PHYTOCHEMICAL CHARACTERIZATION AND NEUROPROTECTIVE ASSESSMENT OF STANDARDIZED EXTRACT OF *Pedalium murex* Linn. LEAVES

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Introduction

I. INTRODUCTION

Research is a process of investigation and interrogation; it is organized, meticulous and humane; research can help to solve real-world obstacles.

"All that man needs for health and healing has been provided by god in nature, the challenge of science is to find it"

"The doctor of the future will no longer treat the human frame with drugs, but rather will cure and prevent disease with nutrition"

- Thomas Edison.

The environment we are alive in is physically, spiritually, emotionally, socially and morally dynamic and challenging. We possess effectual mechanisms to meet every day stress. Occasionally, normal adaptive mechanisms can be over-activated and thus, become nonadaptive. A common result of such over-activation precedes to anxiety, restlessness, depression and memory loss.

I.1. BACKGROUND OF THE PROPOSED WORK

Neurodegenerative diseases (ND) are divergent group of ailments of the central nervous system, including brain, spinal cord and peripheral nerves that have numerous etiologies. It modifies various body's activities such as steadiness, movement, conversation, conscious and heart function. Many of these diseases are hereditary. Due to the pervasiveness, morbidity and mortality of the ND, they symbolize significant therapeutic, societal and monetary encumbrance on the community.[1] Mitochondrial dysfunctions, excitotoxicity and apoptosis have been stated as pathological origin for aging and ND such as Parkinson's disease (PD), Alzheimer's disease (AD), Multiple Sclerosis (MS) and Amyotrophic lateral sclerosis (ALS).[2]

Oxidative stress (OS) is induced by an extreme redox state, involving either excessive genesis of reactive oxygen species (ROS) or malfunction of the antioxidant system. The brain is one of the organs particularly vulnerable to effects of ROS because of its high oxygen demand and its abundant peroxidation-affected lipid cells.[3] ROS includes oxygen-related free radicals and reactive species.[4]

Free radicals are molecules with at least one single electron in the outermost shell; they are highly threatening due to the occurrence of unpaired electron. Any free radical including oxygen can be denoted as a reactive oxygen species (ROS).[5] The most common stated cellular free radicals are hydroxyl (OH·), superoxide ($O_2 \cdot^-$), nitric oxide

(NO·), nitrogen dioxide (NO_2·), peroxyl (ROO·) and lipid peroxyl (LOO·). Molecules like hydrogen peroxide (H_2O_2), ozone (O_3), singlet oxygen ($1O_2$), hypochlorous acid (HOCl), nitrous acid (HNO_2), peroxynitrite (ONOO-), di nitrogen trioxide (N_2O_3), not considered as free radicals, can effortlessly lead to free radical reactions in living creatures.[6] Antioxidant supplementation is one sensible policy to sustain redox homeostasis through straight quenching life-threatening ROS and shielding or strengthening endogenous antioxidative resistance against oxidative stress.[7]

Currently, several antioxidants are manufactured synthetically. They inherit some side effects and toxic properties to human well-being.[8] Research on antioxidant constituents in the herbal foodstuffs has immense interest in the field of medicine. Universal prevalence of ND is increasing, and effective treatment is mandatory to diminish the neuronal damage and oxidative stress. Traditional medicines offer potent pharmacological activity with minimal side effects compared to synthetic drugs to treat such chronic disorders. There is no famous remedy to arrest or saving infection or inflammation-induced brain damage. Despite the huge number of on-going researches, ND remain incurable and the chemicals derived from plants provide healthier protection against a wide range of etiological factors.[9]

I.2. EPIDEMIOLOGY OF NEURODEGENERATIVE DISEASES

In a worldwide study, India has been ranked 143rd among 188 countries on the array of health indicators including its presentation on poor hygiene. As India lags the world in medical professionals and spending on mental health problems, it is understandable that more than 60 million Indian populations suffer from psychological illness.[10]

About 5% of the population agonizes from common mental disorders like depression and anxiety associated problems as per the last report obtainable in 2005. This data was lately quoted by Indian Health and Family Welfare Minister from National commission on macroeconomics and health forum. In India the health budget on mental health care is astoundingly 7.5 times lesser than Bangladesh. There is grave shortage of psychiatrists in India with 3 psychiatrists per million populations which is 18 times lesser than the commonwealth nation's norm of 56 psychiatrists per million persons. Keeping these facts in attention, a new Bill was passed in Indian

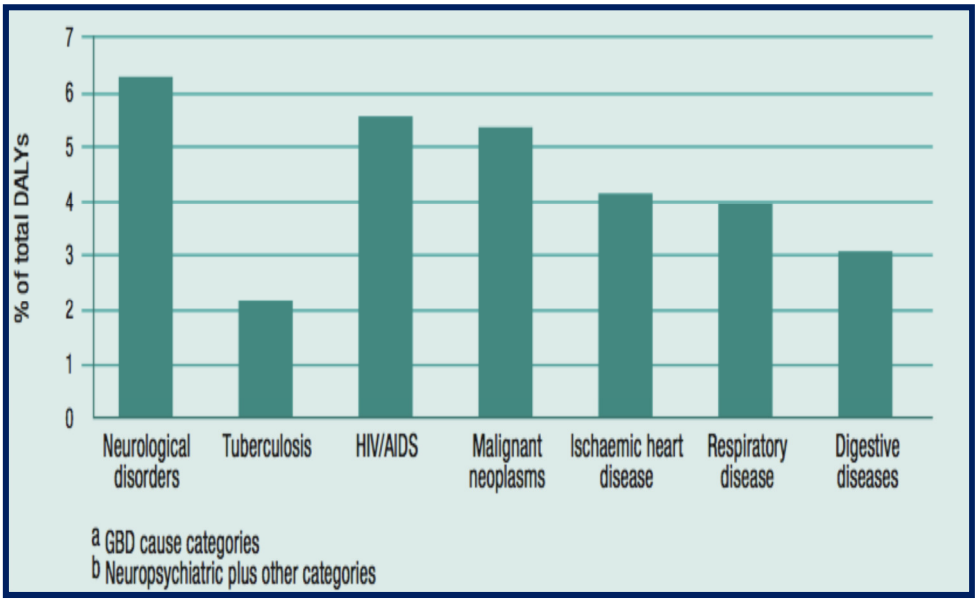
Parliament in August 2016 growing the government funding a little more than earlier.[10]

The occurrence of neurological disorders is increasing in India.[11] Already six epidemiological studies have been directed between the years 1987 and 2004. In accordance to these studies, the frequency of Parkinsonism, peripheral neuropathies and stroke is growing within India. The influence of neurological disorders has been measured by disability adjusted life years (DALY) by the WHO report on neurological disorders.

The DALY is considered by determining the number of years of life lost to premature mortality (YLL) and years alive with disability (YLD). Figure 1 depicts that the problem of neurological disorders is already larger than other chronic non-communicable and infectious illnesses.

Moreover, ND commonly influence aged populations over a extended period of time suggesting that the high DALY is due to the elevated number of years living with disability (YLD). This depicts that there is a large population living with ND, that these disorders are causing elevated levels of illness in the elderly and finally highlights the importance of developing treatments for neurological diseases in both developing and developed countries.[12]

Figure 1: Disability Adjusted Life Years (DALY) For Neurological Disorders



Developing nations including India are passing through a stage of epidemiological evolution with increasing problems of neurological disorders

resulting to transformation of situation with promotion of health care services in preventive and promotive areas. Neurological disorders form an important proportion of global burden of disease.[13,14] Two important papers published by World Health Organization (WHO) and World Federation of Neurology bring to front the public health encounters posed in dealing with neurological disorders specifically in the developing countries with inadequate resources.[12,15]

Occurrence of neurological disorders was determined by house to house surveys in six studies from different areas of the country encompassing of rural population in all studies and urban population in two of them. The basic prevalence rate varied from 967- 4,070 per 100000 population with an average of 2394 per 100000 populaces.[16-21] Based on this data it was assessed that for the current population of 1.27 billion approximately 30 million people agonize from neurological disorders in India.

According to the World Health Organization (WHO) neurological disorders include Epilepsy, AD and other dementias like PD, MS, Migraine, Cerebrovascular Disease, Poliomyelitis, Tetanus, Meningitis and Japanese Encephalitis are accounting for 12% of mortality worldwide among other diseases.[22] They are recurrently denounced, since they are socially overwhelming and can cause cognitive impairment behavioural disorders, depression and suicide.[23,24]

I.3. ROLE OF FREE RADICALS IN NEURODEGENERATION

Oxygen is an element which is vital for life. When cells use oxygen to produce energy and the free radicals are formed because of ATP (adenosine triphosphate) production by the mitochondria. These by-products are usually ROS as well as RNS that result from the cellular redox process. These species play a double role as both toxic and helpful compounds. The faint balance between their two antagonistic effects is clearly significant aspect of life. At low or moderate levels, ROS and RNS exert beneficial effects on cellular responses and immune function. At high concentrations, they produce oxidative stress, a destructive process that can damage all cell structures.[17,22,25-32]

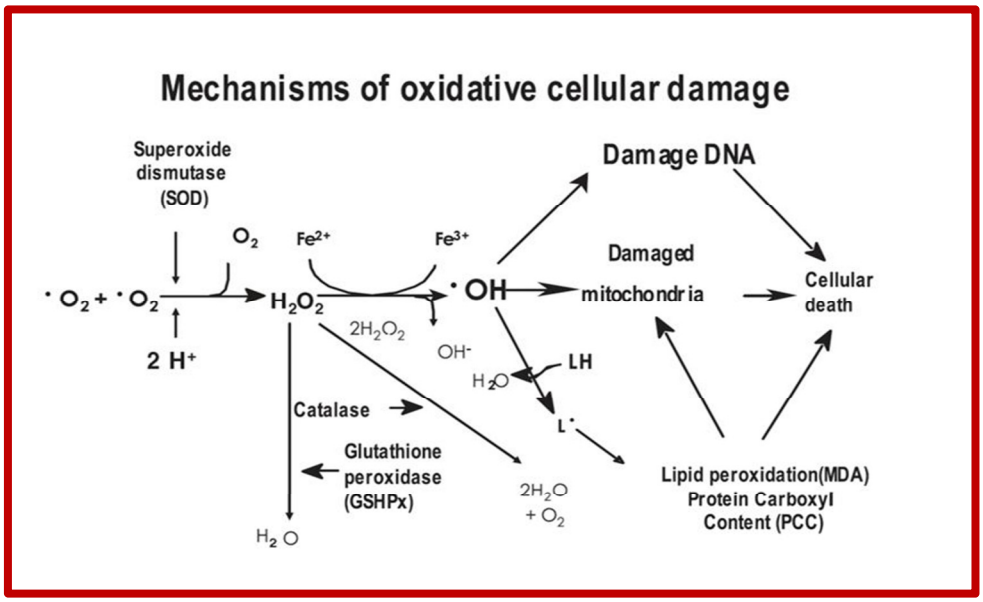
I.4. OXIDATIVE STRESS LEADS NEURODEGENERATION

Biological tissues require oxygen (O_2) to meet their active demands.[33] O_2 is vital for the normal function of eukaryotic organisms. Its role in existence is linked to its high redox potential, which makes it an outstanding oxidizing agent capable of accepting electrons easily from reduced substrates. Dissimilar tissues have different

O_2 demands liable on their metabolic needs. Neurons and astrocytes are the two chief types of brain cells, which are principally responsible for the brain's gigantic consumption of O_2 and glucose; indeed, the brain characterizes only ~2% of the total body weight and yet accounts for more than 20% of the total consumption of O_2.[34] Though, the consumption of oxygen also consequences in the generation of free radicals that may have destructive effects on cells.[35] Despite the essentiality of O_2 for living beings, the state of hyperoxia produces toxicity, including neurotoxicity.[35,36] Oxidative stress ascends as a result of an imbalance between the production of ROS and the biological system's capability to detoxify the reactive intermediates.[37] OS has been concerned in the progression of AD, PD and other ND. OS leading to free radical attack on neural cells contributes dreadful role to neurodegeneration. Toxicity of ROS donates to protein misfolding, glia cell activation, mitochondrial dysfunction and subsequent cellular apoptosis.[38]

Oxidative overload in the neuronal microenvironment causes oxidation of lipids[39], proteins[40], DNA[41] and produces many by-products such as peroxides, alcohols, aldehydes, ketones and cholesterol oxide.[42] Unsaturated lipids are specifically vulnerable to oxidative alteration and lipid peroxidation is a sensitive marker of OS. Lipid peroxidation is the result of attack by radicals on the double bond of unsaturated fatty acids to make highly reactive lipid peroxy radicals that initiate a chain reaction of extra attacks on other unsaturated fatty acids. The chain reaction leads to the creation of breakdown products including 4-hydroxy-2,3-nonenal (HNE) and F2-isoprostanes.[43-46] HNE is able to modify proteins resultant in a multitude of effects, including cessation of neuronal glucose and glutamate transporters, inhibition of Na^+/K^+-ATPases, initiation of kinases and dysregulation of intracellular calcium signalling, that eventually induce an apoptotic cascade mechanism.[31,47,48] These conclusions, together with the current demonstration that HNE is cytotoxic to neurons and that it weakens the function of membrane proteins including the neuronal glucose transporter specify that HNE is a typical marker and a toxin leading to neurodegeneration.[32] The figure 2 signify the mechanism of oxidative cellular damage of cells.

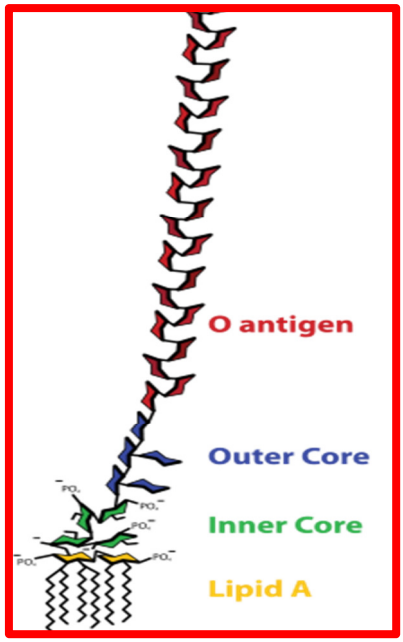
Figure 2: Mechanism of Oxidative Cellular Damage



I.5. LPS INDUCED NEURODEGENERATION

Lipopolysaccharides (LPS) are also recognized as lipoglycans and endotoxins. LPS (Figure 3) are big molecules comprising of a lipid A and a polysaccharide composed of O-antigen, outer core and inner core united by a covalent bond; they are found in the outer membrane of Gram-negative bacteria and provoke strong immune retorts in animals. Neuro-inflammation escorts and often leads to the development of neurodegenerative pathologies such as PD, AD and might be one of the pathogenic influences for neurodegeneration.[50,51]

Figure 3: Structure of Lipopolysaccharides (LPS)

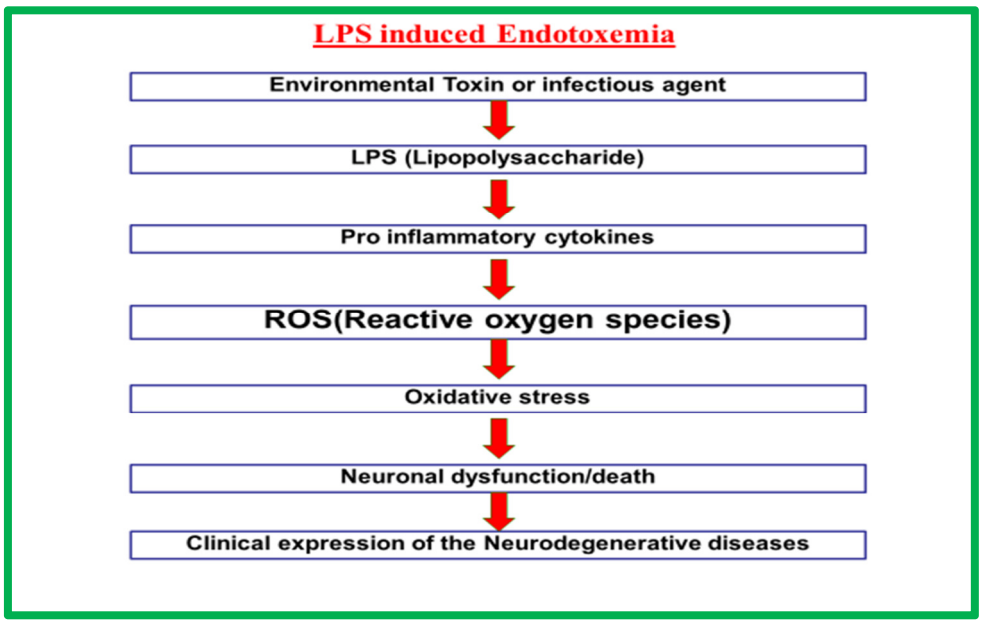


Regular injections of bacterial LPS reduced the density of α7 nAChRs in the brain and brain mitochondria and decreased nucleated cell numbers in the hippocampus and striatum, while inducing astrocytosis, accumulation of Aβ peptides and episodic memory decline-symptoms representative of the early stages of AD.[52] Antibodies are produced *in vivo* by immunization of mice with recombinant extracellular domain of α7 nAChR subunit, α7, facilitated symptoms like those persuaded by LPS but did not cause degeneration in the brain[52] indicating the involvement of specific regulatory processes.[53]

Acetylcholine was shown to weaken the release of pro-inflammatory cytokines, like IL-1 or TNFα, by peritoneal monocytes and macrophages in response to bacterial endotoxin-LPS through α7 nAChRs.[54] This process was first defined in 2000 and called "Cholinergic Anti-Inflammatory Pathway". It was further detected in many organs and tissues including in the brain.[55-61]

The present study was intended to disclose the molecular mechanisms fundamental to the LPS and antibody effects in the brain using a model of LPS-induced swelling. The diagrammatic representation of LPS-induced neurodegeneration pathway is cited in Figure 4.

Figure 4: Pathway of LPS Induced Endotoxemia



I.6. NEUROINFLAMMATION: BEHAVIOURAL CHALLENGES

I.6.a. ANXIETY AND DEPRESSION

Total lifetime incidence rate for anxiety disorders is 24.9%.[62] About 500 million individuals worldwide suffers from mental and behavioural syndromes.[63] Anxiety illnesses like depression are amongst the most predominant psychiatric complaints. Most anxiety ailments formerly appear during infantile and youth. Evidence demonstrates that a huge proportion of kids do not nurture out of their anxiety complaints during adolescence and adulthood.[64]

Anxiety disorders disturb one-eighth of the total population worldwide and have become a very significant area of research attention in psychopharmacology. Anxiety disorders are amid the most recurrent mental sicknesses encountered in medical practice.[61] This represent a varied group of illnesses possibly with no solitary unifying aetiology. Numerous psychodynamic, psychoanalytic, habitual, perceptive, hereditary and biological postulations have been predicted to clarify the aetiology and pathophysiology of anxiety disorders.[65] These are supposed to be biopsychosocial issues that leads to anxiety disorders.[66-68]

Depression is a common disorder of attitude rather than turbulences of mind or perception. It is the most communally sentimental disorder which is escorted by misconceptions and hallucination. In this ailment condition the neurotransmitters levels such as dopamine, acetylcholine, norepinephrine etc., in the brain are elevated. Two types of symptoms of this disease are (i) biological symptoms: obstruction of thought, loss of libido, sleep trouble and loss of appetite (ii) emotional symptoms: moods of guilt, loss of inspiration, ugliness etc. There are two types of depressive syndromes i.e., (i) unipolar depression: mood swings in the same direction; (ii) bipolar depression: depression alternates with mania.

The lifetime frequency of depression is as high as 20% in the global population with a 5:2 female to male ratio. Suicide is a significant danger for death in depression, and the rate of suicide is relatively high between 15 and 24 years of age.[69] Depression is a possible life -threatening disorder that disturbs hundreds of millions of people worldwide. Any age from childhood to late life can suffer due to depression. It is a heavy cost to society as it causes crucial distress and disturbance of life and can be fatal if left untreated. The psychopathological state includes a trio of indicators with low or depressed mood, anhedonia and low energy or fatigue. Other symptoms

such as sleep and psychomotor conflicts, feelings of guilt, low self-esteem, tendency to suicide as well as autonomic and gastrointestinal disorders are also often present.[70]

Behavioural malfunction can be observed in LPS-stimulated animal models together with raised pro-inflammatory cytokine levels and oxidative stress parameters in the hippocampus and other brain parts.[71-74]

I.6.b. MEMORY IMPAIREMENT

Many individuals in the world specifically the senior population suffer from different degrees of learning and memory losses. Although exact reasons have remained anonymous until now, inflammation and OS may at least in part be accountable for learning and memory discrepancies.[75] Neuroinflammation causes mental damage even if it is acutely stimulated by immune stimulatory constituent such as LPS[76]; and its chronic state complies to progression of ND including AD.[77] LPS stimulates a complex collection of behaviours known as "sickness behaviors"[78] and causes alterations in central processes involved in learning and memory.[79,80] In multiple animal models, vulnerability to immune system stimulating pathogens such as viral, bacterial or viral coat proteins and bacterial endotoxins outcomes in learning and memory discrepancies.[80]

LPS gained from the cell wall of gram-negative bacteria has been observed to promote the generation of inflammatory cytokines that in turn lead to surplus production of free radicals and OS. Intraperitoneal injection of LPS leads to neuroinflammation, learning and memory impairments, hippocampus apoptosis and cognitive deficits.[81] Researchers have stated that LPS elevates the induction of mediators such as NO, PGE2 and ROS by stimulating the macrophages.[82] LPS also rises the level of NO by sponsoring the release of pro-inflammatory cytokines such as TNF-α and IL-1β from macrophages and leucocytes.[83] In physiological concentrations, NO helps apoptosis and cell death in high concentrations through inducing the superoxide anion formation in the mitochondria[84] and affects learning and memory processes.[85]

Modifications in the pro-inflammatory cytokines and OS constraints may underwrite to the molecular basis of memory impairment and anxiety and depression-like behaviours.

I.7. ROLE OF ANTIOXIDANT ENZYMES

The antioxidant enzymes includes SOD, GPx and CAT that enable reactions that assist to catalyse the ROS to less toxic molecules[86] (Table 1), thereby playing a prominent role in preventing lipid peroxidation.[87, 88] In a normal state, free radicals are typically detoxified by several internal antioxidant enzymes to less toxic molecules which are then detached by various ways.[89] However, the normal antioxidant defence systems may not be capable to support irresistible production of free radicals and this could result in a lessening in the activity of these enzymes.[90] Hence, increased free radicals and OS in cells can conclude in damaging biological molecules including DNA, proteins and carbohydrates and lead to cell death.[91]

Abnormal protein accumulation epitomizes a unifying biochemical and histo-morphological seal of ND. In maximum cases, these protein collections form microscopically-visible cellular deposits called "inclusion bodies".[92] These include β-amyloid peptides and α-synuclein in PD, tau/phosphorylated tau proteins in AD, superoxide dismutase in amyotrophic lateral sclerosis and mutant huntingtin in HD.[92] It is supposed that the degenerative process is ongoing for many years before symptoms become obvious.[93]

Table 1: Function and Chemical Reactions of Antioxidant Enzymes

Antioxidant enzyme	Function	Chemical reaction
Superoxide dismutase	Catalyzing superoxide Anion to oxygen and hydrogen peroxide	$2O_2^{\cdot} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	Detoxifying hydrogen peroxide to water and oxygen molecule	$2H_2O_2 \rightarrow O_2 + 2H_2O$
Glutathione(GSH)	Electron donor to GDPs in reducing hydroperoxides to water molecules	$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$
Glutathione peroxidase	Reducing hydroperoxides to water molecules	$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$
Glutathione reductase	Catalyzing the reduction of glutathione di sulfide (GSSG) to the sulfhydryl form glutathione (GSH)	$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

$O2^{\cdot}$ (superoxide anion); H_2O_2 (hydrogen peroxide); O_2 (oxygen); H_2O (water molecule); GSSG (reduced glutathione); NADPH (nicotinamide adenine di nucleotide phosphate).

LPS disturbs the functions and chemical reactions of antioxidant enzymes which was improved by herbal medicines. Natural products are the best basis for the discovery of new drugs which were in custom even from the Vedic period. It is recognized that 80% drug molecules are natural products or natural compound inspired.[94] The examination of natural products as a source of innovative human therapeutics reached its top in the western pharmaceutical industry during 1970-1980s which lead to a pharmaceutical landscape heavily influenced by non-synthetic molecules.[95]

I.8. HERBAL MEDICINES OVER SYNTHETIC DRUGS

Most of the artificial drugs act in the brain to produce their ecstatic effects. However, they may also cause impairment due to seizures, stroke and direct toxic effects on brain cells. A brain disorder can also occur due to repeated drug use which leads to variations in the function of multiple brain circuits supervisory to stress, decision-making, pleasures, impulse control, memory, learning and other functions. These changes make it tougher for those with an addiction to practice pleasure in response to normal rewards such as food, positive social connections, etc. Most of the synthetic drugs for brain disorders are prescribed for a long-term use and side and adverse effects can be observed. There is a long list of synthetic drugs for brain disorders moving in the market, most of them with confirmed side effects on brain function or other organs of the body.[96]

Artificial drugs for human brain disorders are expensive symptomatic long treatments, exhibiting grave and inevitable side effects with poor patient agreement. Hence, the Ayurvedic and herbal treatments are favored over synthetic drugs for a series of human brain disorders including AD, PD, depression, epilepsy, schizophrenia, anxiety, etc. Ayurvedic system of medicine has conventionally been used in numerous neurological conditions. The negligible incidence of cost efficiency and side effects of plant products proposes significant boons. Much attention is drawn currently towards the recognized traditional systems of herbal medications for many brain disorders, providing positive hopes for the patients. Ayurvedic plants have capability to cure most of the psychological diseases as given in Table 2. Synthetic drugs, viz. phenytoin (PHT), diazepam, valproate (VPA) etc., are being promoted for the treatment of the epilepsy. Though these agents have new spectrum of efficacy but demonstrate alarming contrary effects.[10] On the other hand, the treatment of epilepsy with herbal medications as adjuvant seems to be more useful and is gaining more acceptance due to their negligible side effects.

Table 2: Potential Ayurvedic Plants for Psychological Ailments

S.No	Botanical name	Family	Major chemical constituents	Ayurvedic recommendations
1	*Acorus calamus*	Araceae	β-Asarone, α-Asarone	• Bark powder enhances memory and cures forgetfulness. • It is beneficial in anxiety and epilepsy when its powder is taken with honey. • Equal weights of its powder and "shunthi" powder (ginger) are recommended to cure facial paralysis.
2	*Allium cepa*	Liliaceae	Dialkenyl sulfides	• Tea from its seeds is beneficial in sleeplessness.
3	*Bacopa monnieri*	Plantaginaceae	Bacosides A, B, C	• Its juice is taken with "kuth" (Costus speciosus root) powder in honey to help in hysteria. • It is also recommended by adding "kuth" and "shankhapushpi" to cure epilepsy and hysteria. • It is very useful in the recovery of memory power.
4	*Brassica nigra*	Brassicaceae	Gallic acid, quercetin	• Its seeds and pigeon's droppings after grinding are applied on forehead. • It helps relieve migraine. • Its fresh oil when

				massaged reduces fatigue and laziness.
5	*Cannabis sativa Linn.*	Cannabinaceae	Flavonoid glycosides	• Its leaves along with asafoetida have been used for epilepsy type problem in women. • It is also useful in treating sleeplessness.
6	*Celastrus paniculatus*	Celastraceae	Asiaticosides	• Its seed powder is used in combination of almond, pepper and cardamom powder to improve memory.
7	*Datura metel*	Solanaceae	Hyoscine, Hyocyamine	• Its seeds are ground with black pepper and given for treating psychosis.
8	*Eclipta alba*	Asteraceae	Widelolactone Glycoside	• After mixing black pepper powder in its juice, it is applied on forehead for relief in migraine.
9	*Ficus benghalensis*	Moraceae	Bengalenosides Leucopelargonidin Glycoside	• Its root bark powder when taken in sugar and cow's milk, improves memory power.
10	*Ficus religiosa*	Moraceae	Kaempferol Sterols	• Extract of branches cures madness.
11	*Glycyrrhiza glabra*	Papilionaceae	Phenolics Glabridin	• Root powder in ghee brings improvements in epilepsy.
12	*Hibiscus rosasinensis*	Lythraceae	Cyanidin Quercetin	• Dried leaves and flowers are powdered together and

				given in sweet milk for improving memory power.
13	*Juglans regia*	Moringaceae	Fattyacids Linoleic Acid	• Walnut seeds are ground in "nirgundi" (Vitex negundo) juice and given as nasal drop for hysteria.
14	*Moringa oleifera*	Fabaceae	Moringine Moringinine	• After grinding the bark, the liquid is squeezed and put into the nostrils or given orally as drink to cure meningitis. • Decoction of its roots is given for epilepsy and hysteria in women.
15	*Mucuna pruriens*	Valerianaceae	L-DOPA Amines Alkaloids	• In Ayurveda, it has been described for use in several illnesses and overall body strength. • Scientifically it has also been found to be effective in Parkinson's disease.
16	*Papaver somniferum*	Papaveraceae	Morphine, Codeine, Thebaine, Papaverine	• Poppy is beneficial in delirium, sleeplessness, convulsion, etc.
17	*Piper nigrum*	Piperaceae	Piperine and related alkaloids	• On empty stomach, pepper powder and "bach" are given to treat hysteria.
18	*Sphaeranthus indicus*	Asteraceae	Sterols Sesquiterpenoids	• It and clove powders are

				given in honey to cure Parkinson's disease.
19	*Sesbania grandiflora*	Fabaceae	Leucocyanidin Cyanidin Triterpenoids	• Sesbane leaves and black pepper are ground in cow urine and made to inhale. • It brings immediate relief from epilepsy.
20	*Solanum surratense*	Solanaceae	Solasodine Solasonine	• Its roots and poppy seeds are grinded in child's urine and put in the nose to be relieved from epilepsy.
21	*Syzygium aromaticum*	Myrtaceae	Carvacrol Thymol Eugenol	• Cloves are grinded in water and the paste is applied on the earlobes to cure migraine.
22	*Terminalia chebula*	Combretaceae	Ethyl gallate Luteolin	• Seeds are grinded in warm water and applied on forehead for relief in migraine.
23	*Vitex negundo*	Verbenaceae	Negundoside	• The powder of its fruits is given in mental disorder.

