NEUROPHARMACOLOGICAL PROFILE OF INDIAN MEDICINAL PLANT

THESIS

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The Tamilnadu Dr. M. G. R. Medical University

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Submitted by

Mr. K. Sujith, M. Pharm.,

Under the guidance of

Dr.(Mrs).V. Suba, M. Pharm, Ph.D.,

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I. INTRODUCTION

1.1 PREVALENCE OF MENTAL DISORDERS

According to the world health report approximately 450 million people suffer from a mental or behavioral disorder, but solely a minority of them receive even the foremost basic treatment, this accounts for 12.3% of the global burden of disease and will increase to 15% by 2020.[1] Drugs acting on the central nervous system were the first to be used by the primitive human and are still the most broadly utilized group of pharmacological agents. Mental ailments are heterogeneous diseases and will probably require a selected arsenal of drugs with different modes of action for successful treatment of their various manifestations.[2] In today's lifetime of stress and strain, there is a general need for agents having neuroprotective and neuropharmacological activity by enhancing learning and memory function of the brain.[3] Stress involves complicated biochemical, neural and immunological mechanisms and plays a crucial role in the progression of a variety of disease states ranging from psychiatric disorders like depression, anxiety, immunosuppression, endocrine disorders including diabetes mellitus, male impotency and cognitive dysfunctions to cardiovascular disease, high blood pressure, peptic ulcers, migraine, allergies, asthma, carcinoma, premature aging, rheumatic diseases and ulcerative colitis.[4] Importantly, stress is also known to interfere with cognitive functions, tending to retard the memory rather than the acquisition of learning.[5,6] Severe stressful conditions are responsible for the etiopathogenesis of various psychosomatic disorders. Homeostasis is controlled by various physiological mediators working in

concert by interacting with receptors placed at various physiological levels and the functional identity of neurotransmitters is challenged during stressful conditions.

Out of various neurotransmitters, nor adrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) are the necessary monoamines which are widely distributed in brain and their practical role is well established during stressful conditions.[7] Cognitive dysfunction, a major health problem in twenty first century and the foremost functionally debilitative side of many neuropsychiatric disorders and neurodegenerative disorders, such as schizophrenic disorder, depression, AD dementia, seizure disorders, head injury and parkinsonism. Memory function is vulnerable to a variety of pathologic processes including neurodegenerative diseases, depression, anxiety and the adverse effects of medication and normal ageing.[8] Learning and memory are generated by an experience dependent and long-lasting modification of the central nervous system.

1.2 MEMORY

In psychology, memory is an organism's capacity to store, retain and retrieve information. Memory is the individual's ability to record sensory stimuli, events, information and keep them over short or long span of time and recall at a subsequent date when needed.[9] In today's stressful and competitive world, troubles like poor memory, smaller retention and slow recall are rather common. Age, stress, emotions are situation that may lead to decrease of memory, amnesia, anxiety, increase in blood pressure, dementia or schizophrenia and Alzheimer's disease.[10]

Memory is a phase of learning and learning has three stages:

- Acquiring, wherein one experts a new undertaking or memorizes verbal material.
- New acquisition is retained for a period of time and
- Remembering, this enables one to duplicate the wise act or memorized material.

In a narrower sense learning merely means obtaining skill. Learning is defined as relatively enduring changes in behavior, those outcomes from experience. Memory is the apart of learning process and it is an essential status for the behavior change to be permanent.[11]

1.2.1 Types of learning and memory

Sensory memory

Sensory memory corresponds roughly to the initial 200–500 milliseconds after a part is perceived. The ability to examine a part and retain in mind what it appeared like with in just a second of observation or learning, is an example of sensory memory.

Short-term memory

Short-term recollection allows recall for a time span of some seconds to a minute without record. Its capability is also restricted to certain limitations. Short-term memory is considered to depend mostly on an acoustic code for retrieving information and to a lesser span on visual code.[12]

Long-term memory

Different types of memory are retained distinct localities of the brain. Long term memory is typically divided up into two major headings: declarative memory and implicit memory.

Declarative memory refers to the memories that are attentively present. Temporal cortex is considered as the location of storage of declarative memory but the precise location of storage remains unclear.

Implicit memory mentions to the use of things or movements of the body, such as how precisely to use a pencil. The encoding of implicit memory probably takes place in cerebellum and the striatum.[13]

1.2.2 Physiology of memory

Multiple stages of cognition processes consist of registration, consolidation and retrieval. The main characteristic of memory formation is its progression from short lived liable form to long lasting stable form. Registration is the process of sensory perception and the ability to act on the information perceived, which involves a change in brain electrical activity and referred to as short term memory. Consolidation is the process of conversion of registered short term information to a long-term memory as a result of physiochemical changes that takes place in neuronal networks. The stored information is created accessible by the method known as retrieval.[14]

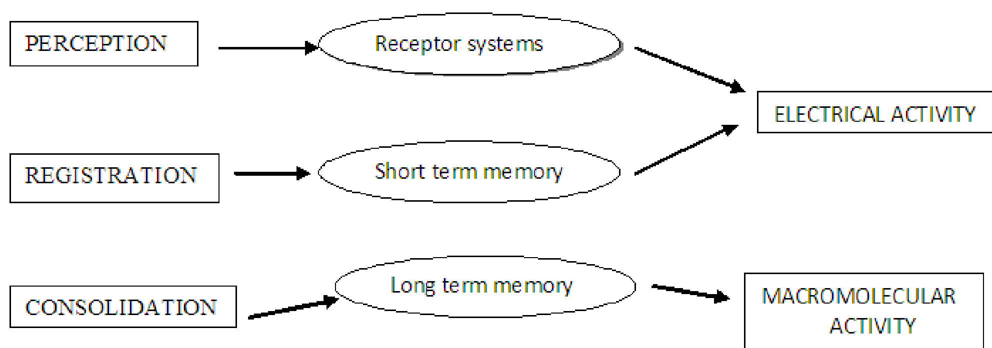


Fig-1 Multiple stages of cognition processes

1.2.3 Brain regions associated with learning and memory

Brain areas such as the hippocampus are concerned with spatial learning and declarative learning, while the amygdala is involved in emotional memory. Learning and memory recollection are attributed to alterations in neuronal synapses that are concerned to be attenuated by long-term potentiation and long-term depression. Patients with amygdalar impairment are no more likely to remember strongly felt described words than nonemotionally charged ones. [15]

Hippocampus

The hippocampus is situated inside the temporal lobe and it receives signals from all association areas of neocortex. The hippocampus contains extremely detailed multimodal information that was processed along different and partially interconnected sensory pathways. Added inputs are obtained from the amygdala and *via* a distinct pathway, from the cholinergic and alternative regulative systems. It has an extensive divergent system of output projections which endows feedback into most of the localities from where it obtains inputs. The hippocampus obtains input from different components of the cortex and drives its output to different components of the

brain. Hippocampal impairment may also origin memory decrease and problems with memory storage. Neurophysiological evidence also directly shows that many of the synapses inside the hippocampus can become changed as an outcome of experience. The sequence of biochemical events in hippocampus engages the activation of NMDA and metabotropic glutamate receptors pursued by alterations in second messengers and biochemical cascade commanded by increased undertaking of protein kinase and subsequent changes in glutamate receptor subunits, binding properties and expanded expression of constitute and inducible transcription factors. Some of the biochemical alterations are structure specific and they are not glimpsed in other brain areas and they appear to be specific in learning.[16]

Dorsal Striatum

The dorsal striatum performances a crucial function not only in learning new answer strategies but furthermore in the control of pre-existing strategies when there is a requirement in the move of strategy. In supplement, a hippocampus mediated system seems to be centre for getting multiple relationships stimuli. These are called as a declarative or relational memory scheme and dorsal striatum is essential for the mediation of stimulus response learning.[17]

Parahippocampal region (PHR)

The PHR obtains inputs from prevalent secondary or association cortical regions and provides the major perform for hippocampal outputs to the cortical association areas. The anatomical verification shows that the PHR has a key place for mediating memory functions of the hippocampal region. Neurophysiological findings

show that the PHR plays a critical function in recognition memory and independent function as an intermediary for cortical-hippocampal interactions.[18]

Basal forebrain

The cholinergic basal forebrain consists of cholinergic cell bodies in the medial septate nucleus, the diagonal band of Broca and the nucleus basalis magnocellularis. Evidently teaching protocol morris water maze deficits were described in rats with nucleus basalis lesions. Hidden-platform acquisition and probe trial deficits were seen in rats with blended basal forebrain lesions of the medial septum or diagonal bands and the nucleus basalis.[19]

Cerebellum

It has been shown that mice with cerebellar damage produce impairment in morris water maze learning. The cerebellum receives input from brain areas engaged in numerous aspects of morris water maze learning including visual cortex, superior colliculus, hippocampus and apart from motor control and acquisition or retention of conditioned reflexes, its specific functions could encompass a variety of cognitive processes as well.[19]

1.3 NEUROTRANSMISSION SYSTEMS INVOLVED IN MEMORY AND COGNITION

Acetylcholine

The most comprehensive and integrated approach towards the discovery of memory and cognition facilitating drugs has been based on the functions of central cholinergic system. Deficient cholinergic functioning result in impairment of

cognitive and memory performance. The brain of persons with severe cognition disorder shows a consistently depleted cortical and hippocampal choline acetyl-transferase (ChAT) and decrease in cell density and number in nucleus basalis of meynert, the key source of cholinergic innervation of human cortex. Cholinergic neurotransmission is regulated by two different classes of receptors, muscarinic and nicotinic receptors.

Pharmacological data apparently show that muscarinic and nicotinic acetylcholine receptors have a function in the encoding of new memories. Ideally, a muscarinic receptor ligand intended as a potential treatment for cognitive impairment secondary to impaired cholinergic neurotransmission should act as a selective central M_1 agonist and M_2 antagonist[20]. Recent studies have revealed that nicotinic ACh ion channel receptors (nAChRs) in the brain modulate the release of neurotransmitters such as acetylcholine, dopamine and other monoamines implicated in learning and memory processes.[21] The concept that cognitive functions are highly reliant on central cholinergic neurotransmission moves back to the early sixties.[22] Though alternative neurotransmitters were renowned to be engaged in learning and memory presentation, the purposes of the cholinergic system in learning and recollection were of prior interest to learning and memory research.[23] Further support for a cholinergic involvement came from investigations which displayed that scopolamine exacerbates the presentation of nucleus basalis lesioned rats whereas acetylcholinesterase inhibitors improve it.[24]

Excitatory amino acid

Glutamate is an endogenous excitatory amino acid present in the brain and has received increasing attention since it's proposed to be involved in neurological and psychiatric disorders. A clear attachment between excitatory amino acids and memory related processes is shown by the engagement of NMDA and AMPA receptors in the initiation of long-term potentiation.[25] AMPA (α-amino-3- hydroxyl-5-methyl-4-isoxazolepropionic acid)-type glutamate receptors exhibit very quick excitatory transmission in the central nervous system. Positive modulation of receptors can potentially increase cognition, countervailing the losses of glutamatergic synapses, promoting synapting plasticity and increasing the production of tropic factors. Ampakines (AMPA receptor modulators) potentiate the encoding of memory in a various animal models.[26] Whereas data accessible show that both long-term potentiation and memory can be altered by blockade of the NMDA receptor, the potentiaton of the glutamatergic signal may have untoward consequences on behavior because high levels of glutamate can produce neurotoxic effects.[27] Therefore there may be only little beneficial effect for memory-enhancing effect of excitatory amino acid agonists.[28]

Serotonin

Serotonergic neurotransmission has been proposed to have a vital role in distinct psychiatric disorders. In supplement, the function of serotonin in learning and memory has furthermore received much interest whereas these data emerge to be rather inconsistent.[29] Serotonin receptor subtypes that have been illustrated to happen in brain regions capable of playing a function in learning and memory encompass

serotonin receptors. There has been increased attention focusing on the role of the serotonergic system in learning and memory over the past decade. There have been a great many reports regarding the existence of serotonergic perturbations associated with age-related cognitive disorders such as Alzheimer's disease.[30,31] Although there is growing evidence from animal experiments indicating a role for serotonin in modulating learning and memory processes, this modulation is not well characterized. Both increases and decreases in performance have been reported following similar experimental manipulations of the serotonergic system.[32]

Dopamine

The distinct investigations have supplied experimental clues that it modulates learning and memory presentation in distinct kinds of tasks. Particularly, the mesocortical dopamine system is expected to have a crucial function in cognitive processes because this neurotransmitter has a significant role in the functions of the prefrontal cortex. Studies in animals with dopaminergic lesions have revealed the learning and memory performance deficits.[33,34] In the field of memory and learning investigations the mesolimbic dopaminergic system has clearly received most of the scientific importance. These regions are well known to play an important function in diverse cognitive processes. The investigations of dopamine function in working memory have concentrated on the D1 and D2 receptors, with prominent verification that suggest a dominant role for the D1 receptor. It could be regarded that dopamine enhances learning methods but interferes with memory processes.[35,36]

1.4 DEMENTIA

Dementia is a syndrome exhibiting impairment in memory, disability in organizing thoughts and reason, the incapability to use language, loss of the power to examine accurately the visual world and these impairments are critically sufficient to origin a decline in the patient's usual grade of functioning. Though some types of memory decrease are usual components of aging, the alterations are obliged to aging are not critical enough to interfere with the amount of function. Diverse diseases can origin dementia, but Alzheimer's disease which is a neurodegenerative disorder which is accompanied with neuronal loss in brain and is considered as widespread cause for dementia. Dementia is of distinct types and it involves loss of memory. The centered cholinergic pathways play a prominent function in learning and memory processes.[37] Dementia mentions to a large class of disorders exhibiting progressive worsening of thinking proficiency and memory as the brain becomes damaged. Incidence of dementia declines in men with age after 90 years but not in women. Dementia is a general term for a range of brain disorders characterized by multiple cognitive deficits that include impairment in memory. Currently, there are around 24 million dementia sufferers globally and it is estimated that this figure will increase to 81 million by 2040.[38] Epidemiological studies in Indian community disclose that dementia is largely a concealed problem.[39] Prevalence rates for dementia boost exponentially with increase in age.[40,41]

1.4.1 Pathophysiology of dementia

Alzheimer's disease (AD) is a degenerative and fatal brain disease, in which the death of brain cells occurs, therefore Alzheimer's disease is given the title as the

most common type of dementia. Alzheimer's disease accounts for 1-5% of the population. It is considered as a major cause of disability among the aged people. Women are the victims of Alzheimer’s disease, with proof disclosing that women with AD display more severe cognitive impairment relation to age matched males with AD and there is also a faster rate of cognitive decline. Alzheimer’s disease, the most common type of degenerative dementia that has been characterized by the progressive impairment of cognitive function and changes in behavior and social adaptability.[42,43] The severity of cognitive dysfunction and memory loss in Alzheimer’s disease is associated with the cholinergic hypofunction.[44] Neuronal loss in the basal forebrain particularly with in the septohippocampal acetylcholinergic systems involved in learning and memory processes constitutes a pathological hallmark of Alzheimer’s disease. Alzheimer’s disease (AD) is a fatal, progressive and disabling organic brain disorder characterized by disturbance of multiple cortical functions, including memory, learning ability, orientation, comprehension, judgment and language.[45] Alzheimer’s disease (AD) is characterized by an acquired intellectual decline manifesting as memory loss together with other cognitive impairments including dysphasia, visuo-spatial dysfunction and disturbances in calculation or concentration. The disease is determined pathologically by the presence of senile plaques, which are accumulations of granular material which include degenerating neurites and glia, with a core formed of amyloid material and neurofibrillary tangles. These pathological changes, despite extensive study, are still poorly understood. Besides the cognitive symptoms AD frequently manifests other behavioral symptoms. These symptoms most commonly include psychomotor agitation, anxiety and depressive symptoms and psychotic symptoms such as delusions. Such symptoms,

together with the cognitive disturbances, are the targets for the treatment of this condition. A large number of clinical trials with agents aimed at treating this condition have been carried out over the past 30 years. The main feature of this analysis is finding agents that could improve the cognitive performance in patients suffering from this disease[46].

1.4.2 Mortality of dementia

A systematic review, reported a positive dose response relationship between the level of cognitive impairment and increasing risk of mortality. A review found increased risk of mortality for moderate levels of cognitive impairment and a two-fold mortality risk for severe cognitive impairment. Most of the evidence on these associations between dementia and mortality are from studies undertaken in developed countries.[47]

Prevalence of dementia

Of the 3.7 million Indian people are aged over sixty years. The prevalence of dementia increases steadily with age and higher prevalence is seen among older women compared with men.[48] It is contended that this cannot be explained by the fact that women live longer in India, because studies of age specific incidence of dementia among older people show no significant differences between gender of the population. It suggest that gender is not a risk factor for AD or other dementia among older people.[49]

Future projections

The future projections estimated in the World Alzheimer Report are based on the assumption that prevalence of dementia is stable over time, but this may not be true. Increase in life expectancy of older people can subsequently increase the prevalence of dementia in the society. The number of people in India with AD and other dementias is expanding every year because of the stable growth in the older population and stable increment in life expectancy resulting in an estimated increase of two fold by 2030 and threefold by 2050.[50,51]

1.4.3 Impact of dementia

Burden of dementia

According to the World Health Organization (WHO) global burden of disease report, dementia is the third leading cause contributors to years if life lost due to disability (YLD) in the elderly in low-income and middle-income countries. The WHO report estimated that dementia is the second highest source of disease burden after tropical diseases. The economic burden of dementia is predicted to increase in the Asian region from 58% in 2005 to 68% in 2030.[52] Evidence suggests that elderly people with dementia in developing countries do not often utilize health services.[52] In addition, around one fifth of older people with dementia (10% to 37% by country) in developing countries are classified as having potentially vulnerable living circumstances. Many need long-term care with primary care services not meeting their needs[53].

Etiology

Risk factors

Advanced age remains the main risk factor for most forms of dementia. Onset before 65 years of age is rare and in the case of AD, often suggests a genetic cause.[54] Single gene mutations at one of three loci (Beta amyloid precursor protein, presenilin-1 and presenilin-2) account for most of these cases. For late-onset of AD, both environmental (lifestyle) and genetic factors are important.[55] The evidence strongly establishes a causal role of cardiovascular risk factors and cardiovascular disease in the etiology of dementia and AD.[56] The latency incidence studies shows smoking increases the risk for AD.[57] Patients presenting with high cardiovascular risk symptoms such as hypertension, hypercholesterolemia, diabetes and if they are also involved in smoking will result in increased susceptibility for dementia incidence whether exposure is measured in midlife or a few years before dementia onset.[58] Recent studies report associations between metabolic syndrome, incident cognitive decline, insulin resistance and impaired executive function.[59,60] Depression is reported as a risk factor and a early presenting symptom in the dementia patients.[61] The findings from these studies supports the hypothesis that atherosclerosis and AD are linked, with several common underlying risk factors such hypertension, increased fat intake, obesity, raised cholesterol and diabetes.[62,63]

1.5 OXIDATIVE STRESS IN THE NERVOUS SYSTEM AND PATHOGENESIS OF NEURODEGENERATIVE DISORDER

Oxidative stress is caused by an imbalance in the pro-oxidant and antioxidant systems that can occur as a result of an increase in oxidative metabolism.[64] High

content of unsaturated fatty acids and iron are present in the nervous system. The high lipid content of nervous tissue and presence of high aerobic metabolic activity will make it more prone to oxidative damage. The adequate increase in the level of iron is a important requirement during the development of brain, but it may also cause injury to the brain cells and release ions of iron, that can cause oxidative stress through the iron-catalyzed formation of reactive oxygen species (ROS).[65] Brain regions that are rich in catecholamines are more prone to free radical generation. The catecholamines can auto-oxidize to free radicals or due to metabolism by the endogenous enzymes such as monoamineoxidases. In substantia nigra (SN), there is a attachment that has been established between antioxidant depletion (including GSH) and tissue degeneration. A number of *in vitro* investigations have shown that antioxidants both endogenous and dietary can defend nervous tissue from damage by oxidative stress.[66] Vitamin-E was found to avert cell death (apoptosis) in rat neurons subjected to hypoxia pursued by oxygen reperfusion.[67] ROS consists of hydrogen peroxide (H_2O_2), nitric oxide (NO), superoxide anions and the highly reactive hydroxyl and monoxide radicals (OH•, NO•). Impaired activated microglia acts as reservoir of ROS.[68] Free radicals have been described for their great assistance to neuronal decrease in neurodegenerative disorders.[69] ROS are particularly active in the mind and neuronal tissue as the metabolism of excitatory amino acids and neurotransmitters can serve as sources of oxidative stress. ROS affects glial cells and neurons and leading to neuronal impairment. It has been reported that deleterious consequences of ROS on human cells may result in oxidative injury that can also lead to programmed cell death i.e. apoptosis.[70] Antioxidants are compounds that remove free radicals and scavenging ROS.[71]Among them, oxidative metabolic reactions and their by products have been

consistently implicated in AD pathogenesis and represent the biological basis for the oxidative stress hypotheses of Alzheimer's disease. Various studies have shown that different biomarkers of oxidative stress mediated events are elevated in the AD brain.[72] The role of abnormal metabolic oxidative reactions in the central nervous system may be considered as pathological cause of sporadic AD. AD brains exhibit constant evidence of reactive oxygen species and reactive nitrogen species (RNS) mediated injury.[73] Oxidative stress will cause protein, DNA, RNA oxidation or lipid per oxidation and this will depend on the substrate attacked by ROS. A simplistic interpretation of this hypothesis is that antioxidant therapies should be beneficial in AD treatment.[74]

1.6 PHARMACOLOGICAL APPROACH FOR MEMORY IMPAIRMENT

Drugs to enhance memory usually work by altering the balance of neurotransmitters in the brain that are engaged in the initial learning of a memory or its subsequent reinforcement. Drugs that produce cognition enhancing capability will increase synaptic plasticity by controlling release of neurotransmitter from the pre-synaptic terminal and increasing sensitivity and specificity of receptors. Drugs may also alter the process at transcriptional and translational level.

Nootropics

Nootropics popularly referred as substances that increase human cognitive abilities. Action of nootropics is due to increase in the brain's supply of neurochemicals, by improving the supply of oxygen to brain and also by stimulating nerve growth. They are furthermore utilized for the remedy of retardation, neural degradation (Alzheimer's and Parkinson's) and in cases of oxygen deficit to avert

hypoxia. Nootropics that are available include piracetam, aniracetam, pramiracetam, oxiracetum and nefiracetam.[75]

Stimulants

Some stimulants can enhance cognition and memory in some persons, but cause psychosis, they usually have a very considerable side-effect profile and are not considered as classical nootropic drugs. There are suggestions from technical community for prevalent use of stimulants such as methylphenidate and amphetamines by the general population to boost brain power.[76]

Dopaminergics

Dopaminergics are compounds that affect the neurotransmitter dopamine or the constituents of the nervous system that use dopamine. Dopamine influence alertness, attention and also exhibit antioxidant property. Quinpirole, a D2 agonist and D- amphetamine improved cognitive performance in various types of learning and memory tasks. It is argued that dopamine enhances learning process but interfere with memory processes.[77]

Cholinergics

Cholinergics are compounds that affect the neurotransmitter acetylcholine or the constituents of the nervous system that use acetylcholine. Increasing the availability of acetylcholine in the brain may improve the memory functions of the brain. Acetylcholinesterase (AChE) inhibitors are the only drugs available which have been associated with symptomatic advantage. An perfect AChE inhibitor `would encompass different characteristics they may be a pseudo reversible or irreversible

inhibitor, produce selective inhibition within the mind, produce inhibition of AChE, increase the levels of acetylcholine (ACh) in AD patients and have reduced toxicity profile. Huperzine A is a powerful acetylcholinesterase inhibitor drawn from Chinese club moss.[78] Rivastigmine is described to have greater effects in the cortex and hippocampus (the major goals for symptomatic remedy) than the striatum, with 80% AChE inhibition. Galantamine is an AChE inhibitor that also acts as a modulator at nicotinic cholinergic receptor sites, enhancing acetylcholine effects at this sites.[79]

Glutamate activators

The AMPA transmitter and AMPA receptors are actually being investigated and they exhibit important memory enhancement and likely alertness enhancement may happen when agonized. The drug category for AMPA system modulation is termed ampakines.[80]

NMDAreceptorantagonists

Memantine, an N-methyl-D-aspartate (NMDA)-receptor antagonist, has been associated with modest improvement in moderate or severe AD in some placebo-controlled studies. While there is no evidence that memantine is capable of modifying the course, it may reduce clinical deterioration in patients with moderate to severe AD.[80]

Cyclic adenosine monophosphate (cAMP)

The increase in the levels of secondary messenger such as cyclic adenosine monophosphate has shown to exhibit memory improvement. The phosphodiesterase -

4 enzyme breaks down cAMP and by decreasing the activity of the enzyme, the levels of cAMP can be increased.[81]

Serotonergics

The effect of serotonin in learning and memory has been receiving greater attention. Stimulation of serotonergic neurotransmission disrupts behavioral performance. 5-HT_3 receptor antagonist such as ondansetron and zacopride shows promise in enhancing cognitive performance. Ipsapirone, a 5-HT_{1A} agonist, improved memory performance in rodents. Antidepressants that produce a rise in the active serotonin levels by inhibiting reuptake mechanism can promote neurogenesis in the hippocampus.[81]

1.7 MAJOR SIDE EFFECTS OF COMMONLY USED NEUROPROTECTIVE

Adverse effects of these neuroprotective drugs which are commonly used for improving memory, mood and behavior has made their use limited and the potential side effects of commonly used neuroprotective drugs is listed in the table-1. Hence it is worthy to explore the use of traditional medicines in the treatment of various cognitive disorders

Table-1 Side effects of commonly used neuroprotectives[82,83]

DRUG	SIDE EFFECTS
Piracetam	Gastric discomfort, excitement, insomnia, dizziness, skin rashes, hyperkinesias, nervousness, depression, diarrhoea, sleep disturbances somnolence and weight gain.
Pyritinol	Gastro intestinal upset, skin rashes, itching, taste disturbances, nausea, vomiting, headache and insomnia.
Donepezil	Nausea, vomiting, diarrhoea, insomnia, fatigue, muscle cramps, headache, dizziness and syncope, bradycardia, convulsions, sinoatrial and atrioventricular block.
Rivastigmine	Peripheral cholinergic side effect, accidental trauma, fatigue, dizziness, headache, somnolence,agitation, insomnia, confusion, depression, nausea, vomiting, diarrhoea, abdominal cramp, upper respiratory tract infection and urinary tract infection.
Co-Dergocrine	Abdominal cramp, nausea, vomiting, headache, blurred vision and skin rashes.
Mesylate	Flushing of the skin, bradycardia and orthostatic hypotension.
Nicergoline	Gastro intestinal disturbances and hypotension.
Cyclandelate	Flushing, headache, dizziness and palpitation.
Pentoxyfylline	Angina, dyspnoea, hypotension, dizziness, anxiety, confusion, cardiac arrhythmias, hepatitis, jaundice and bone marrow suppression.
Isoxsuprine	Vasodilatation, flushing, headache and dizziness.

1.8 BEHAVIORAL PARADIGM FOR EVALUATION OF LEARNING AND MEMORY PROCESSES[84]

Based on the aversive stimuli, behavioral models for studying the neurobiology of learning and memory can be broadly classified in to two types: extroceptive and interoceptive aversive stimuli models.

Exteroceptive aversive stimuli models

Behavior on mazes

- Elevated plus maze
- Radial arm maze
- Radial water maze
- Y-maze
- Morris water maze
- Modular mazes
- Stone T-maze

Avoidance behavior on shuttle box

- Two way active avoidance paradigm
- Retention test Performance of avoidance task
- Step-down type passive avoidance task
- Modified passive avoidance test
- Step-through type passive avoidance learning task

Interoceptive aversive stimuli models

- Brain lesion induced cognitve dysfunction
- Electroshock induced amnesia
- Hypoxic stress induced learning defícts
- Scopalamine induced amnesia
- Dizocilpine-induced memory impairment
- Clonidine induced amnesia
- Triazolam induced cognitive dysfunction
- Clozapine induced cognitive dysfunction
- Lignocaine induced amnesia
- Aluminium induced learning defícts
- Ethanol induced state dependent learning
- Carbon dioxide induced amnesia

1.9 ETHNOPHYTOTHERPIC APPROACH

Herbal medicine is the natural system of medicine that has been practiced for more than 5000 years. The ancient Indian system of medicine (Ayurveda) is replete with medicinal plants as herbal remedies, to prevent and treat diseases through the restoration of a healthy balance of life forces and a harmonious relationship with the environment.[85]Herbs are the major components in all indigenous preparations of traditional medicine and common element in Ayurveda, Homeopathic, Naturopathic and native Indian herbal medicine emphasizes control of disease, restoration of our body systems and it extends the life span and makes healthy life in balance and harmony. From ancient time to present, people throughout the world have sustained

an adequate knowledge of native plants. With the advancement of chemistry and western medicine the active substances from different species are being isolated and used.

According to WHO estimation, 4 billion people, 80% of world population presently use the herbal medicine for specific primary health care and about 25% of the prescription drugs dispensed contain at least one active ingredient of plant origin. The synthetic preparation of some drug is either unidentified or economically impractical, for this reason scientist continue to seek and check for renowned plants and conserve those medicinal properties which have become vital to battle against disease. Medhya herbs promote the intelligence. Their action is more concerned with central nervous system especially the higher cortical centers of brain. In Ayurveda the intelligence is attributed to three powers of the mind, they are the acquisition, the retention and the recollection. Since ages, drugs and natural remedies have been used to enhance memories in people. Memory enhancer herbs enhance the memory and increase blood circulation in the brain. Herbal medicines can be used in the treatment of AD.[86,87]

The recent trends in the pharmacological studies are based on the biochemical and molecular mechanism which leads to the development of CNS active principles from the herbal drugs. Although there is no cure for dementia of AD type at present, alternative pharmacological approach can reduce the symptoms of cognitive impairment and slow disease progression. From the huge array of material medica of the indigenous system, numerous plants have been described to have activity against CNS disorders and therefore proceed as very useful remedies for the alleviation of

human pain. In the search of new therapeutic drugs for the remedy of neurological disorder , medicinal plant are studied worldwide for promising developments in the area of herbal medicines as sources for new therapies for dementia. For example, galanthamine, a commonly used cholinesterase inhibitor, is a herbal derivative.[89] Huperzine A, isolated from the Chinese herb *Huperzia serrata*, also shows promise.[90] Extracts of the herb *Ginkgo biloba* have received much research attention and are currently in widespread use.[91]Experimental reports suggest some herbs may have neuroprotective effects against beta-amyloid.[92]

1.10 HERBAL MEDICINAL PLANTS WITH POTENTIAL COGNITIVE ENHANCEMENT ACTIVITY

The following is the list of cognitive enhancing plants evaluated by researchers and used for memory enhancement and shown in table-2 [93,94]

Table-2 Herbal medicinal plants with potential cognitive enhancement activity

Name of plant	Family	Part used
Abutilon indicum	Malvaceae	Whole plant
Acacia nilotica	Mimosaceae	Leaf
Acanthus ebracteatus	Acanthaceae	Aerial part
Acorus calamus	Araceae	Rhizomes
Aegle marmelos	Rutaceae	Fruit pulp
Albizzia lebbeck	Fabeaceae	Leaves
Alpinia galanga	Zingiberaceae	Rhizomes
Andrographis paniculata nees	Acanthaceae	Aerial part

Name of plant	**Family**	**Part used**
Angelica sinensis	Umbelliferae	Root
Astragalus membranaceus	Fabaceae	Root
Azadirachta indica	Meliaceae	Bark
Bacopa monniera	Scrophulariaceae	Whole plant
Biota orientalis	Cupressaceae	Seed
Boerhavia diffusa	Nyctaginaceae	Whole plant
Butea superba roxb	Leguminosae	Root barks
Buxus sempervirens	Buxaceae	Whole plant
Camelliasinensis	Theaceae	Leaf
Capsella bursa pastoris	Brassicaceae	Whole plant
Carthamus tinctorius	Asteraceae	Flower
Carum carvi	Apiaceae	Radix
Cassia fistula	Leguminosae	Root
Caulis spatholobi	Leguminosaae	Whole herb
Celastrus paniculata	Celastraceae	seeds
Centella asiatica	Umbelliferae	Whole plant
Chamaecyparis pisifera	Cupressaceae	Whole plant
Clitoria ternatea	Fabeaceae	Aerial part,root
Commiphoris whighitti	Burseraceae	Gum resin
Convolvulus pluricaulis	Convolvulaceae	Whole plant
Crocus sativus	Iridaceae	Pistil
Crataegus laevigarta	Rosaceae	Whole plant

Name of plant	**Family**	**Part used**
Curcuma longa	Zingiberaceae	Rhizomes
Cymbopogon schoenanthus	poaceae	Whole plant
Desmodium gangeticum	Fabeaceae	Laves ,roots
Dioscorea bulbifera	dioscoreaceae	Whole plant
Dipsacus asper	Dipsacaceae	Root
Embelia ribes burm	Myrsinaceae	fruit ,rooot
Emblicaofficinalis gaerth	Phyllanthaceae	Whole plant
Equisetum arvense linn	Equisetaceae	Stems
Euphorbia antiquorum	Euphorbiaceae	stem
Euphoria longan	Sapindaceae	Fruit
Evodia rutaecarpa	Rutaceae	Fruit
Evolvulus alsinoides	Convolvulaceae	Whole plant
Ficus religiosa	Moraceae	Whle plant,bark
Gastrodia elata	Orchidaceae	Root
Ginkgo biloba	Ginkgoaceae	Whole plant
Glycyrrhiza glabra	Fabeaceae	rhizome
Heteropterys aphrodisiaca	Malpighiaceae	Root
Hibiscus sabdarifffa linn	Malvaceae	calyces
Huperzia saururus	Lycopodiaceae	Aerial part
Ilex paraguariensis	Aquifoliaceae	Leaves
Indigo naturalis	Apiaceae	Whole herb
Lantana camara	Verbenaceae	aerial parts

1.11 MARKETED HERBAL PREPARATIONS CLAIMING NEUROPROTECTIVE ACTION

Following is list of marketed herbal preparation claiming to have neuroprotective action and are shown in table -3.[95]

Table-3 Marketed herbal preparations claiming neuroprotective action

Brand name	Manufacturing Company	Ingredient
Cebrotone Syrup	Fem Care	*Conscora decussate, Withania somnifera, Herpestis monniera, Celastrus paniculatus, Nordostychus jatamanasi, Acorus calamus*.
Mentat	Himalaya	Brahmi, Mandukaparni, Ashwagandha, vishnukrantha, jatamanasi, vacha Haritki, Amalki, tgara, Arjuna, Shatapushapa, Shyonka, Ela, Salabmisri, Lavanga, Vibhitaki.
Bilovas	Zydus cadila	*Ginkgo biloba.*
Cerestar	Ranbaxy	*Ginkgo biloba.*
Ginkocer	Emcure	*Ginkgo biloba, Panax ginseg*, Garlic.
Iqmem	Lupin	Vacha, Jyotismati, Bramhi, Shankhapushapi, Mandukaparni.
Baco Mind	Natural Remedies	*Bacopa monnieri.*
Vacha ghan	Chaitanya Pharma	*Acorus Calamus.*

II. AIM AND OBJECTIVE OF THE STUDY

Memory impairments lead to various diseases like amnesia, Alzheimer's disease. Most of the aged persons are affected by memory impairments and the number of persons affected by memory impairment is increasing recently. Currently in memory impairments patient's responds to the existing treatments, however the magnitude of improvement remains disappointing. Then, the need for newer, better-tolerated and efficacious treatment remains high.

