"Evaluation of *Cynodon dactylon* L. and *Sida rhombifolia* L. on cognitive dysfunction in rats"

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For the award of the degree of

Doctor of Philosophy

In the

Faculty of Pharmacy

By

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(Registration No: KLEU/Ph.D./17-18/D01217013)

Under the Guidance of

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Mrs. Laxmi A. Pattanashetti

ABSTRACT

Background

Most common form of dementia is Alzheimer's disease, is an incurable disease affecting elder population. For the management of dementia symptoms, symptomatic medications are administered; however, persistent use of these medications is linked with undesirable effects. Traditional neurotherapeutics are in the pipeline of modern therapy to halt the progression of AD.

Medicinal plants *Cynodon dactylon* L. and *Sida rhombifolia* L. have been reported with pharmacological activities viz, hemorrhages, dysentery, hyperdipsia anti-inflammatory, wound healing, hepatoprotective. Traditionally, selected plants are claimed as brain and heart tonic, however preclinically both the plants have limited reports on the management of dementia hence, current study explored to evaluate the efficacy in treatment of dementia in rats.

Aims & Objectives:

Evaluation of effect of "hydro-ethanolic extract of *Cynodon dactylon* L. and *Sida rhombifolia* L. against cognitive dysfunction integrated by *in-silico* methods and experimental models (*in-vitro, in-vivo*).

Methodology:

Reported phytoconstituents of both selected plants were predicted for their targets, enriched by STRING; expected regulated pathways were traced related to KEGG and molecular docking was performed using Autodock vina, and Autodock 4. Later the phytochemical-profile of plant extracts were confirmed via total phenolic and flavonoid

content assay. The phytochemical analysis was performed using LC_MS analysis. Antioxidant activity was carried out using '2, 2-diphenyl-1-picrylhydrazyl', 'hydrogen peroxide' assay by *in-vitro* methods. The *in-vivo* study was performed in the scopolamine induced amnesia model. Scopolamine (1mg/kg) was administered intra- peritonially for 30 days, meanwhile hydro-ethanolic extract of *Cynodon dactylon* therapeutic doses (100, 200, 400 mg/kg), *Sida rhombifolia* (100, 200, 400 mg/kg), Donepezil (3 mg/kg) were administered orally for 15-days. Response to treatment was assessed by cognitive models namely "Morris Water Maze, Elevated Plus Maze, Passive Avoidance Task", Histological changes, determining Acetylcholinesterase (AchE), Beta-amyloid 1-42, Malondialdehyde (MDA), reduced Glutathione (GSH) levels.

Results:

Bio actives of *C. dactylon* primarily modulated acetylcholinesterase activity MAO-B and MAO-B pathways, by connecting with ACHE, BCHE, ADORA2A targets, whereas *S. rhombifolia* regulated cholinergic, Ca+2, dopaminergic, gap junction pathways. Reports of *In-silico*, *in-vitro* pharmacology revealed that hydro-ethanolic extract showed highest free radical scavenging activity, and acetylcholinesterase enzyme inhibition. Along with this *in-vivo* reports support the ameliorative effect on scopolamine induced amnesia in rats by modulated cognitive functions promoting memory enhancing activity (long term, short term, and fear aggravating task) significantly. Neurological hallmarks; acetylcholinesterase enzyme, and beta amyloid $_{1\text{-}42}$ were significantly decreased exploring neuroprotection.

Conclusion:

The anti-amnesic activity of *C. dactylon* could be due to via acetylcholinesterase enzyme inhibition signaling pathway, enhancing antioxidant potential, and decreasing insoluble beta amyloid content. However *S. rhombifolia* proposed cognitive improvement through Ca+2,

cholinergic, dopaminergic, gap-junction signalling pathways and also ameliorates the cognitive dysregulation caused by scopolamine. Both medicinal plants depicted multiple molecular pathways that had links with specific proteins and targets in competence to modify the disease.

Keywords: Alzheimer's Disease, Dementia, *Cynodon dactylon* L, *Sida rhombifolia* L, Acetylcholinesterase, Beta-amyloid 1-42, network pharmacology, molecular docking.