Lately the world is precisely looking towards brain curative properties of traditional drugs, including Ayurveda, for a reliable cure with least side effects. Numerous Indian medicinal floras has been widely used in the Indian traditional system of medicine for the treatment of ND due to

- **Potent pharmacological activity**
- **Low toxicity and less time**
- **Economic viability and renewable sources**
- **Long history of use, better patient tolerance, public acceptances**

- **Cultivation and processing conditions-environmental friendly**
- **Avoid Environmental pollution by the chemical industry**

Amongst widely used medicinal herbs in indigenous systems of medicine is *Pedalium murex* Linn. (*P. murex*) which belongs to *Pedaliaceae* family. These leaves are a good source of natural antioxidant and extensively consumed as vegetables due to its high nutritional values.[97] Epidemiological researches have recommended optimistic associations amid the consumption of phenolic-rich foods or beverages and the prevention of diseases. These effects have been accredited by antioxidant components such as plant phenolics, phenylpropanoids and flavonoids midst of others.[98] *P. murex* Linn. possesses many of the curative properties with other rasayana and its neuroprotective effect in diverse neurodegenerative models is controversial and yet to be recognized. Thus, an effort has been made to estimate the neuroprotective effect of ethanol extract of *P. murex* Linn. leaves against LPS-induced endotoxemia in rats.

Aims & objectives

II. AIMS AND OBJECTIVES

Pedalium murex Linn. (*P. murex*) which belongs to the family *Pedaliaceae* and commonly known as "*anai nerinji*" is one of the most frequently used medicinal herbs in indigenous systems of medicine. Epidemiological studies have proposed positive links between the consumption of phenolic-rich foods or beverages and the prevention of diseases. These effects have been attributed to antioxidant components such as plant phenolics, flavonoids and phenylpropanoids among others. *P.murex* contain a varied array of essential oils rich in phenolic compounds and a wide range of other natural products including polyphenols such as flavonoids and anthocyanins.

Due to the pervasiveness, morbidity and mortality of the neurodegenerative diseases and they embody significant medical, social and financial burden on the society. The etiology of neurodegenerative diseases remains enigmatic; however, defects in energy metabolism, excitotoxicity and for oxidative damage is increasingly compelling, it is likely that there is a complex chemistry between these mechanisms. Despite the great number of ongoing researches, neurodegenerative diseases remain incurable.

Rasayans and adaptogens have been stated to improve physical and mental health, increase the nonspecific resistance of body and also promote physiological functioning and cognition. *P.murex* shares all the medicinal properties with other rasayans in the group and its neuroprotective effect in different neurodegenerative models is debatable. At present the pharmacological therapy of neurodegenerative disorders are limited to symptomatic treatments that do not alter the course of the underlying disease. Hence, the attempt has been made to evaluate the neuroprotective effect of *P. murex* against lipopolysaccharide (LPS) - induced neurotoxicity models in rats.

Furthermore, the analysis of *in vitro* herbal antioxidant, reductive index, free radical scavenging ability and estimation of phytochemicals in *P. murex* and the *in vivo* examination of brain biochemical markers and histopathological study of the hippocampus of the brain may help in understanding the mechanism of its neuroprotective activity.

OBJECTIVE

To contribute further to the knowledge of Indian traditional plants and it's sacred and rich history, the objective of the present study will subject the traditionally well-known leaves of *P. murex* in hot continuous extraction using n-hexane, chloroform, ethyl acetate, 90% v/v ethanol in soxhlet apparatus and the dried extract will be used to evaluate the possible neuroprotective effect of *P. murex* against LPS-induced endotoxemia in rats.

The work is designed to be carried out in the following phases:

PHASE I: PHYTOCHEMICAL STUDIES

1) Phytochemical studies

- **a)** Collection and authentication of leaves of *P. murex*.
- **b)** Extraction of leaves of *P. murex*.
- **c)** Preliminary phytochemical tests of extracts of *P. murex*.

2) *In vitro* antioxidant and free radical scavenging studies

The following *in vitro* antioxidant and free radical scavenging activities of *P. murex* extracts will be determined spectrophotometrically.

- **a)** Total antioxidant activity
- **b)** DPPH radical scavenging activity
- **c)** Nitric oxide radical scavenging activity
- **d)** Superoxide anion radical scavenging activity
- **e)** Hydroxyl radical scavenging activity
- **f)** Reducing power

3) Isolation and characterization studies

- **a)** Isolation of plant constituents by thin layer and column chromatography.
- **b)** Characterization of isolated compounds by IR, ^{1}H NMR, ^{13}C NMR and Mass spectral studies.
- **c)** High performance thin layer chromatography (HPTLC).

4) Quantitative estimation of phyto-constituents

- **a)** Estimation of phyto-constituents.

PHASE II: PHARMACOLOGICAL STUDIES

1) Pharmacological Studies

a) Acute toxicity study
b) Evaluation of neuroprotective activity using the following model.
i) LPS induced endotoxemia.

2) Behavioral studies

a) General behavioral studies

- Changes in body weight
- Changes in food and water intake

b) Behavioral tests for anxiety and depression

- Open-field test
- Elevated plus maze test
- Forced swim test

c) Behavioral tests for learning and memory

- Water maze test
- Radial arm maze test
- Choice reaction task test

PHASE III: BIOCHEMICAL ANALYSIS

Biochemical analysis of the following in the brain hippocampus

a) ***In vivo*** **non- enzymatic antioxidant studies in brain hippocampus**

- Estimation of acetylcholine
- Estimation of nitric oxide
- Estimation of protein
- Estimation of lipid peroxide and malonaldehyde

b) ***In vivo*** **brain hippocampal enzymatic antioxidant studies**

- Estimation of acetyl cholinesterase
- Estimation of superoxide dismutase (SOD)
- Estimation of catalase (CAT)
- Estimation of glutathione reductase (GR)
- Estimation of glutathione peroxidase (GPx)

PHASE IV: HISTOPATHOLOGICAL EXAMINATIONS

The hippocampal and/or other regions of rat brain will be subjected to histopathological analysis.

Review of Literature

III. REVIEW OF LITERATURE

III. a. 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	:	Large Caltrops
Hindi	:	Bada Gokshur
Sanskrit	:	Brihat Gokshur
Kannada	:	Ane Neggilu
Malayalam	:	Ana Nerinnil
Oriya	:	Gokara
Marathi	:	Gokharu
Gujarati	:	Kadva Gokhru

Habitat:

It is distributed geographically in tropical Africa, Ceylon, India, Mexico and Pakistan. It is a common herb which grows throughout India, but it is found commonly along the western and coromandal coasts as a weed of waste places. It is also found in Delhi, Rajasthan, Punjab, Tamil Nadu, Gujarat and Deccan peninsula.

Description:

Large Caltrops is a shrubby, stiff-stemmed herb native to India and grown for reputed medicinal and other uses. It is diffused, annual, much branched, spreading, succulent and glandular up to 60cm in height. Roots are like turmeric in colour. Leaves are simple, opposite, ovate or oblong-obovate, 1-4.5cm long, irregularly and coarsely crenate-serrate. Yellow flowers 1.5-2 cm across, stalk 1-2 mm long, increasing up to 4mm in fruit. Sepals are 2 mm long; Teeth linear, scaly outside, persistent. Petals are fused into a broad tube, 1-3 cm long; lobes obtuse. Stamens 0.5-1cm long; anthers are kidney shaped. The four-angled seed consist of 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 and Dysurea infections.
- Leaves are antibilious. Seeds are demulcent, diuretic, tonic, mucilaginous and aphrodisiac. Used in male impotence, gonorrhea and incontinence.

III.b. PUBLISHED LITERATURES OF *PEDALIUM MUREX*

1. Patel PK *et al.,* (2016) studied the antiurolithiatic effect of *Pedalium murex* fruit extract in ethylene glycol-induced nephrolithiasis in rats. Ethanolic extract of *Pedalium murex* Linn. showed significant improvement in renal function and kidney weight in prophylactic groups as compared to ethylene glycol controls. No effects were shown in urinary oxalate, urine volume and any other serological parameters. Calcium oxalate crystallization was significantly reduced in all the *P. murex* treated groups ($P < 0.05$). Calcium oxalate and phosphate mineralization were also inhibited by 33% and 57% respectively. Ethanolic extract of *P. murex* fruits possessed significant activity for prevention of renal calculi.[99]

2. Vaya Rajkumar *et al.,* (2016) had studied antiulcer activity of fresh juice of the leaves of *Pedalium murex* Linn. The results obtained from the study showed that fresh juice of the leaves of *P. murex* possessed anti-ulcer effect on ethanol induced ulcers. In ethanol stress induced model, there was a decrease in ulcer index, total acidity, total volume of gastric secretion, total protein and an increase in glutathione content and pH of gastric secretion when compared with control. In the study famotidine was used as a standard. Therefore, the ethanolic extract of leaves of *Pedalium murex* was regarded as a favorable antiulcerogen.[100]

3. Sarada K *et al.,* (2016) had designated *in vitro* antioxidant potential of ethanol extract of *Pedalium murex* Linn. (Pedaliaceae). The study was compared with standard. The data suggested that *Pedalium murex* Linn. possessed high scavenging activity and had the property to modulate endogenous oxidative stress and was effective nutraceutical to abrogate oxidative aims at phytochemical characterization and was used to investigate the *in vitro* antioxidant activity of ethanol extract of mangrove associated plant *Pedalium murex* Linn. using DPPH model. Total phenolic and flavonoid contents were also determined. A significant correlation existed between the concentration of the extract and percentage of inhibition of free radicals. The extract showed potent DPPH radical activity with IC_{50} values 81.75 stress in the body.[101]

4. Prabhakaran D *et al.,* (2016) had investigated antimicrobial activity of *Pedalium murex* Linn. (Flowers). The purpose of the study was to examine the antimicrobial effect of the sample isolated from the ethyl acetate fraction of flowers of *Pedalium*

murex Linn. This compound was shown to possess antimicrobial activity against bacteria and fungi. Six bacterial strains such as *Salmonella typhi, Escherichia coli, Enterococcus faecalis, Bacillus cereus, Bacillus substilis, Lacto bacillus* and two fungal strains *Curvularia lunata and Candida albicans* used for study by disc diffusion method. The antibacterial activity of the compound, isolated from ethyl acetate fraction was almost comparable with the standard solvent control Chloramphenicol. The antifungal activity was almost comparable with the standard solvent control Fluconazole. From this study, it was concluded that *Pedalium murex* Linn. (flowers) revealed antimicrobial activity against various human pathogenic bacteria.[102]

5. Buvanaratchagan *et al.,* (2016) had evaluated antibacterial activity of *Pedalium murex* Linn. fruit and its influence on dermatological infections. The study was undertaken to evaluate the anti-microbial activity of various extracts (petroleum ether, acetone, methanol and water) of *Pedalium murex* Linn. against some gram positive and negative organisms which caused dermatological infections. Methanolic and acetone extract of *Pedalium murex* Linn. showed significant antimicrobial activity.[103]

6. Anandalakshmi K *et al.,* (2015) had studied characterization of silver nanoparticles by green synthesis method using *Pedalium murex* Linn. leaf extract and their antibacterial activity. In this study, an aqueous extract of fresh leaves of *Pedalium murex* Linn. was used for the synthesis of silver (Ag) nanoparticles. Different biological methods gained recognition to produce silver nanoparticles (AgNPs) due to their multiple applications. The use of plants in the green synthesis of nanoparticles emerged as a cost-effective and eco-friendly approach. Characterization of nanoparticles were done using different methods which included ultraviolet–visible spectroscopy (UV–Vis), Fourier transform infrared (FTIR), powder X-ray diffraction (XRD), field emission scanning electron microscope (FE-SEM), energy dispersive X-ray analysis (EDAX), fluorescence emission spectroscopy, transmission electron microscope (TEM), dynamic light scattering (DLS), zeta potential and anti-bacterial activity. UV–visible spectrum of the aqueous medium contained silver nanoparticles which showed absorption peak at around 430 nm. Fourier transform infrared spectra had shown that the biomolecule compounds were responsible for the reduction and capping material

of silver nanoparticles. XRD study showed that the particles were crystalline in nature, with a face-centered cubic (fcc) structure. The size and stability were detected using DLS and zeta potential analysis. The anti-bacterial activity of AgNPs against generally found bacteria was assessed to find their potential use in silver containing antibacterial product.[104]

7. Ajit N Solanki *et al.,* (2015) had described antihepatotoxic effect of ethanolic extract of *Pedalium murex* Linn. fruits. The fruit powder of *P. murex* was extracted with 20% ethanol. Acute oral toxicity study of ethanolic extract of fruits of *P. murex* was performed on female Swiss albino mice. Antihepatotoxic effect of ethanolic extract of fruits of *P. murex* was assessed in ethanol induced hepatotoxic rats. LD_{50} value of extract was found more than 5 gm/kg on the basis of acute toxicity study. Administration of ethanol (3.76gm/kg, bid, po) for 25 days produced significant changes in biochemical (increase in serum alanine transaminase, aspartate transaminase, total bilirubin, cholesterol, triglyceride) and histological (damage to hepatocytes) parameters of liver as well as endogenous antioxidant enzymes (SOD, GSH), reflecting liver damage. Pre-treatment with ethanolic extract of fruits of *P. murex* (200 and 400 mg/kg, p.o) 1 hr before ethanol administration for 25 days significantly improved the biochemical, histological changes and endogenous antioxidant enzyme levels induced by ethanol. Results suggested that the ethanolic extract of fruits of *P. murex* protected the livers of rats against ethanol induced hepatic damage.[105]

8. Muhammad Imran *et al.,* (2015) had reviewed phytochemical and pharmacological potentials of *Pedalium murex* Linn. and its traditional medicinal uses. The aim of the study was to assess the pharmacological and phytochemical aspects of the *Pedalium murex* Linn. and its traditional medicinal uses from different parts of the plant. Flavonoids, phenolic compounds, glycosides, carbohydrates, reducing sugars, phytosterols, tannins, triterpenoids, alkaloids, xanthoproteins, aromatic oil, stable oil, saponins and resins are the main phytochemical groups that was found in different chemical extracts of *P. murex*. Pharmacological activities of *P. murex* proved its importance for medicinal uses.[106]

9. Dhivya M *et al.,* (2015) had identified antioxidant activity and immune modulatory activity of *Pedalium murex* Linn. The study was carried out for the characterization, physical, chemical, microbial and immunological strength of the development of immune system for human era. The phytochemical compounds were analysed. Twelve male Wister albino rats (200-220 gm) two months of age were used as experimental animals and were divided into four groups. In this animal model studies assessment of immune modulatory activity was carried out by various haematological and serological tests like determination of phagocytic activity and spleen weight. The study presented that different doses (100, 200 mg/kg, b.w/day) of the ethanolic extract of the fruits showed significant activity and increased phagocytic response and spleen weight. The results were compared with control and standard drug (septilin). The activity reported was dose dependent and set of data were analysed using one-way analysis of variance (ANOVA) followed by Dunnett's test multiple comparison test values **P.[107]

10. Abirami P and Rajendran A, (2015) had investigated the evaluation of antidermatophytic activity of *Pedalium murex* Linn. In the investigation, a different solvent extracts of *Pedalium murex* Linn. were screened against two dermatophytes viz., *Trichophyton rubrum* and *Microsporum gypseum.* The prominent zone of inhibition was observed in methanol and petroleum ether extract of *Pedalium murex* Linn. against in *Microsporum gypseum* at 200 µg/mL concentration. The distilled water and methanol extracts of *Pedalium murex* Linn. showed significant activity against *Trichophyton rubrum*. The standard drugs griseofulvin, fluconazole and ketoconazole were used as the positive control and zones of inhibition were from 10 to 20 mm.[108]

11. Vedhi C *et al.,* (2015) had reported biosynthesis of gold nanoparticles using cold and hot water extract of *Pedalium murex* Linn. leaf. Biosynthesis of gold nanoparticles was done using cold and hot water extract of *Pedalium murex* Linn. leaf. Prepared gold nanoparticle was analysed by UV-visible spectroscopy, cyclic voltammetry, XRD, SEM and TEM. UV studies showed well-built surface plasmon resonance absorption peak at 540 nm. The band gap energy of 2.95 eV and 2.90 eV was achieved for gold nanoparticles prepared by cold and hot water extract respectively. The cyclic voltammetric behaviour of both types of nanoparticles was studied at different pH. The XRD spectra for deposited thin

film samples confirmed the crystalline nature and highly stable gold nanoparticles. SEM image showed that the nanoparticles were semi-spherical, and their sizes were controlled within the range of 180 nm to 200 nm. Hexagonal, triangular, and spherical nanoparticles were seen in the transmission electron micrographs for both types of particles. The selected-area electron diffraction patterns revealed that the sample is semi crystalline in nature.[109]

12. Anandanayaki S and Uma C, (2014) had reported antimicrobial effect of medicinal plant evaluation of antidermatophytic activity of *Pedalium murex* Linn. against different microorganisms. *Pedalium murex* Linn. showed broad spectrum antimicrobial activity against three fungal strains *Aspergillus niger, Aspergillus flavus, Candida albicans* and five bacterial strains *Escherichia coli, Staphylococus epidermis, Klebseilla pneumonia, Citrobactor diverses, Enterococus faecalis*. The ethanolic extracts were tested against selected test bacteria and fungi through disc diffusion assay where amoxicillin was used as standard. The results showed that alcoholic extract possess good antimicrobial activity against selected test bacteria and fungi. The results offered a scientific basis for traditional use of the various extract of *Pedalium murex.*[110]

13. Shanmuga Sundaram R *et al.,* (2014) had reported evaluation of potential central protective role of ethanol extract of evaluation of antidermatophytic activity of *Pedalium murex* Linn. in acute and chronic unpredictable stress induced models in SD rats. Animals were divided into five groups each for AS and CUS models. The animals were subjected to AS (immobility-induced stress) and CUS paradigms (immobility + forced swimming + dark phase) for 10 days followed by a battery of behavioural and biochemical analyses. The changes in food and water intake, body weight and general behaviour were measured for 4 weeks. In addition, the effect of EEPM on the antioxidant enzyme systems [superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH)] in whole brain of animals, *in vitro* antioxidant and free radical scavenging activities were also screened. Results: AS and CUS induced anxiety, depression and impairment of cognition and memory. Pre-treatment with EEPM (200 mg/kg and 400 mg/kg p.o.) for 30 days, significantly reduced stress-induced anxiety and related mood disorders. The normalization of SOD, CAT and GSH levels further substantiated the protective

role of EEPM. The herb was found to be effective in preventing CUS than the AS model.[111]

14. Thangadurai Chitra *et al.,* (2013) had revealed laboratory and field efficacy of *Pedalium murex Linn.* and predatory copepod, *Mesocyclops longisetus* on rural malaria vector, *Anopheles culicifacies*. To test the potentiality of the leaf extract of *Pedalium murex Linn.* 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 Linn.* and *M. longisetus*.[112]

15. Siva V *et al.,* (2012) had determined evaluation of antipyretic activity of *Pedalium murex* Linn. against brewer's yeast-induced pyrexia in rats. The aqueous and ethanolic extracts of *Pedalium murex* Linn. (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 b.w p.o) produced a significant ($p<0.05$) dose dependent inhibition of temperature elevation compared with the standard drug paracetamol (150 mg/kg b.w). At doses of 200 mg/kg b.w, the aqueous extract significantly ($P<0.001$) decreased yeast induced pyrexia in rats. These results indicated that leaf extracts of *Pedalium murex* Linn. possessed potent antipyretic effects and thus pharmacologically justifying its folkloric use in the management of fever.[113]

16. Sharma V *et al.,* (2012) had analysed a comparative study of ethanolic extracts of *Pedalium murex* Linn. fruits and sildenafil citrate on sexual behaviours and serum testosterone level in male rats during and after treatment. Findings provided experimental *in vivo* and *in vitro* evidence that the ethanolic extract of *Pedalium murex* Linn. fruits possessed aphrodisiac property. Studies lend growing support for the traditional use of *Pedalium murex* Linn. as a sexual stimulating agent and offer a significant potential for studying the effect on male sexual response and its

dysfunctions. The findings justified the concept of rasayana as rejuvenate tonics and support their role in prevention or delay of the aging process.[114]

17. Patel DK *et al.,* (2012) had reported aphrodisiac activity of ethanolic extract of *Pedalium murex* Linn. fruit**.** The study represented 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 up to 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 behaviour pattern. The increased pregnancy rate in the drug treated groups was due to the healthy viable sperm and enhancement of sexual desire of the rats.[115]

18. Patel K *et al.,* (2012) had enumerated aldose reductase inhibitory activity of alcoholic extract of *Pedalium murex* Linn. fruit. Physicochemical investigation of ethanolic extract of fruit of the *Pedalium murex* Linn. (EEPM) was carried out. EEPM was screened for aldose reductase inhibitory activity using rat lens AR enzyme. HPTLC fingerprinting analysis of EEPM was performed in chloroform: methanol (8:2) solvent system.[116]

19. Methekar Chandrika *et al.,* (2012) had scrutinized a comparative antitussive activity of *laghu gokshura Tribulusterrestris* Linn. and *brihat gokshura* [*Pedalium murex* Linn.] *panchanga* in Swiss albino mice. It was used in the treatment of *Kasha*, in the present study a comparative antitussive 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 (2mg/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* had shown moderate antitussive activity, among them *Brihat Gokshura* was found to be better. Hence, non-availability of root samples of these plants and to prevent destructive harvesting, whole plants can be used in the treatment of *Kasa*.[117]

20. Rajashekar V *et al.,* (2012) had studied biological activities and medicinal properties of gokhru (*Pedalium murex* Linn.) and Bada Gokhru (*Pedalium murex Linn.*) 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 gave a bird's eye view mainly on the biological activities of some of this compound isolated, pharmacological actions of the extracts, clinical studies and plausible medicinal applications of gokhru along with their safety evaluation.[118]

21. Muruganantham Sermakkani *et al.,* (2011) had explored evaluation of phytochemical and antibacterial activity of *Pedalium murex* Linn. root. In this study, petroleum ether, chloroform, acetone and methanolic extract of Pedalium *murex* L. root was subjected to preliminary phytochemical studies 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 pyogenes* and *Enterococcus faecalis* than the gram-negative bacteria.[119]

22. Kivalina 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. Botanically 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. was commonly used and the drug was frequently substituted. Pharmacognostical study had been carried out to identify the distinguishing features for both morphological and microscopic of the fruits of *Tribulus terrestris* Linn and *Pedalium murex* Linn. This knowledge helped to reduce the problem of substitution of the genuine drug.[120]

23. Patel D K *et al.,* (2011) had outlined *Pedalium murex* Linn.: an overview of its phytopharmacological aspects. Different parts of the plant were 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 included 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 had been investigated for antiulcerogenic, nephroprotective, hypolipidemic, aphrodisiac, antioxidant, antimicrobial and insecticidal activities. From all these reports it was concluded that the plant was found to have a better profile with potential natural source for the treatment of various range of either acute or chronic disease.[121]

24. Thamizhmozhi 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 material medica (krithikar and basu) studies were 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.[122]

25. Jalaram H *et al.,* (2011) had elucidated *in vitro* antioxidant activity of aqueous fruit extract of *Pedalium murex***.** To understand the mechanisms of pharmacological actions, the *in vitro* antioxidant activity of aqueous extract of fruits of *Pedalium murex* Linn. 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.[123]

26. Srinivas P *et al.,* (2011) had evaluated antioxidant activity of *Pedalium murex* Linn. 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.[124]

27. Patel D K *et al.,* (2011) had estimated *Pedalium murex* Linn. fruits: a comparative antioxidant activity of its different fractions. Here the antioxidant activities of *Pedalium murex* Linn. 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.[125]

28. Banji D *et al.,* (2010) had composed scrutinizing the aqueous extract of leaves of *Pedalium murex* for the antiulcer activity in rats. The antiulcer efficacy of the aqueous extract of leaves of *Pedalium murex* Linn. on ethanol induced gastric lesions was investigated. This has been substantiated by ascertaining the content of total acid, acid volume, total protein, ulcer index and glutathione. Ulceration was induced in 36 hrs fasted rats by the administration of 80% ethanol (1mL/kg) orally. The reference standard (famotidine, 3 mg/kg) and aqueous extract of leaves of *Pedalium murex* Linn. in doses of 50, 100, 200 mg/kg was given to different groups, one hr 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. Pre-treatment with aqueous extract of leaves of *Pedalium murex* particularly at a dose of 200mg/kg in a single schedule and 100mg/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* Linn. was regarded as a favourable anti-ulcerogen which could be attributed to its content of flavonoids and mucilage.[126]

29. Mukundh N *et al.,* (2008) had assessed antihyperlipidemic activity of *Pedalium murex* Linn. fruits on high fat diet fed rats. The main objective of the study was to investigate about the antihyperlipidemic potential of the ethanolic extract from the fruits of *Pedalium murex* Linn. at doses of 200 and 400 mg/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.[127]

30. 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* Linn. 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 anti-feedant activity of this plant. Qualitative analysis of *Pedalium murex* Linn. root extract revealed that it contains phytochemical such as steroids, terpenoids, phenolics, saponines, tannins and flavanoids. Phenol, 2-(5,6-dimethyl pyridinyl) 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* Linn. by using GC-MS. *Pedalium murex* Linn. impact was more than the neem based bio pesticide neem gold. Hence this plant was explored as biopesticidal plant soon.[128]

31. Bhakuni R S *et al.,* (1992) had computed flavonoids and other constituents from *Pedalium murex* Linn. Two new compounds isolated from the fruits of *Pedalium murex* Linn. were characterized as 2′,4′,5′-trihydroxy-5,7-dimethoxyflavone and triacontanyldotriacontanoate by physico-chemical methods. Luteolin, rubusic acid, nonacosane, tritriacontane, triacontanoic acid, tritriacontanoic acid and sitosterol-β-d-glucoside had also been isolated and identified.[98]

32. Shukla Y N *et al.,* (1983) had worked on heptatriacontan, tetratriacontanyl octacosanoate and other constituents from *Pedalium murex* Linn. Two new

compounds isolated from the fruits of *Pedalium murex* Linn. were characterized as heptatriacontan-4-one and tetra triacontanyl octa cosanoate by spectral studies. Pentatriacontane, sitosterol, hexatriacontanoic acid, hentriacontanoic acid, ursolic acid and vanillin had also been isolated and identified.[129]

Materials & Methods

IV. MATERIALS AND METHODS

PHASE-I: PHYTOCHEMICAL STUDIES

IV.1. Collection of Plant

The leaves of *Pedalium murex* Linn. was collected from surrounding areas of Komarapalayam & Amaravathi Nagar, Namakkal District, Tamilnadu, India.

IV.2. 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/1934) and the specimen was preserved in Pharmacognosy Lab, JKKNCP.

IV.3. Extraction Procedure

The leaves of *Pedalium murex* Linn. were precisely washed with tap water and dried underneath dimness in room temperature for one week. Then they were crushed into powder and stored in room temperature. The pulverized materials were passed through sieve no. 40 and 80. The crushed materials of identical size present between those two sieves were collected and packed in an airtight container for supplementary use. About 2kg of shaded and dried plant leaves of *Pedalium murex* Linn. was extracted in soxhlet successively with n-hexane, chloroform, ethyl acetate and 90% v/v ethanol. Each extract was evaporated using rotary vacuum evaporator. The extract obtained with each solvent was weighed and the percentage yield was calculated in terms of dried weight of the plant leaves. The consistency and color of the extract were noted. All the solvents used for this work were of analytical grade.