People from different regions of the world have used herbal medicine to alleviate affective disorder for several years. In addition, the exploration for novel pharmacotherapy from medicinal plants for memory impairments has progressed significantly in the past decade. When we reviewed the literature for *Anacyclus pyrethrum*, there are reports that the roots of the plant has traditional claim on memory improvement, rejunator and nervine tonic and also there are scientific evidence on anti-inflammatory, local anaesthetic, aphrodisiac, antimicrobial and immunomodulatory activities. In order to substantiate the traditional medicinal claim of the plant root the present study is aimed to investigate scientifically through following experimental methods.

- To prepare different extracts of roots of *Anacyclus pyrethrum* and subject them to preliminary phytochemical analysis.
- To evaluate *invitro* anticholinesterase activity on these extracts to select the suitable extract based on the ability to inhibit acetylcholinesterase activity for further *invivo* screening procedure.

- To subject the selected extract for the identification of phytoconstiuents through TLC and HPTLC methods.
- To investigate acute and subchronic toxicity of selected extract to assess the toxic effect of extract.
- To evaluate neuropharmacological activity of the selected extract.
- To investigate the effect of extract on cognition by using various interoceptive and extroceptive cognition behavior models.
- To study the effect of extract on the levels of neurotransmitters, cholinesterase enzyme, enzymatic and non enzymatic antioxidants.
- To evaluate antioxidant potential of the extract by using *invitro* and *exvivo* antioxidant experimental methods.

III. LITERATURE REVIEW

Suba *et al.,* (2002)[96] studied the effect of methanolic extract of aerial parts of *Barleria lupulina* on central nervous system activity. The methanolic extract in a dose dependent manner showed reduction in generally behavioural pattern (spontaneous activity, alertness, awareness, pain response and touch response). The extract showed a major reduction of the exploratory behavioural profile (Y-maze check, head dip test) and conditioned avoidance response. Preliminary investigation showed that the methanolic extract has prominent effect on central nervous system activity.

Santanu Bhadra *et al.,* (2012)[97] subjected *Maesilea quadrifolia Linn* to qualitative and quantitative phytochemical screening and its anti-cholinesterase potential was checked by TLC bioautography and other screening procedures using *invitro* acetylcholinesterase and butyrylcholinesterase studies. The study disclosed that the extract comprises diverse categories of phytoconstituents including steroids, saponins, alkaloids and other polyphenols. These findings propose that *Maesilea quadrifolia Linn* is a promise lead as AChE and BChE inhibitor, which may be helpful in the treatment of Alzheimer's disease.

Ilavarsan *et al.,* (2011)[98] evaluated acute toxicity study and 90 days repeated dose toxicological assessment of *Ricinus communis* root extracts in Wistar albino rats. The extracts did not produce any toxic symptoms or mortality in the oral toxicity study. In the 90 days (sub-chronic toxicity) repeated dose toxicity study the extracts were administered (1000 mg/kg) daily through oral route. The sub-chronic toxicity study demonstrated non-toxic nature of the root extracts due to non significant

changes in body weight, food, and water intake, hematology, biochemical and histopathology evaluations also demonstrate the non-toxic nature of the root.

Joshi and Parle., (2006)[99] assessed the nootropic effect of different doses of *Zingiber officinale* extract by employing elevated plus maze and passive avoidance paradigm. They administered extract at a dose of 50 and 100 mg/kg, p.o for eight days to juvenile and aged mice. The dose of 100 mg/kg of extract considerably improved learning and recollection in juvenile mice and furthermore inhibited the amnesia induced by diazepam (1mg/kg, i.p.) and scopolamine (0.4mg/kg, i.p.). It also reversed aging induced amnesia of mice. Extract significantly increased acetyl cholinesterase inhibition and thus providing the evidence for its use as restorative agent in the treatment of dementia glimpsed in the elderly. The underlying mechanism of its activity may be due to antioxidant and acetyl cholinesterase inhibition property.

Santos Junior *et a*l., (2005)[100] assessed cognitive enhancement effect of hydroalcoholic extract of *Equisetum arvense* L in elderly rats. Chronic treatment of extract at dose of 50mg/kg, i.p., advanced both short and long-term retention of inhibitory avoidance task and improved the cognitive presentation in reference and working memory type of morris water maze. The cognitive enhancement property of the extract may be due to its antioxidant activity.

Papandreou *et al*., (2009)[101] investigated effect wild blueberry extract on memory improvement in mice. Results displayed that the extract at dose of 60mg/kg treated mice displayed a significant enhancement in learning and memory. Extract administration furthermore resulted in decreased lipid peroxidation products and higher brain contents of ascorbic acid in extract treated groups respectively and also

the levels of glutathione increased in the extract treated group. They observed the cognitive enhancement activity in adult mice after short-term supplementation with the blueberry extract which is closely related to antioxidant properties and inhibition of AChE.

Kaur *et al*., (2008)[102] studied the cognitive effect of green tea extract in rats. Rats were administered orally with 0.5% green tea extract for a time span of eight weeks and then assessed by passive avoidance, increased plus maze paradigm and alterations in acetylcholinesterase activity. Green tea extract considerably enhanced learning and memory in older rats, with increase in retention latency to enter in passive avoidance test. The green tea treatment produced a considerably increase in number of entries in the enclosed arm by the juvenile and old rats in the elevated maze test. Decrease in acetylcholinesterase activity was seen in the cerebrum of green tea treated aged rats when compared to juvenile rats. These outcomes proposed the use of green tea has a prominent effect on learning, memory and behavior.

Sreemantula *et al.,* (2005)[103] evaluated adaptogenic and nootropic property of aqueous extract of *Vitis vinifera* in scopolamine induced amnesia in trained avoidance response using Cook's pole climbing apparatus. The cognition as evaluated using parameters such as acquisition, retention and recovery in rats were seen to be dose dependent. They attributed the nootropic activity to centered cholinomimetic property apart from its free fundamental scavenging mechanism.

Vyawahare *et al*., (2006)[104] assessed the distinct doses of alcoholic extract of *Clitoria ternatea* for its anti-amnesic activity using scopolamine induced amnesia in rats utilizing passive avoidance and step down passive avoidance task model. The

results showed that dose of 500mg/kg was more effective and it inhibited the scopolamine induced memory loss in both passive avoidance and step down passive avoidance task model.

Kumar et al., (2009)[105] studied the cognitive performance effect of ethanolic extract of *Mangifera indica L.* The experimental method used to study the effect on cognitive performances was step down passive avoidance task and increased plus maze task in mice. Chronic treatment for seven days of extract and vitamin C administration significantly inhibited the aging and scopolamine induced memory loss of memory in the models. The outcomes suggested that the extract comprised pharmacologically active constituents that have memory enhancing property.

Zhang *et al.,* (2008)[106] studied the enhancing effects of tenuifolin on learning and memory using step down type passive avoidance test in mice. The amount of cortical acetylcholine esterase (AChE) activity and hippocampal neurotransmitters in aged mice were also determined and improvement in the levels of norepinephrine (NE), dopamine (DA), reduced activity of AChE was exhibited. There were no changes in level of serotonin (5-HT) in aged mice. These actions on improving cognitive performance of tenuifolin may be due to increase in the levels of NE, DA in the hippocampus and due to decrease in the activity of AChE in the cortex.

Joshi and Parle., (2007)[107] investigated the effects of *Phyllanhus amarus* on cognitive performance and cholinesterase activity. Elevated plus maze and passive avoidance paradigm were used to analyse learning and memory parameters. They administered different dose levels of aqueous extract of *Phyllanhus amarus* for eight days to mice. Extract produced a concentration dependent improvement in scores of

memory in mice and also potentially inhibited the amnesia induced by scopolamine (0.4mg/kg, i.p.) and diazepam (1mg/kg, i.p.) and also decreased the level of acetyl cholinesterase in brain.

Kim *et al*., (2006)[108] studied the effect of gomisin A on the cognitive impairments induced by scopolamine. The cognition improving effect of gomisin A was analysed using a passive avoidance test, Y-maze test, and the morris water maze test in rodents. Drug induced amnesia was produced by treating animals with scopolamine (1mg/kg, i.p). The administration of gomisin A (5mg/kg, p.o.) significantly inhibited scopolamine induced cognitive dysfunction in mice by the passive avoidance test and Y-maze test and they also improved escape latency in the morris water maze test. *In vitro* study showed gomisin A to inhibit acetylcholinesterase activity in a dose dependent manner. These results also reveal that that gomisin A could be beneficial in cognitive impairment treatment and it may be attributed to enhancement of the cholinergic nervous system.

Vasudevan and Parle., (2007)[109] studied the effects of Anwala churna on cognition, cholesterol levels and brain cholinesterase activity in rodents. Anwala churna was treated in different dose for fifteen days to different groups of mice. Elevated plus maze and passive avoidance apparatus served as the exteroceptive behavioral models for testing cognition. There was decrease in the level of brain cholinesterase and total cholesterol after the administration for 15 days. Anwala churna may be beneficial remedy for the management of Alzheimer's disease due to its beneficial effects such as memory enhancing property, cholesterol lowering property and anticholinesterase activity.

Dhingra *et al*., (2004)[110] investigated the effects of *Glycyrrhiza glabra* on learning and memory in mice using elevated plus maze and passive avoidance paradigm. Aqueous extract of *Glycyrrhiza glabra* was administered for 7 successive days in different groups. The aqueous extract of liquorice significantly enhanced cognitive performance of mice and significantly inhibited the amnesia induced by diazepam (1mg/kg i.p.) and scopolamine (0.4mg/kg i.p.). Memory enhancement effect may be due to the anti-inflammatory and antioxidant effect. Hence liquorice may show beneficial effect on learning and memory which may be due to enhancement of cholinergic-transmission in brain.

Chintawar *et al.,* (2002)[111] evaluated the effect of n-butanolic fraction extracted from dried leaves of *Albizzia lebbeck* on cognitive performance using passive shock avoidance and the elevated plus maze experimental paradigms. The retention ability of the normal and amnesic mice improved significantly. They have also analysed the effects of extract on levels of serotonin (5-HT), noradrenaline and dopamine. The serotonin, gamma aminobutyric acid (GABA) and dopamine contents of brain were also determined to establish their correlation to the behavior with neurotransmitter levels. The data obtained revealed the importance of monoamine neurotransmitters in the nootropic action of butanolic fraction of *Albizzia lebbeck.*

Joshi and Parle., (2006)[112] investigated the nootropic acitivity of calyces of *Hibiscus sabdariffa* Linn in mice. Amnesia was induced by the administration of scopolamine. The aqueous extracts of calyces of *Hibiscus sabdariffa* significantly inhibited amnesia induced by scopolamine (0.4 mg/kg, i.p). Extract at a dose of 100 mg/kg and 200 mg/kg significantly decreased the transfer latencies and increased

step down latency in the aged mice when compared with piracetam treated groups. Extract significantly reduced acetylcholinesterase activity in mice. The results reveal that the aqueous extract of calyces of *Hibiscus sabdariffa* Linn may be beneficial as memory restorative agent in the management of dementia.

Joshi and Parle., (2007)[113] studied the effect of aqueous extract of *Desmodium gangeticum* in attenuating scopolamine induced amnesia in mice. The amount of acetylcholine present in the whole brain and acetylcholinesterase activity in different regions of the mouse brain such as cerebral cortex, midbrain, medulla oblongata and cerebellum were analysed. Treatment with the extract at a dose of 100mg/kg and 200mg/kg, p.o. for seven days showed the ability to inhibit scopolamine induced amnesia in mice. Extract improved the levels of acetylcholine content in brain and inhibited acetyl cholinesterase activity in groups treated with piracetam. Therefore aqueous extract of *Desmodium gangeticum* may be used in the management of dementia and Alzheimer's disease.

Touqee Ahmed and Anwarul Hassan., ***(2009)***[114] evaluated the inhibitory effect of curcuminoids on acetylcholinesterase and also the reversal of scopolamine induced amnesia to determine the beneficial effect of turmeric in Alzheimer's disease. The *in vitro* and *ex vivo* models of AChE inhibitory activity was used to analyse the effect on memory in rats. Curcuminoids inhibited AChE in the *in vitro* assay and the results of ex *vivo* AChE assay showed dose dependent inhibition in frontal cortex and hippocampus. Curcuminoids also showed significant activity when they were studied at fixed dose to reverse scopolamine induced amnesia. These data indicate that curcuminoids and all components except curcumin possess pronounced AChE

inhibitory activity. Curcumin showed a weak activity in the *in vitro* assay and has not shown a predominant effect in the *ex vivo* model.

Ilkay orhan and Mustafa aslam., (2009)[115] evaluated the memory enhancing activity of the hydroalcoholic extracts of Lamiaceae species. *Salvia triloba*, *Melissa officinalis* and *Teucrium polium* were assessed for their *in vivo* antiamnesic activity, *in vitro* anticholinesterase and antioxidant property. Scopolamine induced amnesia was analysed in mice by using passive avoidance. The anticholinesterase effect was determined by spectrophotometric method and antioxidant activity was evaluated by scavenging effect against 2,2-diphenyl-1-picrylhydrazyl (DPPH). The extracts showed significant antioxidant activity and in the anticholinesterase assay *Teucrium polium* had the highest inhibition against acetyl cholinesterase.

Julio Rubio *et al.,* (2007)[116] studied the effect of aqueous and hydro alcoholic extracts of Black Maca *(Lepidium meyenii)* on scopolamine induced memory impairment in mice. The study was designed to evaluate the effect of aqueous and hydroalcoholic extracts of Black Maca. The extracts were administered for 35 days to study the effect on memory impairment induced by scopolamine in male mice. Memory and learning parameters were evaluated using the water morris maze and the step down avoidance test paradigms. Both extracts of Black Maca significantly reversed the scopolamine induced memory impairment. Black Maca extracts inhibited AChE activity and there was no effect on MAO activity. These results reveal that Black Maca enhances scopolamine induced memory deficits.

Konrath EL *et al*., (2012)[117] evaluated anticholinesterase and the antioxidant activities of the Lycopodiaceae species extracts and they were analysed by using

in vitro and ex vivo models. The *in vitro* antioxidant effects were determined by analyzing its effect on 2-deoxyribose degradation, nitric oxide (NO) interaction, 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical scavenging activity. After an administration (25 and 10mg/kg i.p.) of the extracts in middle-aged mice, the antioxidant effects were analysed by estimation of thiobarbituric acid reactive substances test (TBARS). The antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) were assessed to determine the effect of the extract on antioxidant status. Results of the study revealed the effect of Lycopodium *clavatum* and *Lycopodium thyoides* commonly used in folk medicine may be due to their AChE inhibitory activity and antioxidant property. Due to the above beneficial effect they may be effective in the treatment of Alzhemiers disease.

3.2 DESCRIPTION OF MEDICINAL PLANT *ANACYCLUS PYRETHRUM*

Scientific name	:	*Anacyclus pyrethrum*
Family	:	Asteraceae

Botanical Classification

Kingdom	:	Plantae
Division	:	Angiospermae
Class	:	Dicotyledoneae
Sub class	:	Asteridae
Order	:	Asterales
Genus	:	*Anacyclus*
Species	:	*Pyrethrum*
Tribes	:	Anthemideae
Synonyms	:	*Anthemis pyrethrum* L.
		Anacyclus depressus Mairs
		Anacyclus freynii Porta & Rigo
		Pyrethrum radix

Vernacular Names [118]

English	:	Spanish Pellitory, Pellitory
Sanskrit	:	Akallaka
Tamil	:	Akkaraka, Akkarakaram
Hindi	:	Akalkara
Malayalam	:	Akikaruka, Akravu

Bengali	:	Akarakara
Gujrati	:	Akkalkaro, Akkalgaro
Kannada	:	Akkallakara, Akallakara, Akalakarabha, Akkallaka
Marathi	:	Akkalakara, Akkalakada
Oriya	:	Akarakara
Punjabi	:	Akarakarabh, Akarakara
Telugu	:	Akkalakarra
Urdu	:	Aqaraqarha

Habitat

Pellitory root is regarded as the most popular herb of India and Arab. This drug entered in to India during Muslim regime. It has been for the first time described in Godo Nigraha as akallaka, Letter in Sargadhaara samhita and Bhava Prakash Nigantu it is described as akarakarabha.[119] The plant is native to Asia and North Africa, introduced into south Europe. In India it is cultivated in a few regions especially at elevations of 900m at Katra (Jammu and Kashmir), Bengal and Himalayan region.[120,121] Herb is also distributed in the hilly regions of Kerala . The small shoots grow with the beginning of the rains. The root is pungent in taste and the person feels hot when it is chewed and a burning sensation is felt on the tongue. Akarakarbha is an important medicinal plant which is used in both Unani and Ayurveda. The roots of *Anacyclus pyrethrum* are commonly used and belong to the Family Asteraceae.

Plant description

Botanical description of herb

It is a perennial and procumbent herb. Before rising errect the stems lie on the ground for part of their length. Each bears one large terminal flower, the disk being yellow and the ray's white, tinged with purple beneath. The leaves are smooth, alternate, pinnate and pale green with deeply cut segments. Fruit is obovateachene. The root is almost cylindrical, very slightly twisted, tapering and often crowned with a tuft of grey hairs. Externally it is brown and wrinkled with bright black spots. The fracture is short, bark with 1-2 circles of resin ducts, closely adhering to yellowish radiate porous wood in which occur 1-3 rows of resin ducts. The odor is distinct, taste sweetish, pungent, very acrid, tingling and produce sialagogue effect. The fresh root is fusiform very pungent and produce a sensation of cold followed by heat. When dried it has brownish appearance externally and whitish internally. Flowering period is between April-June. [122]

Morphological Description of root [123]

a) Macroscopic

Roots are tough and cylindrical about 7-15cm in length and tapering slightly at both ends. They have few hairy rootlets and occasionally topped by bristly remains of leaves. The external surface is rough with brown in color, shriveled and the bark is about 3mm thick and not easily separable. The odour is slightly aromatic in taste and characteristically astringent and pungent. On chewing gives tingling sensation to tongue and lips and causes excessive flow of saliva.

b) Microscopic

Root - Mature root shows cork consisting of tabular cells and many of which developed as sclerenchyma with a few inner cork cells contain rosette crystals of calcium oxalate. The secondary cortex consists of tangentially, elongated, thin-walled, parenchymatous cells and a few sclerenchymatous cells. They are found scattered in secondarycortex. The secondary phloem consisting of usual elements, cambium 2-5 layered, secondary xylem are very wide consisting of xylem vessels, tracheids and xylem parenchyma. The vessels are pitted with more or less in groups distributed throughout the xylem, more and wider vessels found towards periphery. The xylem fibers are thick walled about 1.37-28.8 μ in width and 53.2 - 231 μ in length having narrow lumen. The medullary rays are numerous which are running straight. The multiseriate, uniseriate rays are very rare starting from primary xylem and reaching up to secondary cortex. The ray cells are thick-walled, radially elongated and inulin are present in cells of glands and are found scattered in secondary cortex, secondary phloem and medullary rays. The calcium oxalate crystals in rosette form are present in secondary cortex, secondary phloem, secondary xylem and medullary ray cells.

Powder - They are ash coloured and under microscope after cleaning with 75% chloral hydrate revealed that it is made up of abundance of stone cells, fibers and crystal of calcium oxalate of varying shape and size. In supplement, vessels types of parenchyma and sieve tube cells also constitute the root powder. Powder triturated with water produce mucilage and appearance of yellow color appears after treatment with 66% sulphuric acid and also appearance of greenish yellow colour if treated with 5% sodium hydroxide. They gave appearance of dark green after

treating with ferric chloride. Oil stain appears if powder pressed between two filter papers. Presence of lignine, suberin, protein, alkaloid, calcium oxalate revealed by histochemical and phytochemical test. In fluorescence analysis, powder treated with distilled water showed dull yellow color in ordinary light and brown in U.V light. When they are treated with acetone it showed light green yellow color in U.V light and light yellow in ordinary light. On treatment with benzene it gave a colorless appearance in U.V light. Treatment with chloroform it gave appearance of brown colour under U.V light and light yellow in ordinary light. When treated with carbon tetrachloride it appeared as dull white in U.V light and in ordinary light it is appeared as colorless. They don't show any appearance of colour when treated with petroleum ether. The ethyl acetate treatment showed creamy color in ordinary light and greenish yellow in U.V light. Treatment with methanol gives dark yellow color in ordinary light and light green in U.V light.[124]

Chemical constituents

Phytoconstituents mainly reported from the plant are

- **Flavonoids:**[125]
    - Flavonol 5-glucoside, Diosmetin 7-glucoside.
- **Alkamide:**[126,127,128,129]
    - Pellitorine.
- **Iso butylamide:** [130,131]
    - Anacycline.
- **A series of amides:**[127,125]
    - Amides containing monosubstituted α β-dian-unsaturation.

- **Polyacetylene**:[132]
    - Artemisia Ketone, triyne-triene.
- **Miscellaneous**:[133,134]
    - Linoleicacid, inulin, tannins, resin, essential oil and sesamine.
    - Manganese, zinc, copper, sodium and potassium,

Ethnobotonical Literature

- *Anacyclus pyrethrum* roots, commonly known as akarkara (pellitory root), have been widely acclaimed in the Ayurvedic system of medicine for their rasayana properties. The rasayana class of medicinal plants in Ayurveda are reported to have a triphasic activity, i.e. the ability to improve health and longevity, enhance memory, intelligence, youthfulness and improve complexion.[135]

- Akarkarabha (*Anacyclus pyrethrum*) are the herbs which are described to act upon majja dhatu and manovaha sotras are considered as useful remedy in Alzheimer's disease in ayurveda. Akarkarabha is used as medicated milk in ayurveda along with brahmi and also used in combination with shankapushpi (*Evolvulus Alsinoides*).[136]

- *Anacyclus pyrethrum* provide pure intelligence and this medicinal herb used dry or cooked with food is always useful and good for sick and for healthy. The roots of *Anacyclus pyrethrum* are regarded as a tonic to the nervous system. It is used to treat paralysis, hemiplegia, cephalalgia, epilepsy and rheumatism, chorea, insanity and a host of other diseases

including irritability of temper in children and in impotence in traditional medicine.[137,138]

- Akarkara has been described in ancient Indian literature i.e. Charak Samhita as 'vajikaran rasayana' which signifies a special category of immunomodulators.[139]

- It is commonly used in folk remedies for the stimulation of salivary glands. It is also useful as a remedy for toothache, paralysis of the tongue and throat muscles and in neuralgic problems of the teeth.[140]

- Roots are utilized as insecticide and also have anti-mycosis property. In combination with cade oil, the powder is used against ringworm.[141]

- When applied to the skin, due to rubefacient and local irritant action it induces heat, tingling and redness. The powder of the root when snuffed up in to the nostrils procures sneezing and eases the headache. Along with hog's lard the powder is made in to an ointment and it takes away black and blue spots due to blows or falls and helps in the treatment of gout and sciatica.[142,143]

- Roots has been found useful along with the root of *Withania somnifera* and *vitis vinifera* in the treatment of epilepsy.[144]

- This herb is also suggested by herbalist for its use in diabetes and to aid in digestion.[145]

- An infusion made from the roots along with lesser galangal (*Alpinia officinarum*) and ginger (*Zingiber officinale* Rosc.) is used as a stimulant and is also reported to be useful in certain stages of typhus fever.[146]

- In Ceylon it is used as an important ingredient in decoction given in typhoid fever, convulsion in children, rheumatism and skin eruption due to impurities in the blood, bronchial diseases and sexual debility.[147]

- *Anacyclus pyrethrum* is used as traditional medicinal plant in Algeria for treatment of diseases such as infection, chronic bronchitis and cough. [148]

- Roots of *Anacyclus pyrethrum* are used traditionally in Egypt for eye, skin disease, dentistry and fever, intestinal disease, for inflammation, in animal bites and in poison.[148]

- In European herbal medicine, it is considered to have a restorative action on the kidneys and it also supports and strengthens their function. It is also been used for nephritis, pyelitis, cystitis, and edema. It counteracts mucus and is useful for suppression of chronic coughs. The leaves are also applied as poultices.[14]

3.4 PHARMACOLOGICAL ACTIVITIES AND CLINICAL TRIALS

***In vitro* cyclooxygenase activity**

MullerJakic *et al.*, (1994)[149] isolated polyunsaturated alkamides from *Achillea species*, *Echinacea angustifolia*, *Anacyclus pyrethrum* and *Aaronsohnia pubescens* (Compositae) and were shown to possess inhibitory activity in *invitro* cyclooxygenase and 5-lipoxygenase assays. Activity shown is due to particular

structure of the alkamides. *Invitro* effects of the alkyl amide from the roots of *Anacyclus pyrethrum* at a concentration of 50 µg/ml was studied on prostaglandin metabolism. Results showed that chemical constituents from the roots of *Anacyclus pyrethrum* produced inhibitory effect on microsomal cyclooxygenase and lipoxygenase.

Antimicrobial effect

Jaswinder Singh *et al.*, (2009)[150] studied *antimicrobial effect of* ethanolic extract of *Anacyclus pyrethrum* dissolved in isopropanol by filter disc diffusion method. The *Bacillus cereus* showed inhibition zone of 20mm, for *Staphylococcusalbus* it was 35mm and for *Staphylococcusaureus* it was 32mm respectively. Results show that extracts has a weak antimicrobial effect according to filter disc diffusion method. Organic extract of the roots show some anti bacterial activities but did not exhibit any antifungal properties.

Insecticidal and Molluscicidal effect

The alkylamides from *Anacyclus pyrethrum* show insecticidal and molluscicidal effects. The solution of pellitorine in purified kerosene when used as a spray for house flies (*Musca domestica* L.) showed paralysing effect. Pellitorine also exhibit some lethal effect on adult yellow mealworms (*Tenebrio molitor*). 3.1% solution of pellitorine in acetone topically applied to mealworms produce immobilization of 45% after 24 hours. Anacyclin, as a 3% solution in acetone, produces only 10% mortality in *Tenebrio molitor.*[134]

5- Lipoxygenase inhibitory activity

The abundance of medicinal plants utilized in the traditional medicine is always provides a promising source for identifying new 5-lipoxygenase inhibitors. *Anacyclus pyrethrum* is also presented as an ideal candidate for showing inhibitory activity on 5-lipoxygenase or cyclooxygenase activity among promising plant species tested. [151]

Effect on glucose and serum cholestrol

Anacyclus pyrethrum is used in patients with insulin-dependent diabetes mellitus to reduce the dose of insulin. It also shows a decrease in the level of plasma glucose and serum cholesterol after 3–6 weeks of oral administration.[144]

Contraceptive activity

In temporary birth control dried stems of *Anacyclus pyrethrum* DC are mixed with seeds of *piper nigrum* in equal proportions and made into powder. The powder is made into a paste with honey and applied into the vagina before coition.[152] The seeds of *Anacyclus pyrethrum* produces miscarriages in pregnant albino rats when treated orally for 10 days after copulation and it was administered at a daily dose of 175 mg/kg body weight. Seeds caused abortion in 15% of albino rats, skeletal and visceral malformation was also common in the fetuses.[153]

Anaesthetic activity

Patel *et al.*, (1992)[154] studied the strength of anaesthesia, its effect on surgery, post-operative recovery and wound healing. Two hundred dental patients were

subjected to a double blind study following oral surgery for comparing the activity of an alcoholic extract of the roots of *Anacyclus pyrethrum* (2% alcohol extract, freshly dissolved in sterile distilled water) with xylocaine. Adequate levels of anesthesia produced was observed in 90 out of 100 patients for the extract and while in xylocaine locally administered patient, anaesthesia was observed in 80 out of 100 patients. The plant extract was found useful and safe at lower concentrations (less than 2%), not showing any side effects and facilitated anaesthesia for prolonged oral reconstructive surgery when compared with xylocaine.

Gopalakrishna *et al*., (1987) [155] evaluated a preliminary study for local anaesthetic activity of aqueous and alcoholic extracts of *Anacyclus pyrethrum* roots in laboratory animals. *Anacyclus pyrethrum extracts* induced anaesthesia in 5 minutes and lasted for 12 hours longer than xylocaine in guinea pig dermis and rabbit cornea experimental models. Aqueous and alcoholic (2%) extracts of roots of *Anacyclus pyrethrum* exhibited local anaesthetic activity in animals for longer duration than xylocaine.[156]

Immunomodulatory activity

Bendjeddou Dalila *et al*., (2003)[157] studied immunostimulating activity of *Anacyclus pyrethrum*, *Citrullus colocynthis* and *Alpinia galanga* of water polysaccharide extracts in rodents. The fractions obtained from *Anacyclus pyrethrum* and *Alpinia galanga* exhibited stimulating effect on the reticulo-endothelial system (RES) and also showed increase in the number of peritoneal exudate cells and spleen cells of mice. In this case, the optimum doses were 50 and 25 mg/kg for the two fractions, respectively. The polysaccharide extracts of *Anacyclus pyrethrum* and

Alpinia galanga showed increase in the proliferation of the murine spleen cells. The results of the study reveal that it showed better *in vivo* effect than *in vitro* effect at doses of 50 and 25 mg/kg for *Anacyclus pyrethrum and Alpinia galanga*. While the *Citrullus colocynthis* extract produce much weaker immunostimulating activity.