LIST OF ABBREVIATIONS

ACh : Acetylcholine, **AChE**: Acetylcholinesterase, **AD** : Alzheimer's disease, **ADORA2A** : Adenosine A2A receptor gene, **Aβ** : Beta amyloid, **ANOVA** : Analysis of variance, **APP** : Amyloid Precursor Protein, **ApoE** :Apolipoprotein E, **ATP**: Adenosine triphosphate (ATP) **AICD**: APP intracellular C-terminal domain, **BChE**: Butyrylcholinesterase, **BACE1**: Beta-secretase 1, **CNS** :Central Nervous System, **CHRM1**: Muscarinic acetylcholine receptor M1,**CHRM3**: Muscarinic acetylcholine receptor M3,**ChEBI** :Chemical Entities of Biological Interest**,CAT**: Catalase, **DPPH**: 2, 2-diphenyl-1-picrylhydrazyl, **DA** : Dopamine, **DSM-IV**: Diagnostic and Statistical Manual of Mental Disorders-IV, **ELT**: Escape Latency Time, **EPM** : Elevated Plus Maze, **FDA**: Food and Drug Administration, **GABA** : Gamma Amino Butyric Acid, **GSH**: Glutathione, **5-HT** : 5- Hydroxy Tryptamine, **HTR2A** : 5-hydroxytryptamine receptor 2A, **IAEC** : Institutional Animal Ethical Committee, **IC50**: Half maximal inhibitory concentration, **KEEG**: Kyoto Encyclopedia of Genes and Genomes, **MWM** : Morris Water Maze, **MDA**: Malondialdehyde, **MAOA** : Monoamine oxidase A,**MAOB** : Monoamine oxidase B, **mg/kg**: Milligram per kilogram, **Min**: Minutes, **ml/kg**: Milliliter per kilogram, **NA** : Nor-adrenaline, **NMDA** : N-methyl-D-aspartate, **NOS**: Nitric oxide synthase, **nm**: Nanometer, **NFT** : Neurofibrillary Tangles, **NOS** : Nitric oxide synthase, **p.o**: per oral, **PCR**: Polymerase chain reaction, **ROS** : Reactive oxygen species, $\mathbf{R^2}$:Coefficient of regression **ROS**: Reactive oxygen species, $\mathbf{R_t}$:Retention time, **s.c.:** Sub cutaneous, **S.E.M** :Standard Error Mean, **SCO**: Scopolamine, **STL** : Step through latency, **TL** : Transfer Latency, **TBARS**: Thiobarbituric acid reactive substances, **TFC**: Total flavonoid content, **TPC**: Total phenolic content, **TRPA1** : Transient Receptor Potential Ankyrin-1, **Uniprot**: Universal Protein Database program.

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1. INTRODUCTION

1.1 Background

Brain has the most challenging task to store memory which includes several neural circuits and neurotransmitter. The two basic components of cognitive skills are learning and memory that allow to store information from our past experiences. Learning is the process of acquiring new knowledge about what is going on in the world, around a person and memory is the process of retrieving that information. Memory impairment is a type of organic brain condition characterized as "a loss of intellectual capacity severe enough to interfere with occupational functioning, conventional social activities, or a person's relationship in the absence of extensive clouding of awareness or physical participation." [1]

Cognition refers to the processing of data in a broad perspective. It refers to a high degree of processing of specialized information, such as creative thinking, memory, perception, motivation, skill-full motions and language. The hippocampus houses neuronal circuitry that is important for cognitive activities including learning and remembering.[2]

Dementia is a broad term to explain the mental dysfunction including loss of memory which is enough to affect and disturb the person's daily tasks.[3] Six regions such as language, learning and memory, perceptual-motor, social cognition and executive function in the brain are influenced in dementia cases. [4]

The presence of intracellular neurofibrillary-tangles, insoluble amyloid-beta (Aβ) formation in the extracellular regions results in synaptic loss. Neuronal death are considered as cardinal hallmarks in gradual decline of cognition in AD patients.[5]

World Alzheimer's Report gives evidence that around 35.6 million (2010) are in burden of dementia which is expected to increase by 65.7 million (2030) and in 2050, 115.4 million may be affected. [6]