IV.4. Preliminary Phytochemical Analysis

All the four extracts of *Pedalium murex* Linn. leaves were subjected to qualitative tests for the identification of various plant constituents.[130-133]

IV.4.1. Test for alkaloids

a) **Dragendorff's test:** 1mL of the extract was added with 1mL of dragendorff's reagent (potassium bismuth iodide solution). An orange red precipitate specified the presence of alkaloids.

b) **Mayer's test:** 1mL of the extract was added with 1mL of Mayer's reagent (potassium mercuric iodide solution). Whitish yellow coloured precipitate designated the presence of alkaloids.

c) **Hager's test:** 1mL of the extract was added with 3mL of Hager's reagent (saturated aqueous solution of picric acid) yellow coloured precipitate indicated the presence of alkaloids.

d) **Wagner's test:** 1mL of the extract was added with 2mL of Wagner's reagent (Iodide in potassium Iodide) formation of reddish brown precipitate suggested the presence of alkaloids.

e) **Tannic acid test:** 1mL of the extract was added with 1mL of 10% tannic acid solution, buff colour indicated the existence of alkaloids.

IV.4.2. Test for Saponins

a) **Foam test:** The extract was diluted with 20mL of distilled water and was shaken in a graduated cylinder for 15min lengthwise. A 1cm layer of foam indicated the presence of Saponins.

b) **Lead acetate test:** 1mL of sample solution was treated with 1% lead acetate solution formation of white precipitate indicated the presence of saponins.

c) **Hemolytic test:** The extract or dry powder was added to one drop of blood which was placed on glass slide. Appearance of hemolytic zone showed the presence of saponins.

IV.4.3. Test for glycosides

a) **Legal's test:** The extract was dissolved in pyridine and sodium nitroprusside solution was added to make it alkaline. The formation from pinkish red to red colour showed the presence of glycosides.

b) **Baljet test:** 1mL of the test extract was added with 1mL of sodium picrate solution and the colour change from yellow to orange colour showed the presence of glycosides.

c) **Keller-Killiani test:** The ethanolic extract of 0.5mL of strong solution of lead acetate was added and filtered. The filtrate was shaken with 5mL of chloroform. The chloroform layer was separated in a porcelain dish and the solvent was removed by gentle evaporation. The cool residue was dissolved in 3 mL of glacial acetic acid containing two drops of ferric chloride

solution. This solution was carefully transferred to the surface of 2mL of concentrated sulphuric acid. A reddish brown layer was formed at the junction of the two liquids and the upper layer slowly became bluish green, darkening upon standing.

d) **Borntrager's test:** A few mL of dilute sulphuric acid was added to 1mL of the extract solution. The filtrate was boiled, filtered and extracted with chloroform and the chloroform layer was treated with 1mL of ammonia. The formation of red colour of the ammonical layer showed the presence of anthraquinone glycosides.

IV.4.4. Test for carbohydrates and sugars

a) **Molisch's test:** 2mL of the extract was added with 1mL of α- napthol solution and concentrated sulphuric acid along the sides of the test tube. Reddish violet colour at the junction of the two liquids indicated the presence of carbohydrates.

b) **Fehling's test:** 1mL of the extract was added with equal quantities of Fehling's solution A and B and upon heating formation of a brick red precipitate indicated the presence of reducing sugars.

c) **Benedict's test:** 1mL of extract was added with 5 mL of Benedict's reagent and boiled for 2 min and cooled. Formation of red precipitate showed the presence of sugars.

d) **Tollen's test:** 1mL of extract was added with 2mL of tollen's reagent and boiled. A silver mirror was obtained inside the wall of the tube which indicated the presence of aldose sugar.

e) **Seliwanoff's test:** The extract was treated with hydrochloric acid and resorcinol which was then heated. The formation of red colour showed the presence of glucose.

f) **Bromine water test:** To little quantity of test extract bromine water was added. Bromine water decolorization indicated the presence of aldose sugar.

IV.4.5. Test for tannins

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

b) **Ferric chloride test:** 1mL of extract was added with 1mL ferric chloride solution, formation of dark blue or greenish black product showed the presence of tannins.

c) **Vanillin hydrochloride test:** 1mL of extract was added with vanillin hydrochloride. Formation of purplish red colour indicated the presence of tannins.

d) **Lead acetate test:** A little quantity of test solution was taken and mixed with basic lead acetate solution. Formation of white precipitate indicated the presence of tannins.

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

f) P**otassium dichromate Test**: The sample solution was treated with 1mL of 10% Potassium dichromate solution and yellowish-brown precipitate was produced which indicated the presence of tannins.

IV.4.6. Test for flavonoids

a) **Shinoda's test:** To the extract solution few fragments of magnesium ribbon and concentrated HCL was added drop wise which gave cherry red colour appearance after few min. It showed the presence of flavonoids.

b) **Alkaline reagent test:** The extract was treated with sodium hydroxide, formation of yellow colour indicated the presence of flavonoids.

c) When a little quantity of extract was treated with lead acetate a yellow colour solution was formed which disappeared on addition of acid. It indicated the presence of flavonoids.

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

IV.4.7. Test for steroids

a) Libermann-Burchard's test: Chloroform solution, 1-2mL of acetic anhydride and 2 drops of concentrated sulphuric acid along the sides of the test tube was added to 2mL of extract. Appearance of bluish-green colour showed the presence of steroids.

b) Salkowsky's test: The extract was dissolved in chloroform solution and 2mL of concentrated sulphuric acid was added. Chloroform layer appeared red color which indicated the presence of steroids.

IV.4.8. Test for Proteins and Amino Acids

a) Biuret test: 1mL of the extract was treated with 4% NaOH and few drops of $CuSO_4$ solution, Formation of purple violet colour indicated the presence of proteins.

b) Ninhydrin test: 1mL of the extract was treated with 3 drops of 5% ninhydrin solution in boiling water bath for 10 min. The appearance of purplish or bluish colour indicated the presence of proteins, peptides or amino acid.

c) Xanthoproteic test: When 1mL of the extract was treated with 1mL of concentrated nitric acid a white precipitate was formed. It was boiled and cooled. And 20% of sodium hydroxide or ammonia was added. Orange colour indicated the presence of amino acids.

d) Millon's test: 1mL of the extract was treated with millon's reagent (mercuric nitrate in HNO_3). A white precipitate turned to brick red indicated the presence of proteins.

IV.4.9. Test for Triterpenoids

a) Knoller's test 2 or 3 granules of tin metal were dissolved in 2mL of thionyl chloride solution. Then one mL of the extract was added into the test tube and warmed, the formation of pink colour indicated the presence of Triterpenoids.

IV.4.10. Test for fixed oils and fats

a) Spot test: A small quantity of extract was pressed between two filter papers. The stain on the filter paper indicated the presence of fixed oils.

b) Saponification test: A few drops of 0.5N of alcoholic potassium hydroxide was added to small quantity of various extracts along with a drop of phenolphthalein separately and was heated on water bath for 1 to 2 hrs. The formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

IV.4.11. Test for Gums and Mucilage

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

IV.5. *In vitro* antioxidant and free radical scavenging studies

IV.5.1. Total antioxidant activity

Total antioxidant activity of *P. murex* extracts was determined according to the thiocyanate method.[134] For stock solution, 20mg of *P. murex* extracts were dissolved in 20mL water. *P. murex* extracts (12.5, 25, 50, 100 and 200 mg) or standard sample in 2.5mL of potassium phosphate buffer (0.04 M, pH 7.0) was added to 2.5mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). Each solution was then incubated at 37°C in the dark. At intervals during incubation, each solution was stirred for 3 min. 0.1mL of the incubation solution; 0.1mL $FeCl_3$ and 0.1mL thiocyanate were transferred to the test tube which contained 4.7mL ethanol. Then the solution was incubated for 5 min. Finally, the peroxide value was determined by reading the absorbance at 500 nm in a spectrophotometer (8500 II, Bio-Crom Gmbh, Zurich, Switzerland). During the linoleic acid oxidation, peroxides were formed, and these compounds oxidized Fe^{2+} to Fe^{3+}. Fe^{3+} ions formed complex with SCN^-, which has a maximum absorbance at 500nm. Hence higher absorbance values indicated higher linoleic acid oxidation. The solutions without the addition of *P. murex* extracts or standards were used as blank samples. Five millilitres of linoleic acid emulsion consisted of 17.5gm Tween-20, 15.5mL linoleic acid and 0.04M potassium phosphate buffer (pH 7.0). On the other hand, 5mL control was composed of 2.5mL linoleic acid emulsion and 2.5mL potassium phosphate buffer (0.04 M, pH 7.0). All data about total antioxidant activity was the average of duplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by the following equation:

Percent inhibition = [A0 − A1/A0] × 100

Where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the sample of *Pedalium murex.*

IV.5.2. DPPH Radical Scavenging Activity

1 milli mole solution of DPPH radical solution in methanol was prepared and then 1mL of this solution was mixed with different concentrations of *P. murex* extracts, the mixture was then vigorously and left for 30 min at room temperature in the dark and the absorbance was measured at 517nm with a spectrophotometer and was calculated.[135-137]

DPPH Scavenging activity % = (Control Absorbance – Extract Absorbance) / (Control Absorbance) x 100.

For control 1.0 mL of methanol was added to 1 mL of 1 mmol solution of DPPH radical solution**.**

IV.5.3. Nitric Oxide Radical Inhibition Assay [138, 139]

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen and produced nitrite ions which were measured by Griess reaction. The reaction mixture (3mL) contained sodium nitroprusside (10mmol) in phosphate buffered saline (PBS) and the varying concentrations of extract and control were incubated in water bath at 25°C for 30 min. After incubation, 1.5mL of mixture was removed and 1.5mL of Griess reagent was then added. The absorbance of the chromophore formed was evaluated using spectrophotometer at 546 nm.

NO Scavenging activity % = [(Control Absorbance – Extract Absorbance) / Control Absorbance] x 100.

For control 1.0mL of buffer was added to 3mL of 10mmol sodium nitroprusside and the rest of the procedure was the same.

IV.5.4. Superoxide Radical Scavenging Assay [140, 141]

The superoxide radical scavenging activity of the *P. murex* extracts was measured according to the literature method and they contained PMS (0.1 mmol/L), NADH (1mmol/L), NBT (1mmol/L) in phosphate buffer (0.1 M/L, pH 7.4) with different concentrations of the extracts which was incubated at room temperature for 5 min and the colour was read at 560 nm against a blank. The scavenging effect was calculated against the control.

IV.5.5. Hydroxyl radical scavenging activity

The free radical damage imposed on the substrate, deoxyribose was in the form of thiobarbituric reactive substance (TBARS) by the method of Ohkawa *et al*.,[142] Deoxyribose assay was used to determine the hydroxyl radical scavenging activity in an aqueous medium. This assay is based on the quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by Fe^{3+}ascorbate, EDTA-H_2O_2 system. The reaction mixture containing $FeCl_3$ (200 µmol), EDTA [1.04 mmol], H_2O_2 (1mmol) and 2-deoxy-D-ribose (2.8mmol) were mixed with or without extract at various concentration (125-2000 µg/mL) in 1mL final reaction volume made with potassium phosphate buffer (20 mmol, pH 7.4) and incubated for 1 hr at 37°C. The mixture was heated at 95°C in water bath for 15 min followed by addition of 1 mL each of TCA (2.8%) and TBA (0.5% TBA in 0.025 M NaOH containing 0.02% BHA). Finally, the reaction mixture was cooled on ice and centrifuged at 5000 rpm for 15 min. Absorbance of the supernatant liquid was measured at 532 nm. The negative control without any antioxidant or extracts was considered 100% deoxyribose oxidation. The percentage hydroxyl radical scavenging activity of test sample was determined accordingly in comparison with negative control. Ascorbic acid was taken as the positive control.

IV.5.6. Reducing power

The reducing power of *P. murex* extracts was determined according to the method of Oyaizu.[143] The different doses of *P. murex* extracts (12.5, 25, 50, 100 and 200 mg) in 1 mL of distilled water were mixed with phosphate buffer (2.5mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5mL, 1%). The mixture was incubated at 50°C for 20min. A portion (2.5mL) of TCA (10%) was added to the mixture, which was then centrifuged for 10 min at 1000xgm. The upper layer of solution (2.5mL) was mixed with distilled water (2.5mL) and $FeCl_3$ (0.5mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater the reducing power.

Based on phytochemical and *in vitro* free radical scavenging results ethanol extract of *P. murex* was selected for the isolation, characterization and quantitative estimation.

IV.6. Thin Layer Chromatography

Thin layer chromatography is a procedure for analytical adsorption chromatography which was first introduced by Stahl (1958) who was mainly responsible for bringing out standard equipment for preparing thin layers. It is an important analytical tool for qualitative and quantitative analysis of a number of natural products and for separation and estimation of different components.

The principle of separation is adsorption. One or more compounds were spotted on a thin layer of adsorbent coated on TLC plate. The mobile phase flowed through because of capillary action (against gravitational force). The component moved according to their affinity towards the stationary phase. The component with lesser affinity towards the stationary phase traveled faster and vice versa which led to the separation of components.

The information provided by a finished chromatography included the "migrating behaviour" of the separated substances. It was given in the form of R_f value (relative to front).

$$R_f = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

R_f value usually lies in the range of 0.1 – 1.[144, 145]

Procedure

Preparation of Plate

The silica gel G (60-120 mesh) (Fischer & Co) was utilized for the preparation of TLC plates. Silica gel G was mixed with sufficient quantity of water and triturated well to make slurry. The prepared slurry was spread on the meticulously cleaned and scratch free glass plates of definite dimension with the help of TLC spreader. The thickness of the absorbent was adjusted to 2 mm throughout the plate. Then the prepared plates were allowed to set for 15- 30 min. The activated plates were stored in a vacuum desiccator for future use. Also prior to the use, the plates were once again activated.

Development of Chromatogram

The saturation of atmosphere of TLC chamber by mobile phase, toluene: ethyl acetate: formic acid (5:4:1) before the start of the experiment was almost important to avoid the flawed results due to tailing effect. The sample EEPM was spotted on the plate 2 cm away from the bottom. The plate was then developed in the chamber by

allowing the plate to run up to ¾th distance of the plate. The plate was then removed, dried up to room temperature and sprayed with suitable spray reagent or kept in iodine chamber for identification of spots.

IV.7. Column Chromatography

The column chromatographic technique is widely used for separation, isolation and purification of the natural products. The principle underlying the separation of the compounds is adsorption at the solid liquid interface. The solid support and the interaction between adsorbent and component must be reversible. The adsorbent was washed with fresh solvent the various components were moved down the column until they were arranged in order of their affinity towards the adsorbent. Those with least affinity moved down the column at a faster rate and were eluted from the end of the column before those with greatest affinity for the adsorbent. By changing the polarity of the mobile phase, the separation was achieved by column chromatography. Characterization of the isolated compounds was carried out by analytical techniques, like UV, NMR and Mass spectroscopy.

Procedure for Preparation of Column

The silica gel 60-120 mesh was made into slurry with selected solvent system, the silica gel was previously activated by heating in hot air oven at 100^{o}C for 1hr.

1. The bottom of the column was plugged by cotton and then the silica gel slurry was poured into the column which was filled with solvent system, toluene: ethyl acetate: formic acid (5:4:1) up to 40 cm height, after that it was set aside for 10 min and allowed to settle.

2. The ethanolic extract of *Pedalium murex* Linn .was mixed with small amount of silica gel and wetted with solvent system and the solvent was allowed to evaporate to set the dry residue.

3. Then the dry residue was charged on column with the help of solvent system, after that cotton was placed over it, in order to avoid the disturbance of the top layer of the adsorbent fresh mobile phase was added to the column.

4. The column was eluted with the selected solvent system by isocratic method and the fractions were collected in clean 100 mL beaker up to 25 mL and the speed of the drops was 20 drops/min.

Each collected fraction was tested for the presence of various constituents by TLC. The number of types of constituent and similar fractions was pooled together.[146-148]

IV.8. Spectral Characterization

IV.8.1. FT-IR Spectroscopy

Isolated compounds were analyzed by IR spectral studies by using KBr pellet technique. In this method, the drug and KBr was mixed at the ratio of 1:100. Then this mixture was pressed into a pellet. The FT-IR spectra were recorded for isolated compounds, by using KBr pellet method in the region of 4000-400 cm^{-1}.

IV.8.2. NMR Spectroscopy

NMR is used to elucidate the structure of an unknown compound, there are three pieces of information which should be considered, the position of resonance of the peak (or chemical shift), the number of hydrogen atoms causing the signal (integration) and the number of peaks constituting the signal (multiplicity). NMR data is used to solve the structure, but it is equally acceptable to use MS or IR data to solve the unknown.

IV.8.3. Mass Spectroscopy

Mass spectrometry (MS) is an analytical technique that is used to measure the mass-to-charge ratio of charged particles. It is used for determining masses of particles, for determining the elemental composition of a sample or molecule and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios.[149, 150]

IV.9. HPTLC Spectral Studies

HPTLC Studies on ethanol extract of *Pedalium murex* Linn.

HPTLC method is a modern sophisticated and automated separation technique derived from TLC. Pre-coated HPTLC graded plates and auto sampler was used to achieve precision, sensitive, significant separation both qualitatively and quantitatively.

High performance thin layer chromatography (HPTLC) is a valuable quality assessment tool for the evaluation of botanical materials efficiently and cost

effectively. HPTLC method offers high degree of selectivity, sensitivity and rapidity combined with single-step sample preparation. In addition it is a reliable method for the quantization of nanograms level of samples. Thus this method can be conveniently adopted for routine quality control analysis. It provides chromatographic fingerprint of phytochemicals which is suitable for confirming the identity and purity of medicinal plant raw materials.[151-153]

Basic steps involved in HPTLC

Extracts used : EEPM.

Application mode : CAMAG Linomet IV.

Development mode : CAMAG Twin Trough chamber.

Sample Application

The sample was dissolved in same solvent and 10 µl quantity of sample was applied on the HPTLC silica merk 60F, 254 graded plate sized 6 cm x 10 cm as narrow bands using CAMAG Linomat 5 injector.

Chromatogram Development

It was carried out in CAMAG Twin Trough chambers. Sample elution was carried out according to the adsorption capability of the component to be analyzed. After elution, plates were taken out of the chamber and dried.

Scanning

Plates were scanned under UV at 254 nm. The data's obtained from scanning were brought into integration through CAMAG software. Chromatographic finger print was developed for the detection of phytoconstituents present in each extract and R_f values were tabulated.

Mobile Phase

Toluene: Ethyl acetate: Formic acid (5:4:1)

IV.10. Quantitative estimation of phytoingredients present in *P. murex* leaves

Based on phytochemical and *in vitro* free radical scavenging results ethanol extract was selected for the quantitative estimation. Total flavonoids, total phenolic compounds and total tannins were estimated with respective procedures.

IV.10.1. Estimation of total phenolic compounds[154]

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 10mL 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 mmol) as standard. The results were expressed as mg of catechol equivalents/gm of extract.

IV.10.2. Estimation of total flavonoid compounds[155]

0.5 mL of the sample is added into a test tube containing 1.25 mL of distilled water. Then 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 was added and the mixture was 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/gm sample.

PHASE-II: PHARMACOLOGICAL STUDIES

IV.11. Animal studies

IV.11.1. Animals

Sprague dawley rats (100-150 gm) were used for the experiments. Animals were obtained from KMCH College of Pharmaceutical Sciences, Coimbatore and maintained at standard housing conditions. A standard commercially available diet was provided with water ad libitum during the experiment. The animals were kept in clean and dry polycarbonate cages and maintained in a well-ventilated animal house with 12hrs light and dark cycle. This study was approved by the institutional animal ethics committee (Reg. No KMCRET/Ph.D/08/2015-16).

IV.11.2. Acute Oral Toxicity Study

Acute toxicity study was performed according to OECD-guidelines 423. Three animals of same sex were used in each group. EEPM was administered to each group at 5, 50, 300 and 2000 mg/kg b.w respectively. The animals were fasted over-night before the administration of extract. Animals were observed regularly for 14 days for any signs and symptoms of toxicity.

IV.11.3. Experimental design

Animals were randomly allotted into six groups with 6 animals in each group. EEPM was administered for a period of 30 days (p.o). Then neurodegeneration was induced with administration of intraperitoneal LPS (1 mg/kg) in normal saline on day 31. Two hours after the administration of LPS, animals were subjected to behavioural tests and finally sacrificed, and the brain was extracted for biochemical analysis.

Group I	Normal group. Animals received 0.1 mL of normal saline orally for 30 days
Group II	Disease control. Single dose of LPS (1 mg/kg)
Group III	Standard group. Dexamethasone (0.5 mg/kg) for 30 days + LPS (1 mg/kg)
Group IV	Pre-treatment group. EEPM 100 mg/kg for 30 days + LPS (1 mg/kg)
Group V	Pre-treatment group. EEPM 200 mg/kg for 30 days + LPS (1 mg/kg)
Group VI	Pre-treatment group. EEPM 400 mg/kg for 30 days + LPS (1mg/kg)

IV.12. Behavioural Studies

IV.12.1. General behavioural Studies

- Changes in body weight
- Changes in food and water intake

IV.12.2. Behavioural Tests for Anxiety and Depression

IV.12.2.a. Open field test

The rats were observed in a square open field arena (68x68x45 cm) equipped with 2 rows of 8 photocells, sensitive to infrared light, placed 40 and 125 mm above the floor respectively. The photocells were spaced 90 mm apart and the last photocell in a row was spaced 25 mm from the wall. Measurements were made in the dark in a ventilated, sound-attenuating box. Interruptions of photocell beams were collected by a microcomputer and the following variables were evaluated.[156]

IV.12.2.b. Elevated plus maze test

The apparatus comprised of two open arms (35x5 cm) and two closed arms (30x5x15 cm) that extended from a common central platform (5x5 cm). The floor and walls of the closed arms were made of wood and painted black. The entire maze was elevated to a height of 50 cm above the ground level. Mice weighing 150-200 gm were housed in a pair of 10 days prior to the test in the apparatus. During this time the rats were handled by the investigator on alternate days to reduce stress. 30 min and 60 min after oral administration of the drug treatment, each mouse was placed at the center of the maze facing one of the enclosed arm.[157] During five min session, number

of entries into open arm and time spent in the open arm was noted. The procedure was conducted preferably in a sound attenuated environment.

IV.12.2.c. Forced swim test

Rats were individually forced to swim inside a vertical Plexiglas cylinder containing 15 cm of water maintained at 25°C. Rats placed in the cylinders for the first time were initially highly active vigorously swimming in circles, trying to climb the wall or diving to the bottom. After 2-3 min of activity they began to subside and to be interspersed with phases of immobility or floating of increasing length. After 5-6 min immobility reached a plateau where the rats remained immobile for approximately 80% of the time. After 15 min in the water the rats were removed and allowed to dry in a heated enclosure (32°C) before being returned to their home cages. They were again placed in the cylinder 24 hrs later and the total duration of immobility was measured during a 5 min test.[158] Floating behavior during this 5 min period had been found to be reproducible in different groups of rats.

IV.12.3. Behavioural tests for learning and memory

IV.12.3. a. Water maze test

The water maze consisted of a circular tank with 100 cm diameter and a wall of 20 cm above the water level. A circular platform was hidden 2 cm below the water level. The water was made opaque using titanium dioxide suspension and was kept at about 23°C during the experiment. Animals were trained for 5 consecutive days with 3 consecutive trials per day and an inter-trial interval of 6-10 min. Each trial started from one of four assigned polar positions with a different sequence each day. The swimming length of platform was measured till it finds the platform.[159]

IV.12.3.b. Radial arm maze test

The apparatus was wooden elevated eight-arm radial maze with the arms extending from a central platform 26 cm in diameter. Each arm is 56 cm long, 5 cm wide and 2 cm height rails along the length of the arm. The maze was well illuminated and numerous cues were present. Food pellets (reward) were placed at the end of the arms. During the test, rats were fed once a day and their body weight maintained at 85% of their free feeding weight to motivate the rat to run the maze. Animals were trained on a daily basis in the maze to collect the food pellets for 24 days. The session was terminated after 8 choices and the rats had to obtain the maximum number of rewards with a minimum number of errors.[160]

IV.12.3.c. Choice reaction Time Task Test

Rats were trained to press either of 2 levers with a continuous reinforcement schedule at a fixed ratio of 1:1. Trials began with differential reinforcement of another behaviour (DRO) period (random, 2-5 sec) during which the animals had to refrain from pressing either of the 2 levers. During the CRT period (maximum 10 sec), the time between sample presentation with the cue lamp on and pressing the correct lever was defined as the CRT and a food pellet reward will be provided through the pellet dispenser. With further lever-pressing responses, a house lamp was illuminated and intertribal interval (ITI; 20 sec) begun. One trial took approximately 30 sec, and each test session consisted of 30 trials. One session was performed every day for 30 days. The variables measured were the number of incorrect lever pressings during the DRO and ITI periods.[161]

PHASE-III: BIOCHEMICAL ANALYSIS

IV.13.1. *In vivo* non-enzymatic antioxidant studies in brain hippocampus

IV.13.1.a. Estimation of Acetylcholine (ACh) content

Extraction and measurement of total Ach content from the hypothalamus of brain was done as in our preliminary study. Acetylcholine was extracted in low temperature with 10% trichloroacetic acid.[162] The Ach content was measured by means of standard three-point bioassay technique using a single channel Physiograph (Bio-Devices) with a strain-gauge coupler and a force transducer.[163] The Ach content was expressed as mol/gm tissue.

IV.13.1.b. Determination of Nitric oxide (NO)

The assay of nitric oxide content in brain homogenates was performed in acid medium and in the presence of nitrite. The formed nitrous acid diazotize sulphanilamide was coupled with N-(1–naphthyl) ethylenediamine. The resulting azo-dye had a bright reddish–purple colour which was measured through spectrophotometry at 540nm.[165]

IV.13.1.c. Estimation of Protein

Protein concentrations of the tissue homogenates were determined by the standard method of Lowry[166] using bovine serum albumin as the standard. As per this method, colour change of the sample solution is in proportion to protein concentration which can be measured using colorimetric techniques. In this case EEPM, brain homogenates was taken and mixed with 10 mL buffer (N/10 Acetic acid and N/10

Sodium acetate). Then it was centrifuged at 2500 rpm and supernatant was collected. About 0.5mL supernatant was taken and 0.5 mL distilled water was added to it, and to this about 5mL of alkaline solution (NaOH+ Sodium potassium tartarate) and 0.5 mL of folin reagent was added. The optical density was measured at 600 nm.

IV.13.1.d. Estimation of brain MDA & Lipid peroxide (LPO)

Ohkawa method was used to estimate the total amount of lipid peroxidation (LPO) product. LPO was estimated in terms of TBARS and malondialdehyde (MDA) was taken to represent the TBARS. The incubation mixture consisted of 0.5 mL of supernatant brain homogenate, 0.2 mL of 8% sodium dodecyl sulphate, 1.5 mL of 20% acetic acid solution (adjusted to pH 3.5 with 1N NaOH / 0.1N HCl) and 1.5 mL of 0.9% aqueous solution of thiobarbituric acid (adjusted to pH 7.4 with 1N NaOH / 0.1N HCl) was made up to 5.0 mL with double distilled water and then heated in boiling water bath for 30 min. After cooling, the red chromogen was extracted into 5 mL of the mixture of n-butanol and pyridine and was centrifuged at 4000 rpm for 10 min. The absorbance of organic layer was measured at 532 nm. 1, 2, 3, 3-tetraethoxypropane (TEP) was used as an external standard and the levels of lipid peroxide was expressed as µmol of MDA / gm protein. The calibration curve of TEP was prepared by the above procedure taking 80-240 nmol of TEP as standard over which linearity was obtained.[142]

IV.13.2. *In vivo* brain hippocampal enzymatic antioxidant studies

IV.13.2.a. Estimation of Acetylcholinestrase (AChE) activity

The total AChE activity in different areas of the brain was assessed as done earlier. The brain tissues were homogenized in a Potter–Elvehjem homogenizer by using 0.1 M phosphate buffer (pH 8) at a temperature of 0°C. The homogenate was centrifuged at 10,000×gm for 5 min at 4°C. The activity of AChE in the aliquot of the homogenate was estimated.[166] The aliquot was mixed with phosphate buffer (pH 8). To this, the substrate acetylthiocholine iodide and dithiobisnitrobenzoic acid (DTNB) reagent was added. Acetyl thiocholine iodide was hydrolyzed to thiocholine and acetate by AChE. Thiocholine reacted with DTNB reagent to produce yellow color. The rate of color development was the measure of the AChE activity. A kinetic profile of the enzyme activity was studied spectrophotometrically at 412 nm at the interval of 15 sec. The enzyme activity was expressed as the mol of substrate hydrolyzed/min/gm tissue.