Bendjeddou Dalila *et al*., (2010)[159] isolated immunologically active polysaccharide from *Anacyclus pyrethrum.* The effect of different fractions on lymphocyte activity was studied. The polysaccharides markedly stimulated the proliferation of murine spleen cells *in vitro* measured by MTT colorimetric assay. Fractionation analysis and alkaline phosphatase inducing activity method, results showed that the mitogenic effect of PSI, PSII, PSIII was predominantly on T cell population but they activated B cells indirectly *via* T cells. Furthermore, the three polysaccharides enhanced the reticuloendothelial system potentiating activity by increasing the clearance rate of carbon particles. These results suggested that the three polysaccharides extracted from *Anacyclus pyrethrum* have immunostimulating activity which could be used clinically for the modulation of immune systems.

Sharma *et al*., (2010)[159] studied immunomodulatory activity of petroleum ether extract of *Anacyclus pyrethrum.* Petroleum ether extract of roots was tested at dose of 50 and 100 mg/kg respectively. The effect of the extract on blood parameters, cyclophosphamide induced immunosuppression, survival rate against *Candida albicans* infection, delayed type hypersensitivity reaction, percentage neutrophil adhesion and phagocytic activity were analysed. The extract treated rats were able to neutralize cyclophosphamide induced myelo suppression as revealed by the normalization of blood parameters. The results thus provide a basis for the use of

Anacyclus pyrethrum as an adaptogen and immunomodulator in the Ayurvedic system of medicine

Effect on central nervous system

Gautam *et al*., (2011) [160] studied anticonvulsant activity of ethanolic extract of *Anacyclus pyrethrum* in albino mice. The ethanolic extract of *Anacyclus pyrethrum* reduced the duration of hind limb tonic extension in a dose dependent manner against maxiumal electro shock model. The ethanolic extract of *Anacyclus pyrethrum* inhibits maxiumal electro shock model induced convulsions.

Fakir *et al* ., (2011)[161] Studied antiepileptic effect of the butanolic acid extract of *Anacyclus pyrethrum* in a model of kainic acid induced status epilepticus in Wistar rats which strengthen its effect as anticonvulsant. The result also indicates that the extract exhibited a significant dose dependent protection against electrically induced seizure in rats.

Badhe *et al*., (2010)[162] studied antidepressant activity of roots of *Anacyclus pyrethrum.* Different models in neuropharmacological studies were performed on Swiss male albino mice. Standard root extract of *Anacyclus pyrethrum* exhibited an increase in ambulatory behaviour which indicates a stimulant effect. AP root extract also produced a significant antidepressant effect in both forced swim test and tail suspension test as revaled due to the reduction in the immobility. AP root extract also reversed the hypothermia produced by clonidine and reserpine. *Anacyclus pyrethrum* root extract inhibited haloperidol-induced catalepsy.

Monika Pahuja *et al*., (2011)[163] studied the effect of hydroalcoholic extract of *Anacyclus pyrethrum* root on its protective effect on seizures, oxidative stress induced by seizures and cognitive dysfunction by using various experimental models of seizures. Reduced glutathione, malondialdehyde levels and cholinesterase activity in the brain were also measured in the experiment. Extract showed protection at 50,100, 250 and 500 mg/kg respectively against pentylenetetrazole induced seizures and maximal electro shock model induced seizures. Extract administration significantly inhibited seizure induced oxidative stress and cognitive impairment in a concentration dependent manner. Thus, extract exhibited protection against seizures, seizure-induced oxidative stress and cognitive impairment in rats.

Effect on mutagenesis

Sukumaran and kuttan., (1995)[126] studied the inhibition of tobacco-induced mutagenesis by eugenol and plant. The inhibitory effects of eugenol, a compound present in many spices such as cloves, cardamom and the extracts of *Anacyclus pyrethrum* and *Spilanthes calva* on tobacco-induced mutagenesis were examined using Ames Salmonella/microsome assay. Eugenol significantly showed inhibiton in tobacco-induced mutagenicity at concentrations of 0.5 and 1 mg/plate. *Anacyclus pyrethrum* extract (1 mg/plate) produced 74.33% inhibition. Eugenol and the plant extracts also inhibited the nitrosation of methylurea in a concentration dependent manner.

Anti-inflammatory activity

Rimbau *et al*., (1999)[164] studied the anti-inflammatory activity of some extracts from plants used in the traditional medicine of North African countries.

Aqueous, ethanol and chloroform extracts from five plants were administered either topically (oedema induced by arachidonic acid in mouse ear) or (subplantar oedma induced by carragenan in rats). Results showed that plants possess anti-inflammatory activity, since at least one extract of each plant was active in one of the experimental models. Results showed that three extracts of *Anacyclus Pyrethrum* showed significant anti-inflammatory activity in both experimental models.

Reproductive activity

Sharma *et al*., (2009)[165] studied the anabolic, aphrodisiac and reproductive activity of *Anacyclus pyrethrum* in male rats. Aqueous extract of the roots was evaluated for its effect on sexual behaviour, spermatogenesis and sperm count. Aqueous extract at doses of 50 and 100 mg/kg on administration in Albino rats produced rise in anabolic and spermatogenic effect. The sperm count and fructose levels in seminal vesicles were markedly caused an increase in the improvement of sexual behaviour of male rats and this was characterised by increased mount and intromission frequency and reduced mount and intromission latency. The extract had a concentration dependent influence on sperm count, sexual behavior and seminal fructose concentration increased significantly.

Inhibitory effects of *Anacyclus pyrethrum* extracts on hepatitis C virus (HCV) protease

Ghazi Hussein *et al., (*2000)[166] studied inhibitory effects of *Anacyclus pyrethrum* extracts on hepatitis C virus (HCV) protease. *Anacyclus pyrethrum* is one of the 152 Sudanese medicinal plants screened for their inhibitory effect on Hepatitis C Virus (HCV) protease. The methanolic extract of *Anacyclus pyrethrum* was

reported to have 27.3% and aqueous extract 45.3% hepatitis C virus (HCV) protease inhibition activity respectively.

Cytotoxicity

Alluri *et al.*, (2005)[167] investigated the cytotoxicity of the *Anacyclus pyrethrum.* Brim Shrimp lethality bioassay was carried out to investigate the cytotoxicity of the *Anacyclus pyrethrum extract* on naupalii larva and the lethality concentration LC_{50} was found as 460μg/ml.

3.5 HERBAL FORMULATIONS

Several preparations and formulations are accessible utilizing the root powder or its extracts alone or in blend with other herbs or drugs.

Anacyclus pyrethrum is used as ingredient in lot of herbal formulations some of them were proved for their good effect in different diseases e.g. Jawarish Zaruni Sada for its nephroprotective, diuretic effect and to increase natriuresis in animal model.[168]

Local application of Rumalya ointment containing *Anacyclus pyrethrum* in the formulation has beneficial effect on Rheumatoid arthritis. [168]

An indigenous drug, Fortege, containing *Anacyclus pyrethrum*, *Argyreiya speciosa* sweet and *Withania somnifera* Dunal has been reported to cure sexual disorders in males, with no side effect.[169]

Anacyclus pyrethrum is also an active ingredient of activit, a herbomineral formulation which has antioxidant activity.[170]

Conclusion from review of literature

From the literature review it was concluded that there is no scientific investigation reported on congnitive potential of *Anacyclus pyrethrum.* Hence I have focusssed to carry out this work by using *Anacyclus pyrethrum* roots.

- Review of literature show that phytomedicines are commonly used in alternative system for treatment of disorders related to learning and memory.
- In traditional practices of medicine, variety of plants has been utilized to treat cognitive disorders such as Alzheimer's disease and other memory related disorders.
- Various studies reveal an ideal target to identify potential new drugs from plant sources, including those for memory disorders.
- *Anacyclus pyrethrum* is reported for traditional uses such as to enhance memory, intelligence and youthfulness.
- *Anacyclus pyrethrum* is also used traditionally in the treatment of paralysis, hemiplegia, cephalalgia, epilepsy, rheumatism, chorea, insanity, toothache, paralysis of the tongue, gout and sciatica, diabetes, typhus fever, rheumatism, skin disease, bronchial diseases and sexual debiliy.
- *Anacycluspyrethrum* shows potent *invitro* cycloxygenase activity, anaesthetic and immunomodulatory activity.

- *Anacyclus pyrethrum* is also reported for anticonvulsant, antinflammatory, anabolic, aphrodisiac, reproductive, insecticidal and molluscicidal activity.
- *Anacyclus pyrethrum* also possesses ability to reduce blood sugar, cholesterol and inhibition of tobacco-induced mutagenesis.
- *Anacyclus pyrethrum* is also used in several preparations and formulations that are in combination with other herbs or drugs.

IV. DESIGN OF INVESTIGATION

The following studies were carried out on roots of *Anacyclus pyrethrum* extract

1. **Collection and authentification of plant extract.**
2. **Preparation of extracts and preliminary phytochemical screening of extracts.**
3. ***Invitro* anti cholinesterase activity of extract**
   - *Invitro* anticholinesterase activity by the spectrophotometric method.
   - *Invitro* anticholinesterase activity by thin layer chromatography bioassay.
4. **Identification and determination of phytoconstituents of extract.**
   - TLC and HPTLC of the extract.
   - Determination of total phenolic content.
   - Determination of total alkaloid content.
   - Determination of total flavonoid content.
   - Determination of total ascorbic acid.
5. **Toxicological investigation of the extract.**
   - Acute toxicity study.
   - Sub chronic toxicity.
6. **Neuropharmacological investigation of the extract.**
   - General behaviour studies.
   - Effect on locomotor activity.

- Effect on motor coordination.
- Assessment of anxiolytic activity in rats using the hole board apparatus and elevated plus maze.
- Assessment of antidepressant activity in rats using forced swim test and tail-suspension test.

7. Effect of the extract on cognitive paradigms

(i) Interoceptive behaviour model

- Scopolamine induced amnesia model
    - Elevated plus maze paradigm.
    - Passive avoidance paradigm.
- Estimation of Acetylcholineserase (AchE) enzyme in rat brain.
- **Estimation of antioxidant status in rat brain.**
    - Estimation of MDA.
    - Estimation of catalase.
    - Assay of super oxide dismutase (SOD).
    - Assay of glutathione peroxidase (GPx).
    - Assay of glutathione reductase (GRD.
    - Estimation of reduced glutathione (GSH).

(ii) Exteroceptive behaviour model

- Social recognition task.
- Elevated plus maze paradigm.

- Estimation of dopamine level, serotonin and glutamate levels in rat brain.

8. *Invitro* antioxidant studies of the extract

- Determination of DPPH radical scavenging activity.
- Determination of hydroxyl radical scavenging activity.
- Determination of hydrogen peroxide scavenging activity.
- Determination of reducing power.
- Determination of nitric oxide scavenging activity.

➢ *Ex vivo* studies

- Lipid peroxidation assay.

V. MATERIALS AND METHODS

4.1 PLANT MATERIAL

The roots of *Anacyclus pyrethrum* used for investigation was collected from hilly regions of Pathanamthitta district of Kerala and the roots of *Anacyclus pyrethrum* was identified and authenticated for their correct botanical identity by Prof. P.Jayaraman, Director, National Institute of Herbal Science, Chennai (Ref. no: PARC/-2009/419) and samples (voucher no: 0997) of the plant has been be deposited in the herbarium of the institute.

Chemicals

Analytical grade chemicals supplied by S.D.Fine Chemicals, Qualigen's Fine Chemicals and Sigma Aldrich Chemicals were used for this research. 5,5-Dithio-bis(2-nitrobenzoic) acid, Acetylthiocholine iodide, Acetylcholinesterase electric eel, Folin-Ciocalteu's reagent, Sodium thiosulphate, Dopamine hydrochloride,Serotonin hydrochloride, Glutamate, O-Phthaldialdehyde, Ninhydrin reagent, Reduced glutathione, Oxidized glutathione, Nicotine adenine dinucleotide phosphate (NADPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxyribose, Piracetam, Scopolamine hydrobromide were obtained from Sigma Aldrich Chemicals. Diethylether, Chloroform, Ethanol, Gallic acid, Quercetin, Ascorbic acid, n-butanol, Heptane Sodiumhydroxide, Iodine, Aceticacid, Sodiumdodecylsulphate, Pyrogallol, Ferricchloride, Ascorbate, Potassiumferricyanide, Trichloro acetic acid,Sodium nitroprusside, Sodium dodecyl sulphate, Thiobarbituric acid were obtained from S.D.Fine Chemicals. Ethylene diamine tetra acetic acid (EDTA), Hydrochloric acid, Hydrogen peroxide, was obtained from Qualigen's Fine Chemicals. All other chemicals were analytical grade obtained, from S.D.Fine Chemicals.

4.2 METHODS

Section - 1 Preparation of extract and preliminary phytochemical screening

The extracts of *Anacyclus pyrethrum* roots were prepared with different solvents in the increasing order of polarity and these extracts were subjected to preliminary phytochemical analysis.

4.2.1 Preparation of extracts

The powdered roots of *Anacyclus pyrethrum* were subjected to successive soxhlet extraction with different solvents such as hexane, chloroform and ethanol in the increasing order of polarity.[171] The obtained solvent extracts were evaporated under reduced pressure using rotary vacuum evaporator. Extracts were weighed and percentage was calculated in terms of the air-dried weight of the root material. The yield of the petroleum ether, chloroform and ethanol extract was found to be 8.53%, 5.66%, 7.81% w/w respectively.

4.2.2 Preliminary phytochemical screening

The extracts of *Anacyclus pyrethrum* root was subjected to preliminary phytochemical screening.[171,172]

1. Test for alkaloids

Treated with dilute Hydrochloric acid and filtered. The filtrate was treated with various alkaloidal agents.

a) Mayer's–Test

Treated with Mayer's reagent and cream colour indicates the presence of alkaloid.

b) Dragendroff's–Test

When little amount of the sample was treated with the Dragendroff's reagent, the presence of reddish brown precipitate reveals the presence of alkaloid.

c) Hager's–Test

Treated with the Hager's reagent and presence of yellow colour precipitate indicates the presence of alkaloid.

d) Wagner's–Test

Treated with the Wagner's reagent, the appearance of brown colour precipitate indicates the presence of alkaloid.

2. Test for carbohydrates

The extracts were treated with 3ml of alpha–Napthol in alcohol and to the sides of the test tube concentrated sulphuric acid was added carefully. Formation of violet colour ring at the junction of two liquids shows the presence of carbohydrates.

a) Fehling's–Test

The extracts were treated with Fehling's solution A and B and heated. Presence of reddish brown colour precipitate indicates the presence of reducing sugars.

b) Benedict's–Test

The extracts were treated with Benedict's reagent and heated and presence of reddish orange colour precipitate indicates the presence of reducing sugars.

c) Barfoed's–Test

The extracts were treated with Barfoed's reagent and heated. Appearance of reddish orange colour precipitate indicates the presence of non reducing sugars.

3. Test for proteins

a) Biuret's–test

When the extracts were treated with copper sulphate solution, followed by the addition of sodium hydroxide solution, appearance of violet colour indicates the presence of proteins

b) Millon's–Test

When the extract was treated with Millon's reagent, appearance of pink colour indicates the presence of proteins.

4. Test for steroids

a) Libermann Burchard Test

When the extracts were treated with concentrated sulphuric acid, few drops of glacial acetic acid, followed by the addition of acetic anhydride, appearance of green colour indicates the presence of steroids.

5. Test for sterols

When the extracts were treated with 5% potassium hydroxide solution, appearance of pink colour indicates the presence of sterols.

6. Test for phenols

a) When the extracts were treated with neutral ferric chloride solution, appearance of violet colour indicates the presence of phenols.

b) When the extracts were treated with 10% sodium chloride solution, the appearance of cream colour indicates the presence of phenols.

7. Test for tannins

a) When the extracts were treated with 10% lead acetate solution, appearance of white precipitate indicates the presence of tannins.

b) When the extracts were treated with aqueous bromine solution, appearance of white precipitate indicates the presence of tannins.

8. Test for flavanoids

a) 5ml of the extract solution was hydrolyzed with 10 % v/v sulphuric acid and cooled. Then, it was extracted with diethyl ether and divided into three portions in three separate test tubes. 1 ml of diluted sodium carbonate, 1 ml of 0.1N sodium hydroxide, and 1ml of strong ammonia solution were added to the first, second and third test tubes respectively. In each test tube, development of yellow color demonstrated the presence of flavonoids.

b) Shinoda's test

The extract was dissolved in alcohol, to that one piece of magnesium followed by concentrated HCl was added drop wise and heated. Appearance of magenta color shows the presence of flavonoids.

9. Test for gums and mucilage

The extracts were treated with 25 ml of absolute alcohol, and then solution was filtered. The filtrate was examined for its swelling properties.

10. Test for glycosides

When a pinch of the extracts were dissolved in the glacial acetic acid and few drops of ferric chloride solution was added, followed by the addition of concentrated sulphuric acid, formation of red ring at the junction of two liquids indicates the presence of glycosides.

11. Test for saponins

Foam test

1ml of the extracts are diluted to 20 ml with distilled water and shaken well in a test tube. The formation of foam in the upper part of the test tube indicates the presence of saponins.

12. Test for terpenes

When the extracts were treated with tin and thionyl Chloride, appearance of pink colour indicates the presence of terpenes.

Section - 2 *Invitro* anticholinesterase activity and identification of phytoconstituents

Invitro anticholinesterase activity was done on the extracts to determine the extracts ability to inhibit acetylcholinesterase activity. The extract is selected on basis of its potency to inhibit acetylcholinesterase and was subjected to TLC and HPTLC analysis and identification of phytoconstiuents.

4.2.3 Determination of anticholinesterase activity

AChE inhibitory activity of the extracts was measured by the spectrophotometric method.[173] Acetylcholinesterase was used, while acetylthiocholine iodide was employed as substrate of the reaction. 5,5-Dithio-bis(2-nitrobenzoic) acid (DTNB) was used for the measurement of the cholinesterase activity.[174] Hydrolysis of acetylthiocholine iodide was determined by the formation of the yellow 5-thio-2-nitrobenzoate anion due to the reaction of DTNB with thiocholines, catalyzed by the enzyme at wavelength of 412 nm utilizing, UV–visible recording spectrophotometer, Shimadzu. Rivastigmine was used as reference. Percentage of inhibition of AChE was determined by comparison of rates of reaction of samples relative to blank sample using the formula

$(E-S)/E \times 100$

E is the activity of enzyme without test sample

S is the activity of enzyme with test sample.

The experiments were done in triplicate. The concentrations of test samples that inhibited hydrolysis of the substrate (acetylthiocholine) by 50% (IC_{50}) were

determined by monitoring the inhibitory effect of extracts with increasing concentrations in the assays.

Thin layer chromatography (TLC) with bioassay detection for AChE inhibition

AChE inhibitory activity of the extracts was measured by the TLC method. Merck Aluminium plate pre-coated with Silica gel 60 F_{254} of 0.2 mm thickness was used as stationary phase. The plant extracts were spotted in the TLC plate, it is developed in the mobile phase toluene: ethylacetate (97:3). The plate was developed and it was dried at room temperature. The plate was sprayed with 30mM acetylthiocholine and followed by 20mM DTNB. The plate was dried at room temperature for 45 minutes and then sprayed with AChE. The plate was observed under visible light after 20 minutes. A positive test indicating AChE inhibiton was colorless spot on the yellow background. [175]

4.2.4 Identification of phytoconstituents

TLC and HPTLC of ethanolic extract of *Anacyclus pyrethrum* [176]

500 mg of the sample was dissolved in water, filtered and made up to 15 ml. The solution was applied on Merck Aluminium plate pre-coated with Silica gel 60 F_{254} of 0.2 mm thickness. The plate was developed in toluene: ethylacetate (97:3) solvent system and Rf values was calculated. The plate was then scanned at 254 nm using Deuterium lamp in Camag HPTLC instrument provided with CAMAG software (HPTLC finger print is enclosed).

Determination of total phenolic content

The total phenolic contents of the ethanolic extract of *Anacyclus Pyrethrum* were determined with gallic acid as a positive standard.[177] Samples (100 µl) were mixed with 2 ml 2% sodiumcarbonate and incubated at 25°C for 2 minutes. 1:1 (v/v) Folin-Ciocalteu's phenol reagent was added after incubation and the contents were mixed vigorously. The mixture was allowed to stand at 25°C for 30 minutes and the absorbance was determined at 720 nm. The above said procedure was performed with standard gallic acid solutions and a standard curve was obtained. The total polyphenolic contents of the extract were expressed in terms of gallic acid equivalents of the extract.

Determination of total flavonoid content

The total flavonoid content was estimated using quercetin as a positive standard and expressed in terms of quercetin equivalents in mg/g of extract.[178] To the tubes containing extract, sodium nitrite (150 µl, 5% w/v) was added. The contents were uniformly mixed and allowed to stand for 5 min at ambient temperature, then 1.5 ml of 10% (w/v) aluminium chloride were added and the mixture was allowed to stand for another 6 minutes. To this 1 ml 1 M sodium hydroxide was added. After 10 minutes, the absorbance was measured at 510 nm.

Determination of total alkaloid content

The total alkaloid content of the ethanolic extract of *Anacyclus Pyrethrum* was determined.[179] The extract was dissolved in 2 N HCl and then filtered. The pH of phosphate buffer solution was adjusted to neutral with 0.1 N NaOH. One ml of this

solution was added to a separating funnel and then 5 ml of Bromocresol green solution along with 5 ml of phosphate buffer were added. The mixture was shaken and the complex formed was extracted with chloroform by vigorous shaking. The extracts were collected in a 10 ml volumetric flask and diluted to volume with chloroform. The absorbance of the complex in chloroform was measured at 470 nm.

Determination of total ascorbic acid

Total ascorbic acid of ethanolic extract of *Anacyclus Pyrethrum* was determined.[180] Blanks, standards and samples were prepared and were precipitated with 10% trichloroacetic acid followed by centrifugation. In 500 µL of supernatant, 100 µL of DTC reagent (2,4-dinitrophenylhydrazine 3%, thiourea 0.4%, and copper sulfate 0.05%) prepared in 9N sulphuric acid, was mixed and incubated at 37°C for 3 hours. After the addition of 750 µL of 65% (v/v) sulphuric acid, the absorbance was recorded at 520 nm. A standard curve was prepared with ascorbic acid standards, and total ascorbic acid was expressed as mg ascorbic acid /g of extract.

4.2.5 Animals

Albino wistar rats of either sex approximately same age group were used after being acclimatized for a week at laboratory conditions. They were provided standard rodent pellet diet (Lipton India) and water *ad libitum*. The animals had free access to food and water and maintained under 12:12 hr light and dark cycle. All experiments were carried out during day time from 09.00 to 17.00 hr. The protocol was approved by institutional animal ethical committee and care of the animals was taken as per guidelines of committee for the purpose of control and supervision in experiments on animals (CPCSEA), representative of animal welfare, Govt of India.

The experimental protocol was approved by the Institutional Animal ethics Committee IAEC Ref.No. 290/04/V/CPCSEA/IAEC/PHA-24-29.

Section - 3 Toxicological investigations

Acute and sub chronic toxicity studies were done on the ethanolic extract of *Anacyclus pyrethrum* to assess the toxic effect of the extract.

4.2.6 Toxicity studies

Acute toxicity study

Acute toxicity studies were performed according to the OECD 423 guidelines as shown in fig -3.[181] Female wistar rats weighing 150-175 gm was selected and divided into groups containing three animals in a group. The animals were fasted overnight, provided only with water. The ethanolic extract at an oral dose of 5 mg/kg, 50 mg/kg, 300 mg/kg & 2000 mg/kg was administered step by step according to the guidelines. The general behaviors were continuously monitored for 1 h after dosing, periodically during the first 24 hours with special attention given during the first 4 hours and then daily thereafter for a total of 14 days. The drug treated animals were carefully observed individually for the toxicity signs and mortality. The parameters such as changes in skin and fur, eyes and mucous membranes, respiratory, autonomic and central nervous system, behavioral pattern, tremors, convulsions salivation, diarrhoea, lethargy, sleep and coma were observed.

Sub chronic toxicity

Sub chronic toxicity studies were carried out according to Organization for Economic Cooperation and Development guideline (OECD408 (1995b) repeated dose 90-day oral toxicity study in rodents).[182] Forty-eight age and weight matched rats were randomly divided in to control and treatment groups. The extracts of *Anacyclus pyrethrum* was administered to the treatment group of rats at the dose of 1000

mg/kg/day by oral gavage, in a volume of 0.5ml/100g body weight for 90days, whereas an equal volume of vehicle was given to the control group. All the animals were weighed once a week. Measurement of food and water consumption was made at least weekly. During the period of administration, toxic manifestation such as signs of toxicity, mortality was monitored daily.

Hematological and biochemical parameters of rats

At the end of period of study, all surviving animals were fasted overnight before anesthetization with ether. The blood sample was carefully collected for blood chemistry and enzyme analysis in to heparinized and dry non-herparinized tubes. Heparinized blood samples were used for hematological study such as total red blood cell count, total white blood cell count, platelet count, hemoglobin, hematocrit, differential leukocyte count. [183]

The serum separated from non- heparinized samples were used for the estimation of biochemical parameters like creatinine[184], urea[185], triglycerides, total cholesterol[186], total protein[187], albumin[188], Aspartate transaminase (AST), Alanine transaminase(ALT) [189,190], Alkaline phosphatase (ALP) [191] and total bilirubin.[192,193]

Histopathological examinations

After blood collection rats were sacrificed for tissue studies. The internal organs like liver, kidney, lungs, brain, heart and spleen were isolated and blotted free of blood, weighed immediately to determine relative organs weights and observed for gross lesions. Histological examination was performed on the tissue preserved in 10% buffered formalin solution with particular emphasis on those which showed gross pathological changes.[194,195]

Section - 4 Effect of the extract on central nervous system

Neuropharmacological activity of the extract were assessed by following experimental methods such as general behavior studies, locomotor activity, motor coordination, anxiolytic, antidepressant activity to study the influence on central nervous system activity.

4.2.7 Neuropharmacological activity

General behaviour studies [196,197]

Evaluation of general behavioral profiles was performed by the method. Alibno wistar rats were divided in to five groups (n=6). Ethanolic extract of *Anacyclus pyrethrum* was administered for first three groups at dose of 50, 100 and 200mg/kg p.o respectively. While the last two groups were administered with diazepam (5mg/kg) as drug control and 2%v/v tween 80 as vehicle control. The animals were under observation for their behavioral changes if any, at 30 minutes intervals in the first one hour and at the hourly intervals for the next 4 hour for the following parameters.

Awareness, alertness and spontaneous activity

The awareness and alertness was recorded by visual measure of the animal's response when placed in a different position and its ability to orient itself without bumps or falls. Animals usually show a moderate degree of inquisitive behavior.

Righting reflex

Rats were treated with the test compounds on the test day. After 15, 30 and 60 minutes, each rat was placed gently on its back on an undulated surface made of white iron and kept at 30°C. If the animal remained on its back for 30 seconds, it was considered as a loss of righting reflex.

Pinna reflex

The reflex is examined by touching the centre of pinna with a hair or other fine instrument. The unaffected rat withdraws from the irritating hair.

Grip strength

The grip strength test is used to assess muscular strength or neuromuscular function in rodents. It was measured by allowing the animal to grasp a pencil placed in horizontal position and noting the time taken by the animal to drop the pencil on the table.

Touch response

The touch response was recorded by touching the rat with a pencil or forceps at the various part of the body (i.e.) on the side of the neck, abdomen and groin.

Pain response

The pain response was graded when a small artery clamp was attached to the base of the tail and response was noted.

Sound response

Albino wistar rats normally utter no sound, so vocalization may indicate a noxious stimulus.

Locomotor activity

Locomotor activity was measured using actophotometer. Rats were divided into five groups consisting of 6 per group. Three groups received the extract at a dose of 50, 100 and 200mg/kg body weight. The other two groups received control vehicle 2%v/v tween 80 and standard drug (Diazepam 2 mg/kg, i.p). Locomotor activity is easily measured using actophotometer which operates on photoelectric cells connected with a counter. When a beam of light falling on the photocell is cut off by the animal a count is recorded and displayed digitally. Each rat was placed individually in the activity cage floor for 10 minutes. The activity score was recorded after 60 min of drug and standard administration.[198]

Effect on motor coordination

Rats were divided into five groups consisting of 6 rats per group. Three groups received the extract at a dose of 50, 100 and 200mg/kg body weight. The other two groups received control vehicle 2%v/v tween 80 and standard drug (Diazepam 2 mg/kg, i.p). All the groups of rats were trained to remain on the rotarod. Only those rats which could balance themselves were taken for the study. The animals were discarded and replaced if they failed to do so. All the groups animals were placed on the rotarod and the fall off time from the rotating rod was noted after 60 minutes of test drug and standard administration.[199]

Assessment of anxiolytic activity in rats using the holeboard apparatus

Anxiety level was also evaluated in rats using a holeboard apparatus. The hole board apparatus consisted of wooden box (40×40×25cm) with 16 holes (Diameter, 3cm) evenly distributed in the floor. The hole board was elevated to the height of 25 cm. The test was performed 60 min after administration of ethanolic extract of *Anacyclus pyrethrum* at a dose of 50,100, 200 mg/kg p.o , control vehicle 2% v/v tween 80 and standard drug (Diazepam 2mg/kg, i.p). The number of head poking during 5 minutes period was recorded. An increase of the hole poking response reveals a positive anxolytic effect.[200]

Assessment of anxiolytic activity in rats using the Elevated plus maze

The elevated plus maze test has been widely validated to measure anxiety in rodents.[201] The elevated plus maze consists of two open arms and two closed arms with the open arm perpendicular to the closed one. Rats were divided into five groups consisting of 6 rats per groups. Three groups received the extract at a dose of 50, 100 and 200mg/kg body weight. The other two groups received control vehicle 2% v/v tween 80 and standard drug (Diazepam 2 mg/kg, i.p) and 30minutes later, the animals were individually placed at the center of the plus maze and observed for 5 minutes. The number of entries and time in seconds spent by the animals in the open arm were noted and compared with the control groups.

Assessment of antidepressant activity in rats using Forced swim test

Rats were divided into five groups consisting of 6 rats per groups. Three groups received the extract at a dose of 50, 100 and 200mg/kg body weight. The other two groups received control vehicle 2%v/v tween 80 and standard drug imipramine (15 mg/kg, p.o). Forced swim test is commonly used pharmacological model to study antidepressant activity.[202] The apparatus consisted of transparent cylinder (50cmhigh x20cmwide) and water at room temperature was filled to 30 cm depth. In pre test, rats are placed in cylinder for 15 min 24 hr prior to 5min swim test. Extracts and standard dose was administered 30 minutes prior to swim test Duration of immobility was recorded during 5min swimming test, a rat was assigned to be immobile when it floated in an upright position and making little movements to hold its head above water. Increase in active response such as climbing or swimming and reduction in immobility are considered as behavioral profile consistent with antidepressant like action.[203]

Assessment of antidepressant activity in rats using Tail-suspension test

Rats were divided into five groups consisting of 6 rats per group. Three groups received the extract at a dose of 50, 100 and 200mg/kg body weight. The other two groups recieved control vehicle 2%v/v tween 80 and standard drug imipramine (15 mg/kg, p.o). Thirty minutes after the administration of extract and standard dose, rats were subjected to the test. A cord of about 50 cm in length was placed in between two metal tripods at a height of 70 cm, to which the rats were fixed by the tail with sticky tape. After the initial period of rapid motor activity the immobility period was measured when the rats became still, for a total duration of 4 minutes. Rats were regarded as immobile when they hung completely without motion.[204]

Section - 5 Effect of the extract on cognitive paradigms

Effect of the extract on cognition will be assessed by extroceptive and interoceptive behavior models.

4.2.8 Interoceptive behaviour model

Scopolamine induced amnesia model

Interoceptive behavior models include impairment of cognition by scopolamine in rats and subject them to elevated plus maze and passive avoidance paradigm. Cholinesterase level, enzymatic and nonenzymatic antioxidant status in rats subjected to passive avoidance paradigm was measured.

4.2.8.1 Elevated plus maze test

Rats were divided into six groups consisting of 6 animals per groups.

Groups- I Control group received vehicle (2%v/v tween 80).

Groups- II Animals of this group received scopolamine.

Groups- III Animals of this group received extract at a dose of 50 mg/kg.

Groups- IV Animals of this group received extract at a dose of 100 mg/kg.

Groups- V Animals of this group received extract at a dose of 200 mg/kg.

Groups- VI Animals of this group received standard drug piracetam (200 mg/kg, p.o).