Alzheimer's disease is a pervasive cause in elderly patients which was recognized by Alois Alzheimer in 1907.Although understanding of the aetiology and pathophysiology of the illness is regularly updated, diagnostic capability and the development of pharmacological medicines that might halt or better prevent the disease remain limited. Alzheimer's illness has officially been declared incurable. Existing medications, despite considerable preclinical and clinical research, only provide minimal symptomatic alleviation to a limited percentage of people and do not address the disease's core causes. The failure is most likely owing to a lack of understanding the cellular as well as molecular pathways associated with AD progression, as well as authorized medications that affect both cholinergic and glutamatergic neurotransmission. Numerous novel medications under development, gives attempt to alter the disease progression via affecting multiple complex brain alterations induced by Alzheimer's disease. Further these alterations might be used to develop novel medications to halt or reduce disease development. It is recently acknowledged that AD is a complex illness shows characteristic features of Senile Plaques (SPs) due to deposition of amyloid (Aβ), Neurofibrillary tangle (NFT) formation, extensive mitochondrial damage, oxidative stress, neuroinflammation, glutamate excitotoxicity, and other pathogenic features. As a result hyperphosphorylation of protein tau is formed and this leads to synaptic loss and neuronal dysfunction. [7, 8]

Many key insights have been gained in recent decades from research in neuropathology, genetics, cellular biology and molecular biology. Several theories have been proposed. The "cholinergic theory of age-related cognitive failure" was reinforced by evidence from 1980s showing cholinergic neurons loss of basal forebrain selectively, which were treated with acetylcholinesterase inhibitors.[9] The "deficient neurotrophic theory" was motivated by the selective cholinergic neurons loss as well as 'Nerve Growth Factor' (NGF). [10] Most compelling idea, the amyloid cascade hypothesis, which was initially postulated more than 25 years ago, is backed up by a slew of evidence.[11]Due to duplication and mutation of Amyloid Precursor Protein especially, genes Presenilin 1 & 2 (PSEN1, 2) that encodes enzyme γ – secretase which processes (Amyloid Precursor Protein (APP). Genetic studies were extremely important, pointing APP in Familial AD (FAD) and Down's syndrome (DS).[12]The "tau hypothesis" emphasized the importance of tau-related disease and its link to dementia. The "inflammatory hypothesis" is supported by recent interest in inducing microglia activation in the AD brain.[13]

Drugs approved by 'US FDA' for the treatment of AD namely; class of cholinergic activators (Ex. Tacrine), Glutamate antagonists (Mementine) & CNS stimulants (Amphetamine) provide partial improvement in cognition. Conversely, on a long term therapy variable side effects are common. In AD other chemical messengers (Noradrenaline, Dopamine and Serotonin) take a role but their efficacy remains indistinct. [14, 15]

To modulate cognition in AD patients, Acetylcholinesterase enzyme (AchE) inhibitors are another alternative to boost Acetylcholine (Ach) levels to improve the cholinergic transmission apart from Ach agonists.[16] One of the non-competitive and

reversible most commonly prescribed AchE inhibitor to treat mild to moderate AD is Donepezil which is chosen as first line treatment has been stated as neuroprotective by its cholinergic and anti-inflammatory pathway mechanisms. However, its protective action only maintained for 36 months due to its peripheral cholinergic side effects on vital organs.[17]

Despite substantial study and advancement in scientific insight into the molecular structure and receptor expression, developments in medical technology and breakthroughs in nanotechnology-based techniques, many cognitive and central nervous system (CNS)-related illnesses remain untreated.

Medicinal herbs have been investigated scientifically for the treatment of many ailments, including CNS problems since ancient times. Nowadays, pharmaceutical companies are converging the secondary metabolites generated by plants in order to access lead compounds. In various research, medicinal plants including *Centella asiatica, Bacopa monnieri, Withania somnifera, Salvia officinalis, Ginkgo biloba, Melissa officinalis, Glycyrrhiza glabra*, and *Tinospora cordifolia* were reported to manage Alzheimer's disease and other disorders.[18]

In the recent trends “Network Pharmacology” has flourished as an emerging tool to address multi-complex diseases by targeting their pathogenesis with specific mechanisms of drug actions. To optimize multiple drug’s molecular mechanisms especially for traditional medicines it has made great contributions to elucidate underlying therapeutic targets.[19]

The perennial grass *Cynodon dactylon L.*has been well documented in the Indian system of Medicine for its various medicinal properties. This grass is

commonly known as Doob, Bermuda grass belongs to the Poaceae family. In the light of traditional insight, *C. dactylon* is used to cure haemorrhages, dysentery, wounds, diarrhoea and hyperdipsia. Fresh juice is used to treat catarrhal ophthalmia, dropsy and anasarca. Decoction is used to treat secondary syphilis and vesical calculus.[20] This grass has been described as a heart and brain tonic in the Unani system of medicine.[21]Nevertheless, scientifically in preclinical literature its potential to treat cognitive impairment and mechanism of action is still not explored.