IV.13.2.b. Estimation of Superoxide dismutase (SOD)

The measurement of SOD involved the inhibition of the formation of blue colored formozan dye from nitro blue tetrazolium (NBT), in the presence of phenazinemethosulphate (PMS) and reduced nicotinamide adenine dinucleotide (NADH). The incubation mixture consisted of sodium pyrophosphate buffer (pH 8.3; 0.052 M; 1.2 mL), PMS (186 µmol), NBT 300µmol) and NADH (780 µmol; 0.2 mL). The reaction was initiated by the addition of NADH; followed by incubation for 90 sec at 37°C. The reaction was terminated by the addition of glacial acetic acid (1 mL), n-butanol (4 mL), shaken vigorously, centrifuged at 4000 rpm for 1 min and the upper butanol layer was read at 560 nm, against butanol blank.[167]

IV.13.2.c. Estimation of Catalase (CAT)

CAT measurement was done based on the ability of CAT to inhibit oxidation of hydrogen peroxide (H_2O_2). 2.25 mL of potassium phosphate buffer (65 mmol, pH 7.8) and 100 µL of the brain homogenate or sucrose (0.32M) was incubated at 25°C for 30 min. H_2O_2 (7.5 mmol; 650 µL) was added to initiate the reaction. The change in absorption at 240 nm was measured for 2-3 min. dy/dx for every minute for each assay was calculated and the results were expressed as CAT units of protein.[168]

IV.13.2.d. Estimation of Glutathione peroxidase (GSH-Px) activity

Lawrence and Burk[169] method was used to measure the activity of GSH-Px. Briefly, a 100 µL aliquot of supernatant was mixed with 700 µL reaction mixture containing 1mmol EDTA, 1mmol NaN_3, 0.2 mmol NADPH and 1mmol glutathione in a phosphate buffer saline and 100 µL (10 U/100 µL) glutathione reductase. The tubes were vortexed and incubated for 5 min at room temperature. After incubation, 100 µL of 0.2 mmol H_2O_2 was added to each tube to initiate the reaction, and the absorbance was recorded at 340 nm, every 30 sec over a period of 3 min, using a Spectronic-20 spectrophotometer (Spectronic Instruments). Changes in the rate of absorbance were converted into nmol of NADPH oxidized/min/mg protein, using an extinction coefficient of 6.22x103 l $mol^{-1}cm^{-1}$.

IV.13.2.e Estimation of Glutathione reductase (GR) activity

Briefly a 100 µL aliquot of supernatant was mixed with 700 µL reaction mixture containing 1mmol EDTA, 1mmol NaN_3, 0.2 mmol NADPH and 1 mmol glutathione in a phosphate buffer saline and 100 µL (10 U/100 µL) glutathione was oxidized. The tubes were vortexed and incubated for 5 min at room temperature. After incubation, 100 µL of 0.2 mmol H_2O_2 was added to each tube to initiate the reaction,

and the absorbance was recorded at 340 nm, every 30 sec over a period of 3 min, using a Spectronic-20 spectrophotometer (Spectronic Instruments). Changes in the rate of absorbance was converted into nmol of NADPH oxidized/min/mg protein, using an extinction coefficient of 6.22×103 l $mol^{-1}cm^{-1}$.[170]

IV.14. HISTOPATHOLOGICAL STUDIES

At the end of behavioural experiments, the rats were deeply anesthetized with a high dose of ketamine (150 mg/kg) and perfused through the ascending aorta with glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) followed by 100 mL of 0.1 % PB containing 10% sucrose. Following perfusion, the brains were removed from the skull, the blocks of forebrain and brainstem was prepared, embedded in paraffin and then sections were cut in the thickness of 30 µm in microtome and collected in PB (0.1 M). Sections were Nissl stained with 0.1% crystal violet[171] for histopathological examination of hippocampus using the light microscope.[172] Sections were photographed and analyzed by image analysis software to assess the neuronal size, shapes, number per high power field.

IV.15. STATISTICAL ANALYSIS

Prism graph pad version 7 was used for the statistical analysis, calculations, and graphic interfaces on a personnel computer. The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnet's test. The results values are expressed as mean±SEM observations from six animals in each group. P values *$P<0.05$.

Results & Analysis

V. RESULTS AND ANALYSIS

The present study was designed to evaluate the phytochemical characterization and neuroprotective effect of *Pedalium murex* against LPS-induced endotoxemia neurodegenerative models in SD rats.

PHASE I: PHYTOCHEMICAL STUDIES

V.1. Extraction of *Pedalium murex* Linn. leaves

Dried crushed leaves of *Pedalium murex* Linn. was extracted with n-hexane, chloroform, ethyl acetate and ethanol (90% v/v) continuously with soxhlet apparatus and the results were tabulated in Table 3.

Table 3. Data showing the extractive values of leaves of *Pedalium murex* Linn.

S.No	Extract	Colour/ Physical nature	Percentage yield (% w/w)
1	n-Hexane	Green/ Waxy Semisolid	4.90
2	Chloroform	Green/ Semisolid	6.20
3	Ethyl acetate	Brownish green/Solid	5.72
4	Ethanol (90% v/v)	Brownish green/ Solid	8.34

V.2. Preliminary phytochemical screening of *Pedalium murex* Linn. leaf extracts

The extracts of *Pedalium murex* Linn. leaves were subjected to qualitative phytochemical screening to identify the active constituents which showed the presence of below mentioned phytoconstituents in Table 4.

Table 4. Preliminary phytochemical screening of *Pedalium murex* Linn. leaf extracts

S. No.	Chemical Test	n- Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract (90% v/v)
1.	**Alkaloids**				
a.	Dragendorff''s Test	-	-	+	-
b.	Mayer's Test	-	-	+	-
c.	Hager's Test	-	-	+	-
d.	Wagner's Test	-	-	+	-
e.	Tannic Acid Test	-	-	+	-
2.	**Saponins**				
a.	Foam Test	-	-	+	+
b.	Lead Acetate Test	-	-	+	+
c.	Hemolytic Test	-	-	+	+
3.	**Glycosides**				
a.	Legal's Test	-	+	-	+
b.	Baljet Test	-	+	-	+
c.	Keller-Killiani Test	-	+	-	+
d.	Borntrager's Test	-	+	-	+
4.	**Carbohydrates**				
a.	Molisch's Test	-	-	+	-
b.	Fehling's Test	-	-	+	-
c.	Benedict's Test	-	-	+	-
d.	Tollen's Test	-	-	+	-
e.	Seliwanoff''s Test	-	-	+	-
f.	Bromine Water Test	-	-	+	-
5.	**Tannins**				
a.	Gelatin Test	-	+	-	-
b.	Ferric Chloride Test	-	+	-	-
c.	Vanillin Hcl Test	-	+	-	-
d.	Lead Acetate Test	-	+	-	-
e.	Potassium Ferric	-	-	-	-

	Cyanide Test				
f.	Potassium Dichromate Test	-	+	-	-
6.	**Flavonoids**				
a.	Shinoda's Test	-	-	-	+
b.	Alkaline Reagent Test	-	-	-	+
c.	Lead Acetate Test	-	-	-	+
d.	Conc.Sulphuric Acid Test	-	-	-	+
7.	**Steroids**				
a.	Libermann-Burchard's Test	+	-	-	-
b.	Salkowsky's Test	+	-	-	-
8.	**Proteins**				
a.	Biuret Test	-	-	+	-
b.	Ninhydrin Test	-	-	+	-
c.	Xanthoproteic Test	-	-	+	-
d.	Millon's Test	-	-	+	-
9.	**Triterpenoids**				
a.	Knoller's Test	-	-	-	+
10.	**Fixed Oil & Fat**				
a.	Spot Test	+	-	-	-
b.	Saponification Test	+	-	-	-
11.	**Gum & Mucilage**	+	-	-	-

+ Present - Absent

V.3. *In vitro* free radical scavenging activities

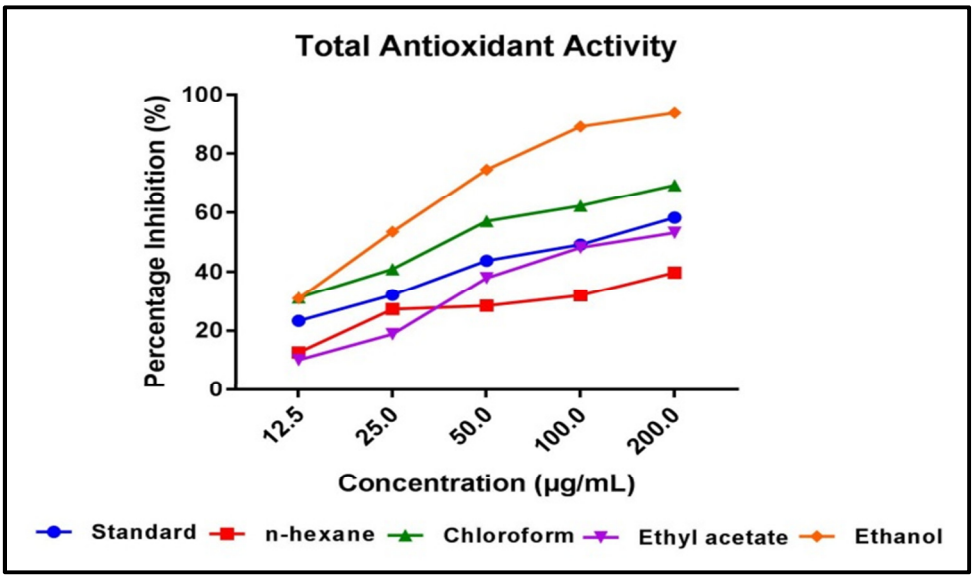
V.3.1. Total antioxidant activity

The total antioxidant activity was estimated by using thiocyanate method. The total antioxidant capacity of ethanol extract of *Pedalium murex* Linn. leaves were found to be higher (93.73% at 200 µg/ml) when compared to other extracts used. The IC_{50} values of total antioxidant capacity of standard ascorbic acid, n-hexane extract, chloroform extract, ethyl acetate extract and ethanol extract were found to be 129.75 µg/ml, >200 µg/ml, 65.41 µg/ml, 154.73 µg/ml and 12.74 µg/ml respectively. Amongst, ethanol and chloroform extracts exhibited significant dose dependent antioxidant activity which were mentioned in Table 5 and Figure 5.

Table 5. % Inhibition and IC_{50} values of total antioxidant activity of *Pedalium murex* Linn. leaf extracts

S.No	Concentration (µg/ml)	Standard (Ascorbic acid)	n-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% inhibition	% inhibition	% inhibition	% inhibition	% Inhibition
1	12.5	23.35	12.67	31.21	10.23	30.99
2	25	32.13	27.33	40.92	18.86	53.55
3	50	43.78	28.42	57.15	37.97	74.63
4	100	49.24	31.97	62.31	48.24	89.15
5	200	58.32	39.75	69.36	53.23	93.73
6	**IC_{50} value**	**129.75**	**278.01**	**65.41**	**154.73**	**12.74**

Figure 5. Total antioxidant activity of *Pedalium murex* Linn. leaf extracts



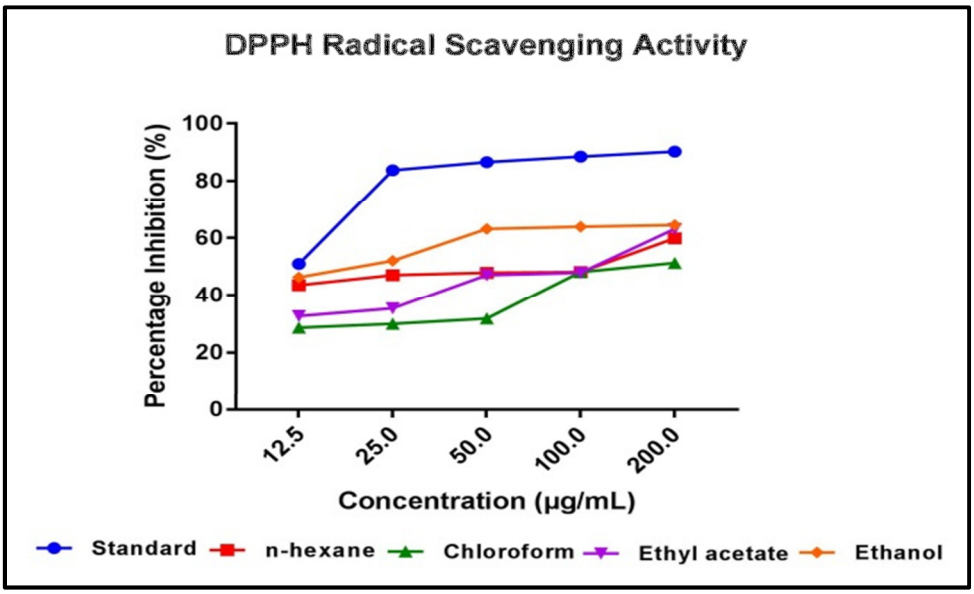
V.3.2. DPPH radical scavenging activity

The DPPH radical is a model for a lipophilic radical chain reaction initiated by the lipid auto oxidation. Among the different extracts used in the study, EEPM exhibited significant dose dependent DPPH radical scavenging activities which were mentioned in Table 6 and Figure 6. Percentage scavenging activity or percentage inhibition was calculated by linear regression method. The IC_{50} values of DPPH radical scavenging activity of standard ascorbic acid, n-hexane extract, chloroform extract, ethyl acetate extract and ethanol extract were found to be <12.5 µg/ml, 86.69 µg/ml, 168.89 µg/ml, 109.19 µg/ml and 21.91 µg/ml respectively.

Table 6. % Inhibition and IC_{50} values of DPPH radical scavenging activity of *Pedalium murex* Linn. leaf extracts

S.N	Concentration (µg/ml)	Standard (Ascorbicacid)	n-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% inhibition	% inhibition	% inhibition	% inhibition	% inhibition
1	12.5	50.95	43.58	28.60	32.62	46.25
2	25	83.75	47.05	29.94	35.29	52.03
3	50	86.62	47.86	31.81	47.05	63.10
4	100	88.53	48.12	48.12	47.86	63.90
5	200	90.28	59.89	51.33	63.10	64.43
6	**IC_{50} value**	**<12.5**	**86.69**	**168.89**	**109.19**	**21.91**

Figure 6. DPPH radical scavenging activity of *Pedalium murex* Linn. leaf extracts



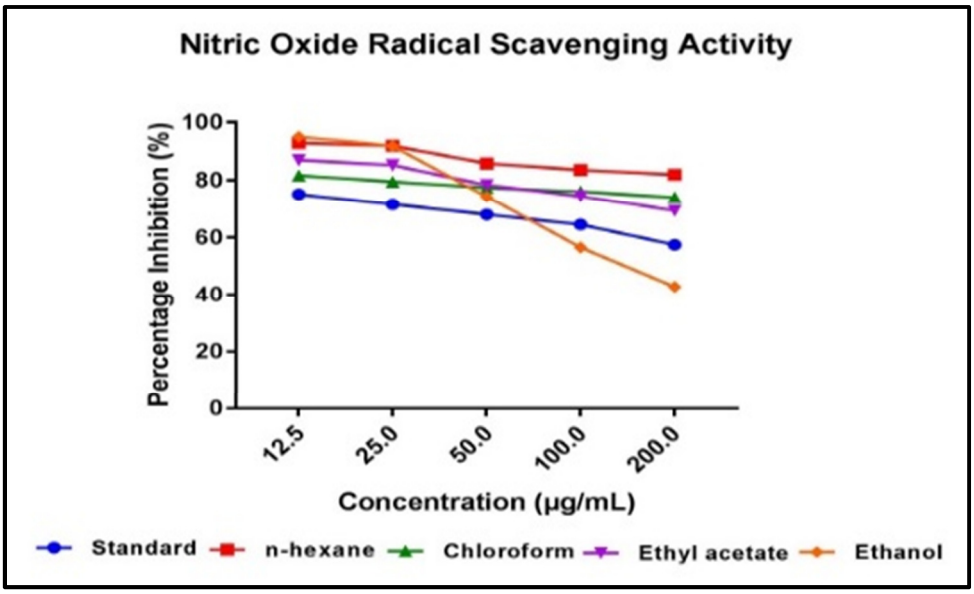
V.3.3. Nitric oxide radical scavenging activity

Nitric oxide is a free radical and excessive generation of nitric oxide radicals leads to some inflammatory disorders. Our study showed crude extracts of *P. murex* leaves scavenge the free nitric oxides in the medium and prevent the formation of nitrite. EEPM exhibited higher nitric oxide scavenging activity which was mentioned in Table 7 and Figure 7. IC_{50} value of standard gallic acid, n-hexane extract, chloroform extract, ethyl acetate extract and ethanol extract were found to be <12.5 µg/ml, >200 µg/ml, >200 µg/ml, >200 µg/ml and 156.13 µg/ml.

Table 7. % Inhibition and IC_{50} values of nitric oxide radical scavenging activity of *Pedalium murex* Linn. leaf extracts

S. No.	Concentration (µg/ml)	Standard (Gallic acid)	n-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% inhibition	% inhibition	% inhibition	% inhibition	% Inhibition
1	12.5	75.17	92.99	81.71	87.05	95.01
2	25	71.63	91.92	79.57	85.27	91.91
3	50	68.08	85.86	77.43	78.38	74.34
4	100	64.53	83.61	76.12	74.58	56.53
5	200	57.44	81.94	74.1	69.35	42.75
6	**IC_{50} value**	**<12.5**	**>200**	**>200**	**>200**	**156.13**

Figure 7. Nitric oxide radical scavenging activity of *Pedalium murex* Linn. leaf extracts



V.3.4. Superoxide anion scavenging activity

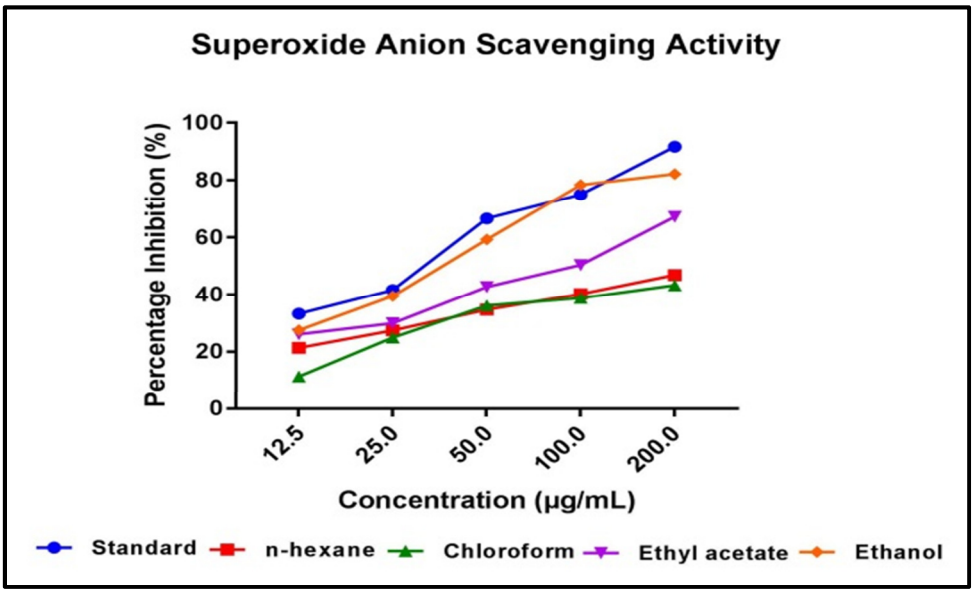
Superoxide anion scavenging activity of extracts of *P. murex* leaves were calculated using the standard curve of ascorbic acid. Ethanol and ethyl acetate extracts exhibited significant dose dependent superoxide anion scavenging activity. The IC_{50} values of ascorbic acid, n-hexane extract, chloroform extract, ethyl acetate extract and ethanol extract

were found to be 37.24 µg/ml, 206.18 µg/ml, 219.22 µg/ml, 109.66 µg/ml and 50.51 µg/ml which were represented in Table 8 and Figure 8.

Table 8. % Inhibition and IC_{50} values of superoxide anion scavenging activity of *Pedalium murex* Linn. leaf extracts

S. No.	Concentration (µg/ml)	Standard (Ascorbic acid)	n-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% inhibition	% inhibition	% inhibition	% Inhibition	% inhibition
1	12.5	33.32	21.34	11.4	26.15	27.56
2	25	41.61	27.48	24.94	29.93	39.42
3	50	66.65	34.73	36.14	42.69	59.28
4	100	75.06	40.19	38.76	50.37	78.34
5	200	91.68	46.91	43.29	67.21	82.17
6	**IC_{50} value**	**37.24**	**206.18**	**219.22**	**109.66**	**50.51**

Figure 8. Superoxide anion scavenging activity of *Pedalium murex* Linn. leaf extracts



V.3.5. Hydroxyl radical scavenging activity

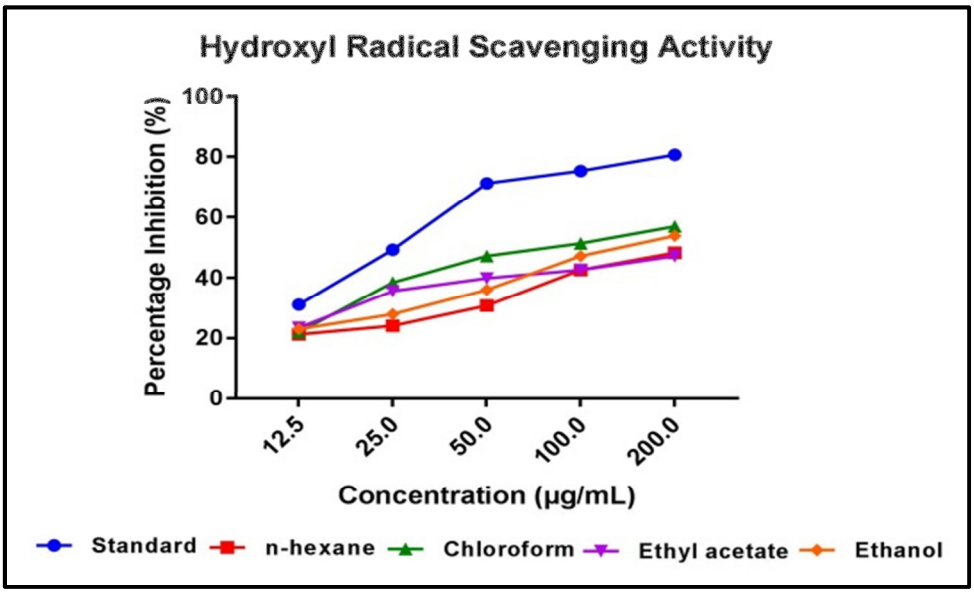
Hydroxyl radical scavenging activities of extracts of *P. murex* leaves were calculated using the standard curve of gallic acid mentioned in Table 9 and Figure 9. From all the four extracts, chloroform and ethanol extracts exhibited significant dose dependent hydroxyl radical scavenging activity. The IC_{50} values of gallic acid, n-hexane

extract, chloroform extract, ethyl acetate extract and ethanol extract were found to be 23.92 µg/ml, 191.80 µg/ml, 124.00 µg/ml, 204.02 µg/ml and 155.40 µg/ml respectively.

Table 9. % Inhibition and IC_{50} values of hydroxyl radical scavenging activity of *Pedalium murex* Linn. leaf extracts

S. No.	Concentration (µg/ml)	Standard (Gallic acid)	n-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% Inhibition	% inhibition	% inhibition	% inhibition	% Inhibition
1	12.5	31.15	21.32	22.02	23.42	23.07
2	25	49.27	24.12	38.46	35.66	27.97
3	50	71.25	30.76	47.20	39.86	36.01
4	100	75.36	42.65	51.39	42.65	47.20
5	200	80.67	48.25	56.99	47.20	53.84
6	**IC_{50} value**	**23.92**	**191.80**	**124.00**	**204.02**	**155.40**

Figure 9. Hydroxyl radical scavenging activity of *Pedalium murex* Linn. leaf extracts



V.3.6. Reducing power

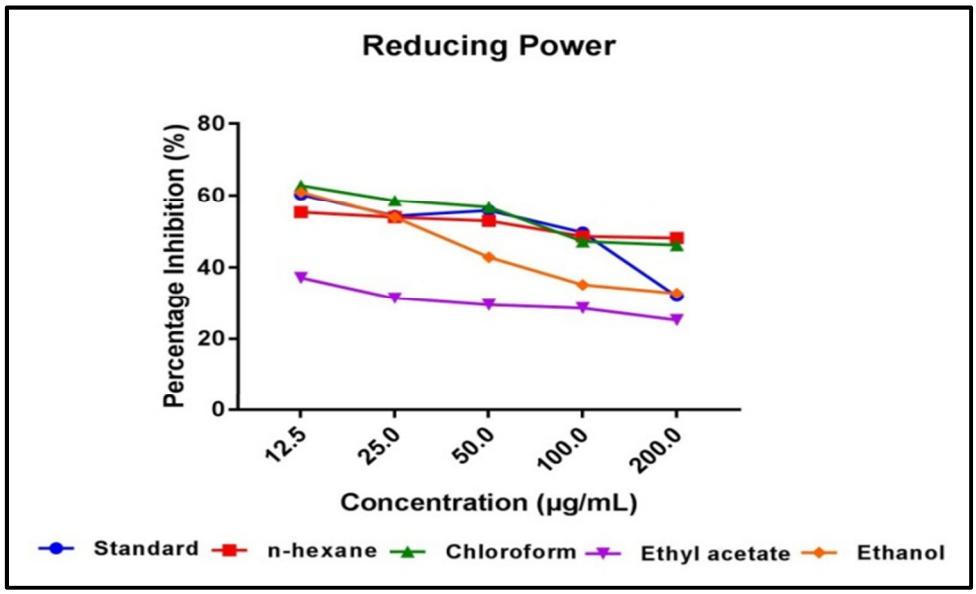
During the reducing power assay, the presence of reductants (antioxidants) in the tested samples would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). The reductive capability and the IC_{50} values of ascorbic acid, n-hexane extract, chloroform extract, ethyl acetate extract and ethanol extract were found to be

80.20 µg/ml, 122.5µg/ml, 127.20 µg/ml, 322.7 µg/ml and 41.58 µg/ml (Table 10 and Figure 10).

Table 10. IC_{50} values of reducing power of *Pedalium murex* Linn. leaf extracts

S. No.	Concentration (µg/ml)	Standard (Gallic acid)	n-Hexane Extract	Chloroform Extract	Ethyl acetate Extract	Ethanol Extract
		% Inhibition	% inhibition	% inhibition	% inhibition	% inhibition
1	12.5	60.30	55.23	62.85	37.14	60.95
2	25	54.19	53.80	58.57	31.42	53.8
3	50	55.72	52.85	56.66	29.52	42.86
4	100	49.61	48.57	47.14	28.57	35.23
5	200	32.06	48.09	46.19	25.23	32.85
6	**IC_{50} value**	**80.20**	**122.5**	**127.20**	**322.7**	**41.58**

Figure 10. Reducing power of *Pedalium murex* Linn. leaf extracts



Based on phytochemical screening and *in vitro* free radical scavenging activities, the ethanol extract of *Pedalium murex* Linn. leaves (EEPM) was selected for quantitative estimation and isolation.

V.4. Thin layer chromatography (TLC)

EEPM was subjected to thin layer chromatography on silica gel G which had shown good resolution of solutes system like Toluene: Ethyl acetate: Formic acid (5:4:1). The different spot developments in each system were identified by means of corresponding detecting agent and R_f values were calculated and presented in Table 11 and Figure 11.

Table 11. Thin layer chromatography of EEPM

Solvent system	No. of spots	Spray reagent	R_f Values
Ethyl acetate: Chloroform (6:4)	2	Iodine vapours	0.62 0.75
Chloroform: Methanol (96:4)	3	Iodine vapours	0.50 0.65 0.69
Ethyl acetate: Formic acid: Water (5:4:1)	4	Iodine vapours	0.62 0.68 0.72 0.76
Toluene: Ethyl acetate: Formic acid (5:4:1)	6	Iodine vapours	0.53 0.65 0.74 0.84 0.90 0.96

Figure 11. Thin layer chromatography of EEPM

V. 5. Column chromatography

On the basis of phytochemical screening and TLC study, isolation of active constituents of EEPM was done by column chromatography through isocratic elution technique with the help of solvent system Toluene: Ethyl acetate: Formic acid (5:4:1) was shown in Table 12 and Figure 12.

Figure 12. Column chromatography of EEPM

Table 12. Column chromatography of EEPM

Fraction No.	Nature of Residue	Analysis by TLC	Colour of the spot	R_f Value
1-4	No residue	---	---	---
5-8	Green	---	---	---
9-12	No residue	---	---	---
13-16	Light green	----	---	---
17-20	Light green	2 spots with tailing effect	Brown Greenish brown	0.67 0.71
21-24	Yellowish green	----	---	---
25-28	Light yellow	1	Yellow	0.51
29-32	No residue	----	----	---
33-36	Light yellow	1	Brown	0.84
37-40	Light yellow	2	Yellow Yellowish green	0.78 0.43

The isolation of the compounds from the Fraction 25-28, 33-36 obtained by column chromatography was selected and named as PM I and PM II. These compounds were subjected to physical and spectral studies for confirming the purity and characterization.

V.6. Isolation of phytoconstituents from ethanol extract

Compound-PM I obtained from 25-28, Compound-PM II obtained from 33-36.

