19.260± 6.19) for 30 days significantly decrease the GSH activity in comparison to stress-treated groups in the brain studied.

Among all the groups, a marked decrease in GSH status was observed with control groups (4.769± 2.61). The results suggest a significant dose dependent decrease GSH status following EEPM pre-treatment for 30 days. Diazepam (16.998± 5.45) significantly diminished the stress effect on GSH status and increased the antioxidant level in all the regions studied.

5.3.2 Effect of EEPM on rat brain anti oxidant system in CUS-treated rats

5.3.2a. Effect of EEPM on SOD (U/mg protein) levels in CUS- treated rat brain

The SOD profile in the rat brain was depicted in Table 20. In comparison to control rats (143.7± 74.36), CUS induction for 10 days resulted in significant reduction of SOD level of brain (16.92± 7.274). Pre-treatment with EEPM (200mg/kg: 107.± 62.87; 400mg/kg: 153.5± 97.15) for 30 days significantly improve the SOD activity in comparison to CUS-treated groups in the brain studied. Among all the groups, a marked increase in SOD status was observed with EEPM 400mg/kg treatment (147.6± 81.64).

The results suggest a significant dose dependent increase SOD status following EEPM pre-treatment for 30 days. Diazepam (129.84± 62.87) significantly attenuated the stress effect on SOD status and increased the antioxidant level in all the regions.

5.3.2b. Effect of EEPM on CAT (U/mg protein) levels in CUS-treated rat brain

Table 20. showed the alteration of CAT levels in various brain regions studied. In comparison to control rats (88.89± 16.66), CUS induction for 10 days resulted in

significant reduction of CAT level of brain (17.85± 3.068). Pre-treatment with EEPM (200mg/kg: 57.9± 58.62; 400mg/kg: 81.43± 34.8) for 30 days significantly improve the CAT activity in comparison to stress-treated groups in the brain studied.

Among all the groups, a marked increase in CAT status was observed with control groups (88.89± 16.66). The results suggest a significant dose dependent increase CAT status following EEPM pre-treatment for 30 days. Diazepam (72.01± 33.97) significantly elevated the stress effect on CAT status and increased the antioxidant level in all the regions studied.

5.3.2c. Effect of EEPM on GSH (mg/g protein) levels in CUS-treated rat brain

The effect of drugs employed on GSH levels are summarized in Table 20. In comparison to control rats (56.267± 29.76), CUS induction for 10 days resulted in significant increase of GSH level of brain (32.948± 4.84). Pre-treatment with EEPM (200mg/kg: 28.001± 8.20; 400mg/kg: 51.477± 29.97) for 30 days significantly decrease the GSH activity in comparison to stress-treated groups in the brain studied.

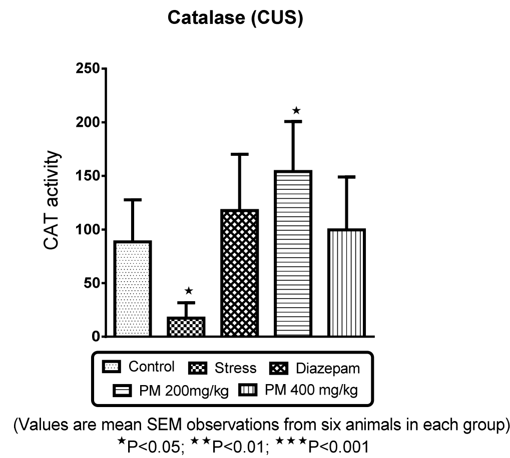
Among all the groups, a marked decrease in GSH status was observed with control groups (4.769± 2.61). The results suggest a significant dose dependent decrease GSH status following EEPM pre-treatment for 30 days. Diazepam (16.998± 5.45) significantly diminished the stress effect on GSH status and increased the antioxidant level in all the regions studied.

Table 20. Effect of EEPM, diazepam on different antioxidant (U/mg protein) levels in CUS-treated rat brain

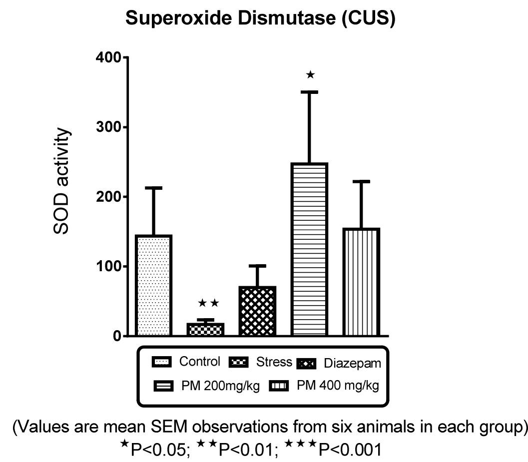
GROUPS	CAT	SOD	GSH
Control	88.59±15.95	143.7±28.18	19.64±0.9595
Stress	17.42±5.834*	16.92±2.657**	28.27±0.7375***
Diazepam	117.6±21.43 ns	69.84±13.81 ns	51.47±0.9506***
EEPM 200	154.0±19.04*	247.0±42.17*	57.23±0.8774***
EEPM 400	99.77±20.10 ns	153.5±27.90 ns	32.05±1.292***