An ancient Indian system of medicine 'Ayurveda' records of the plant genus *Sida* L. is widely described. Approximately 17 species native to India have been used in Rasayana for centuries to cure a variety of diseases, including degenerative disorders. [22] Perennial plant arrow leaf *Sida* scientifically known as *Sida rhombifolia* L. belonging to Malvaceae family has been recognized as hepatoprotective, anti-inflammatory, anti-diabetic, antioxidants and anti-arthritic.[23, 24] For decades, *S. rhombifolia* hot aqueous extract have been utilized in India as tonic, aphrodisiac, as well as to treat cardiac and nerve disorders.[25] Yet, evidence scientifically supporting use of *S. rhombifolia* in management of Alzheimer's disease is limited in preclinical studies.

1.2 Justification for the study

Current therapies do not cure Alzheimer's disease and treat symptoms with numerous adverse effects. So, there is a need for possible alternative treatments for memory deficit.

As per traditional Unani healthcare system, *C. dactylo*n considered as brain tonic. [21] Recently research of *C. dactylon* aqueous extract found to enhance antioxidant status in the rat brain area and activates membrane-bound enzymes (Na+/K+ ATPase and Mg2+ ATPase).[24]This was supported by an earlier report of Rai D K *et al,.*[138]who explained that the aqueous extract of *C. dactylon* ameliorated carbofuran- induced oxidative stress and inhibited acetylcholinesterase enzyme in rat brain. Another report of Poojary R *et al,.*[134]addressed the *C. dactylon* antioxidant potential by scavenging ABTS in scopolamine induced amnesia in rats. Bhalerao *et al,.*[16] explained compelling antioxidant effect of *C. dactylon* ethanolic extract (aerial parts) by DPPH scavenging, nitric oxide inhibition.

Ethanolic extract of *S. rhombifolia* contributes to reducing inflammation and inhibits edema which could have an effect on neuroinflammation as per Logeswari *et al,.* Another study showed scavenging property of *S. rhombifolia* noticed by conversing deleterious effects of hydrogen peroxide as per Dhalwal *et al,.* Root and Dried leaves of *S. rhombifolia* are traditionally used in India for psychological diseases, heart diseases. It is also used as an aphrodisiac and tonic. Based on the above evidences the research was planned to evaluate the influence of *C. dactylon L.* and *S. rhombifolia L.* hydro-ethanolic extracts on amnesic rats induced by Scopolamine (SCO) model and predict the molecular mechanism by system biology (network pharmacology and molecular docking) approaches.

2. AIMS AND OBJECTIVES

To evaluate the anti-amnesic activity of *Cynodon dactylon* L. (whole plant) and *Sida rhombifolia* L. (whole plant) by *in-silico, in-vitro, and in-vivo* studies.

OBJECTIVES

- To assess hydro-ethanolic extract of *Cynodon dactylon* L. and *Sida rhombifolia* L. effects on long term memory, short term memory, fear aggravated task against cognitive impairment induced by scopolamine in rats by using morris water maze, elevated plus maze and passive avoidance task.
- To evaluate the effect of *Cynodon dactylon* L. and *Sida rhombifolia* L. extract on Acetylcholinesterase enzyme, beta amyloid 1-42 level and antioxidant biomarkers and histopathological changes of rat brain (hippocampus and cerebral cortex) in amnesic rats induced by scopolamine.
- To assess the effect of extract/fraction(s) of *C. dactylon* L. and *S. rhombifolia* L. on oxidative stress and acetylcholinesterase enzyme inhibition via in-vitro methods and to predict molecular mechanisms of above chosen plants using in-silico (network pharmacology and molecular mechanism) approaches.

3. Review of Literature

The field of cognitive neuroscience arose from two disciplines: psychology, which sought to establish diligent methods for cognition, behavior, and system neurobiology examinations which attempted to establish neural circuit structure and functions of motor and sensory systems of brain. The discovery of basic experimental procedures for investigating learning and memory was done by Hermann Ebbinghaus in humans for the first time in 1885; Later, Ivan Pavlov also investigated in experimental animals and Edgar Thorndike a few years later – led to the establishment of behaviourism. [26]

3.1 Storage of Memory

Franz Josef Gall was the first to address the topic of how much a mental process may be localized inside the brain. Two key conceptual contributions were made by him. Gall's first aim was to eliminate the mid-brain dualism. Based on anatomical investigations, he claimed that the mind's organ is the brain. Secondly, he identified that the cortex is considered not as homogeneous, rather it comprises various areas that govern different mental activities. As a result, Gall introduced the concept of cortical localization. He claimed that the brain is a complex organ, splits into 27 categories (more were later included), each of which corresponds to different mental ability. Gall's ideas were tested in the late 1820s in France by Pierre Flourens, who was followed by Karl Lashley, who performed numerous experiments in which they isolated different parts of the brain, particularly the cerebral cortex, but unable to recognize any specific area necessary for memory storage.