V.6.1. Characterization of compound - PM I & PM II

Table 13. Chemical tests for compound- PM I & PM II

Chemical Tests	Compound PM I	Compound PM II
Shinoda's test	Positive	Positive
Zinc-hydrochloride reduction test	Positive	Positive

Table 14. Physical characters of compound- PM I& PM II

Parameters	Compound PM I	Compound PM II
Yield	46 mg	30 mg
Physical state and colour	Light yellow crystalline solid	Light yellow crystalline solid
Solubility	Water, Acetone and DMSO	DMSO, Methanol
Melting point	347 ℃	276 ℃
TLC solvent system	Toluene: Ethyl acetate: Formic acid (5:4:1)	Toluene: Ethyl acetate: Formic acid (5:4:1)
R_f value	0.5	0.84

Isolated compounds from the EEPM were tested and it showed the positive test for flavonoids. The physical characters of both the compounds were compared and the results were tabulated above.

Figure 13. Thin layer chromatography of PM I

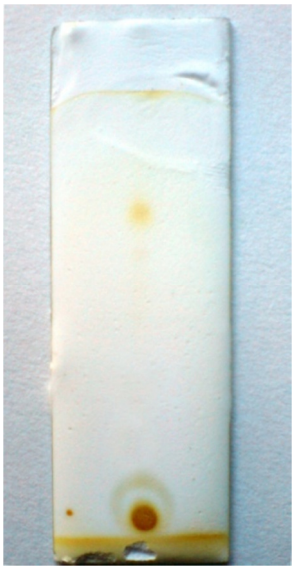


Figure 14. Thin layer chromatography of PM II

V.7. Spectral analysis of isolated compounds

Compound PM-I

Compound **PM-I** was obtained as a light yellow crystalline solid and its molecular formula was established as $\mathbf{C_{15}H_{10}O_5}$ from its Mass spectral data that showed [M-H]- ion at *m/z* 269.05. The molecular formula of **PM-I** was further supported by its ^{1}HNMR and ^{13}CNMR spectral data's. The IR spectra exhibited characteristic bands at 3283.21 cm^{-1} for aromatic–OH groups, 1656.26 cm^{-1} for C=O group, 1171.64 cm^{-1} for C-O group and at 1606.57 cm^{-1} for C=C group.

The ^{1}H NMR spectrum showed the presence of broad peaks at δ 10.36, 10.80 and at 12.95 suggesting the presence of three phenyl hydroxyl groups. The ^{1}H NMR spectrum of **PM-I** also showed the presence of two meta coupled aromatic doublets at δ6.18 and 6.45 corresponds to H-6 and H-8 protons, two doublets at δ6.95 (d,2H,J=6.4Hz) and 7.81 (d, 2H,J= 6.4Hz) for H-3'/H-5' and H-2'/H-6' protons of ring B, and a singlet at δ6.73 corresponding to H-3 proton; characteristic for a 5,7,4'-trisubstituted flavone.

The ^{13}C NMR spectra showed the presence of fifteen carbon atoms (Table 15). Results of spectral data suggested that **PM-I** had structural similarities with 4´, 5, 7-trihydroxy flavone which may have the presence of **Apigenin.**

Table 15. ^{13}C NMR spectral data of the compound PM-I

Carbon	Signal(δ)	Carbon	Signal(δ)
2	162.00	1'	121.73
3	104.26	2'	128.95
4	182.26	3'	116.48
5	164.25	4'	157.83
6	99.37	5'	116.48
7	164.65	6'	128.95
8	94.49		
9	161.68		
10	103.36		

Figure 15. Structure of Apigenin

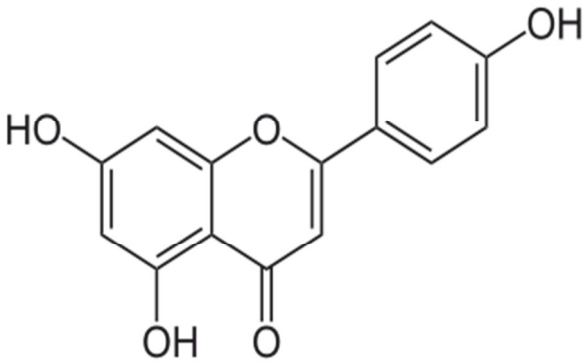


Figure 16. IR spectrum of compound PM I

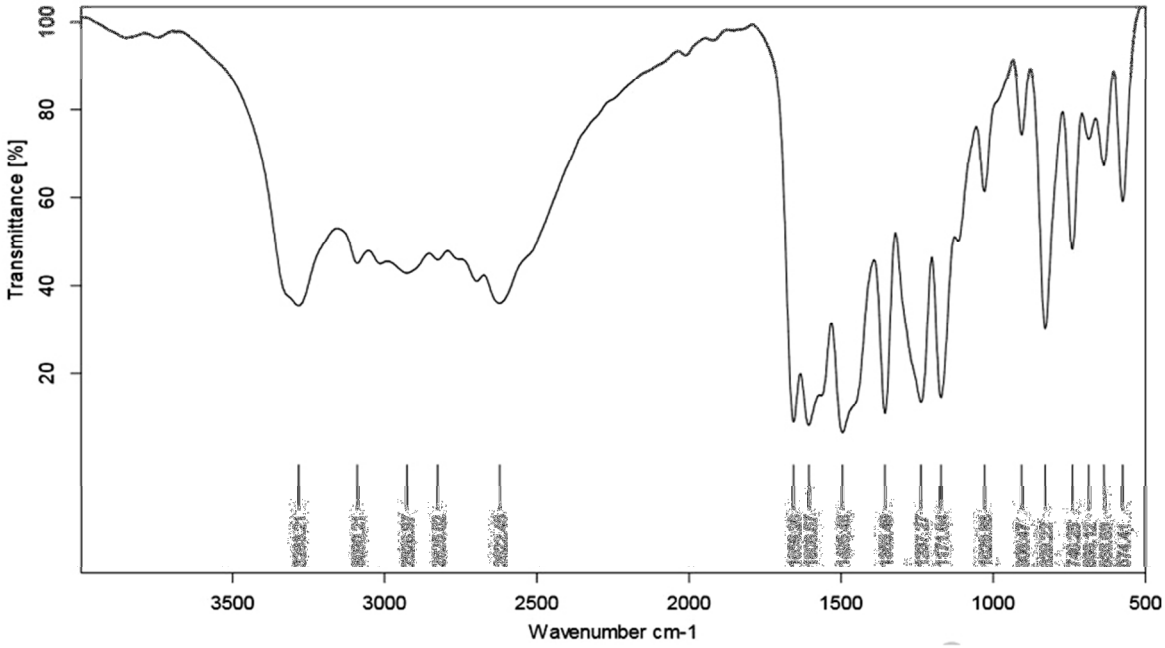


Figure 17. LC-MS of compound PM I

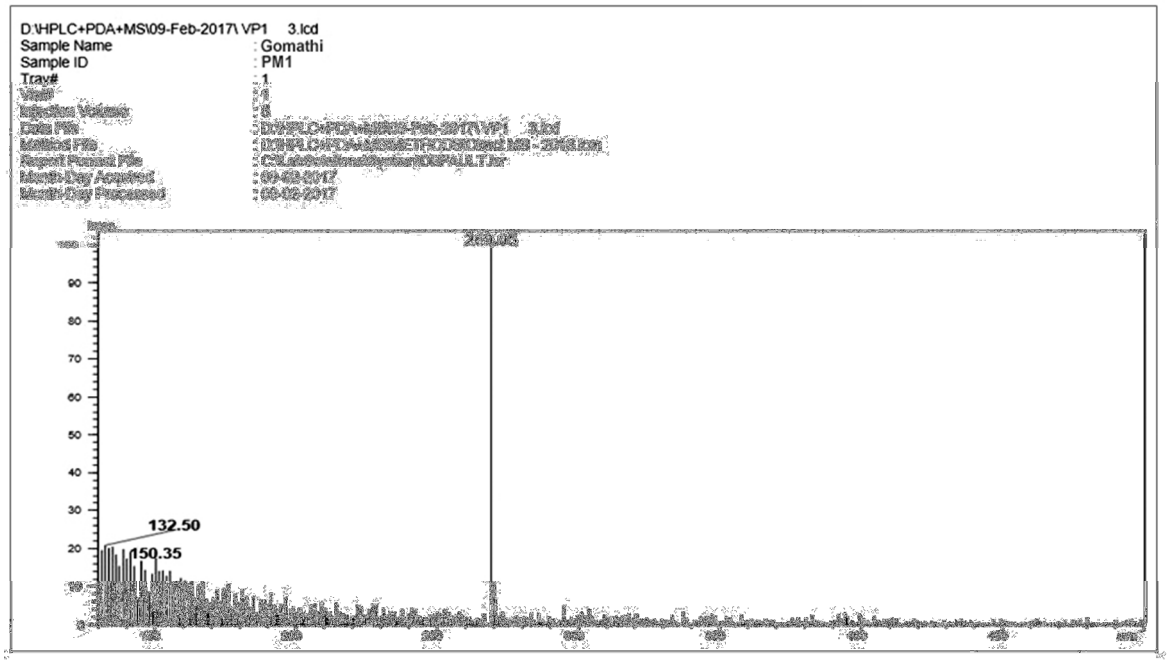


Figure 18. ^{1}H NMR spectrum of compound PM I

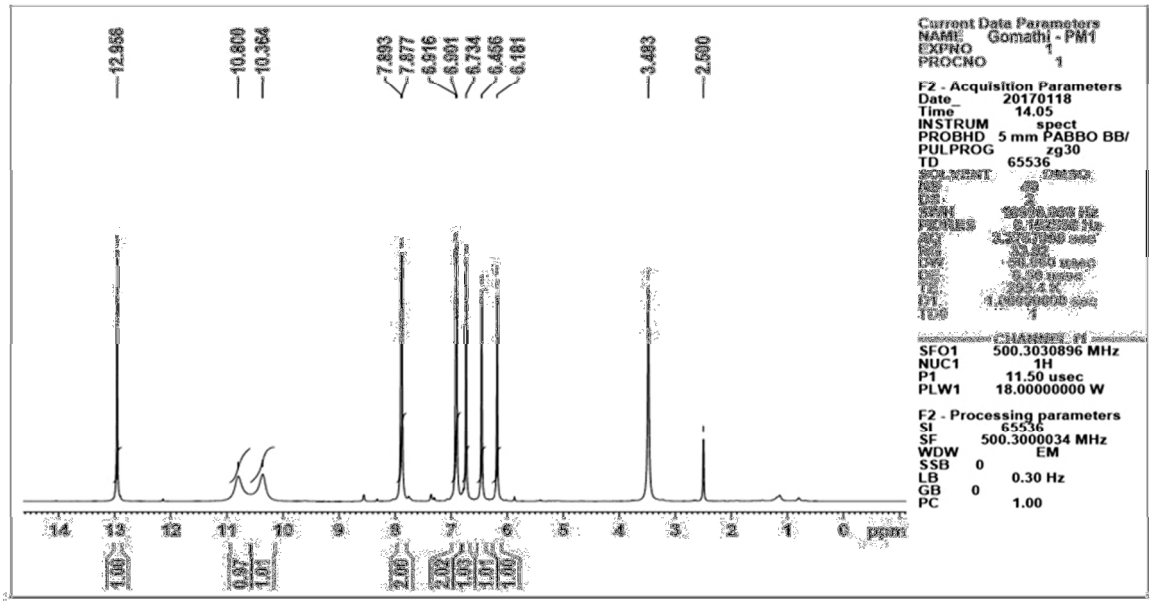
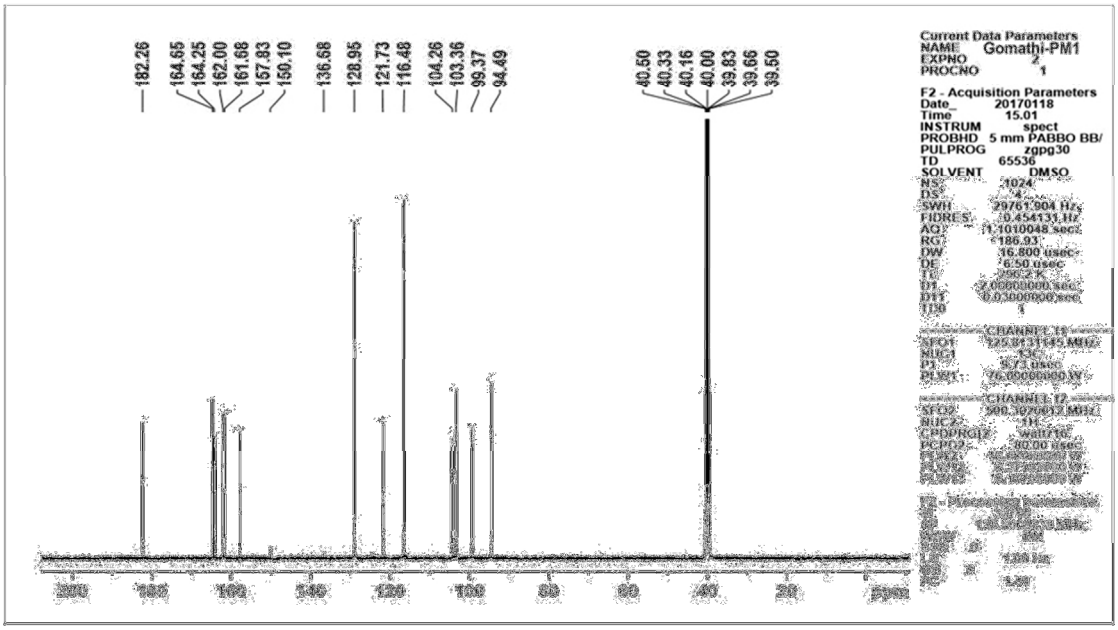


Figure 19. ^{13}C NMR spectrum of compound PM I



Compound PM-II

Compound **PM-II** was also obtained as a light yellow crystalline powder and its molecular formula was established as $\mathbf{C_{15}H_{10}O_6}$ from its Mass spectral data that showed $[M-H]^-$ ion at m/*z* 285 which further supported by its ^{13}C NMR spectral data. The IR spectra exhibited characteristic bands at 3419.89 cm^{-1} for aromatic –OH groups, 1654.29 cm^{-1} for C=O group, 1169.42 cm^{-1} for C-O group and at 1609.54 cm^{-1} for C=C group.

The ^{1}H NMR spectrum of **PM-II** showed the presence of three meta coupled aromatic doublets at δ6.17, 6.43 and 6.87, a singlet at 6.62, one ortho coupled aromatic proton and one ortho and meta coupled aromatic proton appeared as a multiple at δ7.37 are characteristic for a polyphenol. The ^{13}C NMR spectra showed the presence of fifteen aromatic carbons (Table 16). Results of spectral data suggested that **PM-II** had structural similarities with 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one which may have the presence of kaempferol.

Table 16. ^{13}C NMR spectral data of compound PM-II

Carbon	Signal(δ)	Carbon	Signal(δ)
2	162.15	1’	122.25
3	104.40	2’	114.01
4	182.38	3’	146.43
5	164.62	4’	150.39
6	99.57	5’	116.75
7	164.84	6’	119.69
8	94.62		
9	158.01		
10	103.55		

Figure 20. Structure of Kaempferol

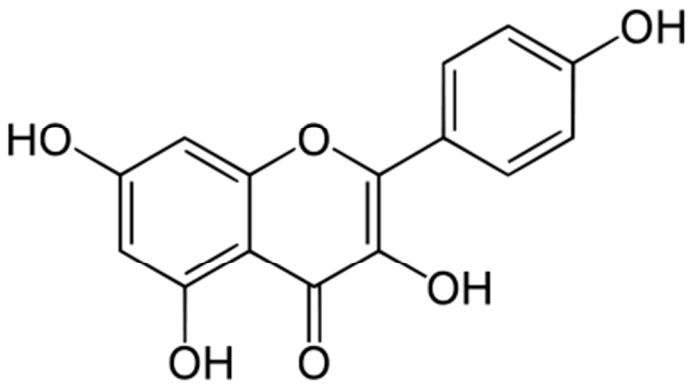


Figure 21. IR spectrum of compound PM II

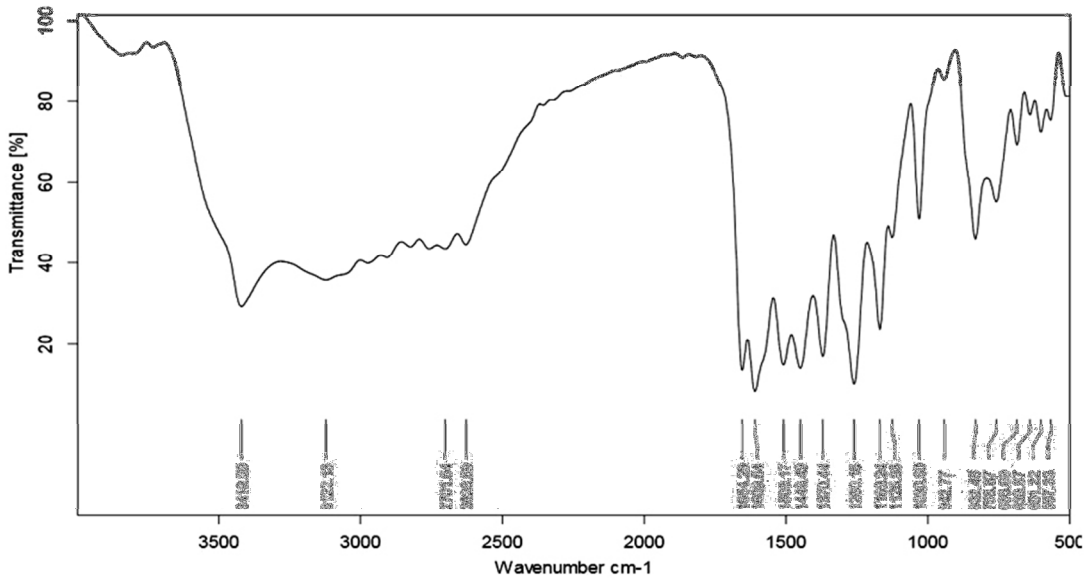


Figure 22. LC-MS of compound PM II

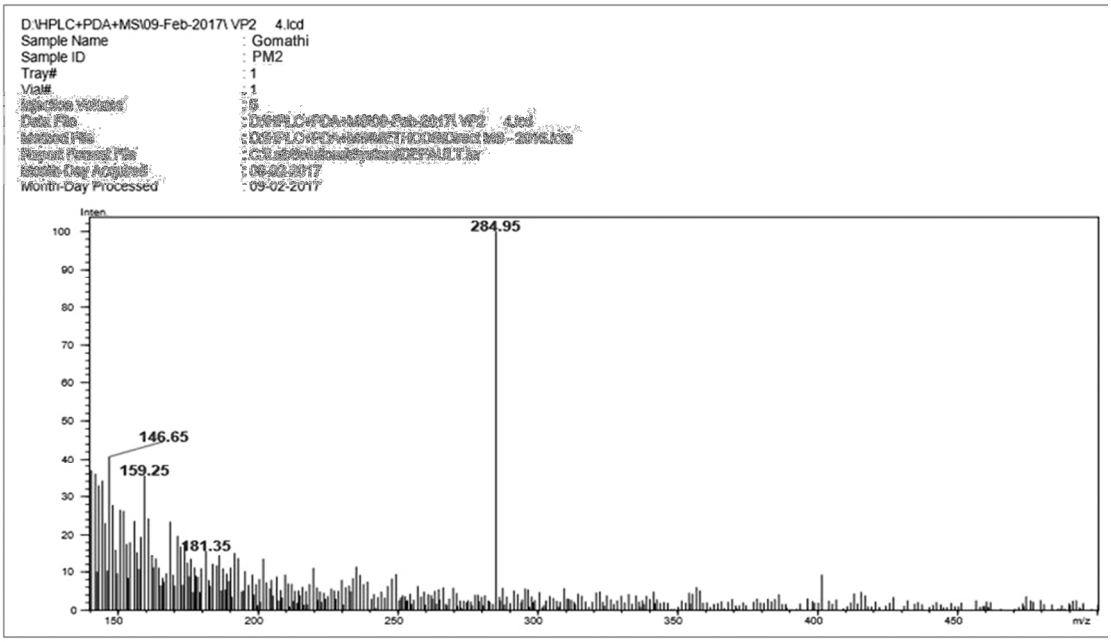


Figure 23. ^{1}H NMR spectrum of compound PM II

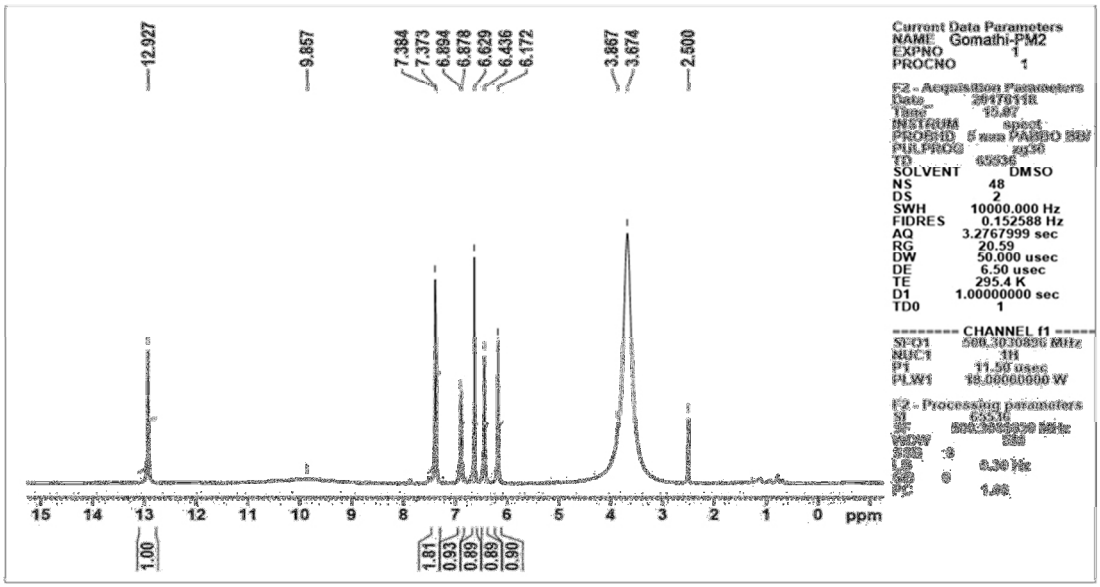
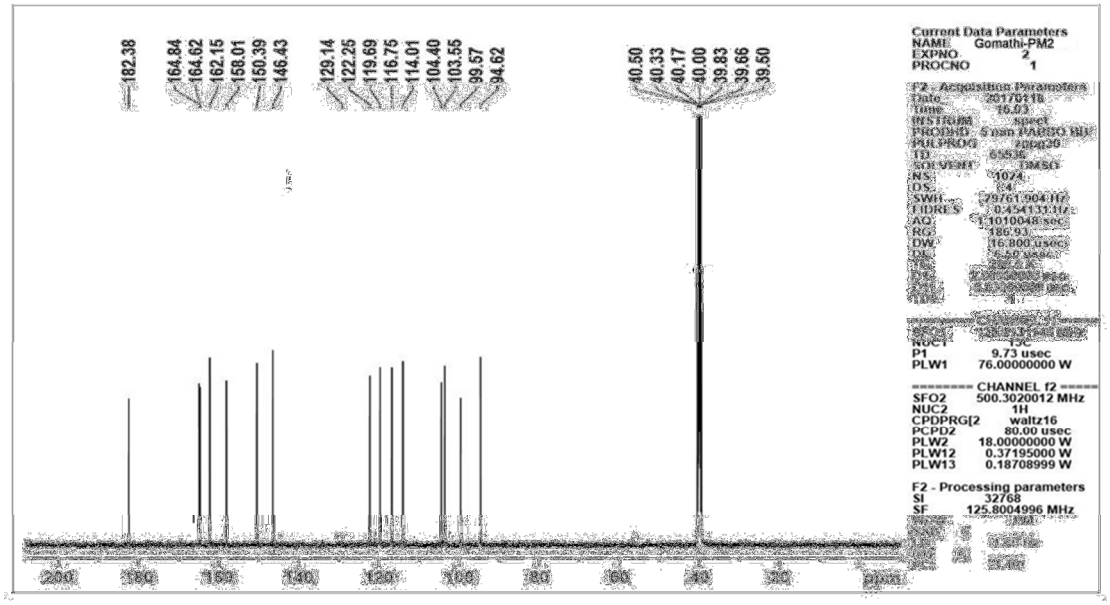


Figure 24. ^{13}C NMR spectrum of compound PM II



V.8. HPTLC Fingerprint profile of phytoconstituents

V.8.1. Flavonoid Profile

Toluene: Ethyl acetate: Formic acid (5:4:1) was used as the mobile phase for the HPTLC analysis of flavonoids. The spray agent 1 % ethanolic Aluminium chloride was used. The results illustrated the presence of 11 different types of flavonoids with 11 different Rf values ranging from 0.01 to 0.96 (Table 17). Yellow, yellowish blue coloured fluorescent zone at UV 366 nm mode present in the given standard and sample track observed in the chromatogram after derivatization, which confirmed the presence of flavonoid in the given standard and sample (Figure 25).

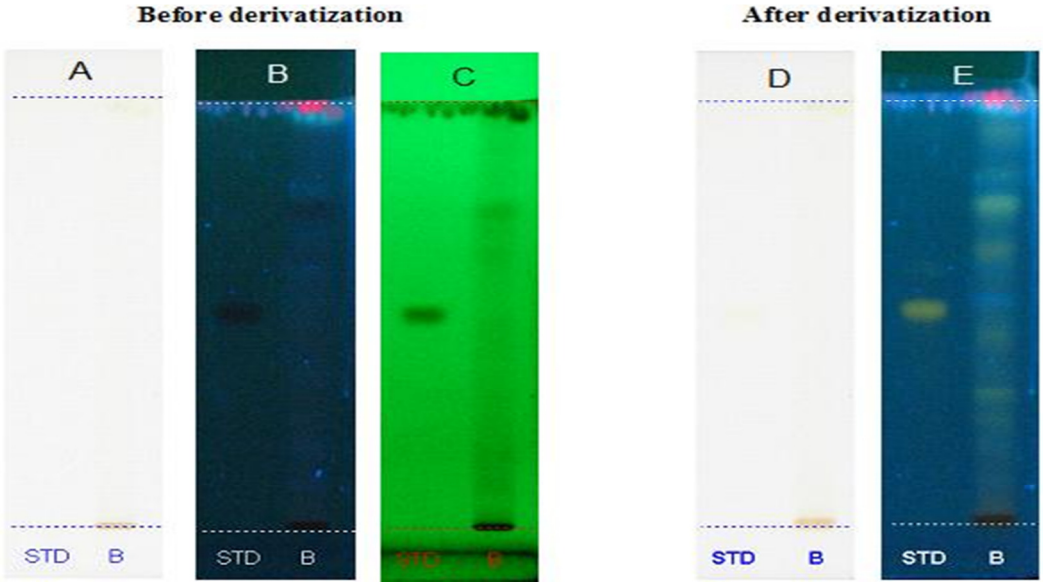
Table 17. Rf values of different flavonoids obtained using HPTLC analysis

Track	Peak	Rf	Height	Area	Assigned substance
Std Rutin	1	0.57	525	20399.8	**Flavonoid standard**
Sample B	1	0.01	21.5	234.4	Unknown
Sample B	2	0.08	615.8	25903.5	Unknown
Sample B	3	0.25	112.7	4178	Unknown
Sample B	4	0.31	93.9	3955.2	**Flavonoid 1**
Sample B	5	0.37	103.1	4397.3	**Flavonoid 2**
Sample B	6	0.50	99.7	4801.9	**Flavonoid 3**
Sample B	7	0.51	101.1	5266.5	**Flavonoid 4**
Sample B	8	0.71	151.3	8343.2	**Flavonoid 5**
Sample B	9	0.81	205.2	8774.3	**Flavonoid 6**
Sample B	10	0.89	26.6	582.2	**Flavonoid 7**
Sample B	11	0.96	26.9	720.2	Unknown

Sample B – 90 % v/v of ethanol extract of *Pedalium murex* Linn. leaves; Std Rutin– Standard Drug

Chromatogram of Flavonoids

Figure 25. HPTLC chromatogram of flavonoids



A-HPTLC plate exposed to day light; B- HPTLC plate exposed to UV 366 nm; C- HPTLC plate exposed to UV 254 nm; D- HPTLC plate showing flavonoid after adding the spray agent. E- HPTLC plate exposed to UV 366 nm.