- In summary, acute stress produced significant changes in anxiety, depressive and cognitive parameters.
- CUS produced significant increase in anxiety levels in elevated plus-maze and depressed the animals in a FST model, and nhanced the impairment in cognitive abilities in an 8- arm radial maze.
- In addition, CUS caused significantly greater behavioral alterations and changes in body weight, food and water intake.
- The changes in body weight, food and water intake produced by CUS were all significantly attenuated by EEPM.

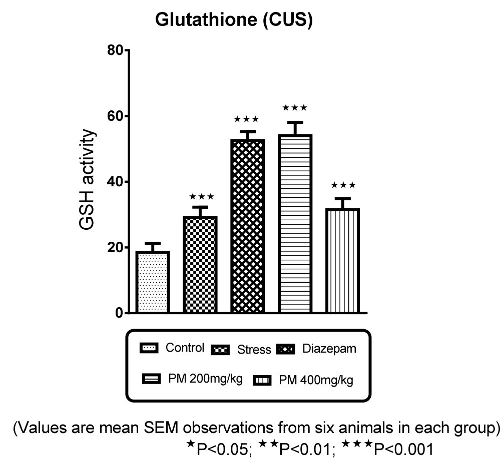
Fig.23 Effect Of EEPM, Diazepam On Different Antioxidant Levels In CUS Treated Rats



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001



(Values are mean SEM observations from six animals in each group)
*P<0.05; **P<0.01; ***P<0.001

DISCUSSION

The study was undertaken to evaluate the effect of ethanol extract of Pedalium murex in acute and chronic stressed rats. Following extraction and preliminary identification of phytoconstituents, the extract was studied for *in vitr*o antioxidant activities (DPPH, nitric oxide, lipid peroxidation, CUPRAC assay, metal chelation) and reducing index.

The animals were subjected to immobilization induced acute stress and, chronic unpredictable stress (CUS) by forced swimming + immobilization + dark phase, for 10 days. All the animals were pretreated with EEPM for 30 days for acute stress model and, 21 days in case of CUS model.

After subjecting the animals to acute and CUS, changes in food intake, water intake, body weight were observed from the time of induction of either acute or chronic stress. Thereafter, the animals were subjected to a battery of behavioral studies, viz., anxiolytic tests using elevated plus maze, depressive test using forced swim test and, impairment of cognition and memory using radial arm maze. Moreover, the perturbations in the levels of brain antioxidant system like SOD, CAT were measured. In addition, the activities of GSH were also measured.

The findings of this 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(acute and CUS). EEPM 400 mg/kg was found to be most effective in attenuating the effects of acute and CUS and, was found to be as potent as diazepam.

The acute 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.

The development of anxiety and depression and, impairment of cognition were evident as observed from decreased activity in EPM, increased periods of immobility in FST and increased errors and trials in an 8 arm radial maze apparatus. 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 (Hliňák & Krejči 1998; Itoh et al., 1990, 1991). Compared to the controls, prolonged start and transfer latencies in stress-treated rats indicate that the ability to escape rapidly from the open arm into the closed arms is somewhat disturbed. 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 stress-treated animals sat motionless. Thereafter, they displayed locomotion accompanied with typical arm- and air-sniffing. Thus, both initial immobility and freezing of stress-treated animals were replaced by a remarkable exploration. It seems 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 spend more time on the open arms of the maze whereas animals with higher anxiety level hide in the closed arms (Martinez et al., 2002; Pellow et al., 1985). 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 (Rodgers et al., 1997). A dissimilar portion of both factors could influence behavioral performance of MSG-treated, EEOS-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 forced swim test, which supports many previous findings. 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 .In this present study, stress produced depression is evident from increased freezing time of rats in FST. 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 assume an immobile posture after initial attempts to escape. In a subsequent immersion, the beginning of the immobility is faster and marked. Porsolt et al., (1978) named this phenomenon "behavior despair" and attributed the animals' response to the development of a depression process. Treatment with anti-depressive medicines reduces the immobility time during the swimming test. The authors presented it as a model of animal depression. In the forced swimming test, which evaluates the depressive behavior doted by Porsolt, a significant decrease of the immobility time was observed after the administration of different doses of EEPM to rats. These

results suggest that EEPM induces an anti-depressive activity in the CNS, in a dose-dependent manner.