Donald Hebb impressed many people later in 1949 who was able to think solemnly regarding the mechanisms of brain underlying memory. Hebb suggested that the neuronal cells work together to present information; these assemblies are scattered throughout wide sections of cortex. Lashley 's conclusive note says that learning could not be restricted to a single brain region. Most lesions will leave a sufficient number of linked cells to ensure that information can still be represented. The concept of dispersed memory storage was brilliant with further research; it has become clear that there is no single memory centre, and that the representation of any single experience involves several sections of the nervous system.

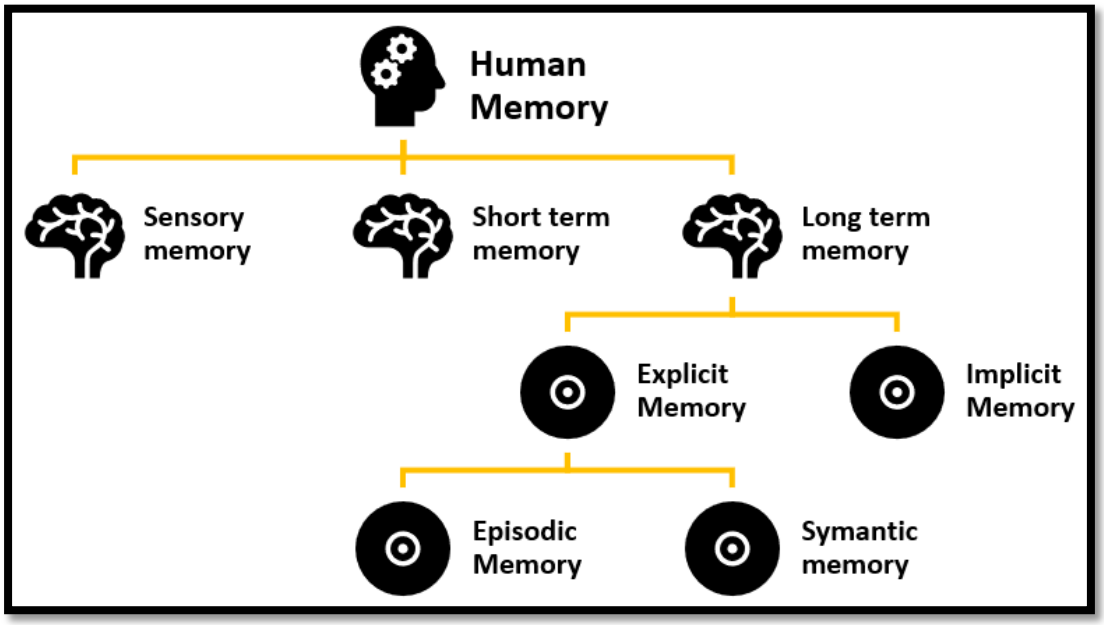
3.2 Different forms of Memory

Memory is adequately separated into explicit and implicit forms from a physiological standpoint.

A.Implicit memory- This is also known as non-declarative or reflexive memory since it does not need awareness. It encompasses, among other things, abilities, habits, and conditioned reflexes, and its retention does not entail processing in the hippocampus, at least in most cases. However, after a task is well understood, explicit memories necessary for tasks like riding a bicycle can become implicit.

B.Explicit memory- This is also known as declarative or recognition memory, is linked to consciousness—or at least awareness—and relies on the hippocampus and other areas of the brain's medial temporal lobes for retention. It is separated into episodic memory and memory for rules, worlds, and language, among other things (semantic memory) as shown in Figure 1.

Figure 1: Different forms of memory.