Figure 26. Densitogram of 90 % v/v of ethanol extract of *Pedalium murex* Linn. leaves showing flavonoids

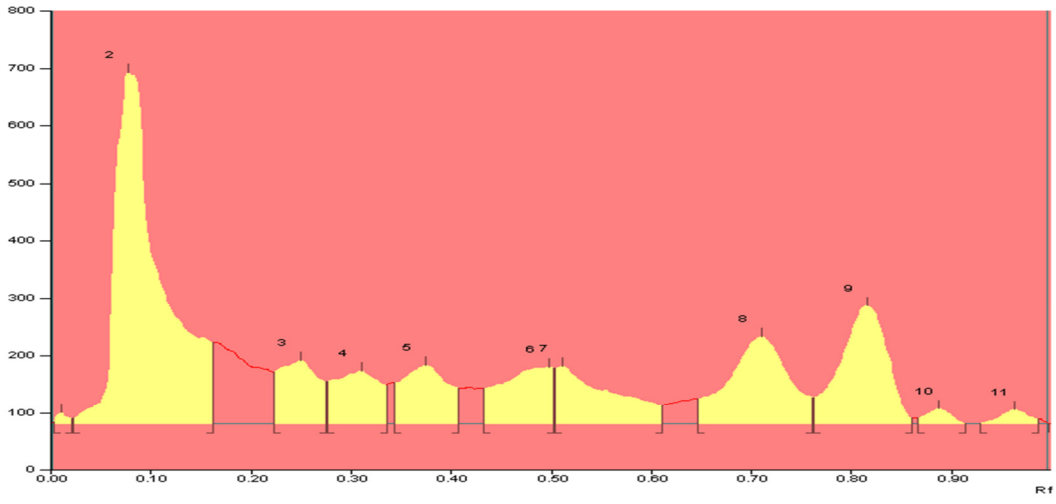


Figure 27. Densitogram of flavonoid standard peak (Scanned at 366 nm)

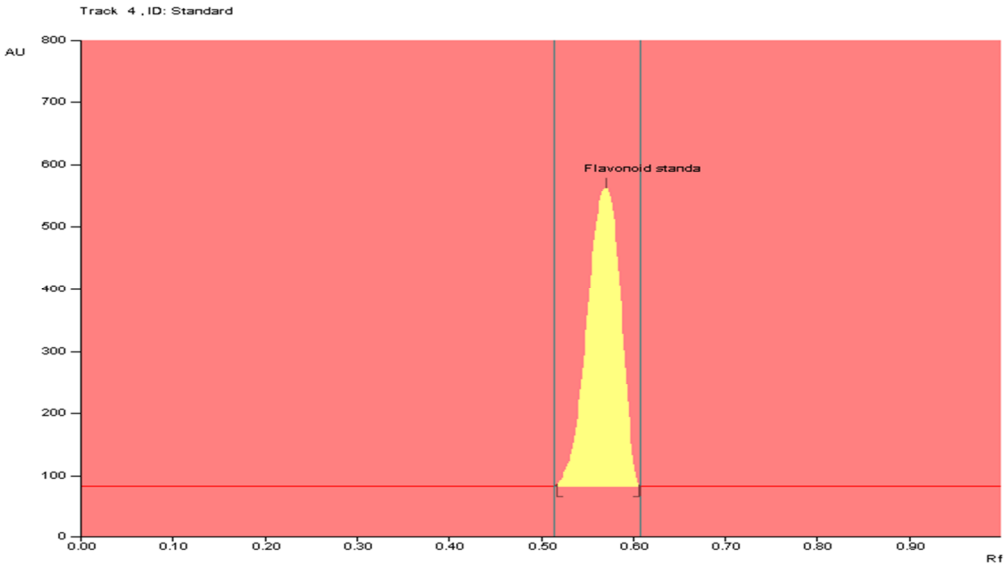
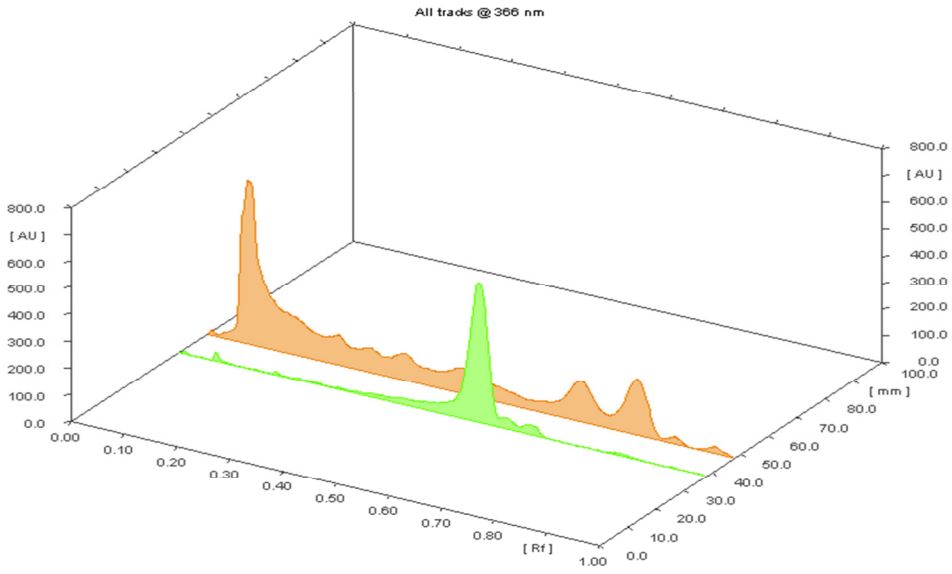


Figure 28. 3D displays of all tracks



V.9. Quantitative estimation of phyto-ingredients presents in *P. murex* leaves

V.9.1. Estimation of total phenolic compounds

The estimation of total phenolic content in EEPM has revealed that 100 µg/ml contains 0.164 gallic acid equivalent of phenolics. The result designates a strong association between anti-oxidative activities of phenolic compounds (R_2 = 0.9264), suggesting that phenolic compounds are possibly responsible for the anti-oxidative activities of *P. murex*.

Phenols exhibit significant anti-oxidant and free radical scavenging ability due to the presence of hydroxyl groups and effective hydrogen providing capability. Thus, therapeutic properties of *P. murex* may possibly be qualified to the anti-oxidant property of phenolic compounds present in EEPM.

V.9.2. Estimation of total flavonoids

Total flavonoid content (expressed as µg quercetin equivalent/ml) was derived from a quercetin standard in the range of 20 -100 µg/ml (R_2 = 0.9805). It was observed for total polyphenols, total flavonoid contents of the ethanolic extract of *Pedalium murex* Linn. (EEPM) was 0.156 µg/ml.

PHASE II: PHARMACOLOGICAL STUDIES

V.10. Acute oral toxicity study

Acute oral toxicity study was done according to the OECD-423 guidelines. Sprague dawley (SD) rats were used for each dose level. The dose levels used were 5, 50, 300 and 2000 mg/kg b.w. for 4 groups (Table 18). Up to the dose level of 2000 mg/kg b.w. there was no significant toxic signs and behaviour was noted in any group for 14 days observational time. Hence 2000 mg/kg b.w. was considered as MTD [Maximum Tolerated Dose]. $1/20^{th}$, $1/10^{th}$ and $1/5^{th}$ of the value of MTD were taken as treatment dose [100 mg/kg - Low dose, 200 mg/kg - Medium dose, 400 mg/kg - High dose] for neuroprotective effect.

Table 18: Animal Grouping - Acute oral toxicity study (OECD-423 Guidelines)

Group	Animals (N)	Dose (mg/kg)
1	3	5
2	3	50
3	3	300
4	3	2000

V.11. Neuroprotective effect of EEPM

V.11.1. Effects of EEPM on general behavior parameters in LPS-induced rats

V.11.1.a. General behaviour

One-week training was performed in rats to prepare them for behavioral study. During the training period only, food and water were administered to rats. The fully trained rats were choice for the study. Following the administration LPS, the animals were observed for general behavior up to 45 min. LPS may result in psychosomatic and physiological fluctuations in behavior, attributable to excitotoxicity. The observations made on general behavior for a period of 45 min following administration of LPS, dexamethasone and pre-treatment with EEPM, is described below.

Rats treated with LPS revealed a continued violent behavior in comparison to the control rats. Pretreatment with EEPM for 30 days, resulted in profound calmness.

V.11.1.b. Food intake

The collective measurement of food intake is shown in Table 19 and Figure 29. In comparison to control group (0.311±0.054 g/g b.w), LPS-treated rats consumed significantly a lesser amount of food (0.188±0.013 g/g b.w). The suppression of food intake was found to be uppermost in LPS-treated group, in comparison to all other

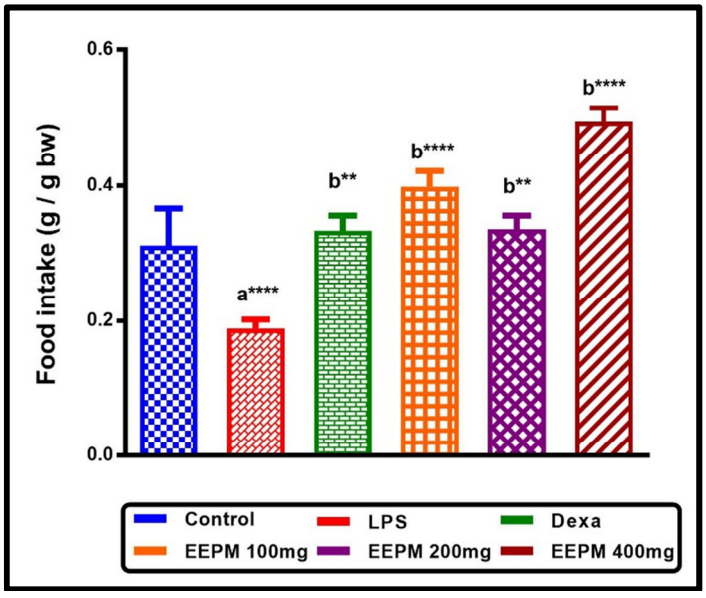
groups. This decline in food intake was associated with an increase in time spent in eating and in meal interval. The food intake of animals pre-treated with different doses of EEPM were measured to be 0.398±0.023 g/g b.w [100 mg/kg], 0.335±0.020 b.w [200 mg/kg], 0.495±0.019 g/g b.w [400 mg/kg] and Dexamethasone-treated group was found to be 0.333±0.022 g/g b.w.

The suppression of food intake in LPS-treated rats significantly antagonized by pre-treatment with EEPM at different dose levels. The food intake was significantly improved in EEPM treated rats.

Table 19. Effect of EEPM on food intake in LPS-treated rats

Groups	Food intake (in g/g b.w)
Control	0.311±0.054
LPS	$0.188\pm0.013^{a****}$
Dexamethasone	$0.333\pm0.022^{b**}$
EEPM 100 mg/kg	$0.398\pm0.023^{b****}$
EEPM 200 mg/kg	$0.335\pm0.020^{b**}$
EEPM 400 mg/kg	$0.495\pm0.019^{b****}$

Figure 29. Effect of EEPM on food intake in LPS-treated rats



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

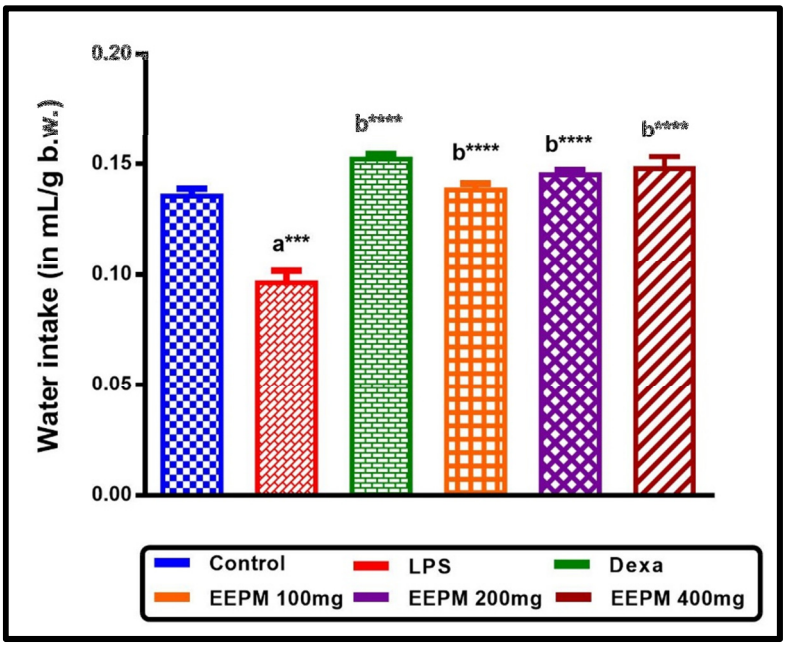
V.11.1.c. Water intake

The results on cumulative intake of water in all the groups of animals studied are shown in Table 20 and figure 30. In comparison to control group (0.136±0.002 mL/g b.w), LPS-treated rats consumed significantly less water (0.0973±0.004 mL/g b.w). The intake of water in EEPM pre-treated rats (100, 200 and 400 mg/kg) was found to be significantly more (0.139±0.001, 0.146±0.0009 and 0.149±0.004 mL/g b.w; respectively), in comparison to control and LPS-treated group. Similarly, dexamethasone-treated rats had consumed 0.153±0.001 mL/g b.w of water in comparison to LPS-treated group. The results indicate that administration of LPS had decreased consumption of water and this effect was antagonized by pre-treatment with EEPM.

Table 20. Effect of EEPM on water intake in LPS-treated rats

Groups	Water intake (mL/g b.w)
Control	0.136±0.002
LPS	$0.0973\pm0.004^{a***}$
Dexamethasone	$0.153\pm0.001^{b****}$
EEPM 100 mg/kg	$0.139\pm0.001^{b****}$
EEPM 200 mg/kg	$0.146\pm0.0009^{b****}$
EEPM 400 mg/kg	$0.149\pm0.004^{b****}$

Figure 30. Effect of EEPM on water intake in LPS-treated rats



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

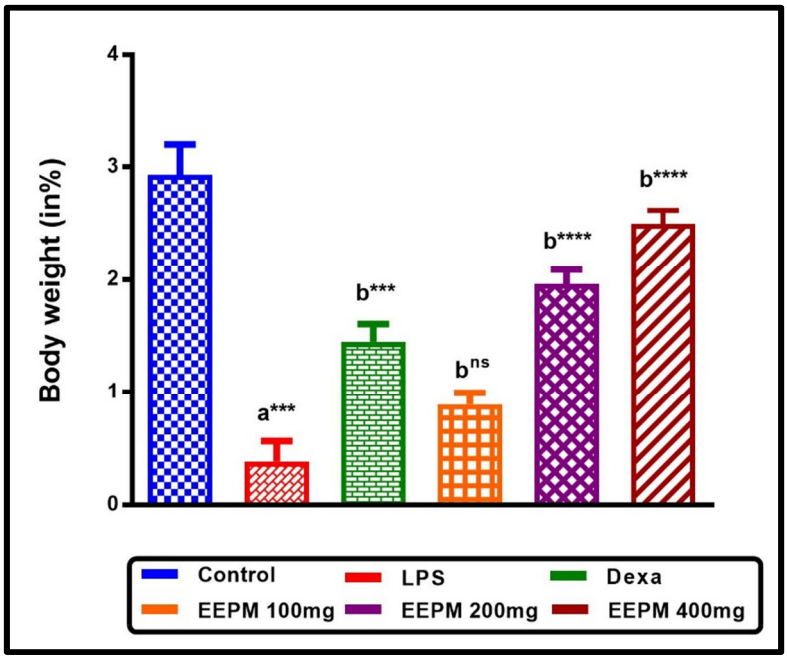
V.11.1.d. Body weight

The results of body mass shown in table 21 and figure 31. In comparison to the control group animals (2.93±0.269 g/g b.w), the LPS-treated animals (0.383±0.182 g/g b.w) were found to be decreasing in body weight. Pre-treatment with EEPM (100 mg/kg: 0.891±0.101 g/g b.w; 200 mg/kg: 1.962±0.129 g/g b.w; 400 mg/kg: 2.498±0.118 g/g b.w), prevented the suppression of rat body growth induced by LPS. EEPM pretreatment was found to improve the body weight of the animals in comparison to control and LPS-treated animals.

Table 21. Effect of EEPM on body weight in LPS-treated rats

Groups	Body weight (g/g b.w)
Control	2.93±0.269
LPS	0.383±0.182^{a***}
Dexamethasone	1.448±0.156^{b***}
EEPM 100 mg/kg	0.891±0.101
EEPM 200 mg/kg	1.962±0.129^{b****}
EEPM 400 mg/kg	2.498±0.118 ^{b****}

Figure 31. Effect of EEPM on body weight in LPS-treated rats



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

V.11.2. Behavioral test for anxiety and depression

V.11.2.a. Open field test

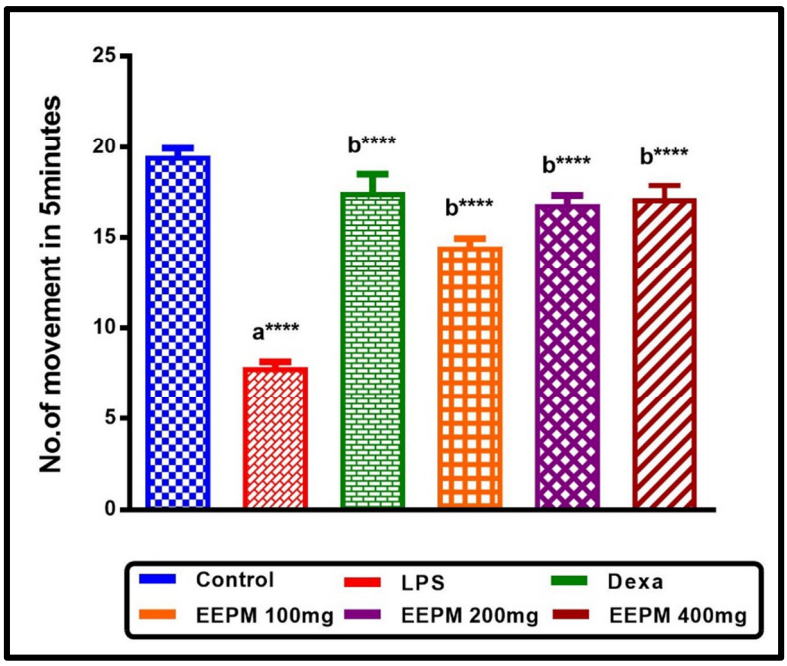
When tested on an open field apparatus, LPS-treated group (7.83±0.307) had significantly decreased in movement levels when compared to control animals (19.50± 0.42). A significant improvement in movement activity was seen in LPS-treated animals administered with EEPM at different doses (100 mg/kg: 14.50±0.42; 200 mg/kg: 16.83±0.47; 400 mg/kg: 17.17±0.70). The movement activity of 400

mg/kg of EEPM showed highly significant activity which was comparable with LPS-treated animals. The results are shown in Table 22 and Figure 32.

Table 22. Effect of EEPM in open field test

Groups	Number of movements (in 5mins)
Control	19.50±0.428
LPS	7.83±0.307^{a****}
Dexamethasone	17.50±0.991^{b****}
EEPM 100 mg/kg	14.50±0.428^{b****}
EEPM 200 mg/kg	16.83±0.477^{b****}
EEPM 400 mg/kg	17.17±0.703^{b****}

Figure 32. Effect of EEPM in open field test



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. (***P<0.05; **P<0.01; ***P<0.001; ****P<0.0001**)

V.11.2.b. Elevated plus maze test

An elevated plus-maze, LPS-treated group had significantly increased in anxiety levels (time spent in open arms [OP]: 7.33±1.25; time spent in closed arms [CL]: 234.2±6.10), when compared with control animals (OP: 42.83±2.72; CL: 234.2±6.10). A significant reduction in anxiety levels were observed in EEPM treated rats at different doses (100 mg/kg: OP: 22.83±2.00 and CL: 192.3±6.79; 200 mg/kg: OP: 32.17±2.79 and CL: 181.8±3.68; 400 mg/kg: OP: 40.17±2.08 and CL: 115.2±4.01). The behavioural effect of 400 mg/kg of EEPM showed excellent activity which was comparable with control animals and the results are shown in table 23, Figure 33 & 34.

Table 23. Effect of EEPM in elevated plus maze test

Groups	**Time spent in arms (in sec)**	
	Open arm entry	**Closed arm entry**
Control	42.83±2.72	172.2±5.00
LPS	7.333±1.25^{a***}	234.2±6.10 ^{a***}
Dexamethasone	30.17±0.70^{b****}	136.7±3.43^{b****}
EEPM 100 mg/kg	22.83±2.007^{b****}	192.3±6.79^{b****}
EEPM 200 mg/kg	32.17±0.79^{b****}	181.8±3.68^{b****}
EEPM 400 mg/kg	40.17±2.08^{b****}	142.2±4.01^{b****}

Figure 33. Effect of EEPM in elevated plus maze test (Open arm entry)

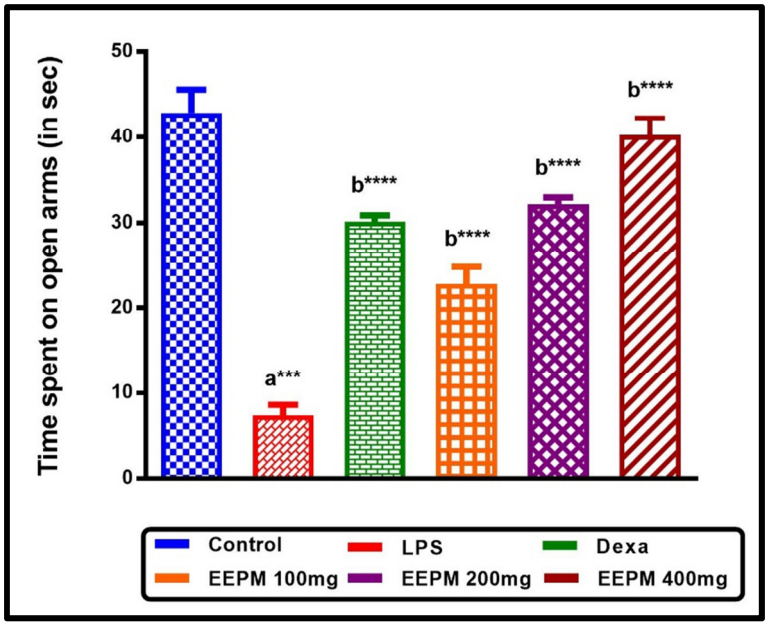
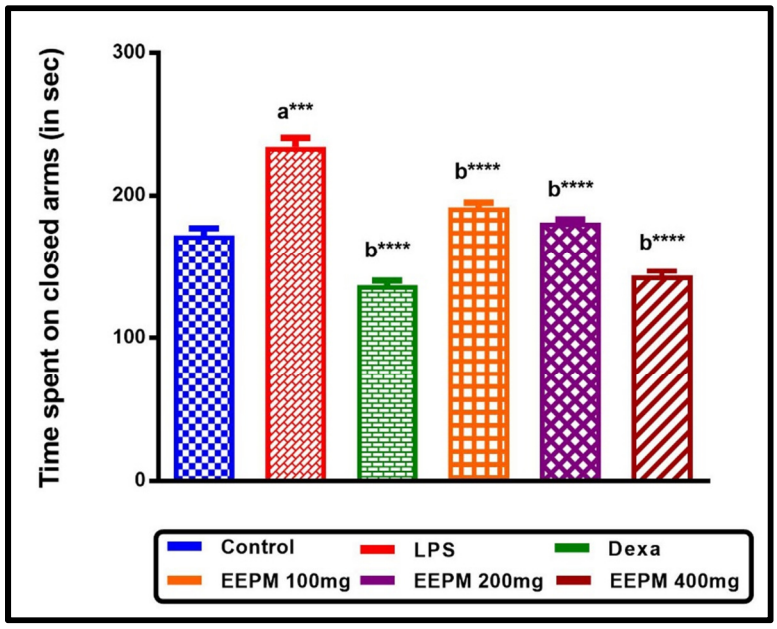


Figure 34. Effect of EEPM in elevated plus maze test (Closed arm entry)



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

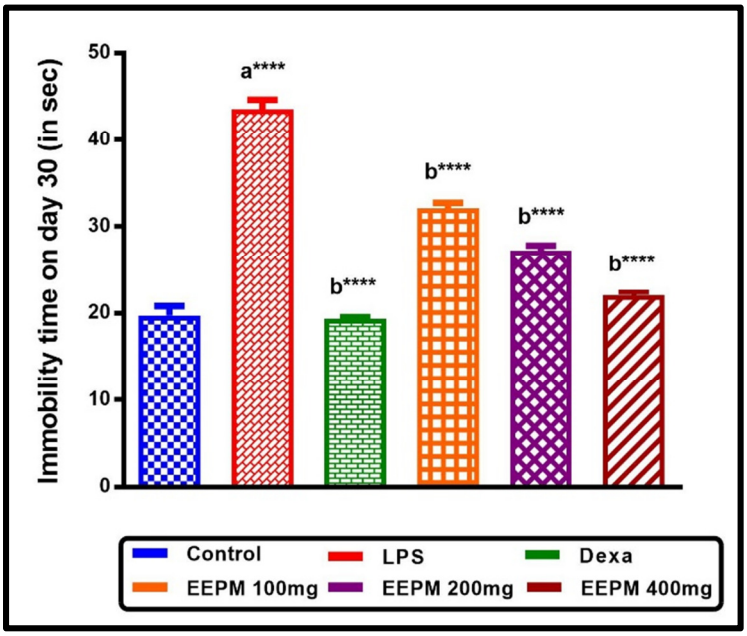
V.11.2.c. Forced swim test

A significant increase immobility time was observed in LPS-treated rats (43.54±1.07), when compared with control animals (19.72±1.06) and decrease in immobility time was seen in EEPM-treated animals at different doses (100 mg/kg: 32.08±0.61; 200 mg/kg: 27.00±0.78; 400 mg/kg: 22.52±0.63) in forced swim test. The results are shown in Table 24 and Figure 35.

Table 24. Effect of EEPM in forced swim test

Groups	Immobility time on day 30 (in sec)
Control	19.72±1.06
LPS	$43.54\pm1.07^{a****}$
Dexamethasone	$19.89\pm0.92^{b****}$
EEPM 100 mg/kg	$32.08\pm0.61^{b****}$
EEPM 200 mg/kg	$27.00\pm0.78^{b****}$
EEPM 400 mg/kg	$22.52\pm0.63^{b****}$

Figure 35. Effect of EEPM in forced swim test



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

V.11.3. Behavioural test for learning and memory

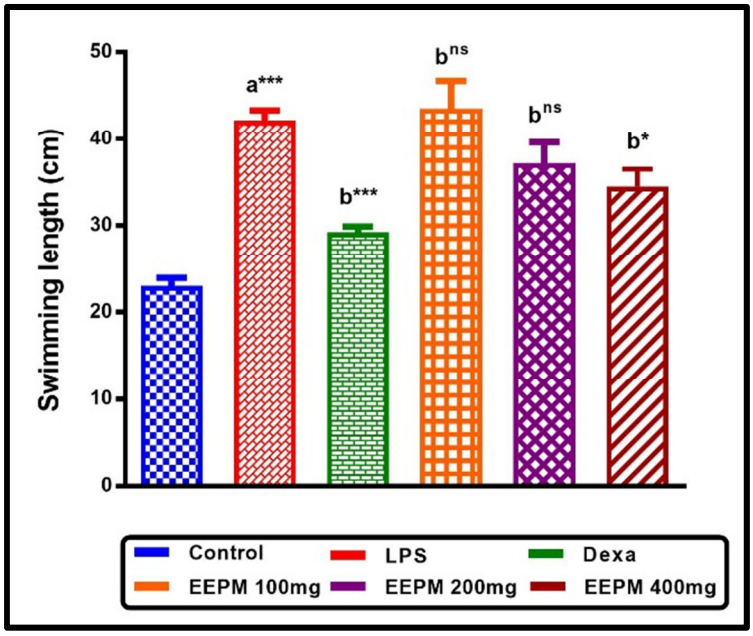
V.11.3.a. Water maze test

Swimming length (cm) was significantly increased in LPS-treated rats (42.17±1.13) when compared to control animals (23.00±0.93). Administration of EEPM (100 mg/kg: 43.50± 3.21; 200 mg/kg: 37.17±2.41; 400 mg/kg: 34.50±1.97) decreased the swimming length. The effect of EEPM was profound in 400 mg/kg, which is statistically significant compared to control group (Table 25 & Figure 36).

Table 25. Effect of EEPM in water maze test

Groups	Swimming length (cm)
Control	23.00±0.93
LPS	$42.17\pm1.13^{a***}$
Dexamethasone	$29.17\pm0.70^{b***}$
EEPM 100 mg/kg	43.50± 3.21
EEPM 200 mg/kg	37.17±2.41
EEPM 400 mg/kg	$34.50\pm1.97^{b*}$

Figure 36. Effect of EEPM in water maze test



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

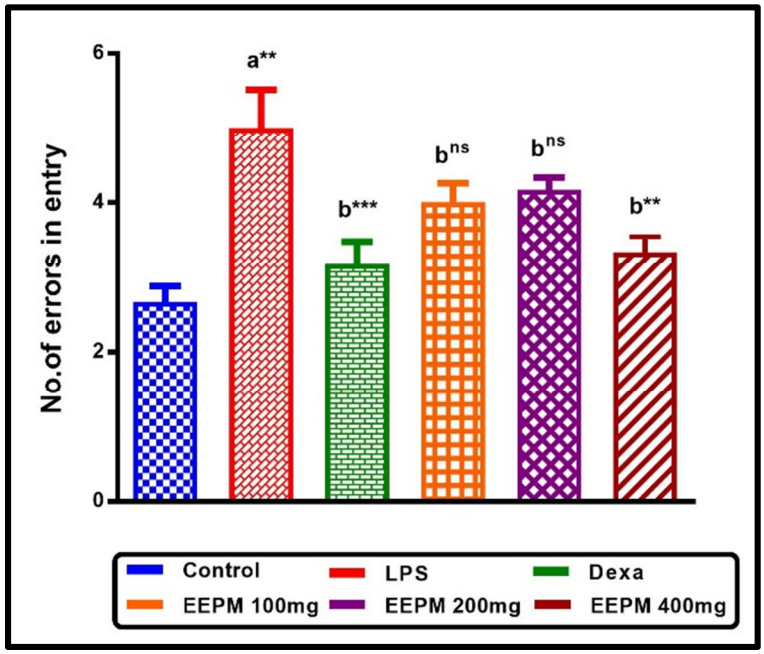
V.11.3.b. Radial arm maze test

LPS-treated group (5.00±0.51) showed significant number of errors in entry when compared to control animals (2.66± 0.21). Administration of different doses of EEPM at 100, 200 and 400 mg/kg decreased the number of errors in entry 4.00±0.36, 4.16±0.16 and 3.33±2.79 respectively when compared with control animals mentioned in Table 26 & Figure 37.