The extract was also found to restore the disturbances in memory and cognitive abilities of rats. 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 prolonged stress. Chronic stress 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 consumption and body weight and, anxiety, cognition and depressive behavior of the rats, according to the tests used to analyze animal anxiety, cognition and depression. These findings provide further support for the similarity between stress-induced immune activation, anxiety and depression.

The depletion in the levels of SOD and CAT and increased activity of GSH following a single and 10 days of acute and CUS, is consistent with previous study in our laboratory. These results clearly show that stress can lead to oxidative stress leading to neurodegeneration. Treatment with ethanol extract of PM, a herb containing among a number of phenolic compounds, flavonoids, tannins, triterpenes,

alkaloids, steroids, fixed and volatile oils, significantly attenuated stress-induced changes.

Recent work suggests that different types of stressful events may sometimes produce qualitatively different patterns of effects in both behavior and physiology.

Stress is a common experience of daily life and all organisms have developed mechanisms to cope with it. Sustained stress can have numerous pathophysiological effects such as activation of neuro–endocrine [limbic-hypothalamic-pituitary adrenal system] (Bonfiglio et al., 2001) and hormonal (corticosterone release) functions (Fuch and Flugge, 1998). Sustained and persistent stressful conditions can precipitate anxiety and affective disorders such as depression, which further lead to the excessive production of free radicals and oxidative burden (Maes, 2011). In an organism, diverse stressors activate a wide spectrum of interacting hormonal and neuronal systems resulting in behavioral (anxiety disorders, decrease in food intake, decrease in sexual behavior, and loss of cognitive function) and physiological responses [activation of pituitary adrenal axis and release of glucocorticoids into the blood stream] (Henry and Stephens, 1977). These stressors are stimulators of arousal and lead to autonomic (changes in body temperature and tachycardia) and behavioral changes; however, when arousal increases to stress-like levels, it results in psychiatric and physical disorders (Hennessy and Levine, 1979).

Stress responses are variable and there are individual differences both physiologically and behaviorally in how an organism perceives a perturbation and in the resulting adaptational /maladaptational processes (Weiner. 1992). According to previous reports stress induces anxiety-like behavior in both humans and animals (Liezmann et al., 2011). Exposure to stress represents an important factor for a

number of neuropsychiatric disorders such as depression, post-traumatic stress disorder, and other anxiety disorders (Lee et al., 2008).

Chronic activation of the stress system would be expected to increase visceral adiposity, decrease lean body (muscle and bone) mass and suppress osteoblastic activity. Antioxidants are known to have great importance in human disease pathology because of their possible action against free radicals. Dietary macronutrients contribute to the antioxidants defense system. These include β-carotene, vitamin C, vitamin E. In view of the vital role of oxidative stress in the pathogenesis of Alzheimer's disease (AD), the potential role of these antioxidant supplements to prevent AD has gained much interest.

7. SUMMARY AND CONCLUSION

The present study was designed to evaluate the possible protective effect of ethanol extract of *Pedalium murex* (EEPM) leaves treatment against acute and CUS-induced neurobehavioral models in SD rats. The neuroprotective effect was assessed using a battery of *invitro* tests, behavioral tests (anxiety, depression and, cognition and memory) and biochemical analysis (SOD, CAT, GSH) of the rat brain. Phytochemical, total phenolic, tannins and flavonoid content of EEPM and, *in vitro* antioxidant studies on EEPM leaves on various antioxidant systems such as lipid peroxidation, DPPH, nitric oxide, CUPRAC assay, metal chelation and reducing index were performed.

Application of acute stress using immobilization for one day and CUS (induced by forced swimming + immobilization + dark phase, for 10 days), resulted in elevated levels of anxiety, depression and changes in memory and cognitive abilities. Pretreatment with EEPM for 30 days prevented the effect of acute and chronic unpredictable stress in rats.

The protective effect of EEPM was evaluated for anxiolytic (elevated plus-maze apparatus and depression was evaluated using forced swim test (FST) in both the acute and CUS-induced models and moreover, effect of EEPM on learning and short-term (spatial) memory was evaluated using a radial arm maze (RAM) test.

To support the neuroprotective effect of EEPM, the implications of EEPM on general behavior, body weight (BW), food intake (FI), water intake (WI) were undertaken in acute and CUS-induced models, respectively, as these behavior could

be profoundly disturbed in anxiety, depression and may impair memory and cognitive abilities.

Also there may be perturbations in the levels of brain antioxidant enzyme system in stressful states. Hence, the effects of EEPM on the levels of glutathione (GSH), catalase (CAT), superoxide dismutase (SOD) were studied in the rat brains.

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