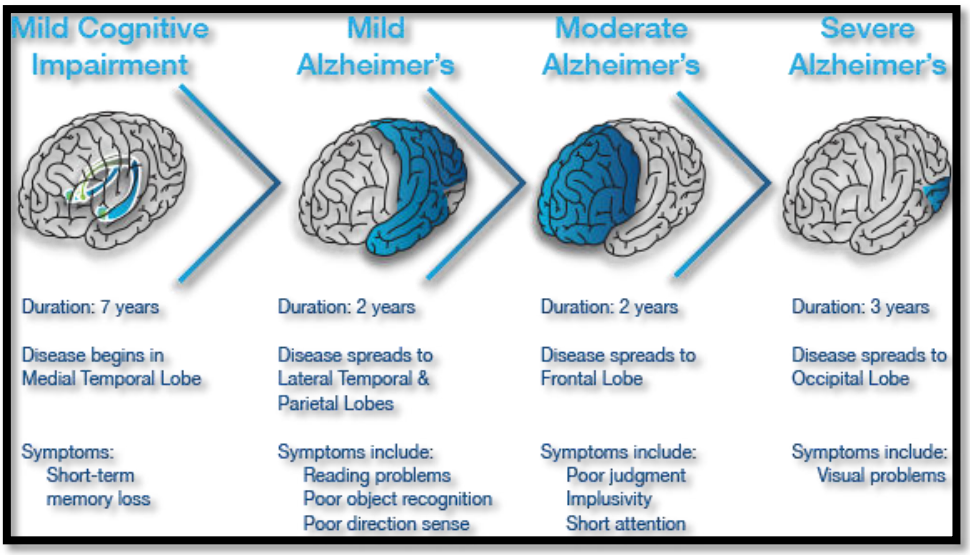
3.3 Dementia

Alois Alzheimer recognized general form of dementia in elder population in 1907. Although understanding aetiology and pathophysiology of the illness is regularly updated, diagnostic capability and the development of pharmacological medicines that might halt or better prevent the disease remain limited. Alzheimer's illness has officially been declared incurable. Existing medications, despite considerable preclinical and clinical research, only provide minimal symptomatic alleviation to a limited percentage of people and do not address the disease's core causes. The failure is most likely owing to a lack of understanding of the cellular and molecular pathways associated in AD progression, as well as authorized medications that modulate cholinergic and glutamatergic neurotransmitters. [7]

Numerous novel medications under progression, on the other hand, attempt to alter the disease condition by affecting multiple wide-ranging brain alterations induced by AD. These modifications might be used to develop novel medications to

halt or reduce disease development. It is now well acknowledged that AD as complex illness, characterised with extensive oxidative stress, formation of neurofibrillary tangle (NFT) and amyloid (a storage depositing senile plaques), mitochondrial damage, glutamate excitotoxicity, neuroinflammation and other pathogenic features. As a result hyperphosphorylation of protein 'tau' is formed and this leads to synaptic loss and neuronal dysfunction. [7,8]

Figure 2: Alzheimer's Disease stages of Progression



3.4 Diagnosis of Dementia

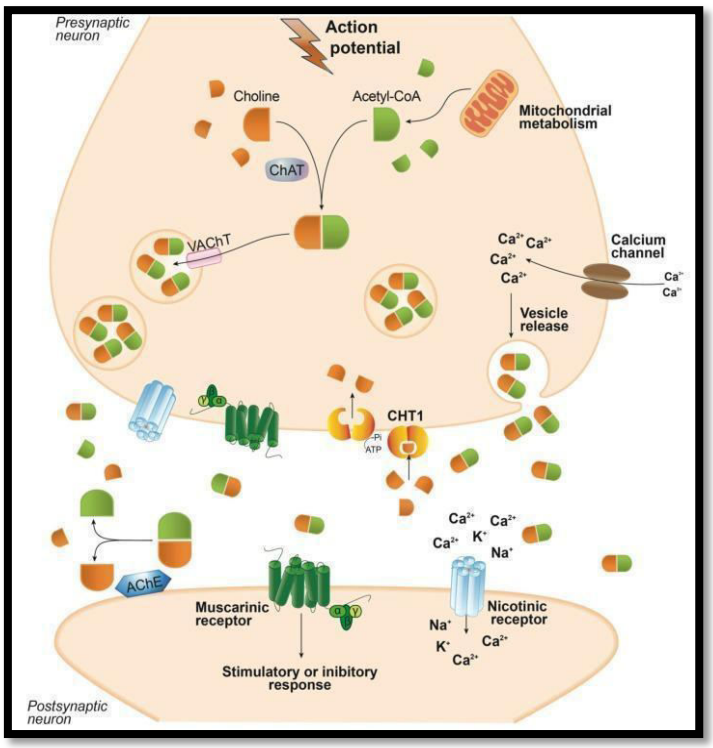
To diagnose dementia and detect loss of cognition and disability of brain function two international standards have been considered viz, "Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV)" and "International Classification of Diseases-10 (ICD-10)". The following details are considered to know the progression of dementia A. Patient history, B. Mini mental state evaluation (MMSE) C. Physical examination. D. Laboratory investigations E. Neuropsychological testing F. Radiology/neuroimaging