Table 26. Effect of EEPM in radial arm maze test

Groups	Number of errors
Control	2.66± 0.21
LPS	$5.00\pm0.51^{a**}$
Dexamethasone	$3.16\pm0.30^{b***}$
EEPM 100 mg/kg	4.00±0.36
EEPM 200 mg/kg	4.16±0.16
EEPM 400 mg/kg	$3.33\pm2.79^{b**}$

Figure 37. Effect of EEPM in radial arm maze test



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

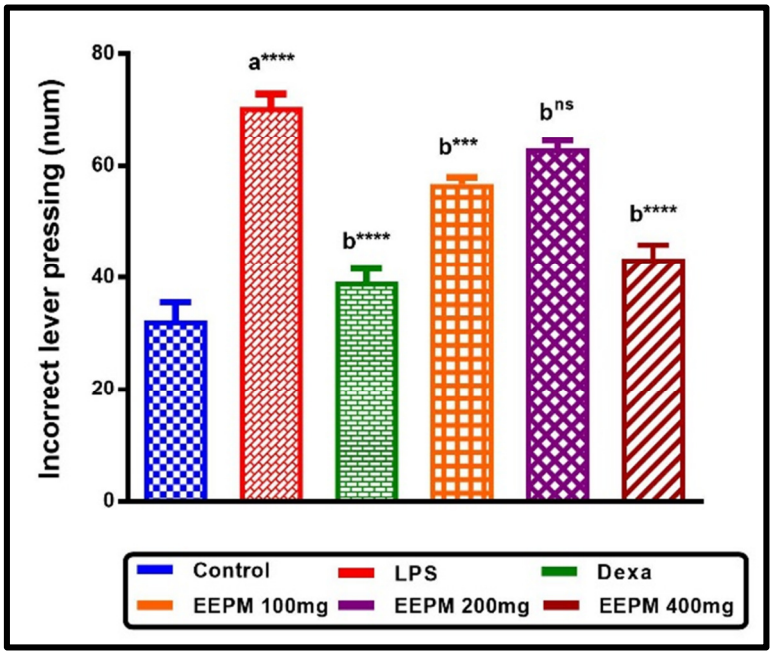
V.11.3.c. Choice reaction time task test

Increased incorrect lever pressing was observed in LPS-treated rats when compared to control animals (32.33± 3.28) in a CRT apparatus mentioned in Table 27 & Figure 38. Incorrect lever pressing was minimized by when EEPM was administered at 100, 200 and 400 mg/kg (56.83±1.13, 63.17±1.42, and 43.50±2.40 respectively). EEPM at 400 mg/kg showed significant activity when compared with LPS-treated group (70.67± 2.27).

Table 27. Effect of EEPM in choice reaction time task test

Groups	No of incorrect lever pressing
Control	32.33± 3.28
LPS	$70.67\pm2.27^{a****}$
Dexamethasone	$39.17\pm2.46^{b****}$
EEPM 100 mg/kg	$56.83\pm1.13^{b***}$
EEPM 200 mg/kg	63.17±1.42
EEPM 400 mg/kg	$43.50\pm2.40^{b****}$

Figure 38. Effect of EEPM in Choice reaction time task test



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

PHASE-III: BIOCHEMICAL ANALYSIS

In vivo enzymatic & non-enzymatic antioxidant levels were measured in the brain hippocampus

V.11.4. *In vivo* non-enzymatic antioxidant studies in brain hippocampus

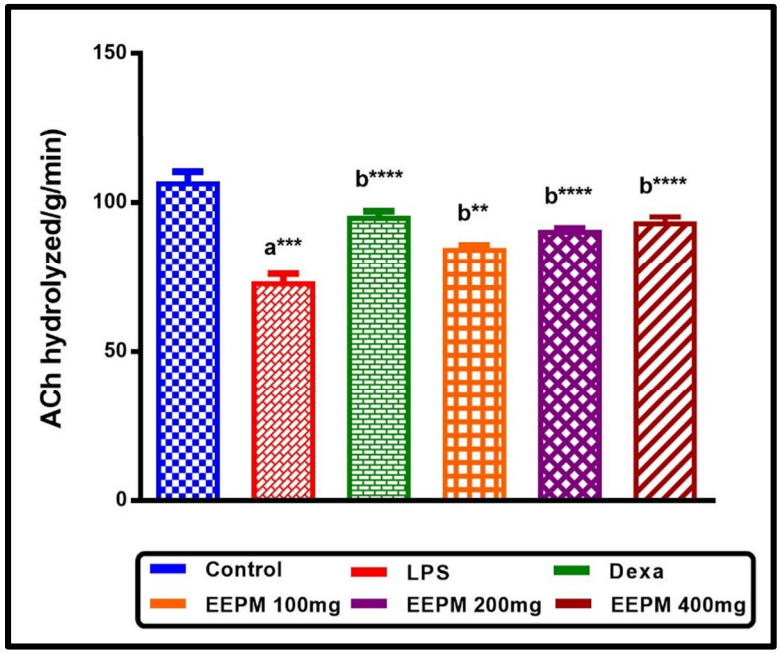
V.11.4.a. Effect of EEPM on Acetylcholine (ACh) levels in LPS-treated rat brain

In comparison to control rats (107.0± 3.24), LPS induction decreased the amount of ACh level in brain (73.33±2.64). Pre-treatment with EEPM at different doses (100 mg/kg: 84.83±0.87; 200 mg/kg: 90.83±0.60; 400 mg/kg: 93.67±1.58) for 30 days significantly increases the ACh level in comparison to LPS-treated groups represented in Table 28 and Figure 39.

Table 28. Effect of EEPM on Acetylcholine (ACh) levels in LPS-treated rat brain

Groups	ACh hydrolysed/g/min
Control	107.0± 3.24
LPS	73.33±2.64^{a***}
Dexamethasone	95.67±1.45^{b****}
EEPM 100 mg/kg	84.83±0.87^{b**}
EEPM 200 mg/kg	90.83±0.60 ^{b****}
EEPM 400 mg/kg	93.67±1.58 ^{b****}

Figure 39. Effect of EEPM on Acetylcholine (ACh) levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

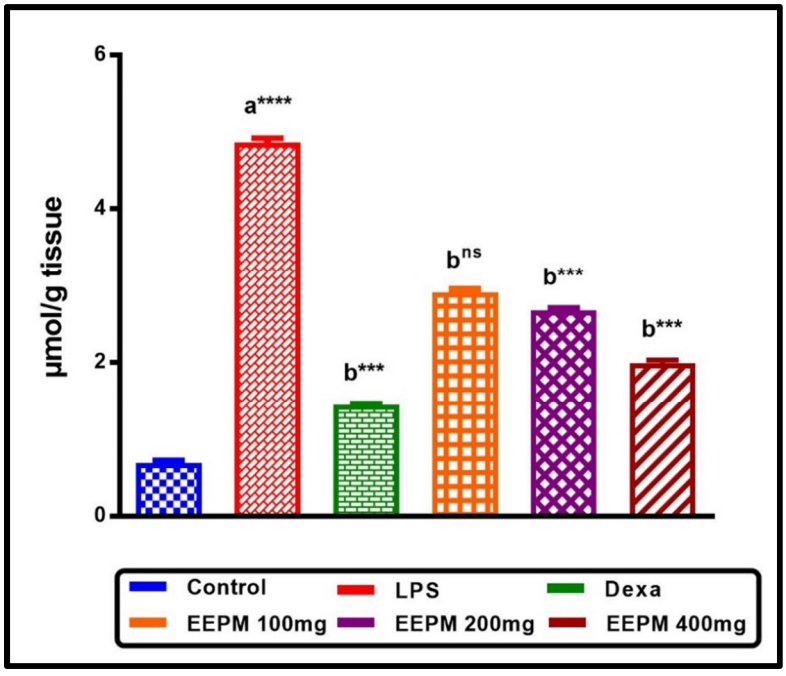
V.11.4.b. Effect of EEPM on Nitric oxide (NO) levels in LPS-treated rat brain

Nitric oxide (NO) acts as signaling molecule in inflammation. The excessive production of NO was observed in LPS-treated rats (4.87±0.10) when compared to control animals (0.68± 0.16) as shown in Figure 41 & Table 31. Treatment with EEPM at different concentrations (100 mg/kg: 2.90±0.13; 200 mg/kg: 2.67±0.82; 400 mg/kg: 1.98±0.55) significantly inhibited the nitric oxide production when compare to LPS-treated rats shown Table 29 and Figure 40.

Table 29. Effect of EEPM on Nitric oxide (NO) levels in LPS-treated rat brain

Groups	µmol/g tissue
Control	0.68± 0.16
LPS	$4.87\pm0.10^{a****}$
Dexamethasone	$1.43\pm0.09^{b***}$
EEPM 100 mg/kg	2.90±0.13
EEPM 200 mg/kg	$2.67\pm0.82^{b***}$
EEPM 400 mg/kg	$1.98\pm0.55^{b***}$

Figure 40. Effect of EEPM on Nitric oxide (NO) levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. (***P<0.05; **P<0.01; ***P<0.001; ****P<0.0001**)

V.11.4.c. Effect of EEPM on Protein levels in LPS-treated rat brain

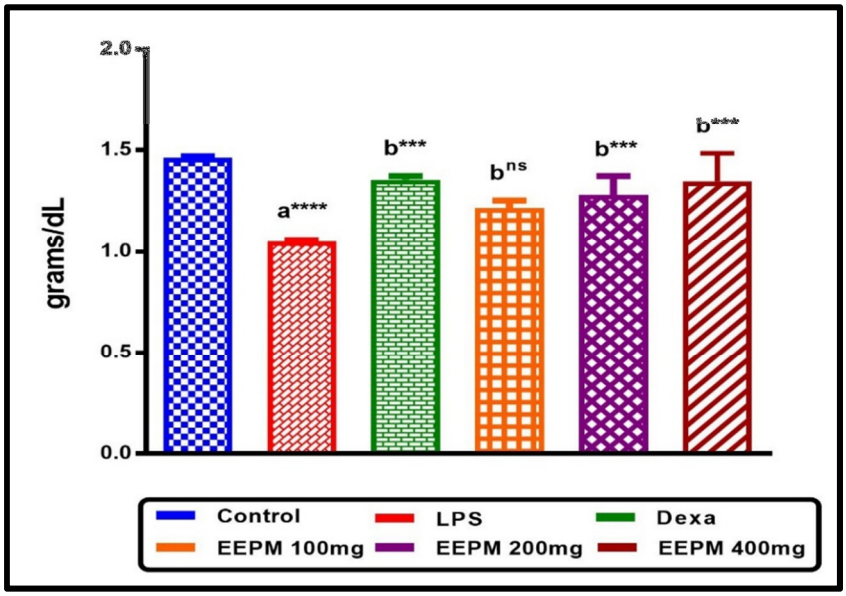
Reduced level of protein (1.04±0.10) was seen in LPS-treated rats when compared to control animal (1.46± 0.03) in Table 30 & Figure 41. Reduced level of protein was recovered in EEPM (100 mg/kg: 1.21±0.02; 200 mg/kg: 1.28±0.09; 400

mg/kg: 1.34±0.12) as like standard dexamethasone treated rats (1.35±0.17), control rats. A significant regaining was seen in EEPM 400 mg/kg.

Table 30: Effect of EEPM on Protein levels in LPS-treated rat brain

Groups	Total proteins g/dL
Control	1.46± 0.03
LPS	1.04±0.10^{a****}
Dexamethasone	1.35±0.17^{b***}
EEPM 100 mg/kg	1.21±0.02
EEPM 200 mg/kg	1.28±0.09^{b***}
EEPM 400 mg/kg	1.34±0.12^{b***}

Figure 41: Effect of EEPM on Protein levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

V.11.4.d. Effect of EEPM on lipid peroxide and malonaldehyde (LPO & MDA) levels in LPS-treated rat brain

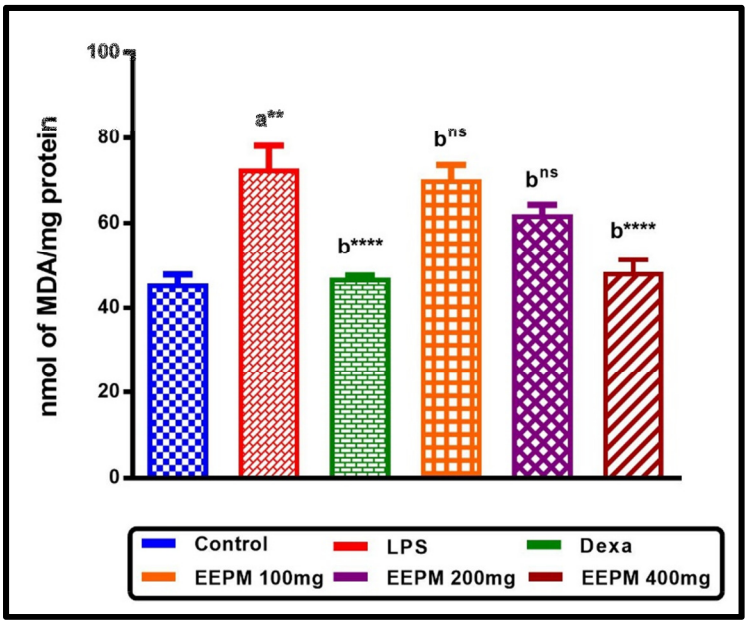
The effect of EEPM in LPS-treated rats on MDA levels are summarized in Table 31 and Figure 42. In comparison to control rats (45.71±2.10), LPS induction resulted in significant increase in MDA level of brain (72.79±5.31). Pre-treatment with EEPM at different doses (100 mg/kg: 70.30±3.27; 200 mg/kg: 62.10±2.23; 400

mg/kg: 48.42±2.85) for 30 days significantly improve the MDA activity in comparison to LPS-treated groups in the brain. Among all groups, a marked decrease in MDA status was observed in LPS-treated groups administered with 400 mg/kg of EEPM.

Table 31: Effect of EEPM on lipid peroxide and malonaldehyde (LPO & MDA) levels in LPS-treated rat brain

Groups	nmol/mg protein
Control	45.71± 2.10
LPS	72.79±5.31^{a**}
Dexamethasone	47.02±0.54^{b****}
EEPM 100 mg/kg	70.3±3.27
EEPM 200 mg/kg	62.1±2.23
EEPM 400 mg/kg	48.42±2.85^{b****}

Figure 42: Effect of EEPM on lipid peroxide and malonaldehyde (LPO & MDA) levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. (***P<0.05; **P<0.01; ***P<0.001; ****P<0.0001**)

V.11.5. *In vivo* enzymatic antioxidant studies in brain hippocampus

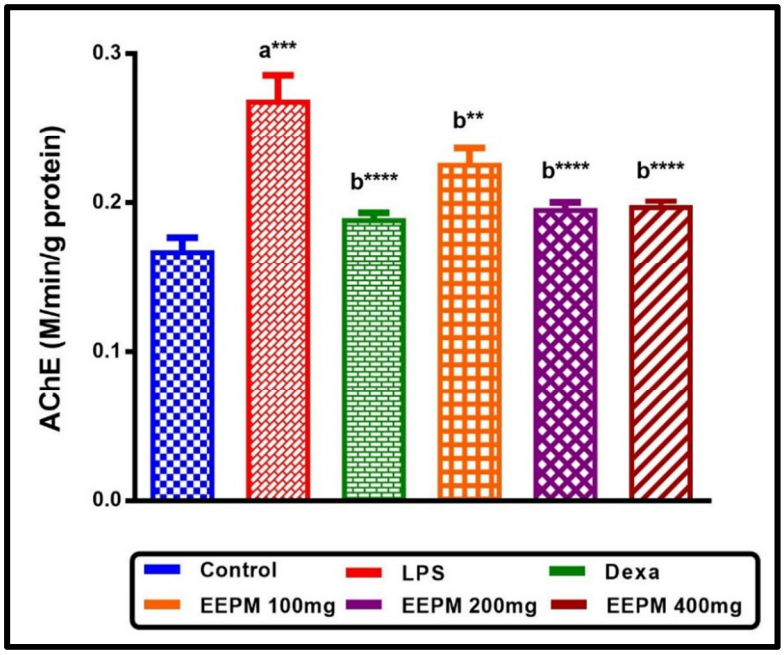
V.11.5.a. Effect of EEPM on Acetylcholinesterase (AChE) levels in LPS-treated rat brain

In comparison to control rats (0.168±0.008), LPS induction increased the amount of AChE level in brain (0.269±0.016). Pre-treatment with EEPM at different doses (100 mg/kg: 0226±0.009; 200 mg/kg: 0.196±0.003; 400 mg/kg: 0.198±0.002) for 30 days significantly decreases the AChE activity in comparison to LPS-treated groups. Among all the groups, a marked decrease in AChE status was observed with 200 mg/kg of EEPM represented in table 32 and figure 43.

Table 32: Effect of EEPM on Acetylcholinesterase (AChE) levels in LPS-treated rat brain

Groups	AChE (M/min/g protein)
Control	0.168±0.008
LPS	$0.269\pm0.016^{a***}$
Dexamethasone	$0.189\pm0.003^{b****}$
EEPM 100 mg/kg	$0.226\pm0.009^{b**}$
0EEPM 200 mg/kg	$196\pm0.003^{b****}$
EEPM 400 mg/kg	$0.198\pm0.002^{b****}$

Figure 43: Effect of EEPM on Acetylcholinesterase (AChE) levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

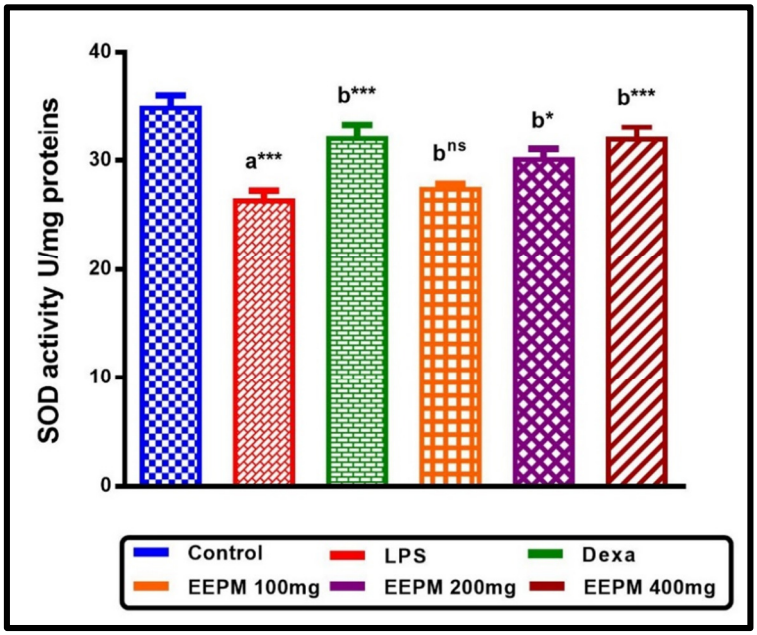
V.12.5.b. Effect of EEPM on Superoxide Dismutase (SOD) levels in LPS-treated rat brain

The SOD profile in the rat brain is depicted in table 33 and figure 44. In comparison to control rats (35.12±0.92), LPS induction resulted in significant reduction of SOD level in brain (26.54±0.70). Pre-treatment with EEPM at different doses (100 mg/kg: 27.59±0.28; 200 mg/kg: 30.28±0.79; 400 mg/kg: 32.16±0.98) for 30 days significantly improve the SOD activity in comparison to LPS-treated groups in the brain. Among all the groups, a marked increase in SOD status was observed with 400 mg/kg of EEPM.

Table 33: Effect of EEPM on Superoxide Dismutase (SOD) levels in LPS-treated rat brain

Groups	SOD activity U/mg
Control	35.12± 0.92
LPS	$26.54\pm0.70^{a***}$
Dexamethasone	$32.20\pm1.14^{b***}$
EEPM 100 mg/kg	27.59±0.28
EEPM 200 mg/kg	$30.28\pm0.79^{b*}$
EEPM 400 mg/kg	$32.16\pm0.98^{b***}$

Figure 44: Effect of EEPM on Superoxide Dismutase (SOD) levels in LPS treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

V.12.5.c. Effect of EEPM on Catalase (CAT) levels in LPS-treated rat brain

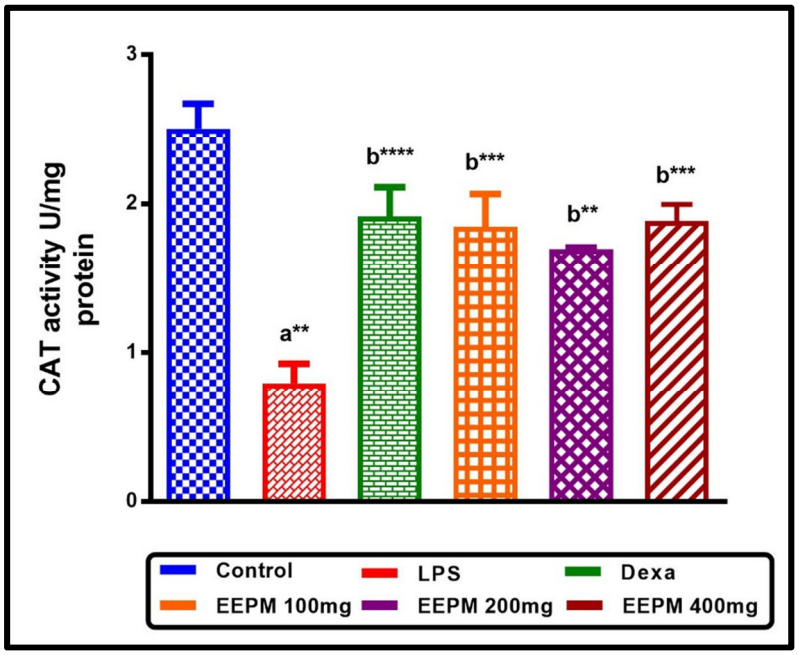
Table 34 and Figure 45 showed the alteration of CAT levels in brain studied. In comparison to control rats (2.50±0.16), LPS induction resulted in significant reduction of CAT level of brain (0.79±0.13). Pre-treatment with EEPM at different doses (100 mg/kg: 1.84±0.21; 200 mg/kg: 1.69±0.01; and 400 mg/kg: 1.88±0.1) for

30 days significantly improved the CAT activity in comparison to LPS-treated groups in the brain studied. Among all the groups, a marked increase in CAT status was observed with LPS-treated groups administered with 400 mg/kg of EEPM.

Table 34: Effect of EEPM on Catalase (CAT) levels in LPS-treated rat brain

Groups	µmoles of catalase/mg protein
Control	2.50±0.16
LPS	$0.79\pm0.13^{a**}$
Dexamethasone	$1.91\pm0.19^{b****}$
EEPM 100 mg/kg	$1.84\pm0.21^{b***}$
EEPM 200 mg/kg	$1.69\pm0.01^{b**}$
EEPM 400 mg/kg	$1.88\pm0.1^{b***}$

Figure 45: Effect of EEPM on Catalase (CAT) levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

V.12.5.d. Effect of EEPM on Glutathione Reductase (GR) levels in LPS-treated rat brain

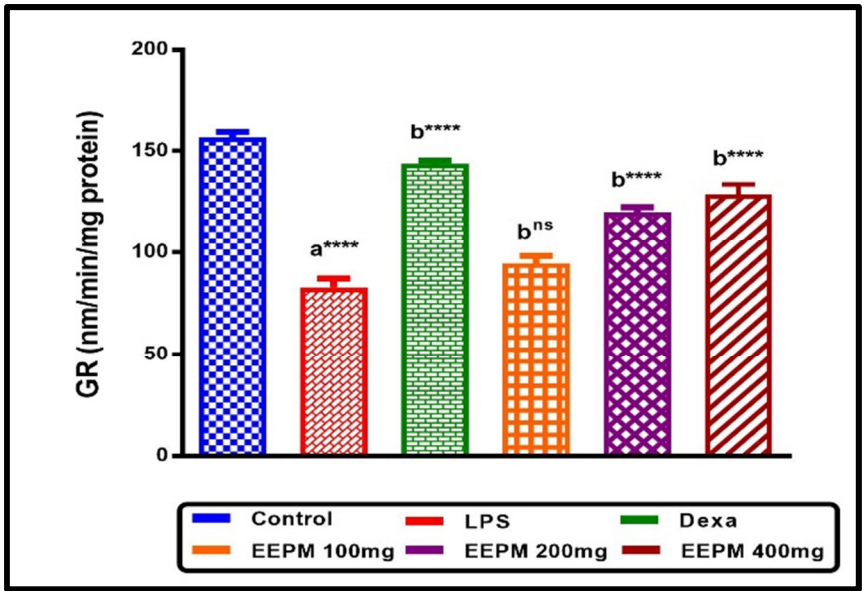
The effect of EEPM in LPS-treated rats on GR levels are summarized in Table 35 and Figure 46. In comparison to control rats (156.70±2.82), A significant decrease

of GR level of brain was seen in LPS-treated rats (82.83±4.43). Pre-treatment with EEPM at different doses (100 mg/kg: 94.50±3.76; 200 mg/kg: 120.0±2.46; 400mg/kg: 128.80±4.95) for 30 days significantly improved the GR activity in comparison to LPS-treated groups in the brain. Among all groups, a marked increase in GR status was observed in LPS-treated groups administered with 400 mg/kg of EEPM.

Table 35: Effect of EEPM on Glutathione Reductase (GR) levels in LPS-treated rat brain

Groups	GR (nm/min/mg protein)
Control	156.70±2.8
LPS	82.83±4.40^{a****}
Dexamethasone	143.70±1.78^{b****}
EEPM 100 mg/kg	94.50±3.76
EEPM 200 mg/kg	120.00±2.46^{b****}
EEPM 400 mg/kg	128.8±4.95^{b****}

Figure 46: Effect of EEPM on Glutathione Reductase (GR) levels in LPS-treated rat brain



Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

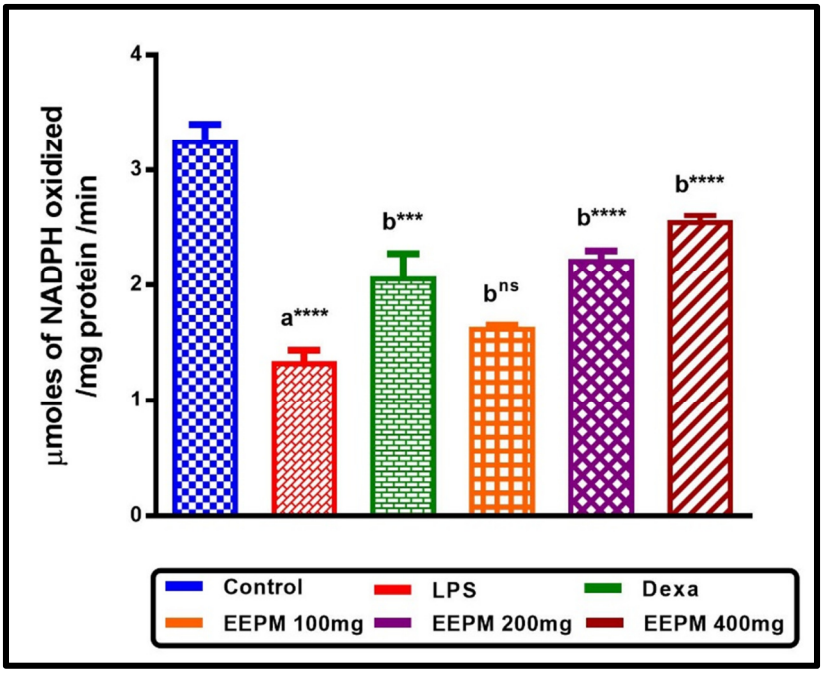
V.12.5.e. Effect of EEPM on Glutathione Peroxidase (GP_X) levels in LPS-treated rat brain

The effect of EEPM in LPS-treated rats on GP_X levels are summarized in Table 36 and Figure 47. In comparison to control rats (3.26±0.13), A significant decrease of GP_X level of brain was seen in LPS-treated rats (1.34±0.09). Pre-treatment with EEPM at different doses (100 mg/kg: 1.64±0.01; 200 mg/kg: 2.22±0.07;400mg/kg: 2.56±0.04) for 30 days significantly improved the GP_X activity in comparison to LPS-treated groups in the brain. Among all groups, a marked increase in GP_X status was observed in LPS-treated groups administered with 400 mg/kg of EEPM.