All the animals were treated for 14 days and at the end of treatment period all the extract treated animals were administered with scopolamine(1mg/kg i.p)[205] and after 60 minutes of administration of extract, except the first groups which served as vehicle control. The elevated plus maze was described as tool for testing memory by

the investigator working in the field of psychopharmacology. On the 14th day each rat was placed at end of the open arm and they are facing away from the central platform. Transfer latency was time taken by the rats to move in to the covered arm with all its four paws, transfer latency was recorded. If the animals did not enter in to one of the covered arms with in 90seconds, it was gently pushed in to one of the two covered arms and transfer latency was assigned as 90seconds. The rat explored the maze for 10seconds and they are placed back to the home cage. Twenty four hours later the transfer latency was recorded again. The measurement of transfer latency on the day 14 served as parameter for acquisition and those on day 15 served as parameter for retention of memory.

4.2.8.2 Passive avoidance paradigm

Rats were divided into six groups consisting of 6 per groups.

Groups- I Control group received vehicle (2%v/v tween 80).

Groups- II Animals of this group received scopolamine.

Groups- III Animals of this group received extract at a dose of 50 mg/kg.

Groups- IV Animals of this group received extract at a dose of 100 mg/kg.

Groups- V Animals of this group received extract at a dose of 200 mg/kg.

Groups- VI Animals of this group received standard drug piracetam (200 mg/kg, p.o).

At the end of treatment period all the animals were administered with scopolamine (1mg/kg i.p) after 60 minutes of administration, except the first groups which served as vehicle control. Passive avoidance behavior is used to assess the long-term memory.[206] The rats were initially trained and was gently placed on the

wooden platform set in the center of the grid floor. When the rat stepped down and placed all its paws on the grid floor, shocks (50 Hz: 1.5mA) were delivered for 15 seconds and the step-down latency (SDL) was recorded. SDL was defined as the time (in seconds) rat takes to step down from the wooden platform to grid floor with all its paws on the grid floor. Animals showing SDL in the range of 2–15 seconds during the training session were selected for the acquisition and the retention test. The acquisition task was carried out 90 minutes after the training session. The retention was tested after 24 hours in a similar manner, except that the electric shocks were not applied to the grid floor observing an upper cut-off time of 300s. Significant increase in SDL value indicate that there is improvement in memory.[207,208]

The animals were euthanized after the experimental period by cervical decapitation and the brains were isolated to determine the anticholinesterase and antioxidant activity of *Anacyclus pyrethrum*

Biochemical estimations

For preparation of homogenate, the fresh whole brain was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% sodium chloride solution. The homogenate was centrifuged at 3000 rpm for 10 minutes and the resultant cloudy supernatant liquid was used for estimation of cholinesterase and *invivo* antioxidant level.

Estimation of Acetylcholineserase (AchE) enzyme in rat brain

Acetylcholineserase (AChE) enzyme activity was estimated.[209]

Reagent

1. Phosphate buffer: 0.1 M (pH 8) containing 5mM EDTA
2. Dithiobisnitro benzoic acid (DTNB)
3. Acetyl thiocholine iodide : 0.075 M

Procedure

A 0.4 ml aliquot of brain homogenate was taken in a cuvette containing 2.6 ml of 0.1M phosphate buffer (pH 8).100µl of the DTNB reagent was added and the absorbance was measured at 412 nm. 20µl of the acetylthiocholine iodide was added. Changes in absorbance were measured and the change in absorbance per minute was estimated.

The enzyme activity is expressed as milli moles/minute/mgtissue.

Estimation of antioxidant status in rat brain

Estimation of MDA

Malondialdehyde (MDA) is a measure of lipid peroxidation and was measured.[210]

Reagents

1) 1.5 ml Acetic acid (20%) pH 3.5
2) 1.5 ml Thiobarbituric acid (0.8%)
3) 0.2 ml Sodium dodecylsulphate (8.1%)

Procedure

Reagents were added to 0.1 ml of processed tissue samples, then heated at 100 °C for 60 min. Mixture was cooled with tap water and 5 ml of n-butanol pyridine (15:1), 1 ml of distilled water was added and vortexed vigorously. The mixture was centrifuged at 4000 rpm for 10 minutes and the organic layer was separated. The absorbance was measured at 532 nm using a spectrophotometer and concentration of MDA was expressed as nmol/g tissue.

Estimation of catalase

Catalase of brain homogenate was estimated according to the method.[211]

Reagents

1) 50 mM phosphate buffer pH 7.0
2) 30 mM hydrogen peroxide in phosphate buffer.

Procedure

0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). Reaction was started after the addition of 1.0ml of freshly prepared 30mM H_2O_2. The decomposition rate of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240nm. Activity of catalase was expressed as units/mg protein.

Assay of super oxide dismutase (SOD)

Reagents

1) 75mM of Tris-HCl buffer (pH 8.2),

2) 30 mM EDTA

3) 2mM pyrogallol

Procedure

To 50 µl of the suspension, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 minutes by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance per minutes at 420 nm. The activity of SOD is expressed as units/mg protein.[212]

Assay of glutathione peroxidase (GPx)

Glutathione peroxidase was assayed by the method.[213]

Reagents:

1. 0.32 M phosphate buffer, (pH 7.0)
2. 0.8 mM EDTA
3. 10 mM sodium azide
4. 3 mM Reduced glutathione
5. 2.5 mM H_2O_2
6. 10% TCA

7. 0.3 M Disodium hydrogen phosphate
8. DTNB solution (40 mg of DTNB in 100 ml of 1% sodium citrate)
9. Reduced glutathione

Procedure

The reaction mixture consisting of 0.2 ml each of EDTA, sodium azide and H_2O_2, 0.4 ml of phosphate buffer, 0.1 ml of suitably diluted tissue was incubated at 37°C at different time intervals and 0.5 ml of TCA was added and the tubes were centrifuged at 2000 rpm. To 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml DTNB were added and the color developed was read at 420 nm immediately.

The activity of GPx is expressed as µmoles of glutathione oxidized / minutes / mg protein.

Assay of glutathione reductase (GRD)

Glutathione reductase was assayed by the method.[214]

Reagents

1. 0.3 M phosphate buffer, (pH 6.8)
2. 0.25 M EDTA
3. 12.5 mM Oxidized glutathione
4. 3 mM NADPH

Procedure

The reaction mixture containing 1 ml of phosphate buffer, 0.5 ml of EDTA, 0.5 ml of oxidized glutathione and 0.2 ml of NADPH was made up to 3 ml with water. After the addition of 0.1 ml of suitably diluted tissue, the change in optical density at 340 nm was monitored for 2 minutes at 30 sec intervals.

The activity of GRD is expressed as n moles of NADPH oxidized / minute / mg protein.

Estimation of reduced glutathione (GSH)

Reagents

1. 0.3 M phosphate buffer, (pH 6.8)
2. 0.25 M EDTA
3. 12.5 mM Oxidized glutathione
4. 3 mM NADPH

Glutathione was measured according to the method.[215]The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5-dithiobis (2-nitrobenzoic acid), and 0.4 ml of distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The concentration of glutathione was expressed as μg/gtissue.

4.2.9 Exteroceptive behaviour model

In extroceptive behaviour model rats were subjected to social recognition task and elevated plus maze paradigms. Neurotransmitters such as dopamine, serotonin and glutamate were determined in rats subjected to elevate plus maze paradigms.

4.2.9.1 Social recognition task

Rats were divided into five groups consisting of 6 animals per groups. Groups received control vehicle 2%v/v tween 80, extracts at a dose of 50, 100, 200mg/kg and standard drug piracetam (200 mg/kg, p.o). Male Wistar rats (350-450g) were used for the experiments and juvenile males (90-110g) were used as social stimuli experimental model. The first day of the experiment, a juvenile rat was introduced in to the adult males cage and the time spent in social investigatory behavior by the adult male within a 5 minute fixed interval was recorded and after 24 h, either the same juvenile or an unfamiliar one was placed again in to the mature males cage and social investigatory behavior was recorded in a 5 minutes interval.[216]

4.2.9.2 Elevated plus maze

Rats were divided into five groups consisting of 6 rats per group.

Groups- I Control group received vehicle (2%v/v tween 80).

Groups- II Animals of this group received extract at a dose of 50 mg/kg.

Groups- III Animals of this group received extract at a dose of 100 mg/kg.

Groups- IV Animals of this group received extract at a dose of 200 mg/kg.

Groups- V Animals of this group received standard drug piracetam (200 mg/kg, p.o).

The elevated plus maze considered is the exteroceptive behavioural model to evaluate learning and memory. The apparatus consisting of two open arms (50cms × 10cms) and two covered arms (50cms × 10cms × 40cms) extended from a central platform (10cms × 10cms) was elevated to a height of 50cms from the floor. On the first training day, each animal was placed at the end of an open arm facing away from the central platform. Transfer latency (TL) was taken as the time taken by the rat to move into any one of the covered arms with all its four legs. TL was recorded on the training day. If the animal did not enter into the one of the arm within 90seconds, it was gently pushed into one of the covered arms and the TL was assigned as 90seconds. The animals was allowed to explore to the maze for 10 seconds and then returned to its home cage. Transfer latency was examined on 14th day and after 24hours on 15th day of drug treatment. Significant reduction in transfer latency value indicates improvement in memory.[217]The animals were euthanized by cervical decapitation and the brains were isolated to determine the levels of dopamine, serotonin and glutamate levels in rat brain homogenate.

Estimation of neurotransmitters

Estimation of Dopamine level [218,219]

Reagents-

1. HCl Butanol
2. Heptane
3. 0.4 M HCl
4. EDTA / Sodium acetate buffer, pH 6.9
5. 5 M Sodium hydroxide

6. 0.1 M Iodine

7. Sodium thiosulphate solution

8. 10 M acetic acid

9. Dopamine standard

Preparation of tissue extract

Weighed a specific quantity of tissue and was homogenized in 3 ml HCl butanol in a cool environment. The sample was then centrifuged for 10 minutes at 2000 rpm. 0.8 ml of supernatant phase was removed and added to an eppendorf reagent tube containing 2 ml of heptane and 0.25 ml 0.1 M HCl. After 10 minutes, shake the tube and centrifuged under same conditions to separate two phases. Upper organic phase was discarded and the aqueous phase was used for dopamine assay.

Dopamine assay

To 0.02ml of the HCl phase, 0.005 ml of 0.4 M HCl and 0.01ml EDTA/ Sodium acetate buffer (pH 6.9) were added, followed by 0.01 ml iodine solution for oxidation. The reaction was stopped after 2 min by the addition of 0.1ml sodium thiosulphate in 5 M sodium hydroxide. 10 M acetic acid was added 1.5 min later. The solution was then heated to 100°C for 6 min. When the sample again reaches room temperature, excitation and emission spectra were read (330 to 375 nm) in a spectrofluorimeter. Compared the tissue values (fluorescence of tissue extract minus fluorescence of tissue blank) with an internal reagent standard (fluorescence of internal reagent standard minus fluorescence of internal reagent blank). Tissue blanks for the assay were prepared by adding the reagents of the oxidation step in reversed order (sodium thiosulphate before iodine). Internal reagent standards were obtained

by adding 0.005 ml distilled water and 0.1ml HCl butanol to 20 ng of dopamine standard.

Estimation of Serotonin Assay

The serotonin content was estimated by the method.[219]

Reagents

1. HCl-n butanol (0.85 ml of 37% HCl in 1 liter of n-butanol).
2. Heptane
3. 0.1M HCL
4. O-phthaldialdehyde

Procedure

The supernatant phase of sample after centrifugation for 10 minutes at 2000 rpm was removed and added to Eppendorf reagent tubes containing 0.2ml of heptane and 0.025ml of HCl 0.1M. After 10 minutes of vigorous shaking the tube was centrifuged under the same conditions as above in order to separate the two phases. The aqueous phase (0.025ml) was taken and 0.025 ml of o-phthaldialdehyde was added. The fluorophore was developed by heating to $100^{o}C$ for 10minutes. After the sample reach the equilibrium with ambient temperature the intensity readings at 360-470nm was taken in microcuvette.

Estimation of Glutamate

The level of Glutamate was estimated by multiple development paper chromatography.[220]

Reagents

1.	N-butanol: acetic acid: water	:	12: 3: 5
2.	Ninhydrin reagent	:	0.25%
3.	Copper sulphate solution	:	0.005%
4.	Standard glutamate	:	2.942 mg of glutamate in 10 ml distilled water.

Procedure

1.0 ml of the supernatant from brain homogenate was evaporated to dryness at 70°C in an oven and the residue is reconstituted in 100 ml of distilled water. Standard solutions of glutamate at a concentration of 2mM along with the sample are spotted on Whatman No. 1 chromatography paper using a micropipette. It was placed on a chamber containing butanol: acetic acid: water (12: 3: 5 v/v) as solvent. When the solvent front reached the top of the paper, it was removed and dried. A second run is performed similarly, after which the papers are dried sprayed with ninhydrin reagent and placed in an oven at 100°C for 4 minutes. The portions which carry glutamate corresponding with the standard are cut and eluted with 0.005% $CuSo_4$ in 75% ethanol. Their absorbance is read against blank at 515 nm in spectrophotometer.

Section - 6 *Invitro* antioxidant studies of the extract

The antioxidant potential of extract was assessed *by* using *invitro* and *exvivo* antioxidant experimental methods to study its beneficial role to protect reactive oxygen species.

4.2.10 *Invitro* antioxidant studies

Determination of DPPH radical-scavenging activity

The free radical-scavenging activity of ethanolic extract of *Anacyclus pyrethrum* was measured in terms of hydrogen donating or radical-scavenging ability using the stable radical DPPH.[221] 0.1 mM solution of DPPH in ethanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in ethanol at different concentrations (25-400µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. Ascorbic acid was used as the reference compound. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample. All the tests were performed in triplicate and the graph was plotted with the mean values.

Determination of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/ ascorbate / EDTA / H_2O_2 system. The reaction mixture contained deoxyribose (2.8mM), $Fecl_3$ (0.1mM), EDTA (0.1mM), H_2O_2 (1mM), ascorbate (0.1mM), KH_2PO_4-KOH buffer (20mM), pH 7.4 and various concentrations (25-

400μg/ml) in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37°C. Deoxyribose degradation was measured at 532 nm.[222]

Determination of hydrogen peroxide scavenging activity

Hydrogen peroxide solution (2mM) was prepared with standard phosphate buffer (pH, 7.4). Extract samples (25-400μg/ml) added to hydrogen peroxide solution (0.6ml). Absorbance of hydrogen peroxide at 230nm was determined after 10min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage scavenging of hydrogen peroxide of plant extract was determined.[223]

Determination of reducing power

Extract samples (25-400μg/ml) in 1ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K_3 $Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50ºC for 20 min. A portion (2.5 ml) of trichloroacetic acid (15%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.[224]

Determination of nitric oxide scavenging activity

Sodium nitroprusside (5mM) in phosphate buffered saline was mixed with different concentrations of the extract. Extract samples (25-400μg/ml) dissolved in ethanol and incubated at 25ºC for 30 min. A control without test compound but with equivalent amount of ethanol was taken. After 30 min 1.5 ml of the incubation

solution were removed and diluted with 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% napthyl ethylene diamine dihyrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine was measured at 546 nm.[225]

4.2.11 *Ex vivo* studies

Lipid peroxidation assay method

Inhibition of lipid peroxide formation induced by Fe^{2+}-ascorbate system in rat brain homogenate was estimated by TBA reaction method. Reaction mixture (0.5ml) containing 25% brain homogenate (0.1 ml) w/v in Tris-HCl buffer (40 mM, pH 7.0), potassium chloride (30 mM), ferrous ion (0.16 mM) and ascorbic acid (0.06 mM) was incubated for 1hr at 37ºC in the presence and absence different concentrations of the extract (25-400μg/ml) and vitamin E (100 μg/ml). For this 0.4 ml of the reaction mixture was treated with sodium dodecyl sulphate (SDS-0.2 ml, 8.1%), thiobarbituric acid (TBA-1.5 ml, 0.8%) and acetic acid (1.5 ml, 2.5% of pH 3.5). The mixture (4 ml) was taken kept in a water bath at 95ºC for 1 hr. After cooling 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and shaken vigorously. After centrifugation, the chromophore was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the result of control and test compounds.[226]

Statistical analysis

The data are expressed as mean ± SEM. Statistical analysis was done using one way analysis of variance (ANOVA) followed by dunnet test.[227]

VI. RESULTS

5.1 PRELIMINARY PHYTOCHEMICAL TESTS

Table - 4 shows the preliminary phytochemical analysis of hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum.* Phytochemical analysis of hexane revealed the presence of phytoconstituents such as carbohydrate, sterols, tannins, phenols, terpenes. Phytochemical analysis of chloroform extract revealed the presence of phytoconstituents such as alkaloids, tannins, terpenes. Phytochemical analysis of ethanolic extract revealed the presence of phytoconstituents such as alkaloids, carbohydrate, tannins, phenols, flavanoids, glycoside and saponins.

5.2 *INVITRO* ANTICHOLINESTERASE ACTIVITY

5.2.1 *Invitro* anticholinesterase activity by the spectrophotometric method

The hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were tested for their *invitro* acetylcholinesterase inhibitory effect at 62.5, 125, 250, 500, 1000 and 2000 µg/ml concentrations. Inhibitory activity on acetylcholinesterase for the hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were evaluated and percentage inhibition was calculated. Hexane extract of *Anacyclus pyrethrum* has not shown any acetyl cholinesterase inhibitory effect and only exhibited mild inhibition at higher concentration. Chloroform extract of *Anacyclus pyrethrum* showed mild acetyl cholinesterase inhibitory effect with increase in concentration of the extract and the IC_{50} value was found to be 627 ± 3.68 µg/ml. Ethanolic extract of *Anacyclus pyrethrum* showed better acetyl cholinesterase inhibitory effect with increase in concentration when compared to the other two extracts tested. The IC_{50}

value of ethanolic extract of *Anacyclus pyrethrum* was found to be 83 ± 1.52 μg/ml and IC_{50} value for rivastigmine was found to be 350±5.95 μM. Results are shown in table-5,6,7,8 and fig -4,5.

5.2.2 Thin layer chromatography (TLC) with bioassay detection for AChE inhibition

TLC bioassay is an easier and rapid means for detection of enzyme inhibition. The hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* were tested for their acetyl cholinesterase inhibition by TLC bioassay detection. The active spots appeared as white spots on yellow background. Hexane extract did not show any white spot on yellow background, but chloroform extract showed one active spot. Ethanolic extract of *Anacyclus pyrethrum* showed more than one active spots compared to other two tested extracts. Ethanolic extract of *Anacyclus pyrethrum* showed better acetylcholinesterase inhibition compared to other extracts. The TLC assay demonstrated AchE inhibitory activity for ethanolic extract of *Anacyclus pyrethrum*. Results are shown in fig-6.

5.3 Identification of phytoconstituents of extract.

5.3.1 TLC and HPTLC

The TLC photo of ethanolic extract of *Anacyclus pyrethrum* is shown in fig-7. TLC of ethanolic extract of *Anacyclus pyrethrum* showed 6 spots with toluene: ethylacetate solvent system and R_f values of various spots were observed at 254 nm and are tablulated in table-9. Fig-8 shows the CAMAG HPTLC densitometric scanning peaks and their R_f values at 254 nm.

5.3.2 Determination of total phenolic, total flavonoid, total alkaloid and total ascorbic acid content in ethanolic extract of *Anacyclus pyrethrum.*

Total phenolic content detected in ethanolic extract of *Anacyclus pyrethrum* was found to be 159.63 ± 0.52 mg gallic acid equivalents/g plant extract, total flavonoid content was found to be 29.65 ± 0.83 mg quercetin equivalents /g plant extract, total alkaloid content was found to be 35.14±0.57 mg/g and total ascorbic acid content detected was found to be 0.22 ± 0.07mg ascorbic acid /g plant extract.

5.4 Toxicological investigation of the extract.

5.4.1 Acute toxicity study

Acute toxicity study revealed non-toxic nature of ethanolic extract of *Anacyclus pyrethrum* in rats. The extract showed mild changes in behavioral pattern at higher dose level of 2000mg/kg body weight in rats. The extract did not produce any toxic symptoms or mortality up to dose level of 2000mg/kg body weight in rats and hence the drugs were considered safe for further pharmacological screening.

5.4.2 Sub chronic toxicity

In the Sub chronic toxicity studies, *Anacyclus pyrethrum* extracts at dose of 1000mg/kg, given orally for 90days did not produce any mortality in rats. No signs of toxicity were observed during the experimental period. Changes in general behavior or other physiological abnormalities were not observed at any point in the present study.

Body weight

Body weight changes were monitored at weekly intervals till 90days.Change in body weights in treated, control female and male groups during the 90-day sub chronic toxicity studies are summarized in fig-9 and fig-10.Percentage increase in weight of treated and control groups was found to be uniform. The extract has not shown any reduction in the body weight, which is an evidence for absence of toxicity.

Relative organ weight

Results of relative organ weight of rats treated with *Anacyclus pyrethrum* have not shown any evidence of drug-related toxicity. There were no significant difference between control and *Anacyclus pyrethrum* extract treated groups in organ weight. Results are shown in table-10

Haematological parameters

The effect of *Anacyclus pyrethrum* extracts administration on haematological parameters of experimental and control rats are presented in table-11. The result indicates that all hematological parameters such as total red blood cell count, total white blood cell count, hemoglobin, hematocrit, platelet count and differential leukocyte count remained within the physiological range in both control and treated groups during the experimental period.

Biochemical parameters

The data of biochemical parameters in treated and control rats are presented in table-12. Sub chronic oral administration of *Anacyclus pyrethrum* extract did not

show any significant changes in biochemical parameters such as creatinine, urea, triglycerides, total cholesterol, total protein, albumin, AST, ALT, ALP and total bilirubin in serum when compared to control groups. There were no statistically significant difference in the hematological and serum biochemical parameters analyzed and are found to be within normal limits.

Histological examination

The histological examination of the various organs was performed in both control and treated groups. All the sampling tissue sections were within the normal limits and revealed normal architecture on comparison with control groups. No alterations were seen in the microscopic examination of internal organ and there were no degenerative or infiltrative lesion observed in the extract treated groups. Histopathology of treated groups revealed following microscopic observation. Liver section shows normal architecture of hepatocytes, mild sinusoidal dilation and normal vessels. Section of spleen shows normal white pulp, red pulp, few congested blood vessels and scattered haemosideris. Histopathology of heart section of treated group show normal cells. Kidney section shows normal glomeruli, tubules, blood vessels and intestitium. Lungs section shows normal alveoli and septae. The bronchi and bronchioles show normal lining with peribronchial collections and lymphocytes. Brain section shows normal cerebellum, focal areas show normal choroid plexus. Pathological examination of tissues indicated that there were no detectable abnormalities as shown in fig-11 and fig-12.

5.5 NEUROPHARMACOLOGICAL INVESTIGATION OF THE EXTRACT

5.5.1 General behaviour studies

Rats treated with ethanolic extract of *Anacyclus pyrethrum* and were submitted to general behavioural profile studies did not show any difference in their behaviour. They were alert, with normal grooming, touch response, sound response, pain response, motor activity and grip strength were normal. The animals showed no signs of depression during the observation period. However, the standard drug diazepam caused a significant depression of all these responses compared with the ethanolic extract of *Anacyclus pyrethrum*. The results are shown in table-13.

5.5.2 Locomotor activity

The ethanolic extract of *Anacyclus pyrethrum* in a dose level of 50mg/kg, 100mg/kg and 200mg/kg, p.o did not produce statistically any significant reduction in locomotor activity as compared to the control animals receiving only the vehicle. Diazepam treated groups revealed a statistically significant ($p < 0.01$) decrease in locomotor activity as compared to the control. Results are shown in table-14 and fig-13.

5.5.3 Effect on motor coordination

There was no statistically significant decrease in the time of falls within 3 minutes after the treatment with ethanolic extract of Anacyclus *pyrethrum* at dose level of 50mg/kg, 100mg/kg and 200mg/kg, p.o and result reveals that *Anacyclus pyrethrum* has no muscle relaxant property. However diazepam treated groups

showed statistically significant ($p < 0.01$) decrease in the time of falls as compared to the control. Results are shown in table-15 and fig-14.

5.5.4 Assessment of anxiolytic activity in rats using the holeboard apparatus

The statistical analysis of data obtained indicated that the groups treated with ethanolic extract of *Anacyclus Pyrethrum* at dose level of 50mg/kg, 100mg/kg and 200mg/kg, p.o did not reveal significant increase in number of head pokings as compared to the control groups. However diazepam treated groups showed significant ($p < 0.01$) increase in exploratory activity as compared to control groups. Results are shown in table-16 and fig-15.

Effect of ethanolic extract of *Anacyclus Pyrethrum* and diazepam on anxiety using the Elevated plus maze

The results obtained indicate that the groups treated with ethanolic extract of *Anacyclus Pyrethrum* at dose level of 50mg/kg, 100mg/kg and 200mg/kg, p.o did not produce significant increase in number of entries in open arm compared to the control groups. However diazepam treated groups showed statistically significant increase ($p < 0.01$) in entries in open arm. Results are shown in table-17 and fig-16.

5.5.5 Assessment of antidepressant activity in rats using Forced swim test

The effect of ethanolic extract of *Anacyclus pyrethrum* at dose level of 50mg/kg, 100mg/kg, 200mg/kg, p.o and impiramine on active behaviours in forced swim test of rats is shown in table-18. Ethanolic extract significantly ($p < 0.01$) shortened the immobility time in dose dependent manner in comparison to the control

values. However impiramine treated groups showed significant reduction ($p < 0.01$) in immobility time as compared to the control. Results are shown in fig-17.

Assessment of antidepressant activity in rats using Tail-suspension test

The effect of extract at dose level of 50mg/kg, 100mg/kg and 200mg/kg, p.o and impiramine on assessment of antidepressant activity in rats using tail-suspension test are shown in table-19. Ethanolic extract significantly ($p < 0.01$) reduced the period of immobility time in dose dependent manner in comparison to the control values. However impiramine treated groups showed significant reduction in immobility time as compared to the control. Results are shown in fig-18.

5.5.6 Effect of the extract on cognitive paradigms

5.5.6.1 Interoceptive behaviour model

Scopolamine induced amnesia models

Elevated plus maze

The effect of vehicle, scopolamine, control, *Anacyclus pyrethrum* at dose level of 50mg/kg, 100mg/kg, and 200mg/kg and piracetam were evaluated at end of day 14. Transfer latency on 14th day of drug treatment reflected learning behavior of animals, where as transfer latency of next day reflected retention of information or memory. Scopolamine hydro bromide (1mg/kg i.p) groups showed a significant ($p < 0.01$) increase in transfer latency values on acquisition as wells as on the retention days as compared with vehicle control rats, indicating impairment in learning and memory. The *Anacyclus pyrethrum* at dose level of 50, 100 and 200mg/kg orally demonstrated significant ($p < 0.001$) decrease in transfer latency in a dose dependent manner on transfer latency on 14th day and 15th day in elevated plus maze test as compared to

scopolamine control and successfully reversed memory deficit induced by scopolamine. Piracetam used as positive control at a dose of 200mg/kg also improved learning and memory in rats and reversed the amnesia induced by scopolamine. The results obtained was statistically significant (p<0.01) and results are shown in table-20 and fig -19.

Passive avoidance paradigm

Step down latency of the second day, 15th day of drug treatment reflected long term memory of animals. Scopolamine (1mg/kg i.p) decreased step down latency in acquisition and retention test indicating impairment in memory. *Anacyclus pyrethrum* at a dose of 50 mg/kg, 100mg/kg and 200mg/kg of the extract orally administered for 14 days significantly (p<0.01) increased step down latency in dose dependent manner and reversed scopolamine induced amnesia. The groups of rats which were treated with piracetam (200mg/kg p.o) for 14 days showed statistically significant improvement (p<0.01) in memory and reversed amnesia induced by scopolamine. Results are shown in table-21 and fig-20.

Estimation of the cholinesterase level in the brain homogenate of scopolamine induced amnesia in rats

Effect on brain cholinesterase activity

Ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg ,100mg/kg and 200mg/kg p.o significantly (p<0.01) reduced the levels of cholinesterase as compared to scopolamine treated groups by Ellman's kinetic calorimetric method, which is considered as indicator of inhibition of cholinesterase activity in rat brain after 14

days of treatment. Piracetam at dose of 200mg/kg p.o significantly ($p<0.01$) reduced the levels of cholinesterase and results indicated in table-22 and fig -21.

Estimation of the antioxidant enzyme level in the brain homogenate of scopolamine induced amnesia in rats

Effect of ethanolic extract of *Anacyclus pyrethrum on* MDA level in the brain

Scopolamine treatment significantly ($p <0.01$) increased the brain MDA level compared to control groups. Standard drug Piracetam and ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg, 100mg/kg and 200mg/kg p.o treatment, ($p < 0.01$) significantly decreased brain lipidperoxide level in dose dependent manner when compared to their corresponding scopolamine treated groups, respectively. Results are shown in table-23 and fig-22.

Effect of ethanolic extract of *Anacyclus pyrethrum on* catalase level in the brain

Scopolamine treatment significantly ($p < 0.01$) decreased brain catalase level as compared to control groups. Ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg, 100mg/kg and 200mg/kg p.o treatment increased the catalase level significantly ($p < 0.01$) in dose dependent manner when compared to the corresponding scopolamine treated groups respectively. Piracetam treatment also significantly ($p < 0.01$), increased the catalase level compared to the corresponding scopolamine treated groups respectively. Results are shown in table-24 and fig-23.

Effect of ethanolic extract of *Anacyclus pyrethrum on* super oxide dismutase level in brain

Scopolamine treatment significantly ($p<0.01$) decreased brain super oxide dismutase level as compared to control groups. Treatment with standard drug piracetam and ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg, 100mg/kg and 200mg/kg p.o respectively increased the super oxide dismutase level significantly ($p<0.01$) in dose dependent manner when compared to the corresponding scopolamine treated groups. Results are shown in table-25 and fig-24.

Effect of ethanolic extract of *Anacyclus pyrethrum on* glutathione peroxidase (GPx) level in brain

Piracetam and ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg, 100mg/kg and 200mg/kg p.o treatment did not show significant change in the glutathione peroxidase level when compared to the corresponding scopolamine treated groups. Results are shown in table-26 and fig-25.

Effect of ethanolic extract of *Anacyclus pyrethrum on* glutathione reductase (GRD) level in brain

Scopolamine treatment significantly ($p<0.01$) decreased brain glutathione reductase level as compared to control groups. Ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg, 100mg/kg and 200mg/kg p.o treatment increased the glutathione reductase level significantly ($p < 0.01$) in dose dependent manner when compared to the corresponding scopolamine treated groups respectively. Piracetam treatment also increased the glutathione reductase level significantly ($p < 0.01$) when

compared to the corresponding scopolamine treated groups. Results are shown in table-27 and fig-26.

Effect of ethanolic extract of *Anacyclus pyrethrum on* reduced glutathione level (GSH) in brain

Scopolamine treatment significantly ($p<0.01$) decreased brain reduced glutathione level as compared to control groups. Piracetam and ethanolic extract of *Anacyclus pyrethrum* at dose of 50mg/kg, 100mg/kg and 200mg/kg p.o treatment increased the reduced glutathione level significantly ($p < 0.01$) in dose dependent manner when compared to the corresponding scopolamine treated groups respectively. Results are shown in table-28 and fig-27.

5.5.6.2 Exteroceptive behaviour model

Social learning test

In the social learning test, results demonstrate that extract of *Anacyclus pyrethrum* significantly ($p < 0.01$) decreases the investigation time of the same juvenile rat in the forgetting procedure, indicating that the extract enhances short-term social memory in rats and showed statistically significant ($p < 0.01$) reduction in duration of exploration of the familiar partner in the second session of the test in dose dependent manner when compared to control group. Similar results were produced in rats treated with standard drug piracetam. The facilitation of social memory, demonstrates that the extract displays memory-enhancing properties even when administered orally. Results are shown in table-29 and fig-28.

Elevated plus maze

Transfer latency reflected retention of learned task or memory. *Anacyclus pyrethrum* treated animals at a dose of 50mg/kg, 100mg/kg and 200mg/kg, p.o showed dose dependant significant decrease ($p < 0.01$) in transfer latency on 14th day and 15th day when compared to the control groups. Higher dose of *Anacyclus pyrethrum* at 200mg/kg p.o significantly enhanced learning and memory when subjected to elevated plus maze test. Piracetam 200mg/kg p.o treated for 14days decreased the transfer latency on 14th day and after 24 hrs on 15th day as compared to the control groups, indicating improvement in learning and memory. Results are shown in table-30 and fig-29.