3.5. Pathogenesis

3.5. 1. Cholinergic hypothesis

Neurotransmitter "Acetylcholine" has a crucial role in the neurological system. Cytoplasm present in cholinergic neurons forms acetylcholine from acetyl-CoA and choline with the help of enzyme choline acetyltransferase and with the help of vesicular acetylcholine transporter the Ach is released into synaptic vesicles. Depolarization leads to exocytosis and allows ACh to move towards synaptic cleft to bind with its receptors (muscarinic and nicotinic). However, hydrolysis of ACh may occur by enzyme 'acetylcholinesterase' (AChE) generates choline and acetate which gets reintroduced into the presynaptic terminal by "high-affinity choline transporter" (CH1). In cases of AD, nucleus basalis of Meynert are extensively damaged due to cholinergic transmission loss. [27] as featured in **Figure 3.**

Figure 3: Schematic diagram of Acetylcholine neurotransmission.



Role of acetylcholinesterase enzyme

This enzyme is versatile present in neuro-muscular junction, cholinergic synapse, which mediates catabolic breakdown of acetylcholione to choline and acetate, in turn terminates crucial functions of acetylcholine neurotransmitter. [28, 29]

AchE has diverse functions as such cell proliferation, adhesion, stem cell diffentiation, synaptogenesis, and is associated with homeostasis of amyloid plaque. Over expression leads to abnormalities of these functions specifically results in acetylcholine breakdown, increased content of insoluble amyloid production and deposition this gives wide implication to address the hallamrk of AD. [30, 31, 32]

Vital researches were carried out in which AchE inhibitors alleviate, memory dysfunction by regulating the amount of acetylcholine at synaptic cleft [33, 34, 35], prevents apoptosis in hippocampal region. [36, 37]

Through cholinergic muscarinic and nicotinic receptors, acetylcholine (ACh), the primary neurotransmitter in the central nervous system, controls a wide range of physiological reactions, including inflammation and higher cognitive processes [38, 39.]

As a result, a plethora of experimental data points to the existence of both muscarinic and nicotinic receptor subtypes at presynaptic and postsynaptic locations, exerting significant impacts on the regulation of synaptic plasticity. Five distinct subtypes of cholinergic muscarinic receptors—M1–M5—have been identified [40], and they are expressed differently at various anatomical sites, such as hippocampus pyramidal and nonpyramidal cells [41, 42, 43]

Pirenzepine has been created based on the varied selectivity and binding properties of numerous ligands, such as scopolamine, for the subtype of muscarinic receptor (M1-M5) [44, 45]

While presynaptic M2 and M4 receptors function as autoreceptors via Gi/o activating adenylcyclase to reduce neurotransmitter release, postsynaptic cholinergic muscarinic M1, M3, and M5 act via G-protein coupled receptors (Gq) activating phospholipase C (PLC) to regulate neurotransmission [46, 47 40]

The brain's impact on cognitive functions including learning and remembering 40 Muscarinic M1 and M4 receptors predominate in the hippocampus over M2, M3, and M5 receptor subtypes, and they have been linked to memory functions [47, 48, 49, 50]

Numerous signalling pathways are activated by muscarinic cholinergic receptors, which modulate vital brain processes such neuronal excitability, synaptic plasticity, and the control of ACh release.