Table 36: Effect of EEPM on Glutathione Peroxidase (GP_X) levels in LPS-treated rat brain

Groups	µmoles of NADPH/mg protein/min
Control	3.26± 0.13
LPS	1.34±0.09^{a****}
Dexamethasone	2.07±0.20^{b***}
EEPM 100 mg/kg	1.64±0.07
EEPM 200 mg/kg	2.22±0.07^{b****}
EEPM 400 mg/kg	2.56±0.04^{b****}

Figure 47: Effect of EEPM on Glutathione Peroxidase (GP_X) levels in LPS-treated rat brain



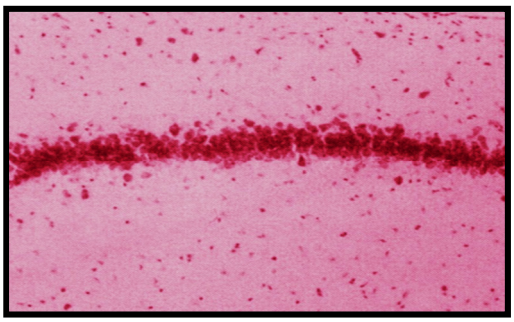
Values are expressed as mean±SEM, n=6 in each group. One-way ANOVA followed by Dunnett test was performed. **a**-Group I (Control) was compared with Group II (LPS control). **b**-Group II was compared with treated groups III, IV, V, VI. **(*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001)**

V.12.6. Histopathological studies

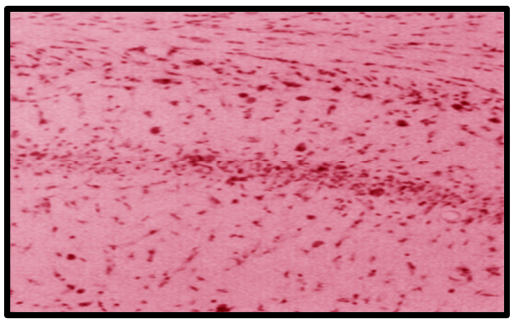
The histological changes of brain sections of rats treated with LPS, EEPM and Dexamethasone are shown in Figure 48. Gross structural anatomy of CA1 hippocampal neuronal cells were seen in the brain sections of control rats. The degeneration and disarray of neuronal damage (CA1 hippocampal), were seen in LPS-treated rats. In rats treated with EEPM (100 mg/kg; 200 mg/kg; 400 mg/kg) significantly prevent the neuronal damage and restore the brain neuronal anatomy. Brain sections of rats treated with dexamethasone showed CA1 hippocampal neuronal cells. The result showed that the endotoxin LPS-caused brain damage, are prevented by EEPM at higher doses (200 mg/kg and 400 mg/kg). EEPM at lower doses (100 mg/kg) is found to be less effective.

Figure 48: Effect of EEPM on histopathological studies in LPS-treated rat brain

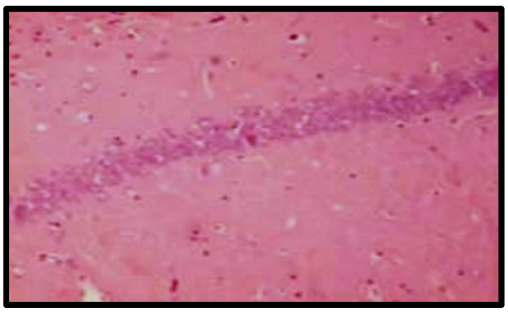
CONTROL



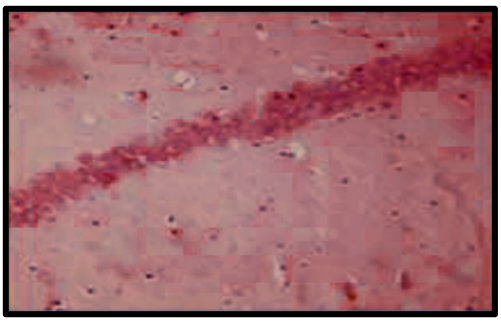
LPS



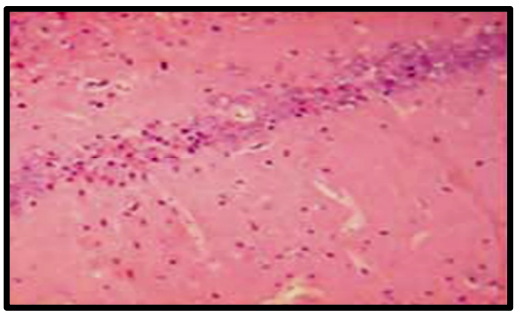
Dexamethasone



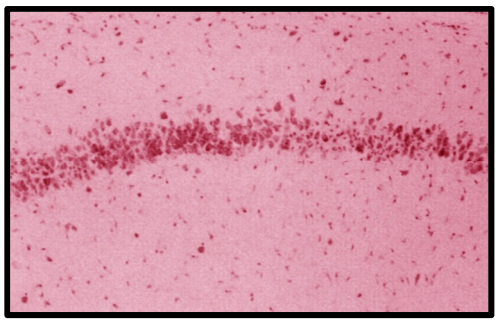
EEPM 100 mg/kg



EEPM 200 mg/kg



EEPM 400 mg/kg



Discussion

VI. DISCUSSION

Ayurveda, the world most prehistoric documented medical science is the first to advocate that healthy body cannot be attain without healthy mind and give the novel concept of psychosomatic disorders and psychiatric diseases.[173] Survey says that world is not threatened by spreading communicable diseases but by raising incidences of non-communicable diseases that spoil mind and thereby causing ailment in body. Stress and stress related disorders like mood disorders and personality disorders including anxiety and depression become the key problem of metropolitan cities.[174] Learning and memory is one of the most intensively studied subjects in the field of neuroscience.[175] Learning is the process of acquisition of information and skills, while subsequent retention of that information is called memory. Learning and memory together called as cognition.[176] Currently available psychoactive drugs, mainly anxiolytics and anti-depressants do not often properly meet the therapeutic demands of patients suffering co morbid psychiatric conditions, and the drawbacks of such drugs in terms of unwanted side effects, incredible benefits and moderate costs.[177] A variety of herbal extracts and their components have been demonstrated to exert neuroprotective effects associated with antioxidant activities,[178,179] either by directly stimulating antioxidant response genes or by potentiating the bodies' own natural antioxidant defense systems. *P. murex* shares numerous therapeutic properties with other 'rasayana' and its neuroprotective effect in different neurodegenerative models is arguable and yet to be established. In this study we had evaluated the herb *P. murex* leaf for LPS-induced endotoxemia along with antioxidant potential using ethanol solvent extract. The plant *P. murex* is a well-known herbal medicinal plant used in the Indian medications for treating quite a lot of diseases.

VI.1. Phytochemical studies

The plant *P. murex* leaves were collected, cleaned and dried. Dried leaves were extracted with 4 different solvents with increasing polarity such as n-Hexane, Chloroform, Ethyl acetate and finally 90% v/v of Ethanol (hydro alcoholic) in soxhlet apparatus. 90% v/v of Ethanol extraction given more extractive value – 8.34% w/w when compared to other extracts. Preliminary phytochemical screening revealed, n-hexane extract contains the compounds includes fixed oils and fats, steroids, gum & mucilage. Chloroform extract contains glycosides, tannins, phenolic compounds. Ethyl acetate extract contains alkaloids, carbohydrates, proteins, saponins. Ethanol

extract contains flavonoids, glycosides, tannins, carbohydrates, phenolic compounds, triterpenoids and saponins.

Constant generation of free radicals occurs in living system and it causes extensive damages to the cells, tissues and organs which may lead to various disease states, especially degenerative disorders and inflammatory diseases. Antioxidants resist cells and prevent the diseases through scavenging the free radical molecules, inhibiting lipid peroxidation's and many other mechanisms.

The total antioxidant capacity of ethanol extract was found to be higher (93.73% at 200 µg/ml) when compared to other solvents used. The total antioxidant capacities of different solvent extracts of leaves of *P. murex* at various concentrations (12.5-200 µg/ml) were in the following order: ethanol>chloroform> ethyl acetate > n-hexane.

The DPPH scavenging activity of *P. murex* leaf extracts were evaluated using ascorbic acid (positive control). DPPH reduction is directly proportional to the antioxidant content in the extract. Higher the antioxidant contents produced higher DPPH reduction. Ethanol extract exhibited the maximum DPPH scavenging activity of 64.43% at a concentration of 200 µg/ml with IC_{50} value of 21.91 µg/ml and it showed the antioxidant activity. The scavenging activity of *P. murex* extracts were in the following order: ethanol >n-hexane >ethyl acetate > chloroform.

Ethanol extract exhibited higher nitric oxide scavenging at the concentration of 200 µg/ml. The superoxide anion activity of extracts of leaves of *P. murex* in this method is due to either inhibiting or scavenging the radicals. The order of super oxide anion scavenging activity of *P. murex* leaf extract in the study was found to be, ethanol >ethyl acetate >n-hexane > chloroform. The hydroxyl radical activity of extracts of leaves of *P. murex* in the order of antioxidant potency of *P. murex* leaf extracts in our study was found to be, chloroform >ethanol >n-hexane. In reducing power n-hexane and ethanol extracts exhibited higher reducing power when compared to other extracts and the order of reducing power of *P. murex* leaf extracts in our study was found to be, ethanol >n-hexane>chloroform> ethyl acetate.

Based on extractive values, preliminary phytochemical studies and *in vitro* free radical scavenging activities, the ethanol extract of *Pedalium murex* Linn. leaves (EEPM) was carefully chosen for isolation and animal studies.

Thin layer chromatography has confirmed the results of preliminary phytochemical screening through staining. TLC is an analytical method used to

identify the secondary metabolites of plant extracts and to separate the active constituents of these metabolites. Retention factor of the compound was calculated.[180] Many solvent systems were tried to attain a good resolution. Toluene: Ethyl acetate: Formic acid (5:4:1) exhibited six spots in iodine vapors, R_f values were 0.53, 0.65, 0.74, 0.84, 0.90 and 0.96 (Table 11). Solvent systems play a crucial role in separation and isolation. Finally, the solvent system Toluene: Ethyl acetate: Formic acid (5:4:1) was selected for column chromatography.

Based on TLC, Toluene: Ethyl acetate: Formic acid (5:4:1) solvent system used for CC, with wet packing and isocratic elution method (Table 12). Fraction 25-28, 33-36 obtained from CC was selected and named as PM I and PM II. Physical and chemical characters of the compounds where analyzed & it showed the positive tests for flavonoids.

IR spectra of the isolated compound PM I was carried out and data showed the presence of C-H stretching at 2926.97 cm^{-1}, Aromatic O-H stretching at 3283. 21cm^{-1}, C=C stretching at 1606.57 cm^{-1}, carbonyl (C=O) stretching at 1656.26 cm^{-1}, C-O stretching at 1171.64 cm^{-1}. IR spectra of the isolated compound PM II was carried out and data showed the presence of phenolic OH at 3419.89 cm^{-1}, C=O stretch at 1654.29 cm^{-1}, carbonyl (C=C) stretching at 1609.54 cm^{-1}, C-O stretching at 1169.42 cm^{-1}.

The mass spectrum of PM I showed the m/z 269.05 suggesting approximate molecular weight as 270 and the spectrum gave the fragment ion peaks at 150.35, 132.50 and of PM II showed the m/z 284.95 suggesting approximate molecular weight as 285 and the spectrum gave the fragment ion peaks at 181.35, 159.25, 146.65.

Protocol of the ^1H NMR spectrum showed the presence of broad peaks at δ10.36, 10.80 and at 12.95 suggesting the presence of three phenyl hydroxyl groups. The ^1H NMR spectrum of **PM-I** also showed the presence of two meta coupled aromatic doublets at δ6.18 and 6.45 corresponds to H-6 and H-8 protons, two doublets at δ6.95 (d,2H,J=6.4Hz) and 7.81 (d, 2H,J= 6.4Hz) for H-3'/H-5' and H-2'/H-6' protons of ring B, and a singlet at δ6.73 corresponding to H-3 proton; characteristic for a 5,7,4'-trisubstituted flavone.

Protocol of the ^{1}H NMR spectrum of **PM-II** showed the presence of three meta coupled aromatic doublets at δ6.17, 6.43 and 6.87, a singlet at 6.62, one ortho coupled aromatic proton and one ortho and meta coupled aromatic proton appeared as a multiple at δ7.37 are characteristic for a polyphenol.

The ^{13}C NMR spectra showed the presence of fifteen carbon atoms (Table 15). Results of spectral data suggested that **PM-I** had structural similarities with 4´, 5, 7-trihydroxy flavone which may have the presence of Apigenin.

The ^{13}C NMR spectra showed the presence of fifteen aromatic carbons (Table 16). Results of spectral data suggested that **PM-II** had structural similarities with 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one which may have the presence of Kaempferol.

HPTLC finger print profiling and spectral data revealed that higher levels of flavonoids were predominantly found in the EEPM. These phytochemicals exhibited various pharmacological and biochemical activities when ingested in animals. Quantitative estimations had shown, total flavonoids and total phenolic components were almost found in the EEPM when compared to standard.

VI.2. Acute oral toxicity study

The present study indicates various doses 5 mg, 50 mg, 300 mg, and 2000 mg of EEPM did not produce any signs of acute toxicity in normal rats. Based on the acute toxicity study results the doses were fixed at 100mg/kg as low dose, 200mg/kg as medium dose and 400 mg/kg as high dose for the neuroprotective studies.

VI.3. Neuro protective activity

EEPM leaves (100 mg/kg, 200 mg/kg and 400 mg/kg) were decreased the neurodegeneration in LPS-induced rats which was compared with control rats. The current study has been undertaken to evaluate the neuroprotective activity of EEPM on LPS-induced neurodegeneration in rats. The effect of EEPM also evaluated on the behavioral studies especially for anxiety & depression, learning & memory, *in vivo* non- enzymatic [acetylcholine (ACh), nitric oxide (NO), lipid peroxide (LPO), protein] and enzymatic [acetyl cholinesterase (AChE), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT)] antioxidant studies were performed in rat's brain hippocampus.

VI.4. Behavioral studies

After subjecting the animals to LPS-induced endotoxemia, changes in food intake, water intake and body weight were observed from the time of induction. Thereafter, the animals were subjected to a battery of behavioral studies, viz., anxiolytic tests using elevated plus maze test, open field test, depressive test using forced swim test and impairment of cognition and memory using water maze test, radial arm maze, choice reaction time task test.

The development of anxiety & depression and impairment of cognition were evident as observed from decreased activity in elevated plus maze, increased periods of immobility in forced swim test, open field test, increased errors and trials in an 8-arm radial maze apparatus, decreased swimming length in water maze test, increased levels of incorrect lever pressing in choice reaction time task.

To support the neuroprotective effect of EEPM, the implications on general behaviour, body weight (BW), food intake (FI) and water intake (WI) were also analyzed, as these behaviors could be disturbed in depression, anxiety and cognitive models, employed. The suppression of food, water intake in LPS-treated rats significantly antagonized by pre-treatment with EEPM 400 mg/kg dose level which was compared with control rats. In comparison to the control group animals (2.93±0.269 g/g b.w), the LPS-treated animals (0.383±0.182 g/g b.w) were found to be declining in body weight. EEPM 400 mg/kg improves the body mass.

The open-field test has been used widely to assess emotionality and locomotor performance. [181,182] Open-field test was used to exclude these false effects that could be associated with hyperkinesia. The main difference between antidepressants and psycho-stimulants is that antidepressants do not increase general motor activity.[183] In our study, administration of EEPM increased the locomotor activity at doses that produced an antidepressant like effect, indicating that the specific actions of this extract on the behavioral model are predictive of antidepressant activity. In addition, the antidepressant effect of EEPM was influenced by changes in locomotor activity. The locomotion activity of 400 mg/kg of EEPM (400 mg/kg: 17.17±0.70) showed highly significant activity which was comparable with LPS-treated animals (Table 24 and Figure 33).

The elevated plus maze is an etiologically valid animal model of anxiety because it uses natural stimuli viz. fear of a novel open space and fear of balancing on a relatively narrow, raised platform that can induce anxiety in humans.[184] The ratio of

open/closed area entries and the time spent reflect a specific effect on anxiety. In the present study, oral administration of EEPM (100 mg/kg: OP: 22.83±2.00 and CL: 192.3±6.79; 200 mg/kg: OP: 32.17±2.79 and CL: 181.8±3.68; 400 mg/kg: OP: 40.17±2.08 and CL: 115.2±4.01) exhibited an anxiolytic like effect in rats, since it increased the number of entries and the time spent on open arms and decreased the time spent in closed arms in the elevated plus maze test. In agreement with previously published reports, diazepam increased the percentage of time spent on open arms and the number of entries into the open arms.[185]

Moreover, the anti-immobility effect produced by EEPM shared some pharmacological mechanisms with established antidepressant drugs in this investigation and showed dose dependent antidepressant effect. Again, forced swim test increase the cortisol level of mice by altering hyperactivity.[186] EEPM showed antidepressant effect which was probably due to reduction of the corticosterone concentration in rats exposed to forced swim test. In fact, hyperkinesia also causes false positive effect in forced swim test by shortening the immobility time in EEPM-treated animals at different doses (100 mg/kg: 32.08±0.61; 200 mg/kg: 27.00±0.78; 400 mg/kg: 22.52±0.63) when compared with LPS-treated rats (43.54±1.07).

Mazes are traditionally used to evaluate spatial learning and memory. Spatial memory is a form of short term memory utilizing neuro circuitry that provides temporary storage and manipulation of information necessary for complex cognitive tasks such as language comprehension, learning and reasoning.[187] Its impairment is analogous to memory disorder in Alzheimer's dementia.[188] EEPM pre-treatment significantly reduced escape latency in Morris water maze performance, facilitating learning and memory processes integral to spatial navigation. The reduction in distance travelled with lower doses of EEPM does not meet the criteria of a classic nootropic agent as the effect disappeared at higher doses.[189] However, distance travelled can be reduced either via improved cognition or a change in swimming behaviour influenced by serotonergic transmission.[190] In the present investigation, a significant nootropic effect was observed with respect to escape latency with all doses of EEPM. We believe that at higher doses, flavonoid components in EEPM may have exerted an inhibitory effect on swimming behaviour via serotonergic mechanisms,[191] and will explore this in future work.

In behavioral neuroscience trial, radial 8 arm-maze (RAM) task is widely used. [192,193] These RAM tests are useful in evaluating the effect of drugs, stress and

various other environmental factors on learning and memory. Working memory and reference memory are the two variables that report the physiological status of the brain.[194] Thus, LPS-treated rats with the EEPM (100, 200 and 400 mg/kg) exhibited an improvement of working memory as compared to LPS alone-treated rats in radial arm-maze task.

The choice reaction time task represents the first step of cognition and memory and is related to attention and immediate memory retention ability.[195] The incorrect lever pressing was more in LPS-treated rats (70.67± 2.27). Attention capacity would be recovered by EEPM pre-treatment rats (100, 200 and 400 mg/kg). EEPM at 400 mg/kg (43.50±2.40) showed significant activity when compared with LPS-treated group.

VI.5. *In vivo* antioxidant activity

The detoxification of ROS in brain involves the co-operative action of all intracellular antioxidant enzymes. Superoxide dismutase (SOD) is the first line of antioxidant enzymatic defense catalyzes the conversion of superoxide radicals to less toxic H_2O_2. Then catalase (CAT) metabolizes H_2O_2 to water.

When this mechanism is saturated, the second line of antioxidant enzymatic defense mainly GPx that regulated by selenium availability is activated.[196] GPx are a family of selenium-containing enzymes that responsible for etoxification of H_2O_2 and lipid peroxides at the membrane level into less reactive species using cellular GSH as substrate thus preventing the progressive formation of free radicals and provide the cell important protection against oxidative stress and LPO.[197] Many cells contain both CAT and GPx, while the brain GPx seems to be the major importance.[198]

Our data illustrated that LPS-treated rats were significantly ($P<0.001$) decreased the activity of the antioxidant defense enzymes (CAT, SOD, GPx, GR) in a dose dependant as compared with that of the control rats. While EEPM pre-treatment rats were significantly enhanced the activity of these antioxidant enzymes in brain tissue as compared to the control group. This depression of antioxidant enzyme activities reflects failure of the antioxidant defense mechanisms to overcome the influx of ROS induced by LPS exposure that leads to the accumulation of free radicals and facilitate the enhancement of LPO, which in turn increases the oxidative damage to the brain tissue.[199] LPO level was increased in LPS-treated rats which was minimized in EEPM treated rats.

Acetylcholinesterase (AChE) is an important cholinesterase enzyme present in the neuromuscular junctions and cholinergic synapses in the CNS. AChE terminates the signal transmission by hydrolyzing acetylcholine (ACh), a neurotransmitter that conducts nerve impulses across neuromuscular junctions in the nervous system.[200] ACh is also implicated in brain plasticity and disease.[201] Therefore, AChE is considered as a key enzyme in detecting the neurotoxicity. The obtained results in LPS-treated group was significantly ($p<0.001$) inhibited the enzymatic activity of AChE in dose dependant manner suggested that the impairment of AChE activity could be one of the LPS-neurotoxicity mechanism. However, LPS along with EEPM pre-treatment was able to preserve the AChE activity near healthy state. The inhibition of AChE enzyme was attributed to the occupation of its active sites by pollutants that could lead to decrease the cellular metabolism, disturb metabolic and nervous activity and lead to ionic refluxes and differential membrane permeability in addition to increase in lipid peroxidation.[202] In this context, Podolska and Napierska[203] observed that the inhibition of AChE enzyme causes accumulation of ACh resulting in rapid, uncontrolled twitching of voluntary muscles which eventually leads to cholinergic hyperactivity, convulsion, status epilepticus, paralysis and finally respiratory failure and death. The AChE, NO level was increased AChE, protein level was decreased in LPS-treated rats were significantly reversed by EEPM pre-treatment rats. Oxidative changes to proteins due to NO can lead to diverse functional consequences such as inhibition of enzymatic activities, proteolysis and altered immunogenicity.[204]

VI.6. Histopathological studies

The histological changes of brain sections of rats treated with LPS, EEPM and Dexamethasone are shown in Figure 40. Gross structural anatomy of CA1 hippocampal neuronal cells were seen in the brain sections of control rats. The degeneration and disarray of neuronal damage (CA1 hippocampal), were seen in LPS-treated rats. In rats treated with EEPM (100 mg/kg; 200 mg/kg; 400 mg/kg) significantly prevent the neuronal damage and restore the brain neuronal anatomy. Endotoxin LPS-caused brain damage, are prevented by EEPM at higher doses (200 mg/kg and 400 mg/kg).

Summary & Conclusion

VII. SUMMARY AND CONCLUSION

Neurodegenerative diseases (ND) are threatening worldwide. Epidemiological studies obviously represent substantial burden on well-being. Previously countless medications are obtainable for ND, but all the drugs are symptomatic relief only and they do not ameliorate the total mechanism of illnesses. ND was traditionally treated with numerous herbal medicines because to minimize side and adverse effects, chemical pollution from synthetic medications. Publics are accepting the herbal medicines due to long history of usage, better patient tolerance, easy availability, cost effective, time consuming.

Huge number of behavioural changes are associated with ND such as depression, anxiety, memory loss, stress and so on. It affects not only adult, even small kids also suffering a lot due to depression and stress. Effective drugs need to cure the ND from nature. Now a day, people are focused on an organic product from herbal plants.

In this present study, we had evaluated the phytochemical characterization and neuroprotective assessment of standardized extract (90% v/v Ethanol) of *Pedalium murex* Linn. leaves against LPS-induced endotoxemia in SD rats. Previously many work had been done on this plant except LPS-induced neurodegeneration along with behavioural and biochemical alterations.

Preliminary phytochemical studies in *Pedalium murex* Linn. leaves confirm the presence of flavonoids, triterpenoids, steroids, tannins, phenolic compounds in four different extracts such as n-Hexane extract, Chloroform extract, Ethyl acetate extract, 90% v/v of Ethanol extract.

In vitro free radical scavenging activity proved that, the 90% v/v of ethanol extract of *Pedalium murex* Linn. leaves (EEPM) were significantly acts against the free radicals. IC_{50} values were represents, EEPM possessed strong antioxidant activity when compared to n-hexane extract, chloroform extract, ethyl acetate extract of *Pedalium murex* Linn. leaves.

From the results of preliminary phytochemical and *in vitro* free radical scavenging studies, EEPM was selected for phytochemical characterization and neuroprotective assessment.

TLC study has been performed on pre-coated silica gel plates for elution. Based on trial and error method, 5:4:1 ratio of Toluene: Ethyl acetate: Formic acid

were used as a mobile phase, iodine vapors used as detecting agent. Six spots were observed. From the TLC study, isolation of active constituents of EEPM was done by column chromatography (CC) through isocratic elution technique with the help of same solvent system. Two compounds were isolated from CC fractions, 25-28, 33-36 and named as PM I, PM II. These two compounds were subjected to physical, chemical and spectral studies to authorizing the purity and characterization of isolated compounds of EEPM.

Spectral data of isolated compounds strongly suggested that PM I showed the structural similarities with 4´, 5, 7- trihydroxy flavone may be Apigenin and PM II showed the structural similarities with 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one which may have the presence of kaempferol. PM I and PM II may be a flavonoids type of compounds.

HPTLC fingerprint profile of flavonoids and steroids showed that more number of flavonoids and steroidal compounds were present in EEPM. Quantitative estimation also recommended the presence of flavonoids and phenolic compounds in EEPM.

EEPM was subjected to animal studies. Acute toxicity studies revealed up to 2000 mg/kg dose of EEPM did not show any toxic signs in animals. The present study proved that endotoxin (LPS) administration reduces the social, exploratory and locomotor activity which was leads the depression, anxiety, cognition impairment such as long-term memory, short-term memory, attention capacity of the animal. LPS completely degenerate the CA1 hippocampal region of the brain.

The data of our present study revealed that LPS significantly decreases the food, water intake and body mass which was recovered EEPM treated rats. In open field test, reduced level of animal's locomotion indicates that the depression and anxiety level was significantly more in LPS-induced rats. EEPM 400 mg/kg significantly increases the movement of animal through diminish the depression. Elevated plus maze test showed repeated animal entries in closed arms represents the immune activation and depression was produced by LPS, which was significantly antagonized by EEPM in different doses. Immobility time was increased in LPS-treated rats in forced swim test, hence EEPM treated rats were anticipated to recover from depression. Moreover, treatment with EEPM different doses attenuated or completely abolished these symptoms of depression and anxiety.

EEPM provides excellent activity against cognitive impairment, increases the learning and memory power of the animals. LPS-treated rats attained long-term memory loss that's why the swimming length was increased due to confusion to reach circular platform of water maze apparatus & EEPM 400 mg/kg significantly recover the memory power of animals. Short-term memory power loss and minimum number of rewards (food pellets) induced by LPS in radial arm maze test. EEPM 400 mg/kg increases the maximum no of rewards and minimum number of errors and enhance the learning, memory power of the animals. Attention capacity was measured in choice reaction time task test. Incorrect lever pressing for reward (food pellets) was increased in LPS-treated rats recovered by EEPM 400 mg/kg.

Antioxidant enzymes includes SOD, CAT, GR, GP_X levels were decreased in LPS-treated rats. An increased level of these antioxidant enzymes was observed after administration of EEPM, which indicates the antioxidant activity. ACh level was decreased and AChE level was increased in LPS-treated rats due to beta amyloid precursor protein degeneration which was reversed by EEPM treated rats. An increased level of nitric oxide and lipid per-oxidation (LPO) and decreased level of protein in LPS-treated rats was antagonized by various doses of EEPM. Histopathological results also support the neuroprotective effect of EEPM against LPS-induced neurodegeneration.

From the above results, we may conclude that EEPM may be a potential candidate for LPS-induced brain damage which may be attributed to the presence of potent antioxidants in EEPM. The results of this study warranted further research on active constituents of EEPM through molecular docking will lead new therapeutic approaches from nature.

Impact of the Study

VIII. IMPACT OF THE STUDY

- The present study emphasized a sum of researchable features that could be followed by phytochemical, *in vitro* antioxidant and free radical scavenging activities and neuroprotective effect assessed through a battery of behavioral and biochemical studies on *Pedalium murex* Linn leaves.

- Innovative level of phytochemical studies such as molecular docking analysis and analytical studies like x-ray crystallography, COSY, NMDR, 2D-NMR are recommended for isolation, identification and purification of various phytoconstituents present in the different solvent extracts of *Pedalium murex* Linn leaf.

- Advanced level of pharmacological studies through pharmacophore modelling are recommended to understand the clear mechanism of neuroprotective effect of *Pedalium murex* Linn leaf.

- It is further advised for pharmacokinetics and acute, sub-acute and chronic toxicological studies to be subjected in animals before human. Clinical trials are suggested before the therapeutic use.

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