Estimation of neurotransmitters

Effect of ethanolic extract of *Anacyclus Pyrethrum* on dopamine levels

Treatment with piracetam and ethanolic extract of *Anacyclus Pyrethrum* at a dose level of 50,100 and 200 mg/ kg, p.o. for 14 days significantly ($p < 0.01$) decreased brain dopamine level in dose dependent manner when compared to their corresponding control treated groups respectively. Results are shown in table-31 and fig -30.

Effect of ethanolic extract of *Anacyclus Pyrethrum* on serotonin levels

Ethanolic extract of *Anacyclus Pyrethrum* at a dose level of 50,100 and 200 mg/ kg, p.o treatment for 14 days significantly ($p < 0.01$) increased brain serotonin level in dose dependent manner in comparison to their corresponding control treated groups. Piracetam also significantly ($p < 0.01$) increased brain serotonin level when

compared to their corresponding control treated groups respectively. Results are shown in table-32 and fig -31.

Effect of ethanolic extract of *Anacyclus Pyrethrum* on glutamate levels

Standard drug piracetam and ethanolic extract of *Anacyclus Pyrethrum* at a dose level of 50,100 and 200 mg/ kg, p.o treatment for 14 days did not reveal any significant change in glutamate levels when compared to their corresponding control treated groups, respectively. Results are shown in table-33 and fig -32.

5.7 *INVITRO* ANTIOXIDANT STUDIES

DPPH Scavenging Reduction

Ethanolic extract of *Anacyclus pyrethrum* exhibited a significant dose dependent inhibition of *DPPH* activity.The IC_{50} value of ethanolic extract of *Anacyclus pyrethrum* and ascorbic acid was found to be 55.83± 1.92µg/ml and 10.38±0.52 µg/ml respectively. Results are shown in table-34 and fig-33

Hydroxyl Radical Scavenging Activity

The scavenging ability of ethanolic extract of *Anacyclus pyrethrum* on hydroxyl radical is shown in table-35 and fig-34. Ethanolic extract of *Anacyclus pyrethrum* was capable of scavenging hydroxyl radical in a dose dependent manner. It exhibited a scavenging activity of 37.80, 48.95, 57.96, 66.81 and 93.20% on hydroxyl radical at dose of 25µg, 50µg, 100 µg, 200 µg and 400 µg/ml respectively.

Hydrogen peroxide scavenging activity

The percentage scavenging activity by ethanolic extract of *Anacyclus pyrethrum* increased in a dose dependent manner. IC_{50} value of ethanolic extract of *Anacyclus pyrethrum* was found to be 38.54± 0.94μg/ml. Hydrogen peroxide scavenging activity was found to increase from 25μg/ml up to 400μg/ml(32.92± 0.83 to 91.27± 0.68 %). Results are shown in table-36 and fig-35.

Reducing power

Increase in absorbance of the extract at 700nm indicates the reducing power of the test sample. Reducing power of ethanolic extract of *Anacyclus pyrethrum* increased with increased concentration of test compound. Results are shown in table-37 and fig-36.

Nitric oxide scavenging activity

The scavenging of nitric oxide by the ethanolic extract of *Anacyclus pyrethrum* was found to be concentration dependent and the IC_{50} value was found to be 32.61±1.68μg/ml. Ethanolic extract of *Anacyclus pyrethrum* was capable of scavenging nitric oxide radical in a dose dependent manner. It exhibited a scavenging activity of 46.81, 55.23, 61.87, 70.34, 82.50 % on nitric oxide radical at dose of 25,50,100, 200, 400 μg/ml. Results are shown in table-38 and fig-37.

5.8 *Ex vivo* antioxidant studies

Lipid peroxidation assay method

The ethanolic extract of *Anacyclus pyrethrum* was effective in inhibiting Fe^{2+} / ascorbate system induced lipid peroxidation in rat brain homogenate in a dose dependent manner. The malondialdehyde generated as result of lipid peroxidation reacts with thiobarbituric acid and was found to be inhibited in presence of the extract. It exhibited inhibition of 28.17, 36.27, 45.75, 63.04, 73.95% at dose of 25, 50, 100, 200, 400 μg respectively. Vitamin-E (100 μg) exhibited 69.23% inhibition of lipid peroxide formation. Results are shown in table-39and fig-38.

Table - 4 Preliminary phytochemical screening

S.No.	Constituents	Hexane extract	Chloroform extract	Ethanolic extract
1.	Alkaloids	-ve	+ve	+ve
2.	Carbohydrates	+ve	-ve	+ve
3.	Steroids	-ve	-ve	-ve
4.	Sterols	+ve	-ve	-ve
5.	Protein	-ve	-ve	-ve
6.	Tannins	+ve	+ve	+ve
7.	Phenols	+ve	-ve	+ve
8.	Flavonoids	-ve	-ve	+ve
9.	Gums and Mucilage	-ve	-ve	-ve
10.	Glycosides	-ve	-ve	+ve
11.	Saponins	-ve	-ve	+ve
12.	Terpenes	+ve	+ve	-ve

-ve-indicate the absence of compound

+ve-indicate the presence of compound

Table - 5 *In vitro* anticholinesterase activity of hexane extract of *Anacyclus pyrethrum*

S.No.	Hexane extract (µg/ml)	% acetylcholinesterase inhibition	IC_{50} Value((µg/ml)
1.	62.5	0	--
2.	125	0	
3.	250	0	
4.	500	0	
5.	1000	7.25 ± 1. 02	
6.	2000	9.5 ± 2. 32	

NI-Non inhibition

Values are Mean ± SEM of 3 replicates.

Table - 6 *In vitro* anticholinesterase activity of chloroform extract of *Anacyclus pyrethrum*

S.No.	Chloroform extract (µg/ml)	% acetylcholinesterase inhibition	IC_{50} Value (µg/ml)
1.	62.5	10 ± 1.43	627± 3.68
2.	125	15 ± 1.27	
3.	250	23 ± 1.31	
4.	500	47 ± 0. 98	
5.	1000	55 ± 1.21	
6.	2000	57 ± 1. 83	

Values are Mean ± SEM of 3 replicates.

Table - 7 *In vitro* anticholinesterase activity of ethanolic extract of *Anacyclus pyrethrum*

S. No.	Ethanolic extract (µg/ml)	% acetylcholinesterase inhibition	IC_{50} Value (µg/ml)
1	62.5	47± 1.42	83 ± 1.52
2.	125	55± 2.35	
3.	250	69± 1.74	
4.	500	74± 1.66	
5.	1000	81± 1.35	
6	2000	84 ± 2. 27	

Values are Mean ± SEM of 3 replicates.

Fig - 4 *In vitro* anti cholinesterase activity of ethanolic extract of *Anacyclus pyrethrum*

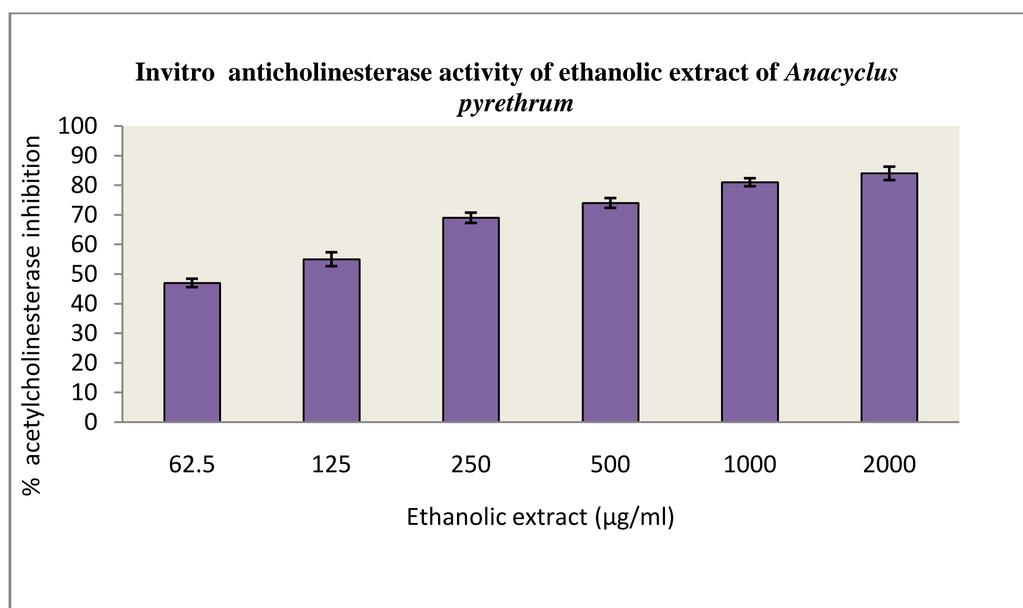


Table - 8 *In vitro* anticholinesterase activity of rivastigmine

S. No.	Rivastigmine (µM)	% acetylcholinesterase inhibition	IC_{50} Value(µM)
1.	62.5	15± 1.23	350±5.95
2.	125	32± 1.71	
3.	250	39± 0.97	
4.	500	73 ± 1.34	
5.	1000	75 ± 1.07	
6.	2000	80 ± 0. 68	

Values are Mean ± SEM of 3 replicates.

Fig - 5 *In vitro* anti cholinesterase activity of rivastigmine

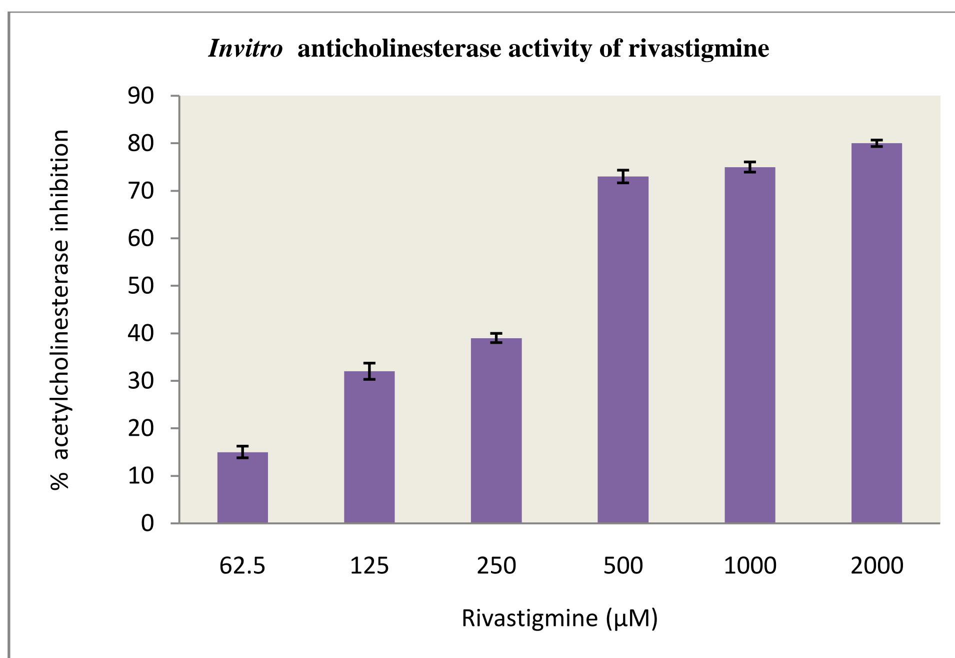


Fig - 6 Thin layer chromatography (TLC) with bioassay detection for AChE inhibition

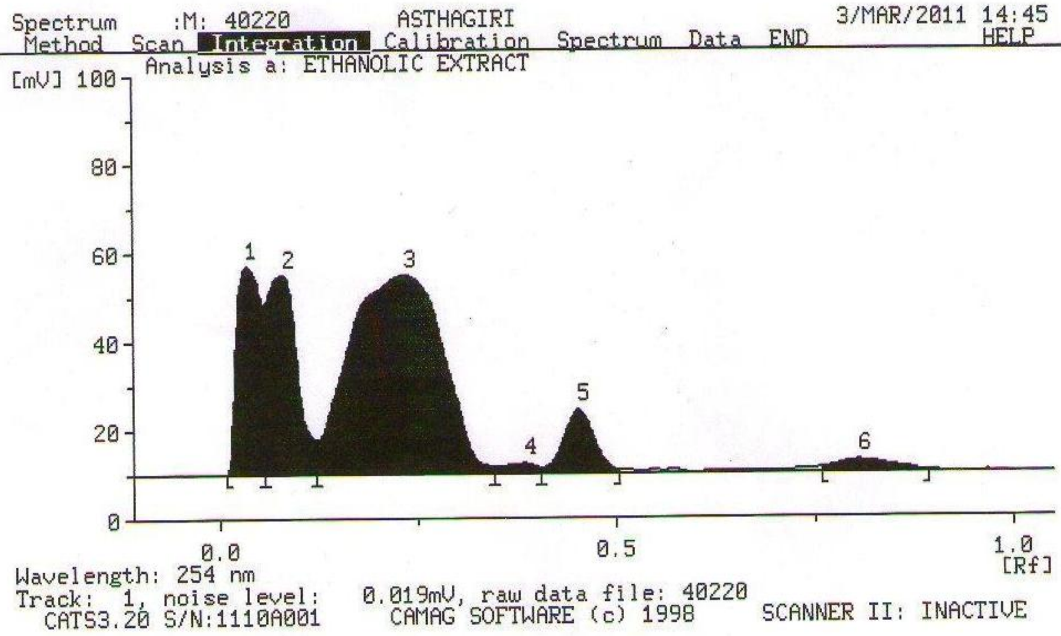
Fig - 7 Thin Layer Chromatography of ethanolic extract of *Anacyclus pyrethrum*

Solvent system: Toluene: Ethylacetate (97:3v/v)

Table – 9 Thin Layer Chromatography-Rf values of ethanolic extract of *Anacyclus pyrethrum*

Extract	Solvent system	Number of spots		Rf value
Ethanolic extract	Toluene: Ethylacetate (97:3)	6	1	0.03
			2	0.07
			3	0.23
			4	0.38
			5	0.45
			6	0.81

Fig - 8 HPTLC Chromatogram of ethanolic extract of *Anacyclus pyrethrum*



Track 1, Analysis a: ETHANOLIC EXTRACT

Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.00	0.1	0.03	46.9	29.93	0.05	38.3	1132.0	15.18
2	0.05	38.3	0.07	45.2	28.83	0.12	7.9	1390.9	18.65
3	0.12	7.9	0.23	45.1	28.75	0.34	1.7	4240.1	56.86
4	0.34	1.7	0.38	2.3	1.48	0.40	1.4	79.9	1.07
5	0.40	1.4	0.45	14.5	9.24	0.50	0.8	452.6	6.07
6	0.76	1.1	0.81	2.8	1.77	0.89	0.3	161.3	2.16
		Total height =		156.7			total area =	7456.7	

Sub chronic toxicity

Fig - 9 Body weight in treated and control female groups during the 90 days safety assessment

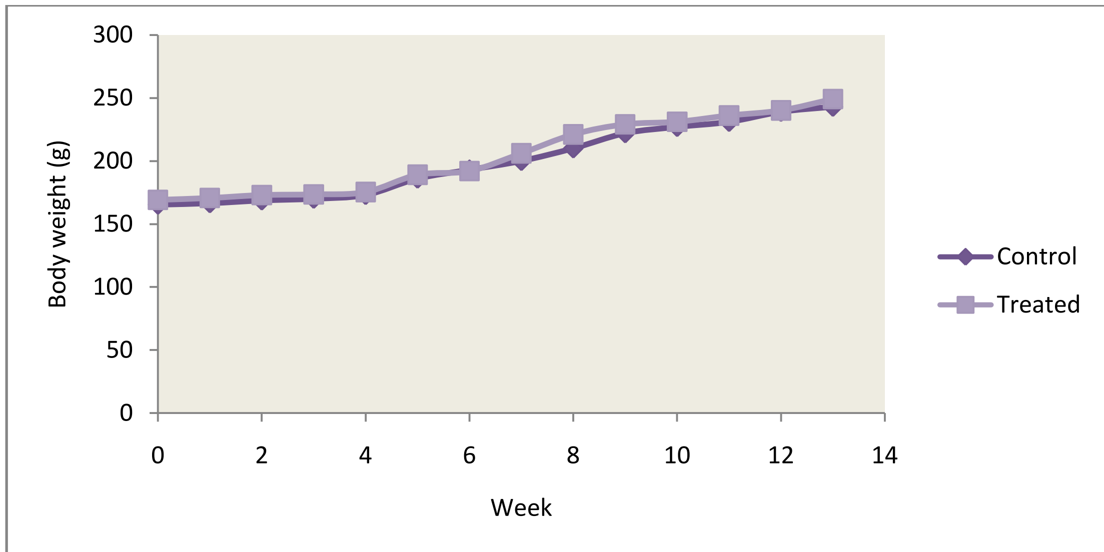


Fig-10 Body weight in treated and control male groups during the 90 days safety assessment

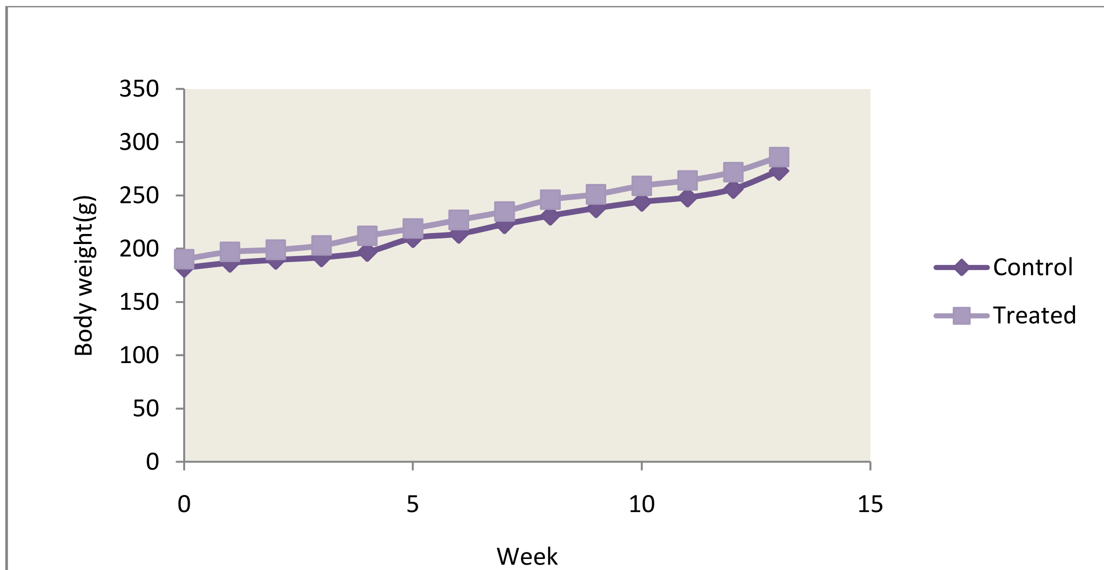


Table - 10 Relative organ weight (g/100g) of control and *Anacyclus pyrethrum* ethanolic extract treated rats (90th day)

Organs	Control		Ethanolic extract *Anacyclus pyrethrum* (1000 mg/kg)	
	Female	**Male**	**Female**	**Male**
Liver	2.66 ± 0.02	2.69 ± 0.06	2.65 ± 0.15	2.63 ± 0.03
Kidney	0.62 ± 0.03	0.60 ± 0.12	0.63 ± 0.02	0.61 ± 0.12
Brain	0.53 ± 0.10	0.49 ± 0.14	0.52 ± 0.10	0.55 ± 0.16
Lungs	0.45 ± 0.03	0.44 ± 0.01	0.45 ± 0.05	0.49 ± 0.02
Heart	0.35 ± 0.02	0.37 ± 0.01	0.38 ± 0.02	0.32 ± 0.01
Spleen	0.22 ± 0.09	0.22 ± 0.07	0.17 ± 0.06	0.23 ± 0.05

Data are expressed as mean ± SEM (n=12)

Table - 11 Hematological parameters of rats in sub chronic toxicity of ethanolic extract of *Anacyclus pyrethrum* (90th day)

Haematological parameter	Control		Ethanolic extract *Anacyclus pyrethrum* (1000 mg/kg)	
	Female	**Male**	**Female**	**Male**
Total R.B.C. count ($\times10^6$/ mm^3)	09.09 ± 1.46	08.13 ± 1.66	8.89 ± 2.05	8.43±1.78
Total W.B.C. Count ($\times10^3$/mm^3)	13.68 ±1.97	09.58 ± 1.45	11.29 ± 1.88	12.63±1.26
Haemoglobin (Hb) (g/dl)	15.82 ±1.94	13.79 ± 1.27	17.62 ± 0.72	16.12±1.33
Hematocrit (%)	42.54±1.36	44.95±1.49	40.12±3.06	41.27±2.47
Platelets ($\times10^3$/mm^3)	652.34±12.34	961.75±16.64	843.35±15.67	893.74±15.35
Neutrophils (%)	17.79±2.03	20.94±3.11	10.06±2.75	14.12±2.72
Lymphocytes(%)	82.43±3.43	77.56±2.45	79±4.55	82.04±3.52
Eosinophil(%)	2.38±0.43	1.82±0.75	1.64±0.25	1.42±0,64
Monocyte(%)	3.10±0.13	1.04±0.10	1.50±0.03	1.20±0.05
Basophil (%)	0.00±0.00	1.01±0.01	0.00±0.00	1.03±0.06

Data are expressed as mean ± SEM (n=12)

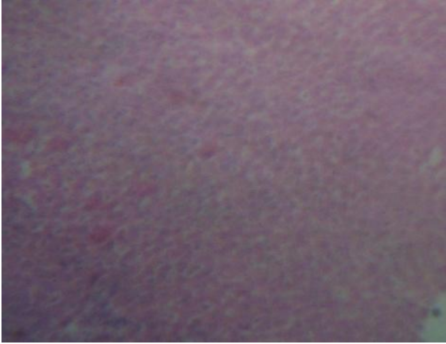
Table - 12 Biochemical parameters of rats in sub chronic toxicity of ethanolic extract of *Anacyclus pyrethrum* (90th day)

Biochemical parameter	Control		Ethanolic extract *Anacyclus pyrethrum* (1000 mg/kg)	
	Female	Male	Female	Male
Creatinine (mg/dl)	0.57 ± 0.07	0.43±0.03	0.64±0.01	0.61±0.05
Urea (mg/dl)	17.14 ± 1.52	20.75±1.31	15.17±1.45	18.12±2.53
Triglycerides (mg/dl)	52.83 ± 4.92	47.25±7.34	54.21±9.03	49.24±5.32
Total Cholesterol(mg/dl)	49.33 ± 2.03	43.00±2.46	58.25±3.95	52.17±3.76
Total protein (g/dl)	7.23 ± 0.24	5.11±0.23	3.57±0.14	4.91±0.94
Albumin (g/dl)	3.64 ± 0.05	4.29±0.03	4.27±0.19	3.25±0.12
AST (IU/L)	124.01 ± 17.6	138.54±19.4	119.6±28.8	112.71±23.84
ALT (IU/L)	61.47 ± 3.19	71.33±6.19	63.47±5.61	72.45±4.02
ALP (IU/L)	114.3 ± 12.0	108.4±15.32	97.54±12.76	104.13±13.52
T. Bilirubin (mg/dl)	0.23 ± 0.05	0.27±0.12	0.31±0.09	0.29±0.03

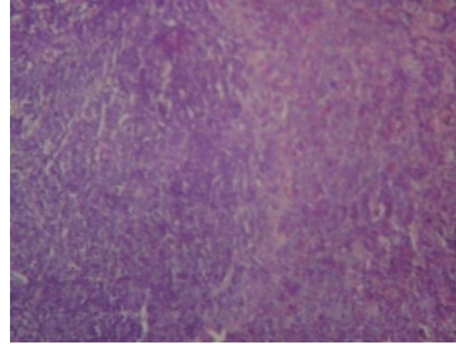
Data are expressed as mean ± SEM (n=12)

Fig - 11 Histopathological examination of control group

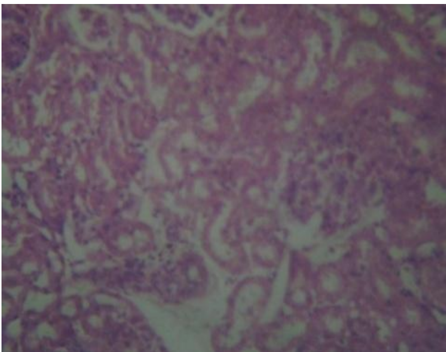
(Photomicrographs showing hematoxylin and eosin stained sections)



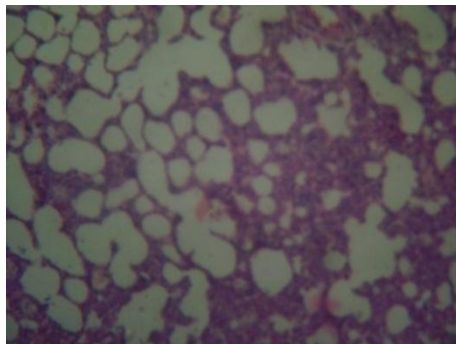
Section of Liver – control



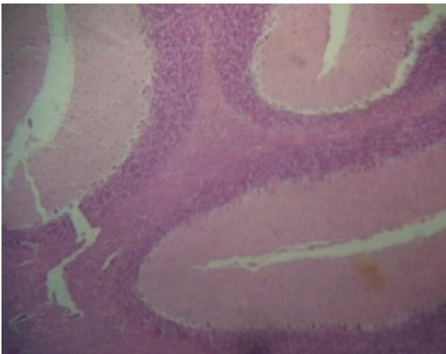
Section of spleen–control



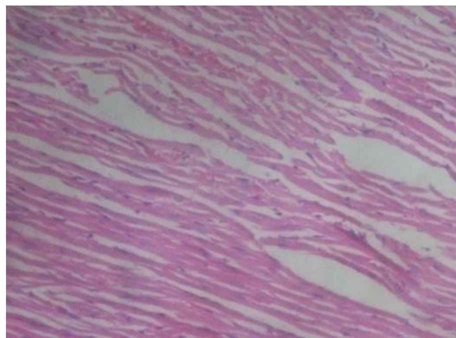
Section of kidney–control



Section of lungs–control



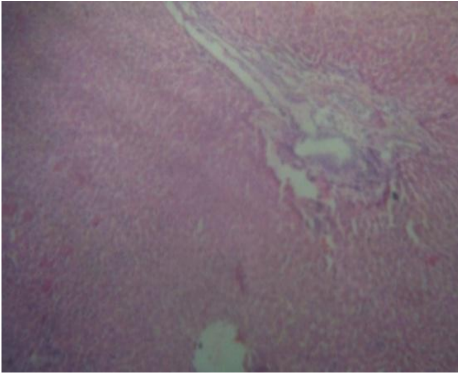
Section of brain–control



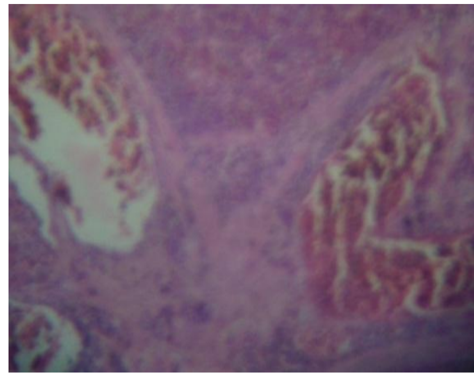
Section of heart-control

Fig - 12 Histopathological examination of treated group

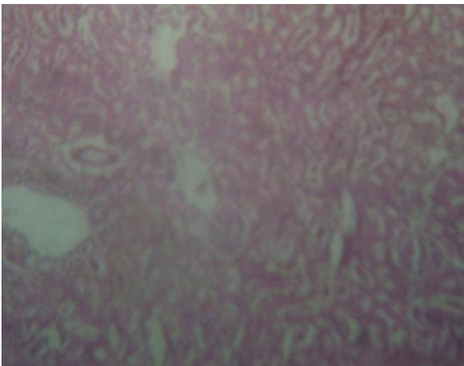
(Photomicrographs showing hematoxylin and eosin stained sections)



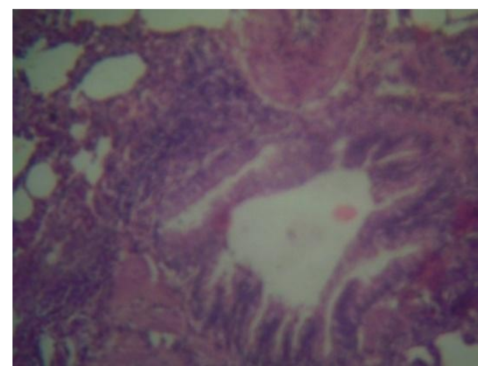
Section of liver -treated



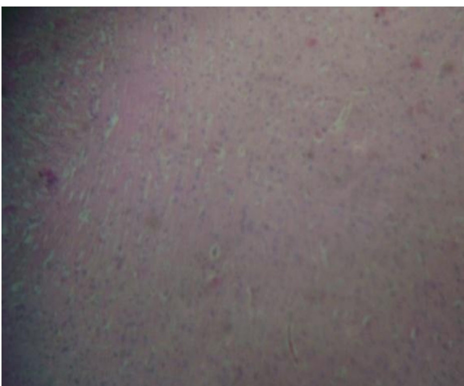
Section of spleen-treated



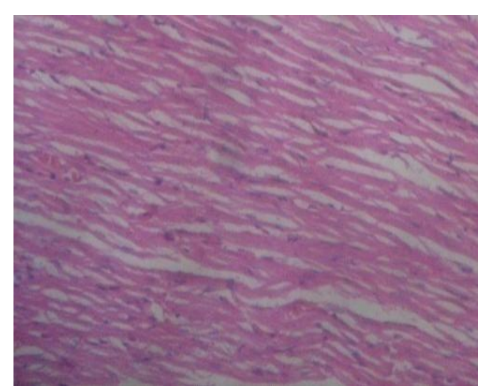
Section of kidney-treated



Section of lungs-treated



Section of brain-treated



Section of heart –treated

Table - 13 Effect of ethanolic extract of *Anacyclus pyrethrum* on general behavioral studies in rats

Behaviour type	Extract (50mg)	Extract (100mg)	Extract (200mg)	Diazepam (5mg/kg)	2%v/v tween 80
Spontaneous activity	_	_	_	++++	_
Alertness	_	_	_	+++	_
Soundresponse	_	_	_	++++	_
Touch response	_	_	_	++++	_
Pain response	_	_	_	++++	_
Righiting reflex	_	_	_	++++	_
Pinna reflex	_	_	_	++++	_
Grip strength	_	_	_	++++	_

No effect (-), slight depression (+), moderate depression (++), strong depression (+++), very strong depression (++++).

Table - 14 Effect of ethanolic extract of *Anacyclus pyrethrum* on locomotor activity in rats using actophotometer

S. No.	Treatment	Locomotor activity (scores)in 10 min	
		Before treatment	**After treatment**
1.	Control (vehicle, p.o)	466.16±11.79	450.83±8.72
2.	EEAP (50mg/kg p.o)	477.66±5.49	483.16±6.49
3.	EEAP (100mg/kg p.o)	491.5 ± 5.60	416.16 ± 13.42
4.	EEAP (200mg/kg p.o)	486.83 ± 12.93	403.33 ± 9.11
5.	Standard(Diazepam 2mg/kg i.p)	499.6 ± 7.98	114.16 ± 10.79*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

Comparison was made between before drug administration and after drug administration

*P< 0.01vs control

Fig - 13 Effect of ethanolic extract of *Anacyclus pyrethrum* on locomotor activity in rats using actophotometer

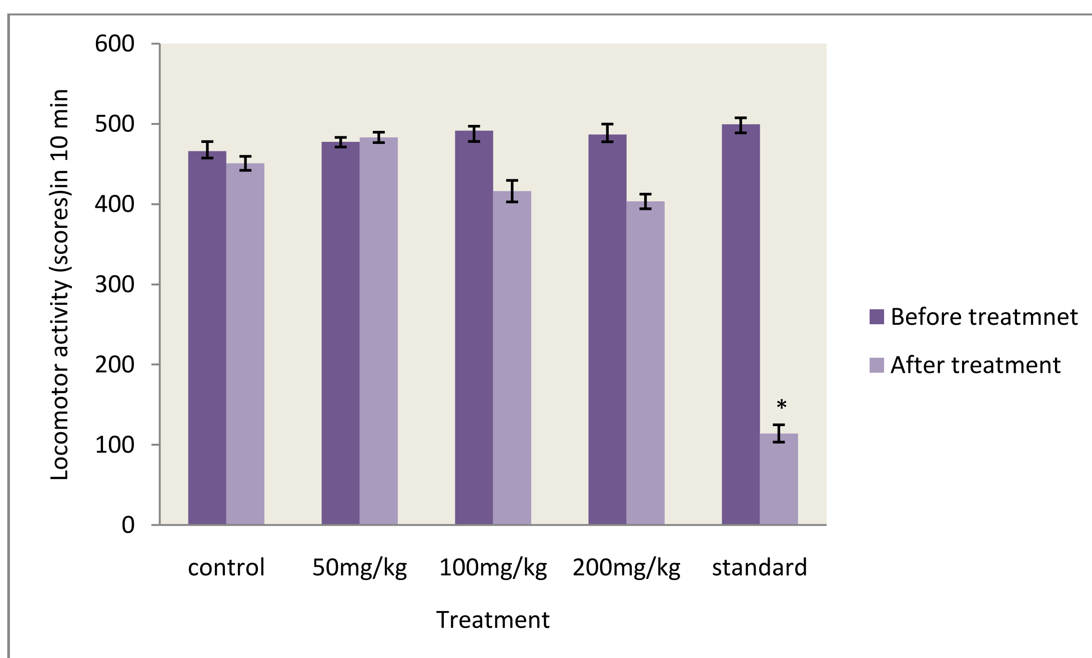


Table - 15 Effect of ethanolic extract of *Anacyclus pyrethrum* on muscle relaxant activity in rats using rota rod

S.No.	Treatment	Fall of time (secs)	
		Before treatment	After treatment
1.	Control (vehicle, p.o)	57.08 ± 6.57	58.33±3.13
2.	EEAP (50mg/kg p.o)	69.25±1.49	67.66±2.61
3.	EEAP (100mg/kg p.o)	58.5±3.34	59.6±1.55
4.	EEAP (200mg/kg p.o)	75.5±1.80	77.0±1.36
5.	Standard (Diazepam 2mg/kg i.p)	59.16±2.70	18.5±1.76*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

Comparison was made between before drug administration and after drug administration

*P< 0.01vs control

Fig - 14 Effect of ethanolic extract of *Anacyclus Pyrethrum* on muscle relaxant activity in rats using rota rod

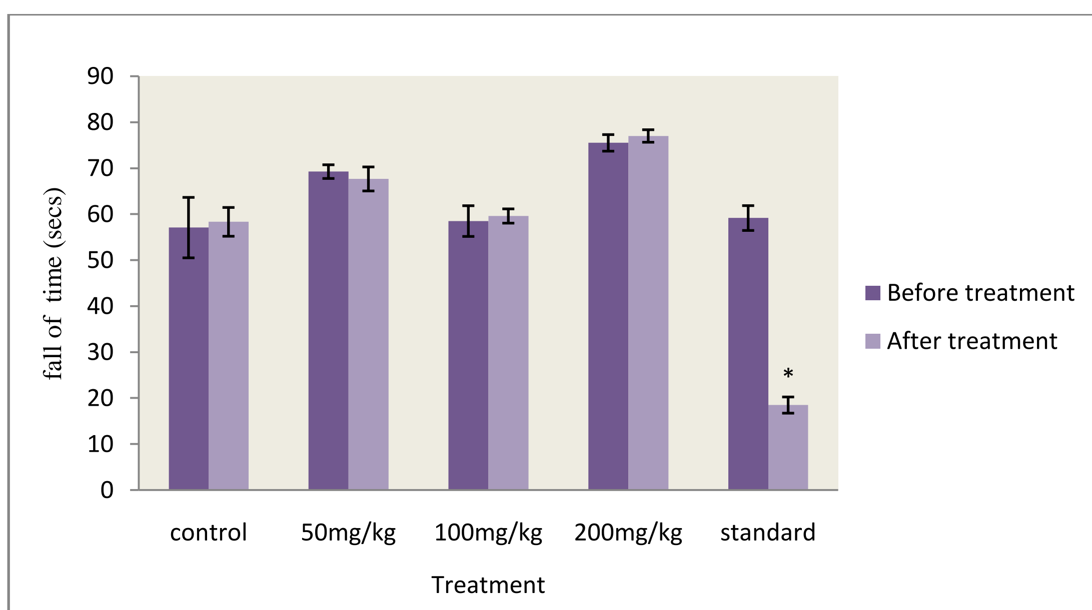


Table - 16 Effect of ethanolic extract of *Anacyclus pyrethrum* on anxiety induced in rats using holeboard

S.No	Treatment	Number of head pokings
1.	Control (vehicle, p.o)	19.33 ± 2.35
2.	EEAP (50mg/kg p.o)	19.81 ± 1.01
3.	EEAP (100mg/kg p.o)	18.06 ± 3.18
4.	EEAP (200mg/kg p.o)	17.16 ± 1.13
5.	Standard (Diazepam 2mg/kg i.p)	48.31 ± 5.30*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P< 0.01vs control

Fig - 15 Effect of ethanolic extract of *Anacyclus pyrethrum* on anxiety induced in rats using holeboard apparatus

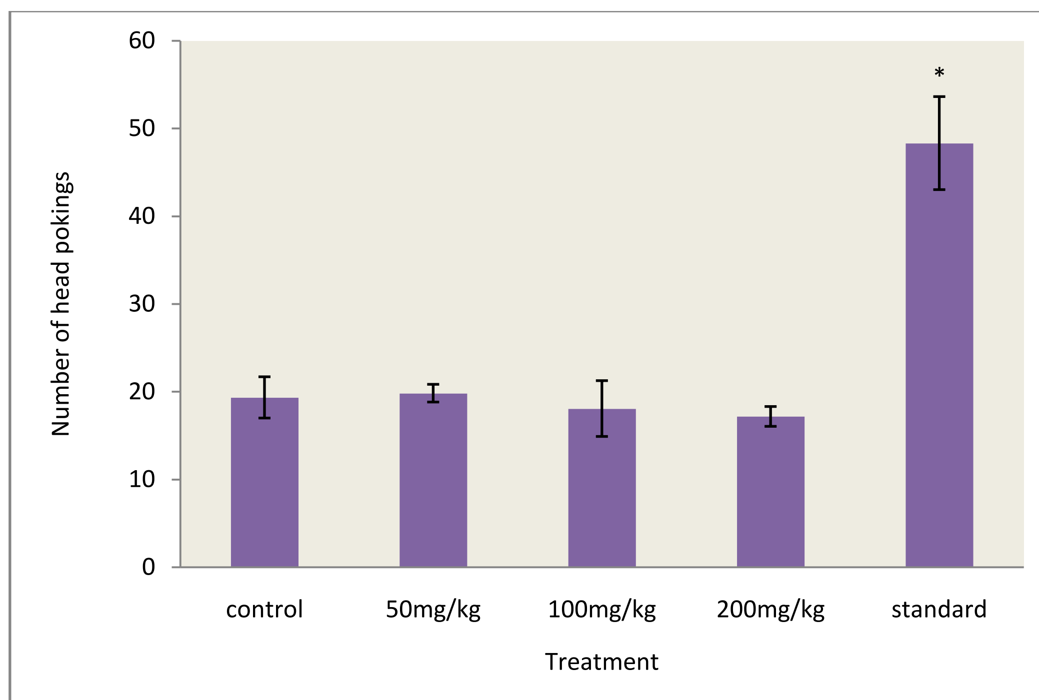


Table - 17 Effect of ethanolic extract of *Anacyclus pyrethrum* on anxiety induced in rats using elevated plus maze

S. No.	Treatment	Time spent in open arm (secs)	Time spent in closed arm (secs)	No of entries in open arm (secs)
1.	Control	44.83±7.23	203.5±9.58	1.6±0.12
2.	EEAP (50mg/kg, p.o)	43.97±6.86 ^{NS}	183.31±7.46	1.42±0.46
3.	EEAP (100mg/kg, p.o)	45.83±5.27 ^{NS}	141.73±11.34	1.83±0.16
4.	EEAP (200mg/kg, p.o)	49.66±3.76 ^{NS}	176.17±6.23	2±0.36
5.	Diazepam(2mg/kg)	105.33±5.32*	115.41±9.63	5.66±0.33

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

P < 0.01 Vs Control

NS- Non significant

Fig - 16 Effect of ethanolic extract of *Anacyclus pyrethrum* on anxiety induced in rats using elevated plus maze

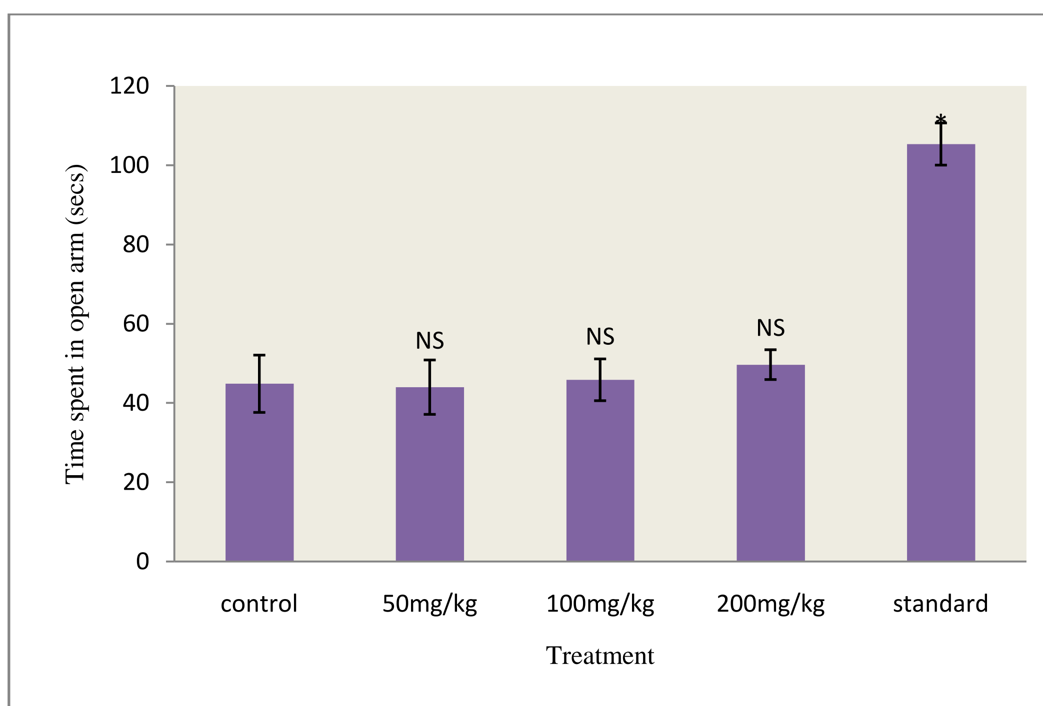


Table - 18 Effect of ethanolic extract of *Anacyclus pyrethrum* on immobility period in forced swim test

S.No.	Treatment	Immobility time (sec)
1.	Control (vehicle, p.o)	135.17±1.63
2.	EEAP (50mg/kg p.o)	95.23±1.90*
3.	EEAP (100mg/kg p.o)	71.33±1.18*
4.	EEAP (200mg/kg p.o)	68.66±0.84*
5.	Imipramine (15mg/kg i.p)	55.32±1.42*

Statistical significance test was done by ANOVA followed by Dunnet's 't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P< 0.01vs control

Fig - 17 Effect of ethanolic extract of *Anacyclus pyrethrum* on immobility period in forced swim test

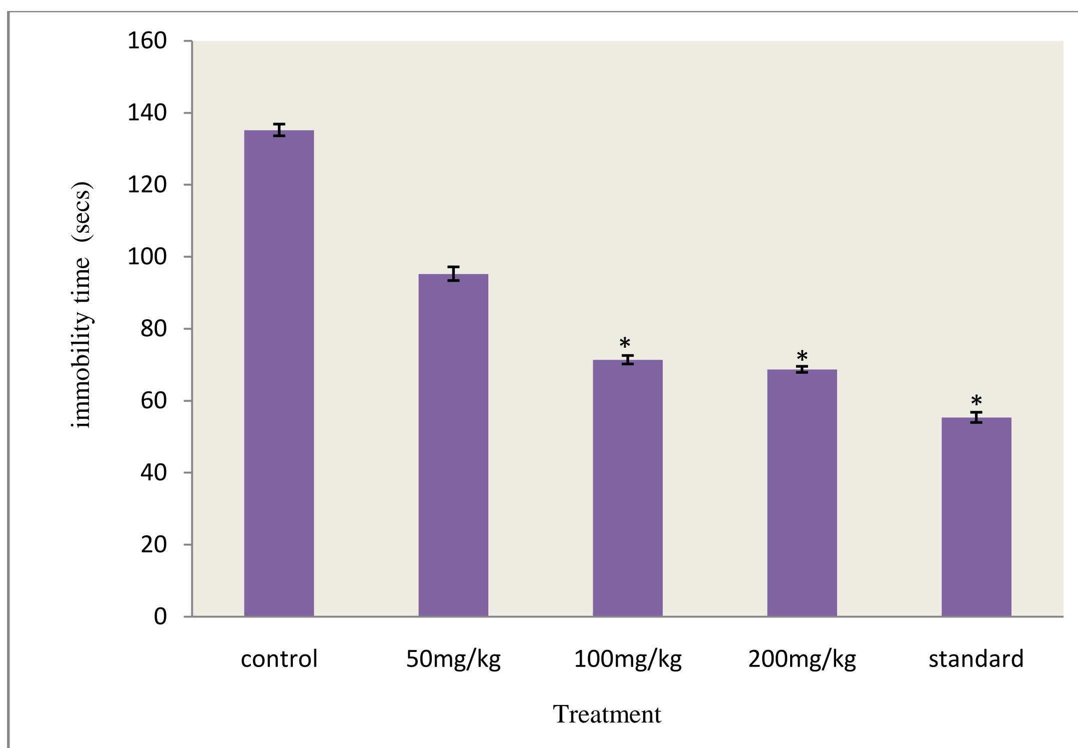


Table - 19 Effect of ethanolic extract of *Anacyclus pyrethrum* on immobility period using tail-suspension test

S.No	Treatment	Immobility time (sec)
1.	Control (vehicle, p.o)	121.26±1.54
2.	EEAP (50mg/kg p.o)	95.31±2.75*
3.	EEAP (100mg/kg p.o)	82.33±1.33*
4.	EEAP (200mg/kg p.o)	68.42±1.57*
5.	Imipramine (15mg/kg i.p)	56.34±0.83*

Statistical significance test was done by ANOVA followed by Dunnet's 't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P< 0.01vs control

Fig-18 Effect of ethanolic extract of *Anacyclus pyrethrum* on immobility period using tail-suspension test

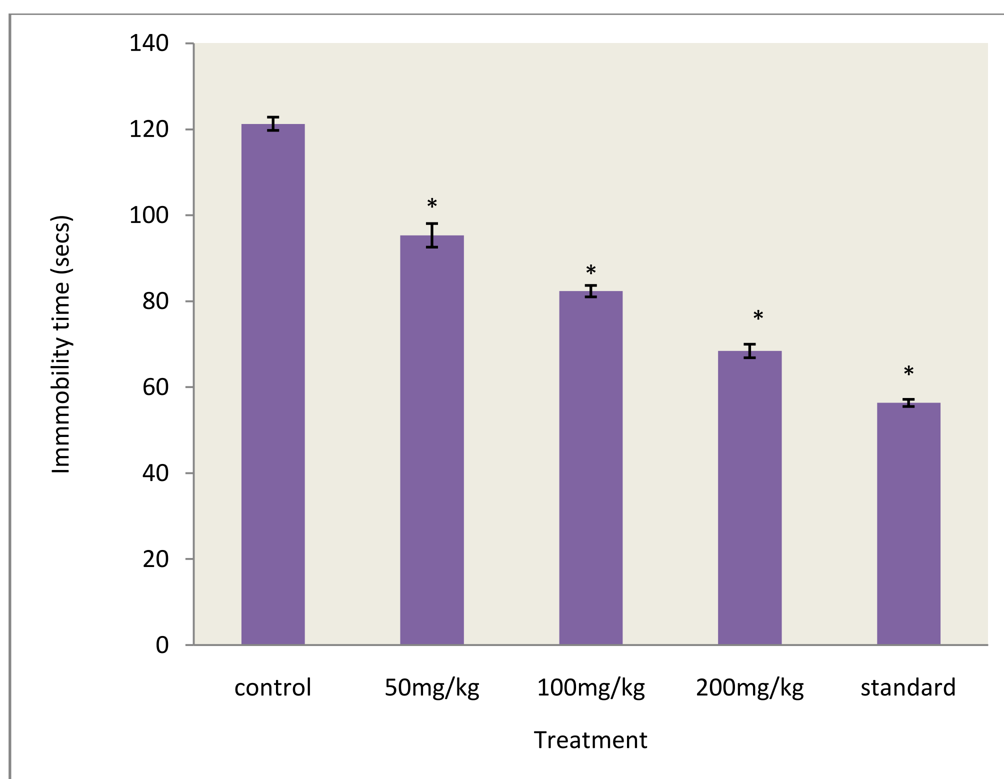


Table - 20 Effect of ethanolic extract of *Anacyclus pyrethrum* on transfer latency (elevated plus maze paradigm) in scopolamine induced amnesia in rats

S. No.	Treatment groups	Transfer latency on acquisition day(secs)(14day)	Transfer latency on retention day (secs)(15 day)
1.	Control(vehicle, p.o)	34.66±2.53	27.33±1.40
2.	Scopolamine hydro bromide	135.33±3.58	133.16±4.85
3.	EEAP(50mg/kgp.o)+Scopolamine	31.53±2.63*	16.83±1.51*
4.	EEAP(100mg/kgp.o)+Scopolamine	14.50±0.88*	9±1.46*
5.	EEAP(200mg/kgp.o)+Scopolamine	15.33±1.054*	6.16±1.13*
6.	Piracetam(200mg/kg)+Scopolamine	26.50±2.43*	7.20±1.18*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

* P < 0.01 Vs Scopolamine- treated groups

Fig - 19 Effect of ethanolic extract of *Anacyclus pyrethrum* on transfer latency (elevated plus maze paradigm) in scopolamine induced amnesia in rats

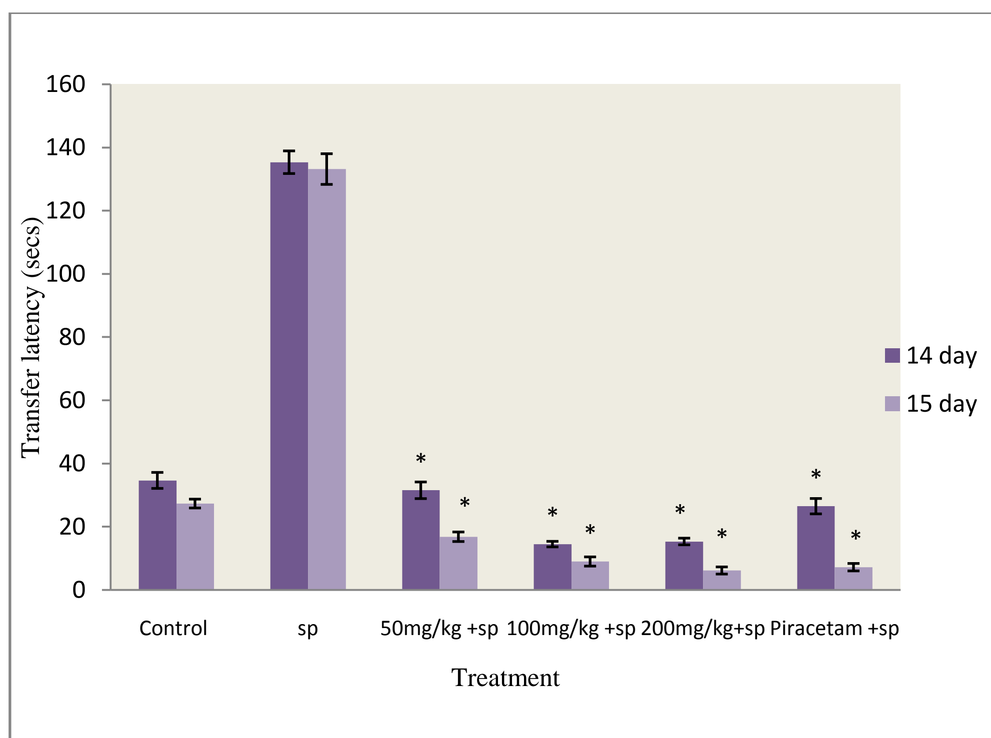


Table - 21 Effect of ethanolic extract of *Anacyclus pyrethrum* on step down latency (passive avoidance paradigm) in scopolamine induced amnesia in rats

S. No.	Treatment groups	Step down latency on acquisition day (secs) (14 day)	Step down latency on retention day (secs) (15 day)
1.	Control(vehicle, p.o)	3.33±0.49	3.16±0.47
2.	Scopolamine hydro bromide	1.18±0.20	1.38±0.15
3.	EEAP(50mg/kgp.o)+Scopolamine	9.63±0.76*	12.83±1.07*
4.	EEAP(100mg/kgp.o)+Scopolamine	13.83±1.13*	18.50±0.562*
5.	EEAP(200mg/kgp.o)+Scopolamine	23.16±0.79*	28.16±0.94*
6.	Piracetam(200mg/kg)+Scopolamine	20.38±0.94*	34.36±1.13*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)Values are mean ± SEM of 6 animals per groups

* P < 0.01 Vs Scopolamine- treated groups

Fig - 20 Effect of ethanolic extract of *Anacyclus pyrethrum* on step down latency (passive avoidance paradigm) in scopolamine induced amnesia in rats

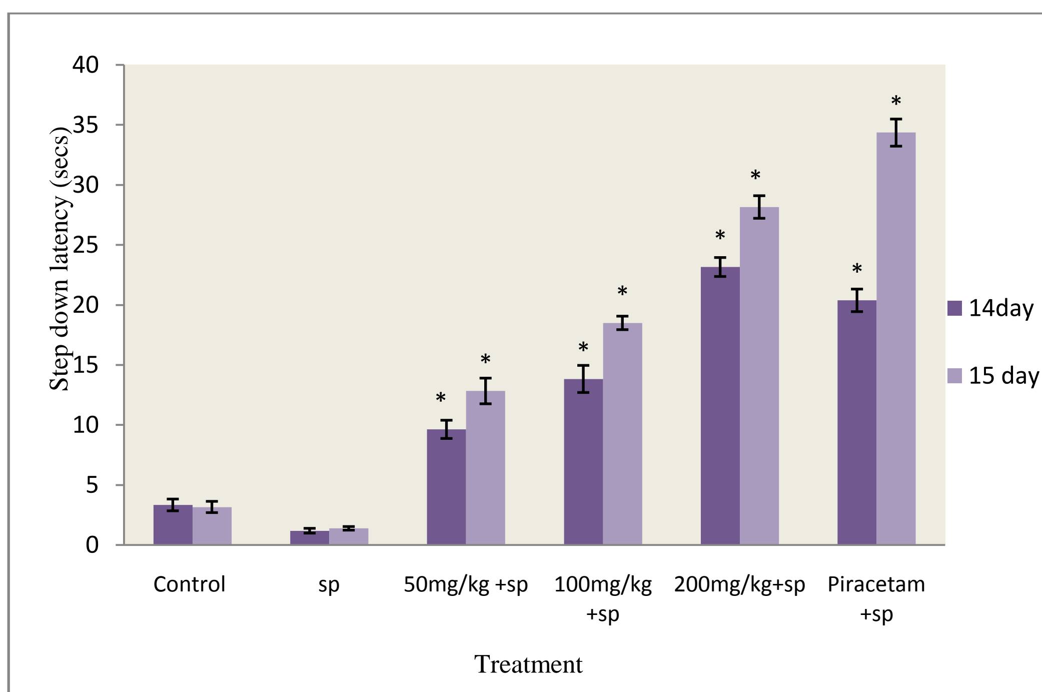


Table - 22 Estimation of the acetylcholinestrease activity in the brain homogenate of scopolamine induced amnesia in rats

S.No.	Treatment groups	Acetylcholinesterase level (m moles)
1.	Control(vehicle, p.o)	47.44±1.72
2.	Scopolamine hydrobromide	68.03±2.08
3.	EEAP(50mg/kgp.o)+Scopolamine	40.58±1.56*
4.	EEAP(100mg/kgp.o)+Scopolamine	36.38±1.20*
5.	EEAP(200mg/kgp.o)+Scopolamine	29.25±1.92*
6.	Piracetam(200mg/kg)+Scopolamine	28.81±2.52*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6) Values are mean ± SEM of 6 animals per groups

* P < 0.01 Vs Scopolamine- treated groups

Fig - 21 Estimation of the acetylcholinestrease activity in the brain homogenate of scopolamine induced amnesia in rats

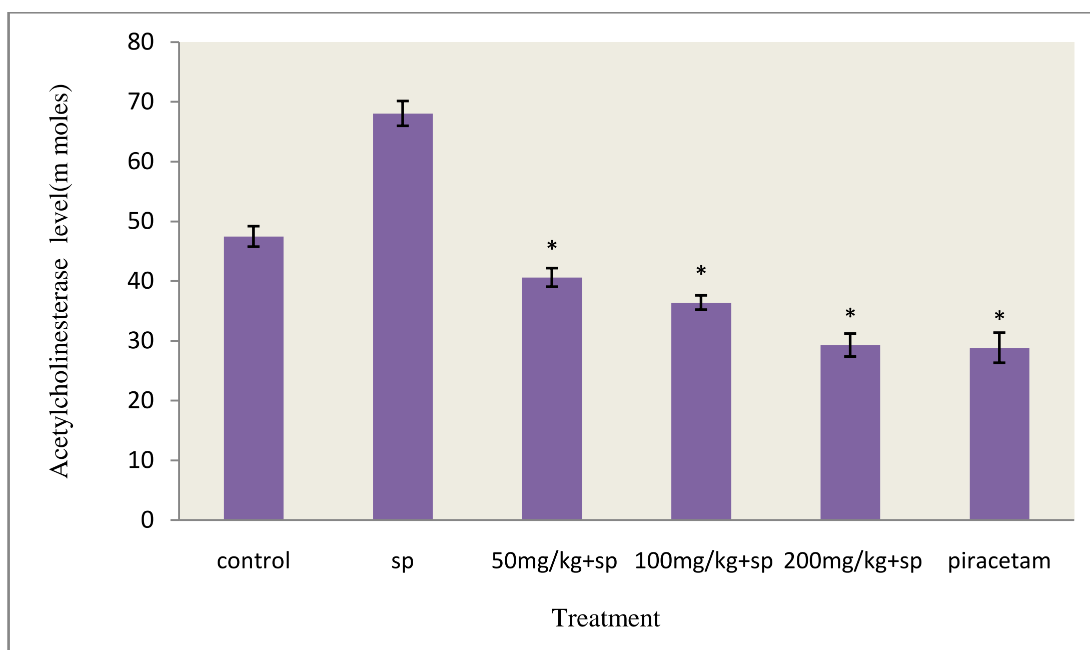


Table - 23 Effect of ethanolic extract of *Anacyclus pyrethrum on* MDA level in the rat brain

Treatment groups	MDA(nmol/g tissue)
Control(vehicle, p.o)	192.7±.7.5
Scopolamine hydrobromide	282.5±15.2
EEAP(50mg/kgp.o)+Scopolamine	252.7±.12.5*
EEAP(100mg/kgp.o)+Scopolamine	212.7±.6.5*
EEAP(200mg/kgp.o)+Scopolamine	197.3±6.3*
Piracetam(200mg/kg)+Scopolamine	190.5±8.4*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P < 0.01 Vs positive Control

Fig - 22 Effect of ethanolic extract of *Anacyclus pyrethrum on* MDA level in the rat brain

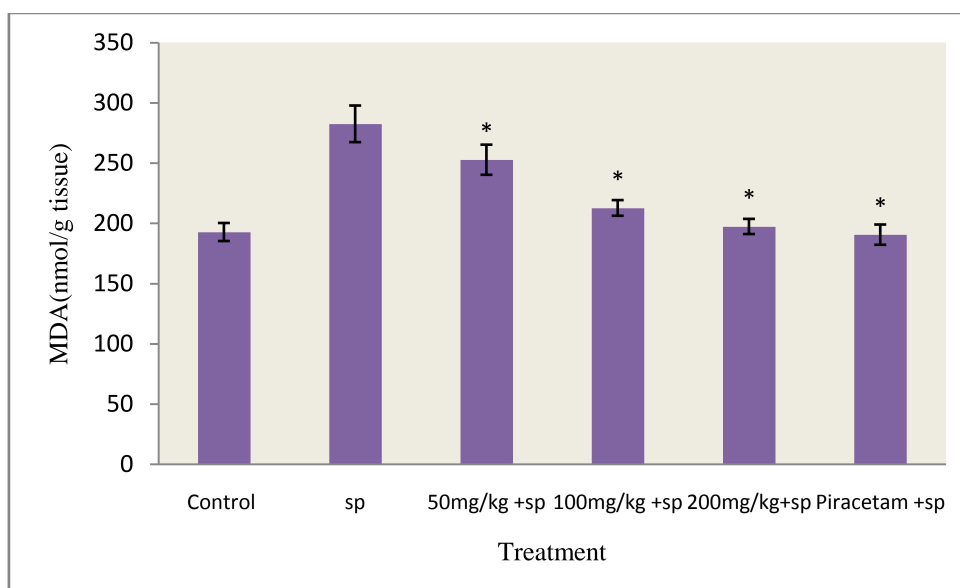


Table - 24 Effect of ethanolic extract of *Anacyclus pyrethrum on* catalase level in the rat brain

Treatment groups	Catalase (units/mg protein)
Control(vehicle, p.o)	15.3±2.5
Scopolamine hydrobromide	9.68±1.4
EEAP(50mg/kgp.o)+Scopolamine	13.42±2.7*
EEAP(100mg/kgp.o)+Scopolamine	23.7±3.4*
EEAP(200mg/kgp.o)+Scopolamine	38.16±1.2*
Piracetam(200mg/kg)+Scopolamine	49.3±6.5*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P < 0.01 Vs positive Control

Fig - 23 Effect of ethanolic extract of *Anacyclus pyrethrum on* catalase level in the rat brain

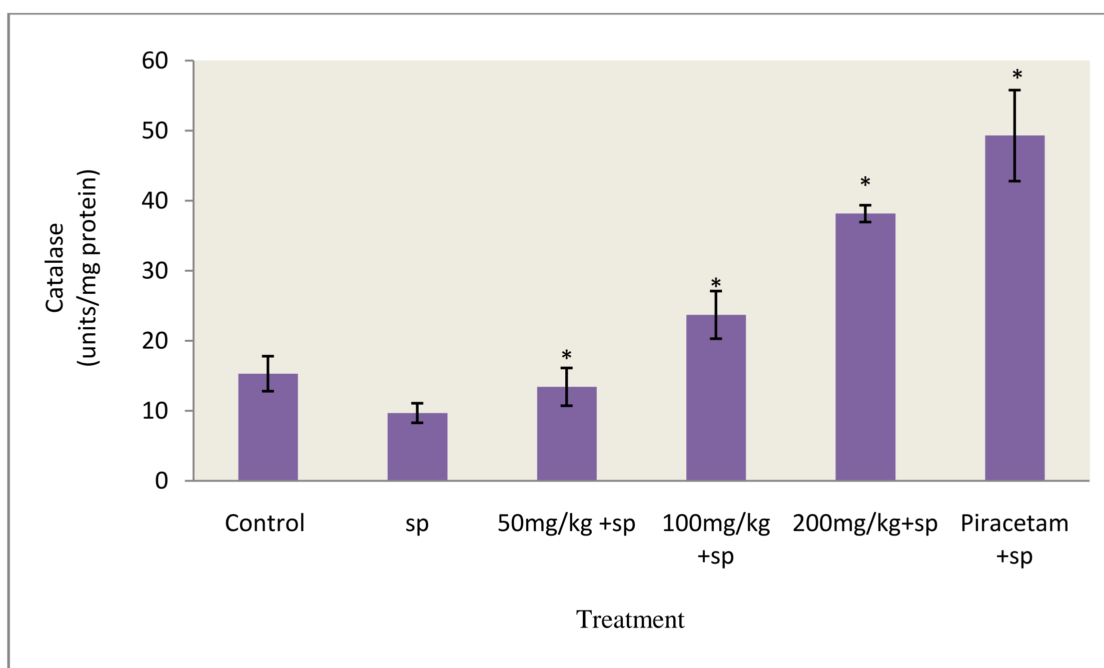


Table - 25 Effect of ethanolic extract of *Anacyclus pyrethrum on* super oxide dismutase level in rat brain

Treatment groups	SOD(units/mg protein)
Control(vehicle, p.o)	5.63±0.31
Scopolamine hydrobromide	1.69±0.12
EEAP(50mg/kgp.o)+Scopolamine	2.02±0.23*
EEAP(100mg/kgp.o)+Scopolamine	2.89±0.72*
EEAP(200mg/kgp.o)+Scopolamine	3.45±0.13*
Piracetam(200mg/kg)+Scopolamine	3.99±0.91*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P < 0.01 Vs positive Control

Fig - 24 Effect of ethanolic extract of *Anacyclus pyrethrum on* super oxide dismutase level in the rat brain

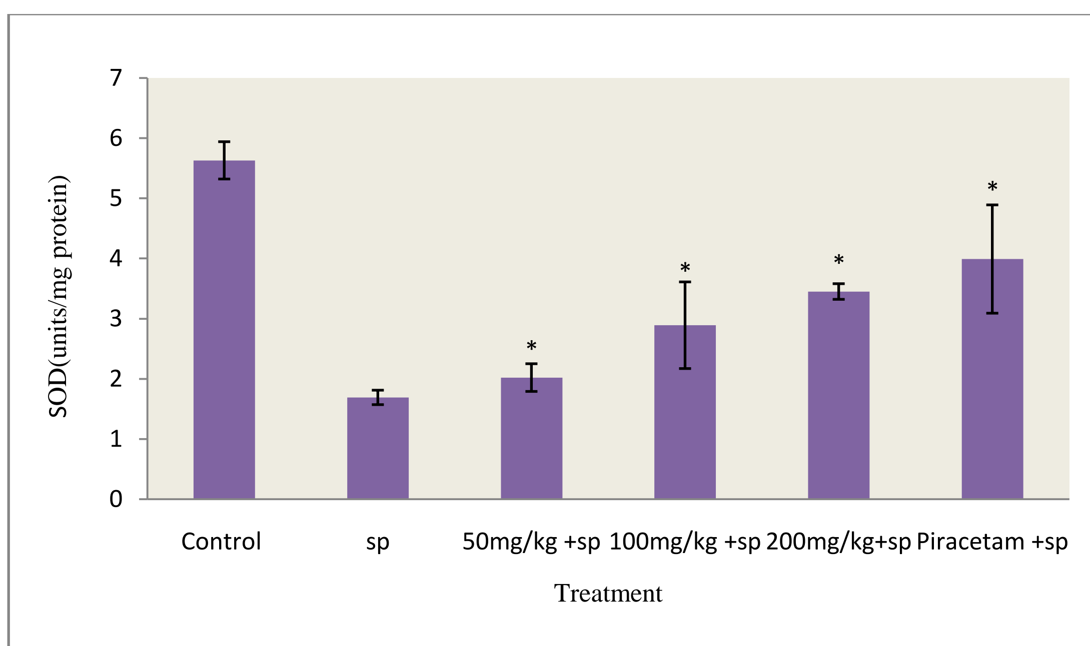


Table - 26 Effect of ethanolic extract of *Anacyclus pyrethrum on* glutathione peroxidase (GPx) level in rat brain

Treatment groups	Glutathioneperoxidase (µmoles / minutes / mg protein)
Control (vehicle, p.o)	27.26 ± 0.70
Scopolamine hydrobromide	26.83 ± 1.04
EEAP(50mg/kgp.o)+Scopolamine	24.14 ± 1.25^{NS}
EEAP(100mg/kgp.o)+Scopolamine	25.17 ± 1.17 ^{NS}
EEAP(200mg/kgp.o)+Scopolamine	27.22 ± 1.2 4 ^{NS}
Piracetam(200mg/kg)+Scopolamine	26.16 ± 1.31 ^{NS}

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

NS- Non significant

Fig - 25 Effect of ethanolic extract of *Anacyclus pyrethrum on* glutathione peroxidase (GPx) level in rat brain

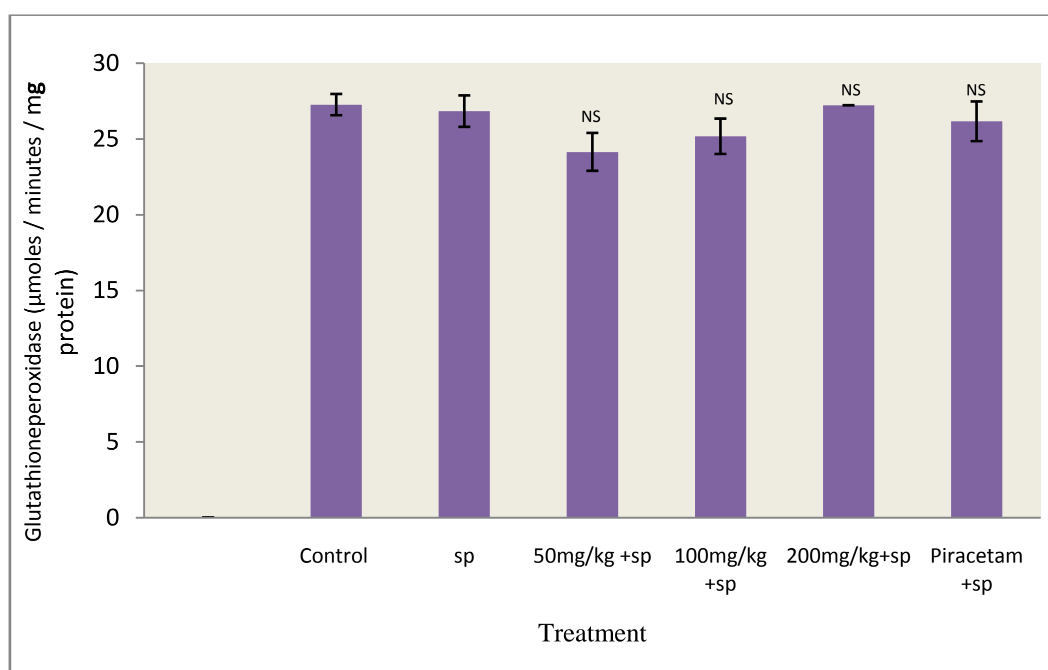


Table - 27 Effect of ethanolic extract of *Anacyclus pyrethrum on* glutathione reductase (GRD) level in rat brain

Treatment groups	Glutathione reductase (Units/min/mg protein)
Control(vehicle, p.o)	31.72 ± 0.81
Scopolamine hydrobromide	25.35 ± 1.39
EEAP(50mg/kgp.o)+Scopolamine	27.95 ± 1.34*
EEAP(100mg/kgp.o)+Scopolamine	30.96 ± 1.47*
EEAP(200mg/kgp.o)+Scopolamine	31.15 ± 1.58*
Piracetam(200mg/kg)+Scopolamine	29.16 ± 1.31*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P < 0.01 Vs positive Control

Fig - 26 Effect of ethanolic extract of *Anacyclus pyrethrum on* glutathione reductase (GRD) level in rat brain

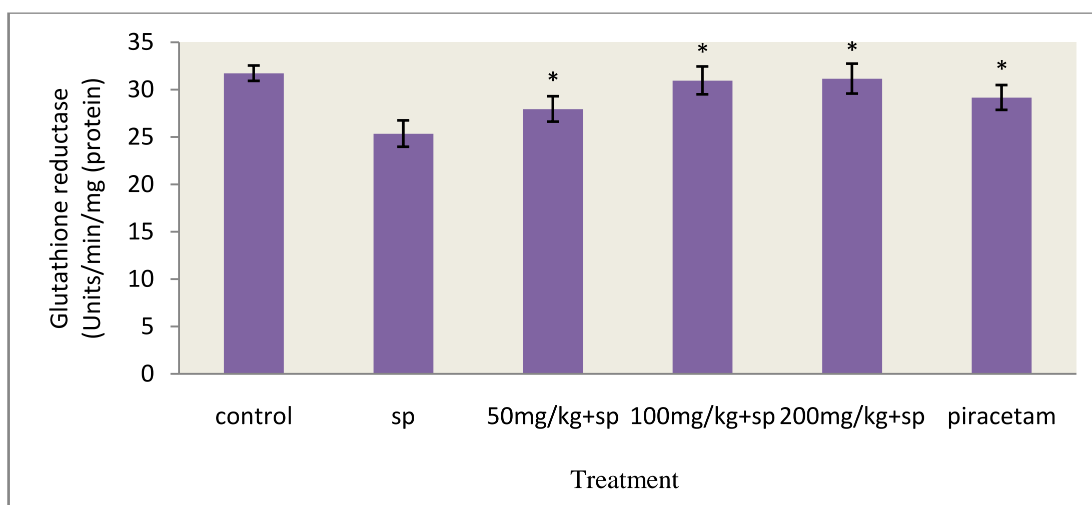


Table - 28 Effect of ethanolic extract of *Anacyclus pyrethrum on* reduced glutathione level in rat brain

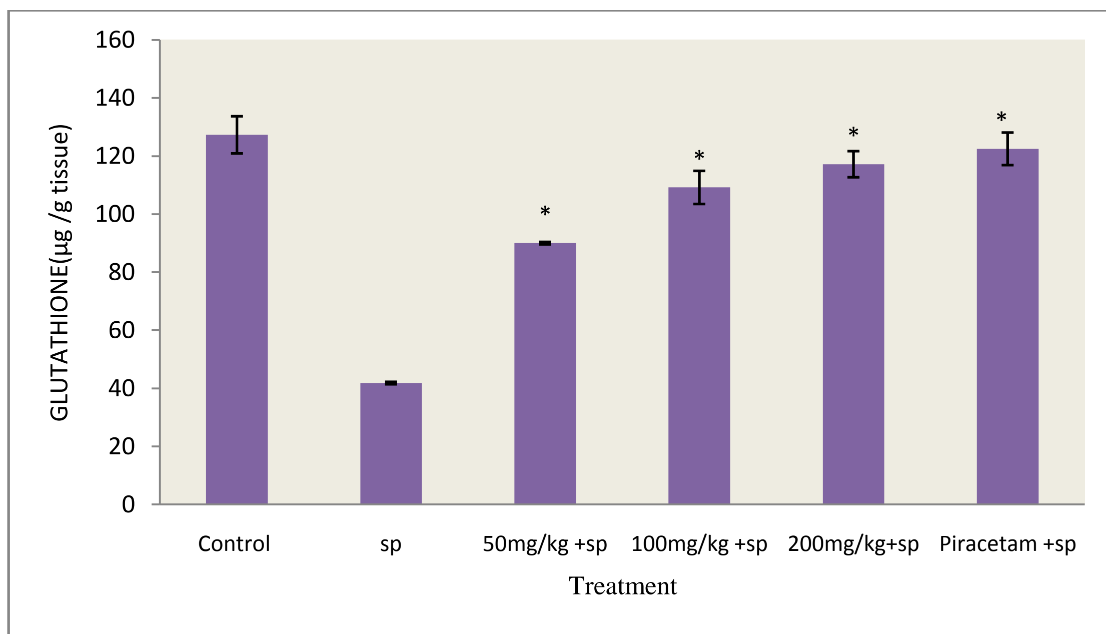
Treatment groups	Glutathione(μ g /gtissue)
Control(vehicle, p.o)	127.3±6.4
Scopolamine hydrobromide	41.83±0.41
EEAP(50mg/kgp.o)+Scopolamine	90.02±0.42*
EEAP(100mg/kgp.o)+Scopolamine	109.2±5.7*
EEAP(200mg/kgp.o)+Scopolamine	117.2±4.5*
Piracetam(200mg/kg)+Scopolamine	122.49±5.6*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P < 0.01 Vs positive Control

Fig - 27 Effect of ethanolic extract of *Anacyclus pyrethrum on* reduced glutathione level in rat brain



Social learning test

Table - 29 Effect of ethanolic extract of *Anacyclus pyrethrum* on social recognition task

S.No	Treatment	Investigation time (secs)	
		First presentation	Second presentation
1.	Control(vehicle, p.o)	59±6.99	54.33±4.25
2.	EEAP (50mg/kg, p.o)	56.33±1.22	24.66±0.88*
3.	EEAP (100mg/kg, p.o)	57.66±3.70	19.33±1.56*
4.	EEAP (200mg/kg, p.o)	57.33±3.35	9±0.73*
5.	Piracetam (200mg/kg)	59±3.27	8.66±0.49*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

* P < 0.01 Vs Control

Fig - 28 Effect of ethanolic extract of *Anacyclus pyrethrum* on Social recognition task

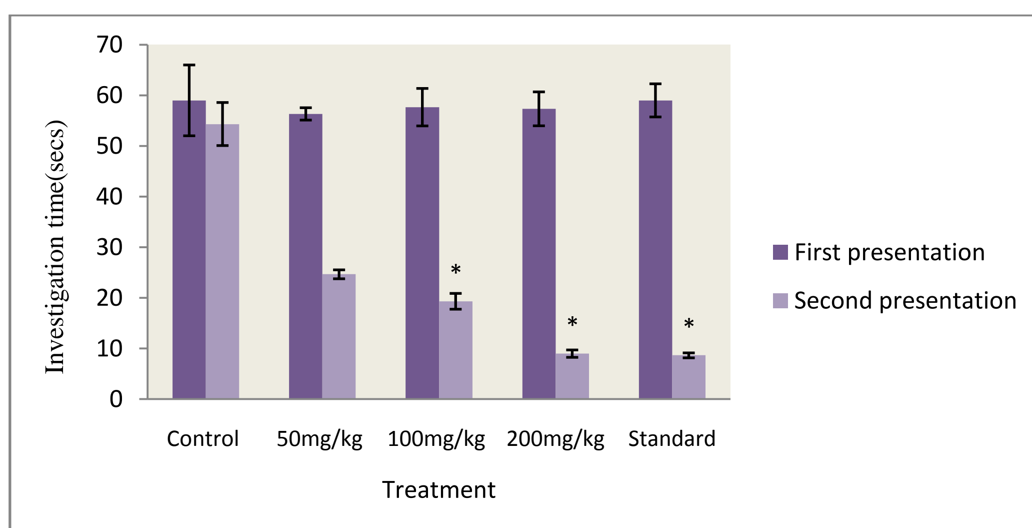


Table - 30 Effect of ethanolic extract of *Anacyclus pyrethrum* on learning and memory using elevated plus maze

S.No	Treatment	Transfer latency (secs)	
		Day 14	Day 15
1.	Control (vehicle, p.o)	24.83±1.35	24.66±1.11
2.	EEAP (50mg/kg p.o)	18.33±1.47*	15.08±0.42*
3.	EEAP (100mg/kg p.o)	16.33±0.80*	15.16±0.70*
4.	EEAP (200mg/kg p.o)	9.16±0.30*	10±0.57*
5.	Piracetam (200mg/kg)	11.16±0.30*	10.33±0.80*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P< 0.01vs control

Fig - 29 Effect of ethanolic extract of *Anacyclus Pyrethrum* on learning and memory using elevated plus maze

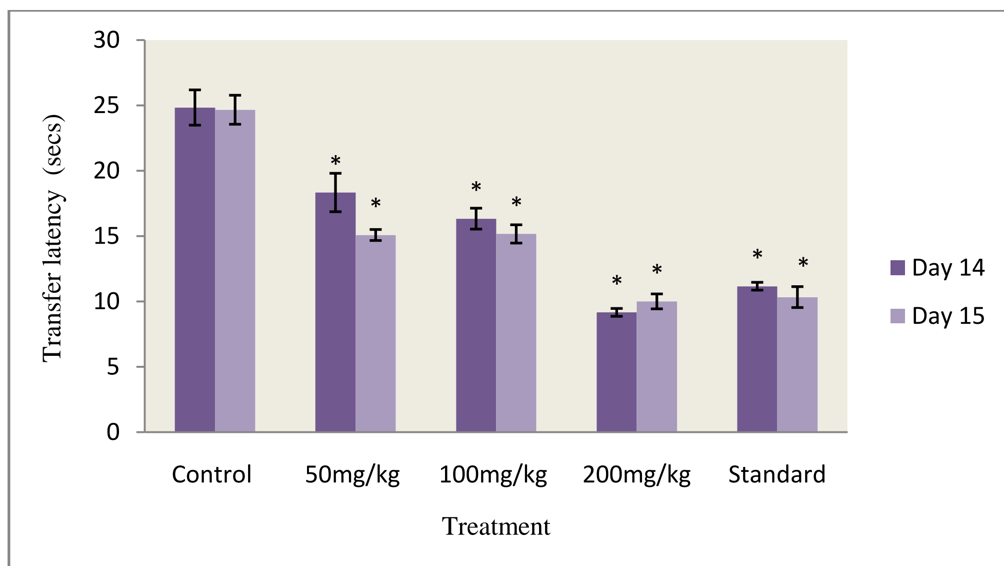


Table - 31 Effect of ethanolic extract of *Anacyclus Pyrethrum* on dopamine levels in rat brain

S.No.	Treatment	Dopamine (ng/mg tissue)
1.	Control (vehicle, p.o)	381.68±9.93
2.	EEAP (50mg/kg p.o)	231.26±4.86*
3.	EEAP (100mg/kg p.o)	112.82±1.23*
4.	EEAP (200mg/kg p.o)	98.75±1.62*
5.	Piracetam (200mg/kg)	133.24±2.83*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)

Values are mean ± SEM of 6 animals per groups

*P< 0.01vs control

Fig - 30 Effect of ethanolic extract of *Anacyclus Pyrethrum* on dopamine levels in rat brain

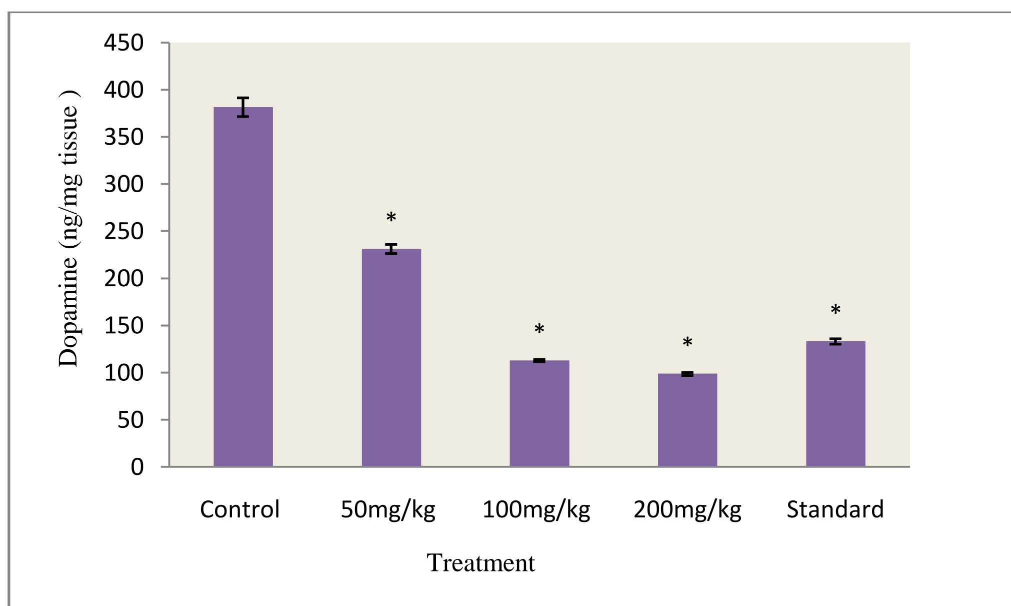


Table - 32 Effect of ethanolic extract of *Anacyclus Pyrethrum* on serotonin levels in rat brain

S.No.	Treatment	Serotonin (ng/mg tissue)
1.	Control (vehicle, p.o)	121.66±3.29
2.	EEAP (50mg/kg p.o)	143.34±3.64*
3.	EEAP (100mg/kg p.o)	160.27±5.33*
4.	EEAP (200mg/kg p.o)	168.3±3.89*
5.	Piracetam (200mg/kg)	174.5±6.55*

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)
Values are mean ± SEM of 6 animals per groups
*P< 0.01vs control

Fig - 31 Effect of ethanolic extract of *Anacyclus Pyrethrum* on serotonin levels in rat brain

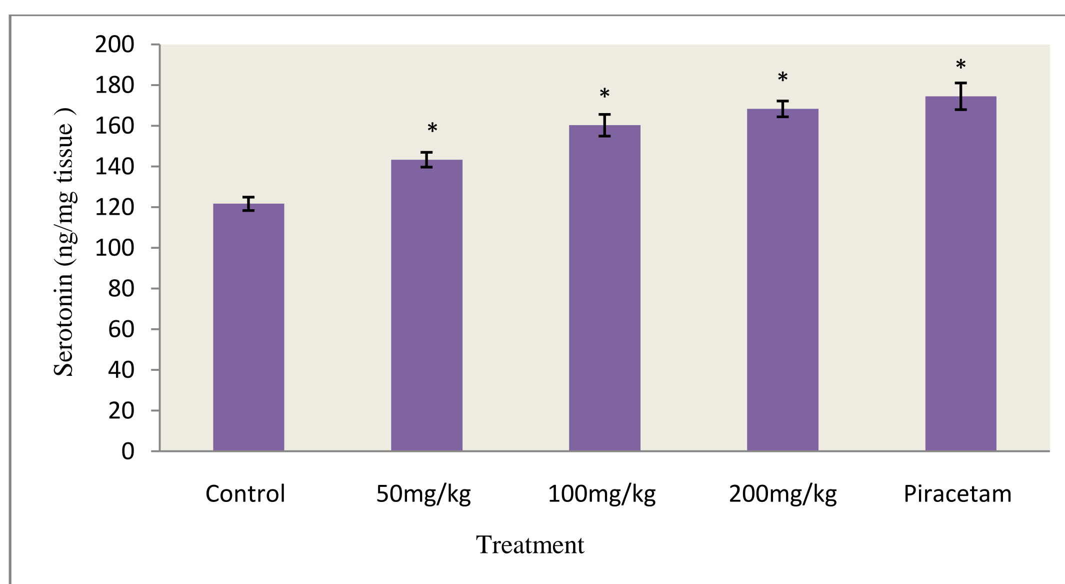


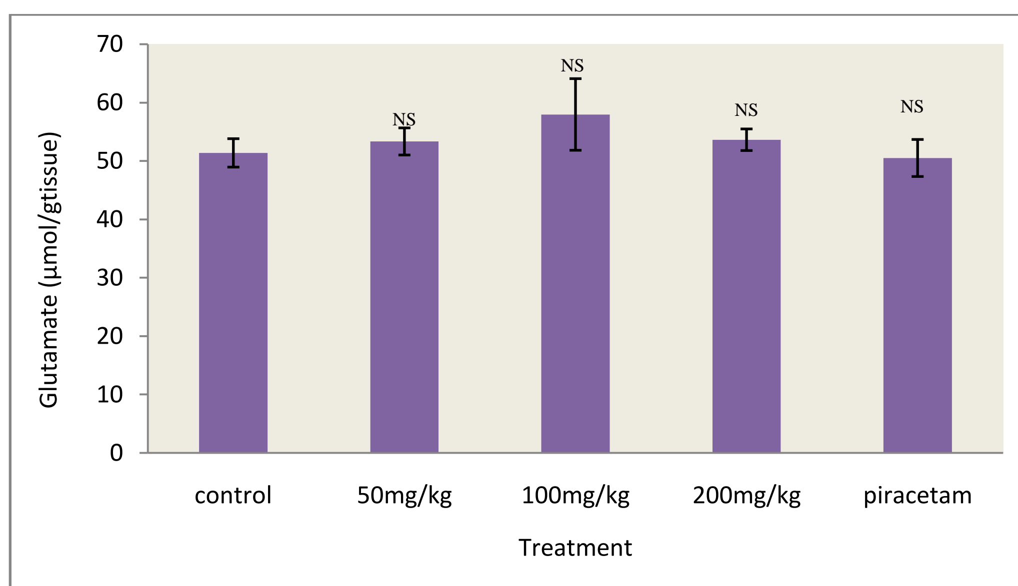
Table - 33 Effect of ethanolic extract of *Anacyclus Pyrethrum* on glutamate levels in rat brain

S.No	Treatment	Glutamate (µmol/gtissue)
1	Control (vehicle, p.o)	51.37±2.43
2	EEAP (50mg/kg p.o)	53.33±2.32 ^{NS}
3	EEAP (100mg/kg p.o)	57.95±6.13^{NS}
4	EEAP (200mg/kg p.o)	53.62±1.85 ^{NS}
5	Piracetam (200mg/kg)	50.50±3.17 ^{NS}

Statistical significance test was done by ANOVA followed by Dunnet's't' test (n=6)Values are mean ± SEM of 6 animals per groups

NS- Non significant

Fig - 32 Effect of ethanolic extract of *Anacyclus Pyrethrum* on glutamate levels in rat brain



***In vitro*antioxidant Studies**

Table -34 Free radical scavenging activity of ethanolic extract of *Anacyclus pyrethrum* by DPPH reduction

Sl. No.	Concentration (µg/ml)	Inhibition (%)	IC_{50} Value (µg/ml)
1.	25	33.71 ± 0.18	55.83± 1.92
2.	50	47.50 ± 0.24	
3.	100	65.52 ± 0.39	
4.	200	72.63 ± 0.32	
5.	400	83.47 ± 0.68	
6.	Ascorbic acid		10.38±0.52

Values are mean ± SEM of 3 replicates

Fig-33 Free radical scavenging activity of ethanolic extract of *Anacyclus pyrethrum* by DPPH reduction

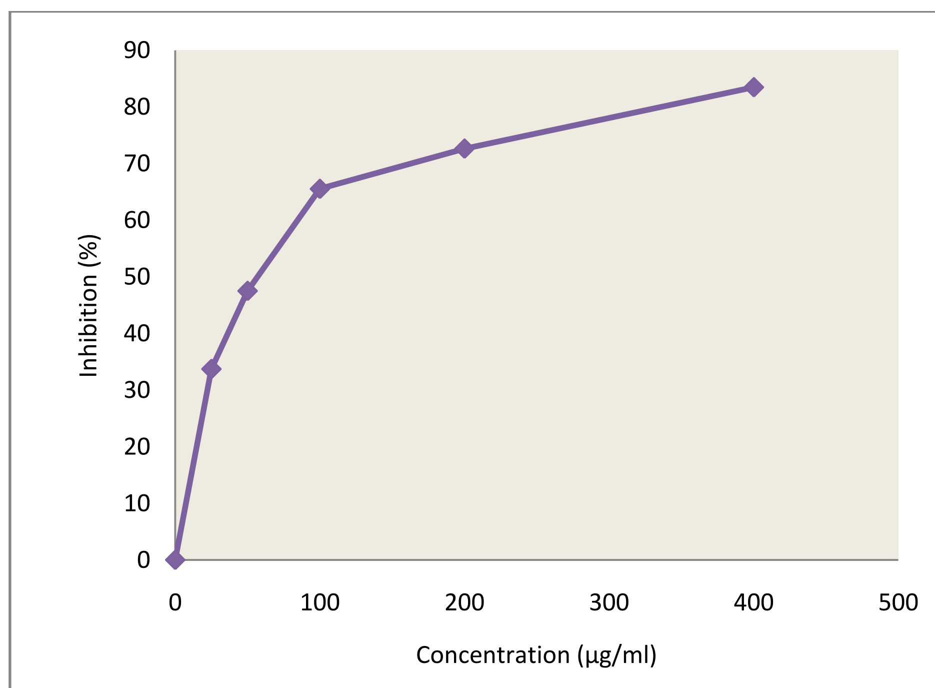


Table – 35 Hydroxyl radical scavenging activity of ethanolic extract of *Anacyclus pyrethrum*

Sl. No.	Concentration (µg/ml)	Inhibition (%)	IC_{50} Value((µg/ml)
1.	25	37.80 ± 0.24	60.14± 0.43
2.	50	48.95 ± 0.46	
3.	100	57.96± 0.31	
4.	200	66.81 ± 0.45	
5.	400	93.20± 0.15	

Values are Mean ± SEM of 3 replicates.

Fig - 34 Hydroxyl radical scavenging activity of ethanolic extract of *Anacyclus pyrethrum*

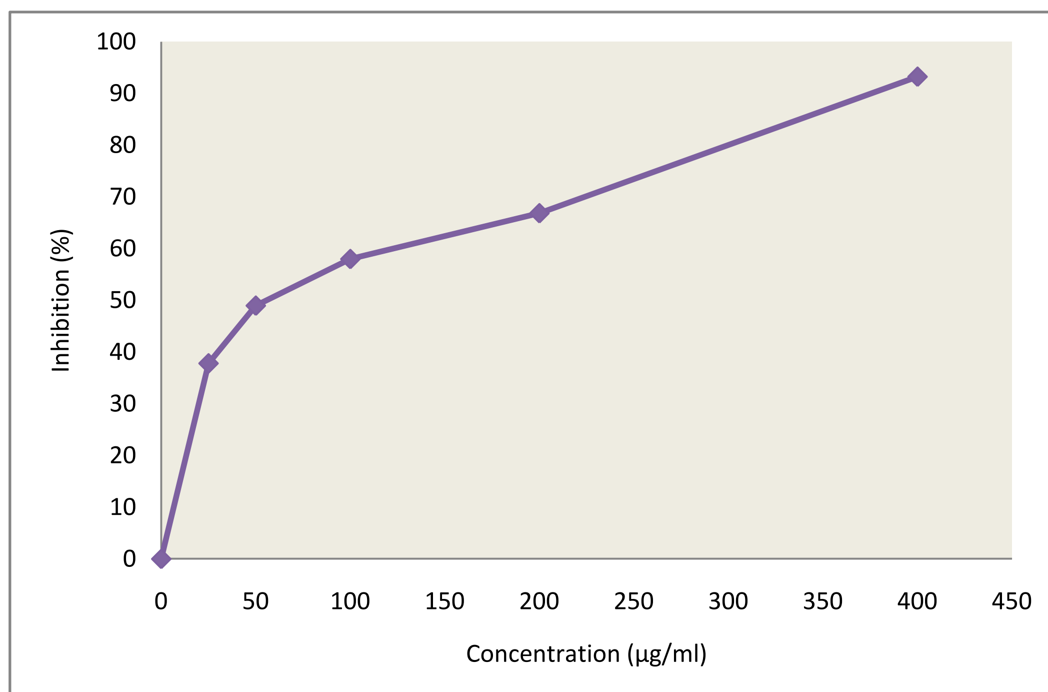


Table – 36 Hydrogen peroxide scavenging activity of ethanolic extract of *Anacyclus pyrethrum*

Sl. No.	Concentration (µg/ml)	Inhibition (%)	IC_{50} Value((µg/ml)
1.	25	32.92 ± 0.83	38.54± 0.94
2.	50	63.14 ± 0.62	
3.	100	74.44± 1.73	
4.	200	87.64 ± 0.29	
5.	400	91.27± 0.68	

Values are Mean ± SEM of 3 replicates.

Fig - 35 Hydrogen peroxide scavenging activity of ethanolic extract of *Anacyclus pyrethrum*

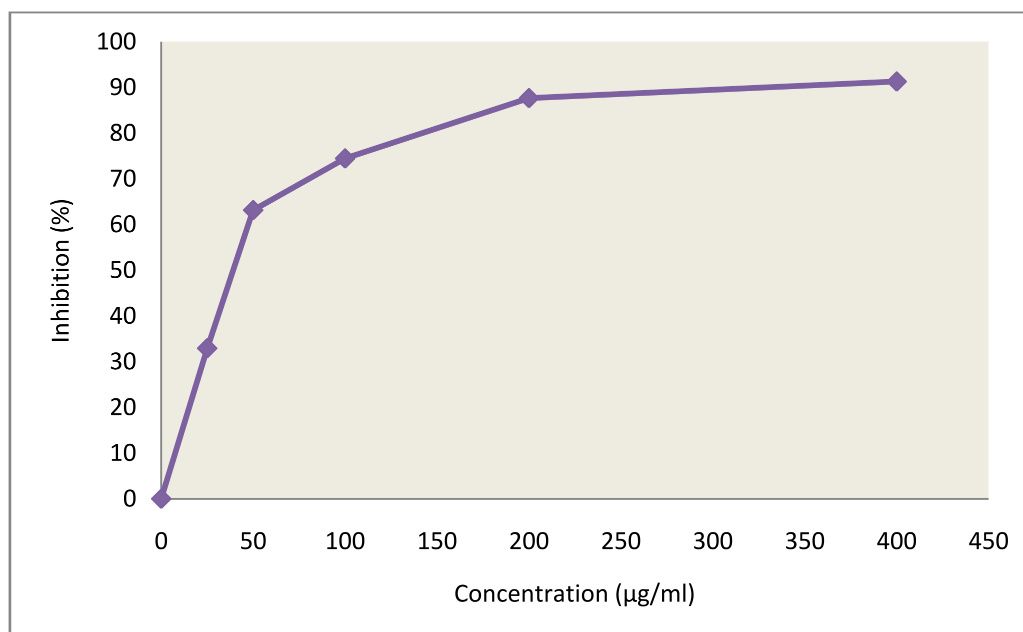


Table - 37 Determination of reducing power of ethanolic extract of *Anacyclus pyrethrum*

Sl. No.	Concentration (µg/ml)	Absorbance
1.	25	0.022 ± 0.001
2.	50	0.078 ± 0.002
3.	100	0.192 ± 0.001
4.	200	0.233 ± 0.012
5.	400	0.241 ± 0.002

Values are Mean ± SEM of 3 replicates.

Fig - 36 Determination of reducing power of ethanolic extract of *Anacyclus pyrethrum*

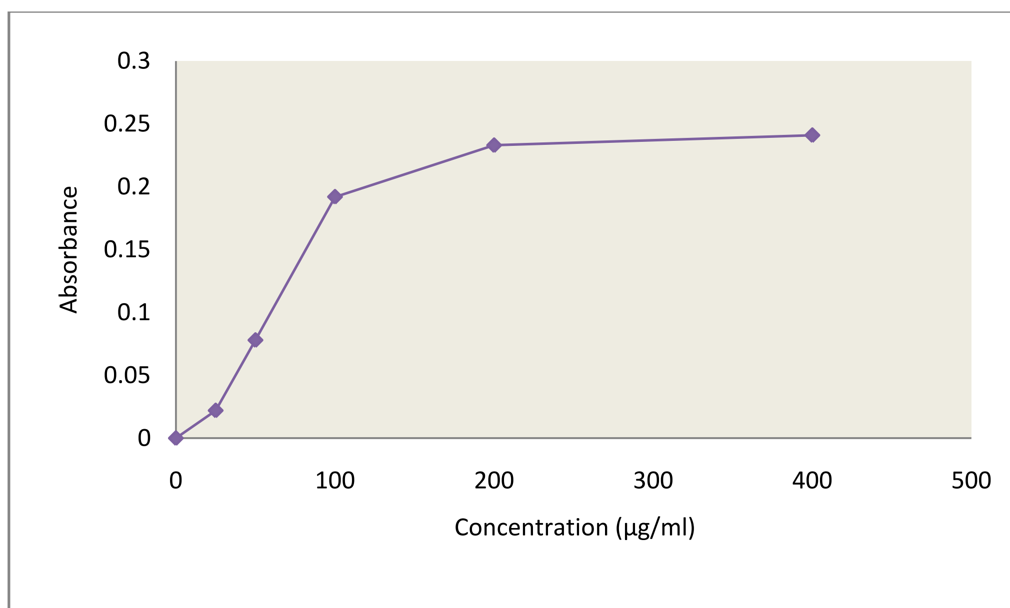
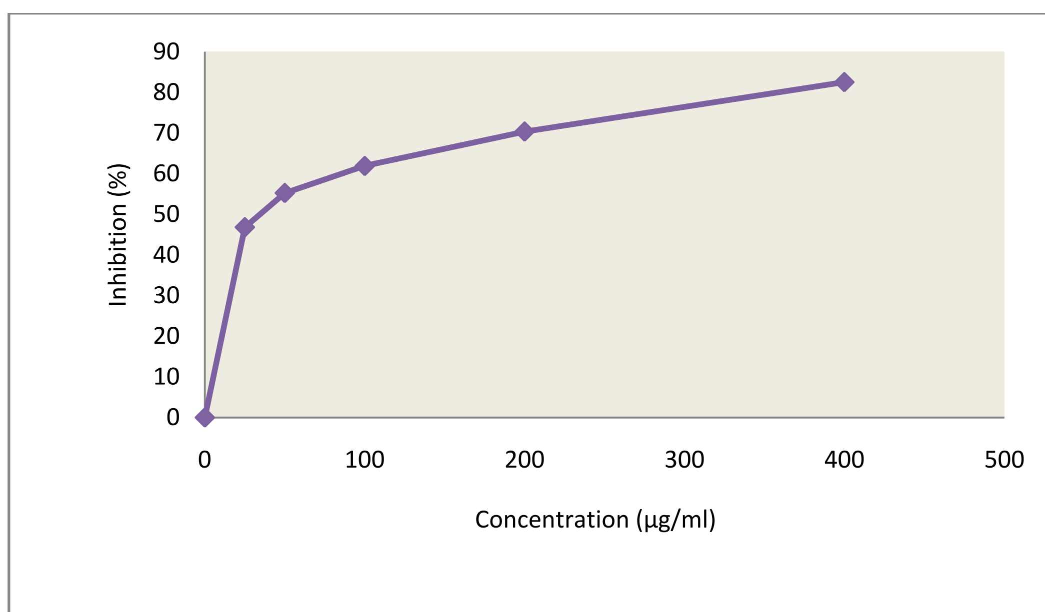


Table –38 Nitric oxide scavenging activity of ethanolic extract of *Anacyclus pyrethrum*

Sl. No.	Concentration (μg/ml)	Inhibition (%)	IC_{50} Value((μg/ml)
1.	25	46.81 ± 0.65	32.61± 1.68
2.	50	55.23 ± 1.50	
3.	100	61.87± 0.33	
4.	200	70.34 ± 1.13	
5.	400	82.50± 1.08	

Values are Mean ± SEM of 3 replicates.

Fig - 37 Nitric oxide scavenging activity of ethanolic extract of *Anacyclus pyrethrum*



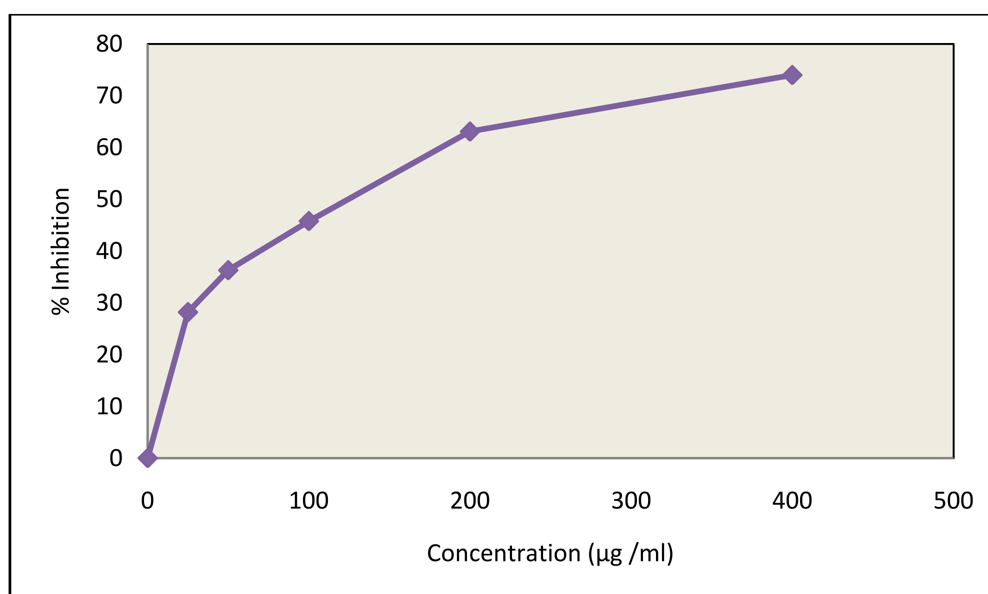
***Exvivo*antioxidant Studies**

Table - 39 Effect of ethanolic extract of *Anacyclus pyrethrum* on inhibition of lipid peroxidation

Sl. No.	Concentration (μg/ml)	% Inhibition
1	25	28.17 ± 0.16
2	50	36.27 ± 0.72
3	100	45.75 ± 0.85
4	200	63.04 ± 0.78
5	400	73.95 ± 0.62
6	Vitamin E (100)	69.23 ± 0.46

Values are Mean ± SEM of 3 replicates.

Fig – 38 Effect of ethanolic extract of *Anacyclus pyrethrum* on inhibition of lipid peroxidation



VII. DISCUSSION

Herbal remedy for human mental illness is much preferred over synthetic pharmaceuticals because of various side effects. Herbal treatment not only advances persevering compliance but furthermore there are possibilities of enhancing the bioavailability of numerous pharmaceuticals. Active constituents extracted from parts of plant origin is beneficial in treatment of mental illness sources have proved to be beneficial.[87] Preliminary phytochemical analysis of hexane, chloroform and ethanolic extract of *Anacyclus pyrethrum* revealed the presence of phytoconstituents.The ethanolic extract of *Anacyclus pyrethrum* has more number of phytoconstituents when compared to other extracts

Recently, the inhibition of acetylcholinesterase enzyme was targeted as a new approach to prevent the progression of Alzheimer disease as it is neurochemically characterized by a consistent deficit in cholinergic neurotransmission. For this reason, symptoms can be treated by the use of agents that retrieve the level of acetylcholine through inhibition of acetylcholinesterase, AChE. In late stages of AD, levels of AChE decline by up to 85% in the brain.[228] The most important strategy to increase cholinergic function is inhibition of acetylcholinesterase. AChE inhibitor is always the target of many Alzheimer dementia drugs.[229] This made us to investigate *in vitro* acetylcholinesterase activity of the extracts by spectrophotometric and TLC bioassay method. This activity is further designed to select the suitable extract with potential acetylcholinesterase inhibitory activity inorder to subject the better extract for *invivo* investigation. The *invitro* acetylcholinesterase inhibitory study showed that chloroform extract at higher dose and ethanolic extract of *Anacyclus pyrethrum*

exhibited dose dependent *invitro* acetylcholinesterase inhibitory effect. From the study, among the different extracts tested for *Anacyclus pyrethrum* it was found that the ethanolic extract of *Anacyclus pyrethrum* indicated higher anti-AChE activity than chloroform extract. Ethanolic extract of *Anacyclus pyrethrum* showed IC_{50} value at 83±1.52µg/ml. *In vitro* analysis confirmed cholinesterase inhibiting properties for the ethanolic extract.

Anacyclus pyrethrum was evaluated by TLC bioautography methods. TLC bioautography of extracts showed that ethanolic extract of *Anacyclus pyrethrum* has significant acetylcholinesterase inhibition compared to other extracts. TLC bioautography of active extract revealed active spots on TLC. The active spots appeared as white spots on yellow background. Ethanolic extract of *Anacyclus pyrethrum* showed more than one active spots compared to other extracts. The TLC assay demonstrated AChE inhibitory activity for ethanolic extract of *Anacyclus pyrethrum*. This inhibition explains the reported memory enhancing effects of this plant. Since ethanolic extract showed better acetylcholinesterase inhibitory activity it was subjected to HPTLC inorder to identify the phytoconstituents.

The demand for phytotherapeutic agents is growing and there is need for their technical validation before plant derived extracts gain broader acceptance and use. These investigations provide a phytochemical basis for these herbs to have vital role in neuroprotection.[86] In HPTLC finger prints of ethanolic extract of *Anacyclus pyrethrum* showed 6 spots. The resolution of spots in TLC and HPTLC shows the presence of various active principles in the extract. The total phenolic, flavonoid, alkaloid and ascorbic acid content in ethanolic extract of *Anacyclus pyrethrum* were

also determined. The ethanolic extract showed better acetylcholinesterase inhibitory activity and it was subjected to further toxicological and pharmacological investigation.

Phytotherapeutic products are often considered as safe because they are natural.[230] However these products may contain bioactive principles with potential to cause adverse effects.[231] In supplement, the poor pharmacovigilance surveillance in this field make it difficult to determine the adverse effects caused by the utilization of phytotherapeutic products.[232] Herbal remedies requires an scientific validation of their efficacies and safety due to their increase use through out the world. Thus all the natural products used in therapeutics must be evaluated for their efficacy and safety tests by the same methods as used for new synthetic drugs.[233] In the present study no mortality was observed during acute toxicity evaluation in the various doses administered. No signs or symptoms of toxicity were observed. The results of the study reveal that the ethanolic extract of *Anacyclus pyrethrum* should be regarded practically as non toxic. The change in body weight has been used as an indicator of potential adverse effect of drugs and chemicals. Results from the body weight of treated groups when compared to control rats also suggest that at sub chronic administration of *Anacyclus pyrethrum* has revealed no effect on the normal growth of rats. The haematological system is the one of the most sensitive targets for toxic compounds and important index of physiological and pathological states in man and animals. In the present study all the haematological parameters remained under the reference range for the rats in both drug treated and control groups. A similar absence of toxic effects was observed in biochemical parameters. There were no significant effect on the levels of AST, ALT, ALP, bilirubin, urea and creatinine, which are good

indicators of liver and kidney functions. This was further confirmed by histological assessment of these organs. There was no treatment related adverse effect of *Anacyclus pyrethrum* extract on the biochemical parameters in rats. However the values were within the normal laboratory range. Results of histological analysis of internal organs revealed that there were no treatment related histopathological findings. All the findings were consistent with normal background lesions in clinically normal rats of age and strain used in this study. Based on the results no observed adverse effect was concluded to groups treated with *Anacyclus pyrethrum* at dose of 1000mg/kg for 90 days. In conclusion the sub chronic oral administration of *Anacyclus pyrethrum* extract at a dose of 1000mg/kg has not produced any significant alteration in the hematological, biochemical parameters and histological observation in albino Wistar rats. This study provides valuable data on toxicity profile of *Anacyclus pyrethrum* roots, which could stand as assurance for medicinal use of this plant for longterm treatment.

General behaviour studies suggest that ethanolic extract does not possess any neurotoxicity The ethanolic extract of *Anacyclus pyrethrum* were found to have no effect on the locomotor activity. Locomotor activity is regarded as an indicator of alertness and a decline in the activity would suggest sedative activity.

Experimental findings suggest the extract did not illustrate any effect on the muscle coordination which was indicated by the outcome with respect to the rotarod experimental test. In our investigation, the extracts did not produce any significant change or increase in the exploratory activity of the rats in the hole board and elevated plus maze method, hence we can conclude that the extract does not possess anxiolytic

activity. Generally most of the anxiolytic agents have an adverse effect on memory as seen with the benzodiazepines, commonly used as anxiolytics.[234]

Alzheimer disease is associated with decline in cognitive abilities, patient also have non cognitive symptoms such as depression, apathy and psychosis that impair learning.[268] The forced swimming test and tail suspension test demonstrated that ethanolic extract of *Anacyclus pyrethrum* clearly acted as antidepressant in rats. The reduction of immobility was comparable to observed effects after administration of reference antidepressant drug imipramine, a putative catecholaminergic involvement in the antidepressant like effects of *Anacyclus pyrethrum* extracts could be suggested. Considering the lack of need of drugs with proven effect in improving learning, specific memory improving and antidepressant effect of *Anacyclus pyrethrum* can be of enormous interest for neurochemical investigation which can unravel the mechanism of action of plant drug with respect to activity.

Alzheimer disease is a neurodegenerative disorder without an effective treatment. Progressive memory loss, dementia, cognitive deficit are curently seen as medical and social problems of disastrous dimension.[235] The administration of antimuscarnic agent scopolamine produces transient memory deficit. Scopolamine amnesia test is commonly used as an important screening test for antiAlzheimer drug.[236] The result of present study suggests that ethanolic extract of *Anacyclus pyrethrum* possess memory enhancing activity in scopolamine induced amnesia model. *Anacyclus pyrethrum* extract treated rats showed decrease in transfer latency in elevated plus maze model paradigm which is an indicative of cognition improvement. In case of passive avoidance paradigm administration of *Anacyclus*

pyrethrum extract for 14 days exhibited prounced effect in reversal of scopolamine induced amnesia which was revealed by increase in step down latency. Acetylcholine is considered as the most important neurotransmitter involved in the regulation of cognitive functions. Cholinergic neurons plays important role in cognitive deficit associated with Alzheimer disease and neurodegenerative disease.[237] It has been demonstrated that impairment in learning, memory and behavior observed in the patients with dementia are caused at least by changes with in cholinergic system.[238] Facilitation of central cholinergic activity improves memory.[239]

In the present study *Anacyclus pyrethrum* inhibited acetylcholinestrease enzyme, there by elevating acetylcholine concentration in the brain homogenate and ultimately improved memory in rats. It had been suggested that the varying degrees of behavioral impairments are associated with aging and age associated neurodegenerative diseases. Oxidative stress due to free radicals generation is responsible for producing the neuronal changes mediating these behavioral deficits.[240] Oxidative stress in brain generates oxygen radicals like superoxide anion, hydroxyl radical and hydrogen peroxide, which act on polyunsaturated fatty acids in brain, there by propagating the lipid peroxidation.[241] The oxidative free radical scavenging enzymes like glutathione, SOD and catalase plays an important role to reduce oxidation stress in brain.

The treatment with scopolamine resulted in a significant increase in lipid damage which was determined by estimation of MDA level, which is a measure of lipid peroxidation and free radical generation. Elevation of brain oxidative status of amnesic rats resembled the clinical situation where considerable studies reported the

incidence of oxidative stress and membrane lipid peroxidation in demented patients.[242] More specifically, the entire brain of patients with Alzheimer's disease (AD) was shown to be subjected to an oxidative challenge.[243] In addition, the overall peroxidation activity in brains of AD patients was significantly elevated compared to normal subjects.[244] Inhibition of MDA by *Anacyclus pyrethrum* suggests its neuroprotective properties.

The status of endogenous antioxidant enzymes were investigated as free radicals mediate oxidative damage. The antioxidant enzymes like SOD, GPx, GRD and GSH serves as the first line of protection against free radical damage during the oxidative stress conditions. Scopolamine induced a significant decrease in the enzymatic activity of antioxidant enzymes like GRD and SOD, but there was only slight change in GPx activity. *Anacyclus pyrethrum* at a dose of 50 mg/kg, 100mg/kg, 200mg/kg and also piracetam inhibited the decrease in the activity of GRD, whereas reduced SOD activity induced by scopolamine was not only restored, but also increased higher than that of normal control rats. Studies have also reported significantly lower levels of SOD activity than that of non-demented control in the cerebellum, frontal cortex and hippocampus.[245]

Intracellular GSH status served as a sensitive indicator of the health of a cell or tissue. Our results indicate that GSH levels decreased significantly after scopolamine treatment. There was a significant reduction in levels of glutathione, a tripeptide found in all cells, which reacts with free radicals to protect cells from superoxide radical, hydroxyl radical and singlet oxygen.[246] Depletion of brain GSH level due to the treatment with scopolamine was restored significantly by *Anacyclus*

pyrethrum. Thus, it can be postulated that *Anacyclus pyrethrum* scavenges ROS and exhibit a protective effect against oxidative damage induced by scopolamine by restoring the activities of glutathione reductase and SOD. SOD is the only enzyme that uses the superoxide anions as the substrate and produces hydrogen peroxide as a metabolite. Super oxide anion is more toxic than H_2O_2 and has to be removed. Pretreatment with *Anacyclus pyrethrum* significantly prevented the reduction of SOD activity in brain during scopolamine treatment. The cognitive enhancing activities of *Anacyclus pyrethrum* might be due to the inhibition of AChE activity and the decrease in ROS by restoring the antioxidative defense system. In this study, the resultant elevation in brain oxidative status after administration of amnestic dose of scopolamine may further substantiate the value of scopolamine induced amnesia as an animal model to test for drugs with prominent therapeutic benefit in dementia.

In addition, *Anacyclus pyrethrum* extract, as an antioxidant medication may be of value for demented elderly patients with elevated brain oxidative status. Since depression usually coexists with dementia, *Anacyclus pyrethrum* extract as an antidepressant medication with added advantage of preventing oxidative stress could be a better alternative for depressed demented patients. This study may also provide more evidence that *Anacyclus pyrethrum* extract, as an antioxidant medication, can be a novel type of antidepressant with memory enhancing properties.

The neurotransmitters play a vital role in cognitive and hence we have estimated the neurotransmitters. Our findings indicated that ethanolic extract of *Anacyclus Pyrethrum* treated rats show remarkable dose dependent reduction in transfer latency, indicating significant improvement in memory, thus demonstrating

nootropic activity. This probably may be attributed to the involvement of neurotransmitters since the building of memory is augmented only when the levels of neurotransmitters are attenuated on repeated administration of the extracts. There is ample evidence demonstrating that the central cholinergic system, serotonergic transmission and dopaminergic function play a vital role in the cognitive function of the brain. Ethanolic extract of *Anacyclus pyrethrum* had significant effect on the brain dopamine concentration and was significantly reduced in dose dependent manner. The decrease observed in the dopamine levels can be correlated to the improved learning and memory process in the drug treated rats. Studies using dopamine agonists as well as electrical stimulation suggested that heightened activity in the dopaminergic system during learning and memory process might lead to retention failure.[247]

A reciprocal relationship between serotonin and dopamine has been demonstrated in many brain areas. Apomorphine, a dopamine agonist administered before and immediately after training on a passive avoidance step-through task, was found to produce marked amnesia when tested for 24 hour retention.Similar results were observed with the electrical stimulation of the substantia nigra.[248] The data on brain dopamine content in present study also support the above reports suggesting that a decreased dopaminergic activity is favourable for improved learning and memory process. It has been indicated that an increase in serotonergic transmission can interfere with learning acquisition and memory consolidation.[249] It was reported that treatments augmenting brain 5-HT activity can both improve and alter performance in aversive learning tasks where multiple 5-HT receptors are involved with functions that usually vary at different stages of information processing.[250] Treatments affecting brain 5-HT activity were nevertheless, reported to play a minor role in memory testing

paradigms since tiapentine, a serotonergic 5-HT re-uptake enhancer did not affect reference spatial memory test. [251] In the present study ethanolic extract of *Anacyclus Pyrethrum* increased the level of serotonin when compared to the control group. This suggest the involvement of serotonergic mechanisms in the cognitive enhancing properties of *Anacyclus pyrethrum* extract since cortical and hippocampal areas of the brain are innervated by serotonergic pathways and are known to be involved in controlling learning and memory processes.[252] Ethanolic extract of *Anacyclus Pyrethrum* at a dose level of 50,100 and 200 mg/ kg, p.o treatment for 14 days did not reveal any significant change in glutamate levels compared to their corresponding control treated groups.

Furthermore, the lack of effect on locomotor activity works to the benefit of the plant illustrating nootropic activity. *Anacyclus pyrethrum* has been demonstrated to ameliorate cognitive processes, not only preventing amnesia induced by pharmacological treatments in elevated plus maze and passive avoidance test , but also by producing facilitation of social memory in a social learning task which demonstrates that the extract displays memory enhancing properties.

Herbal compounds demonstrating antioxidant activity have been reported to ameliorate disturbed cognitive function in both humans and animals.[253] Many actions have been correlated with their ability to scavenge oxygen generated free radicals and to inhibit lipid peroxidation *in vitro.*[254] Free radicals are essential to different biochemical process and indicate a vital part of aerobic life and metabolism. Due to their high reactivity and low stability, reactive oxygen species generates oxidized metabolites and DNA adducts.[255] The ROS causes a progressive functional

deterioration of cells, tissues and organ systems.[256] The unifying theory of oxidative damage provides a clear and widely accepted mechanism responsible for different diseases that ranges from atherosclerosis, inflammations, neurodegenerative disorders and also cancer. Commercially present antioxidants produced through different chemical process usually possess very strong and unspecific antioxidative effects.[257] This position motivates research on naturally occuring antioxidants that can be developed from plants, organisms and which has the potential to be developed with efficient systems that can protect against environmental oxidative stress. [258] The free radical scavenging activity of the roots of ethanolic extract of *Anacyclus pyrethrum* was analysed based on the capability to quench the synthetic DPPH and the extract showed concentration dependent activity.The bleaching of DPPH absorption representative of the capacity of the test compound to scavenge free radical independently.[259]

Hydroxyl radical is a principle contributor for tissue damage. The formation of hydroxyl radical from fenton reaction was determined using 2, deoxy -D-ribose degradation. Studies with ethanolic extract of *Anacyclus pyrethrum* have revealed significant hydroxyl scavenging activity in *invitro*. Ethanolic extract of *Anacyclus pyrethrum* was capable of scavenging hydroxyl radical in a dose dependent manner.

H_2O_2 can generate hydroxyl radical via fenton reaction. In addition H_2O_2 can easily cross the cell membrane and exerts an injurious effect of tissue through a number of different mechanisms, such as perturbing intracellular Ca^{2+} monostat, increased intracellular ATP inducing DNA damage and inducing aptotosis. Hydrogen peroxide though not very reactive, but it can occasionally it can be toxic to cell

because it can give rise to hydroxyl radical in the cells. Thus the removal of H_2O_2 is very important for antioxidant defence in cell or food systems. H_2O_2 can cross membranes and may oxidize a number of compounds. Ethanolic extract of *Anacyclus pyrethrum* was capable of scavenging hydrogen peroxide in a dose dependent manner.

Sodium nitroprusside serves as a chief source for NO radicals. The absorbance of the chromophore produced during diazotization of the nitrite with sulphanilamide and further coupling with napthylethylene diamine was measured at 546 nm. The chromophore formation is not complete in the presence of ethanolic extract of *Anacyclus pyrethrum*, as it scavenges the NO thus produced from the sodium nitroprusside. Nitric oxide is a potent regulator of many physiological processes and control of cell mediated toxicity. It is a free radical which as a vital roles as an effector molecules in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumour activities.

Scavengers of nitric oxide compete with oxygen leading to the reduced production of nitric oxide. Our finding suggests that the phenolic compounds present in the extract might be responsible for nitricoxide scavenging effect. Hence the scavenging activity of ethanolic extract of *Anacyclus pyrethrum* increases in dose dependent manner.[260]

Reducing power of ethanolic extract of *Anacyclus pyrethrum* increased with increased concentration of test compound. The reducing capacity of a compound may provide a significant indication of its potential antioxidant activity.[261]The reducing ability is usually affiliated with the presence of reductones, which breaks the free

radical chain by donating a hydrogen atom. The extract had reductive ability which increased with increasing concentrations of the extract.

Oxidative stress can lead to peroxidation of cellular lipids and lipid peroxidation (LPO) has been implicated in the pathogenesis of number of diseases including neurodegenerative disorders. It is well found that bioenzymes are very much suspectible to LPO which is regarded to be the crucial part of much toxic as well as as degenerative process. Ethanolic extract of *Anacyclus pyrethrum* inhibits the rate of lipid peroxidation by a reduction in the red color complex formed reflecting its anti lipid peroxidative potential.

The extract showed a significant protection against Fe^{2+}/ascorbic acid induced lipid peroxidation that could be caused by absence of ferryl-perferyl complex. It is generally assumed that ability of the plant phenolic compounds such as flavanoids to chelate ions in the LPO system is very important for their antioxidant property. Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups. Total amount of phenolic, flavonoid and ascorbic acid compounds in extract was investigated. Recent reports reveal alkaloids can be used as good source of natural antioxidants.[262] The preliminary phytochemical analysis of *Anacyclus pyrethrum* root also revealed the presence of alkaloids and tannins. In conclusion, the high antioxidant potential of the *Anacyclus pyrethrum* root extract may due to their phytochemical constituents and provides a justification for memory enhancing and antidepressant activity.

The present findings indicate improvement of learning acquisition and observed antidepressant property of *Anacyclus pyrethrum* root extract, there by

validating its claim as a nervine tonic in the Indian system of medicine. These findings suggest the possible neuroprotective role for *Anacyclus pyrethrum,* therefore it seem that *Anacyclus pyrethrum* may prove to be useful as anti Alzheimer agent in view of its memory enhancing property observed in the present study. In traditional medicine, it is conventional to utilize herbs for the achievement of treatment purposes and simultaneously to enhance beneficial effect without causing severe side effects. *Anacyclus pyrethrum* could be considered as a therapeutic agent to prevent or slow down the development of neurodegenerative diseases such as AD at an early stage. *Anacyclus pyrethrum* extract has potential therapeutic effect on improving antiamnesic activity in rats through inhibiting lipid peroxidation, augmenting endogenous antioxidant enzyme and altering the levels of neurotransmitters and decreasing the acetyl cholinesterase activity in brain. Further study is warranted for its potential use in human.

VII. SUMMARY AND CONCLUSION

Medicinal herbs are indispensible part of traditional medicine practiced all over the world due to easy access, low cost, least risk and low side effect profile. *Anacyclus pyrethrum*, Family Asteraceae is used in traditional system of medicine and it is regarded as a tonic to the nervous system. The plant roots are reported for antibacterial, anti-inflammatory, immunostimulating and aphrodisiac activities. As root of this plant has potential health benefits, it can be better utilized for nutraceutical and functional food formulations. The present work was undertaken to study neuropharmacological role of *Anacyclus pyrethrum* in consideration to memory improvement activity to treat the diseases related to cognition and use in learning and memory processes.

- In phytochemical analysis ethanolic extract of roots of *Anacyclus pyrethrum* showed more number of phytoconstituents.
- Ethanolic extract of *Anacyclus pyrethrum* was found have significant inhibition on acetylcholinesterase activity by spectrophotometric and TLC plate bioassay detection for AChE inhibition.
- TLC and HPTLC of ethanolic extract of *Anacyclus pyrethrum* were done. The good resolution of spots in TLC and HPTLC shows the presence of various active principles in the extract.
- Total phenolic, flavanoid, ascorbic content in ethanolic extract of *Anacyclus pyrethrum* was quantified.

- In the acute toxicity study, ethanolic extract of *Anacyclus pyrethrum* were found to be non-toxic and did not produce any adverse effects upto a dose level of 2000 mg/kg bodyweight.

- In the chronic toxicity study for 90 days extract treated groups showed no changes in body weight, food and water intake as compared to control group. There was no treatment related adverse effect of *Anacyclus pyrethrum* extract on haematological, biochemical parameters and histopathological studies in rats.

- General behaviour studies on ethanolic extract of *Anacyclus pyrethrum* has not shown any deviation in treated rats from normal behaviour and suggests that ethanolic extract does not possess any neurotoxicity.

- The experimental observation using actophotometer shows that extracts of *Anacyclus pyrethrum* did not produce significant effect on the locomotor activity.

- Experimental findings on ethanolic extract of *Anacyclus pyrethrum* suggest the extracts did not demonstrate any effect on the muscle coordination, as indicated by the findings with respect to the rotarod experiment.

- Study of exploratory activity using hole board apparatus and elevated plus maze in rats reveals that ethanolic extract of *Anacyclus pyrethrum* treated rats does not possess anxiolytic activity.

- The antidepressant evaluation models using forced swim test and tail suspension test demonstrated that ethanolic extract of *Anacyclus pyrethrum* clearly posses an antidepressant action in rats.

- Effect of ethanolic extract of *Anacyclus pyrethrum* on scopolamine induced interoceptive behavior models revealed the antiamnesic potential of *Anacyclus pyrethrum.*

- In the present study *Anacyclus pyrethrum* inhibited acetylcholinestrease enzyme, there by elevating acetylcholine concentration in the brain homogenate and ultimately improved memory in rats treated with scopolamine.

- Decreased activities of enzymatic and non-enzymatic antioxidants together with increased levels of lipid peroxidation in rat brain seen in scopolamine induced interoceptive behavior models in rats and were brought back to normal levels by the administration of ethanolic extract of *Anacyclus pyrethrum*.

- Rats treated with ethanolic extract of *Anacyclus pyrethrum* in extroceptive behaviour models for learning and memory using social test model demostrated that ethanolic extract of *Anacyclus pyrethrum* display short term social memory enhancing property.

- Rats treated with ethanolic extract of *Anacyclus pyrethrum* in extroceptive behaviour models for learning and memory using elevated

plus maze revealed significant improvement in memory thus demonstrating nootropic activity.

- Repeated administration of the extracts showed significant changes in neurotransmitter level. There was decrease in dopamine level and increase in serotonin level when compared to control group and there were no effect on level of glutamate on repeated administration of extract.

- Ethanolic extract of *Anacyclus pyrethrum* showed good antioxidant property, when studied by the *in vitro* experimental methods such as DPPH scavenging activity, hydroxyl radical scavenging activity, hydrogen peroxide scavenging activity, nitric oxide scavenging activity and also reducing power of the extract is associated with its antioxidant property.

- *Exvivo* methods such as lipid peroxidation assay also suggest antioxidant potential of the extract.

In conclusion, the present study revealed ethanolic extract of *Anacyclus pyrethrum* have memory enhancing property, antidepressant activity and neuroprotective role which may be due to its anticholinesterase potential, effect on neurotransmitters such as dopamine and serotonin level and its antioxidant profile, therefore it seem that *Anacyclus pyrethrum* may prove to be useful in treatment of dementia of Alzhemier diseases. Further studies are to be warranted for its safety, efficacy and mechanism of action for its therapeutic potential.

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