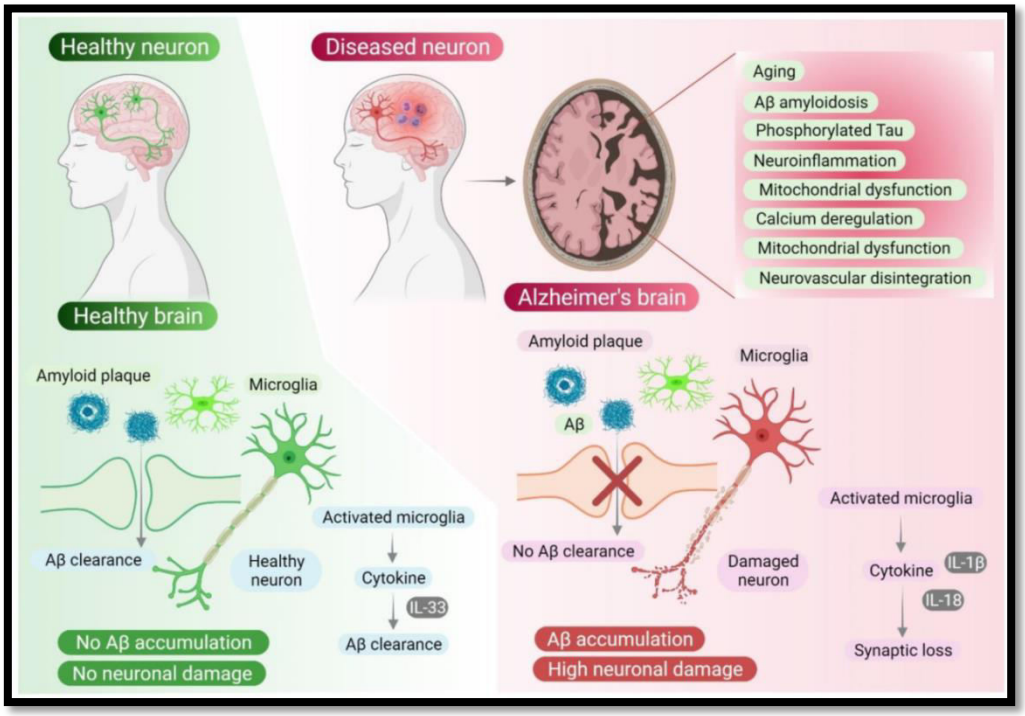
Numerous studies indicate the role of the muscarinic M1 receptor, suggesting it as a viable target for neurological illnesses like AD that cause cognitive impairment [50, 51, 52, 53]

Additionally, muscarinic and nicotinic receptor functional abnormalities are linked to a number of neurodegenerative diseases, including Alzheimer's disease. The neurobehavioural response to ischemia in the complex brain is the consequence of a cascade of cellular and molecular processes that are carefully controlled by a dysregulated cholinergic system that promotes delayed neuron

3.5.2. *Amyloid β and AD*

A fundamental membrane glycoprotein found in regions of the brain is called amyloid precursor protein (APP) (CNS). It can be sequentially processed by two different proteolytic processes,such as α and β pathways. The majority of the time, 'α—secretase' and 'γ-secretase' cleave APP in a sequential manner across the route. The cleavage of APP by α-secretase is not amyloidogenic, meanwhile the route results in the production of Aβ by β pathway . In this pathway, the 99-amino-acid C-terminal portion of APP is left inside the membrane when APP is initially released into extracellular space by –β secretase. After that, C99 is converted to 38–43 amino acids by γ-–secretase, releasing the intracellular C–terminal domain of Aβ and "APP intracellular C-terminal domain" (AICD). [54] A-cleavage often results in A-40, but it can also yield A-42, which is more hazardous. [55, 56] as shown in Figure 4.

Figure 4: Schematic presentation of Aβ role in neuron



Oxidative stress and beta amyloid $_{1-42}$

Deposition of insoluble Aβ and neurofibrillary tangles are associated with oxidative damage and abnormal function of biometals viz, iron, zinc and copper mounts the evidence of neurodegeneration. Copper has role in producing highly reactive hydroxyl radical and reports do suggests that amyloid plaque contain high content of copper which addresses length of Aβ fragment. Additionally, the neocortex, amygdala, and hippocampus, which are the brain areas most commonly impacted by AD pathology, were shown to have significant amount of zinc. [57, 58, 59, 60]

This binding of zinc produces the formation of poisonous, fibrillary, Aβ aggregates with a highly organised conformational state of Aβ (1–40). As a result, the immune/inflammatory reaction to non-soluble Aβ-plaques involves the disturbance of zinc homeostasis followed by an uncontrolled release of zinc from the brain, which is typical of oxidative stress. Thus, unchecked zinc or Aβ build-up results in cytotoxicity and oxidative stress caused by zinc and Aβ , respectively. [61, 62, 63]

The oxidation of brain proteins might impact enzymes crucial to neuron and glial processes, protein oxidation by free radicals may be important in AD. This is true for two enzymes that are particularly vulnerable to oxidative modification, glutamine synthetase and creatine kinase, which are both significantly downregulated in AD brains due to altered glutamate concentrations and increased excitotoxicity, respectively. However, oxidative impairment of creatine kinase may result in decreased energy metabolism in AD. [64, 65, 66, 67]

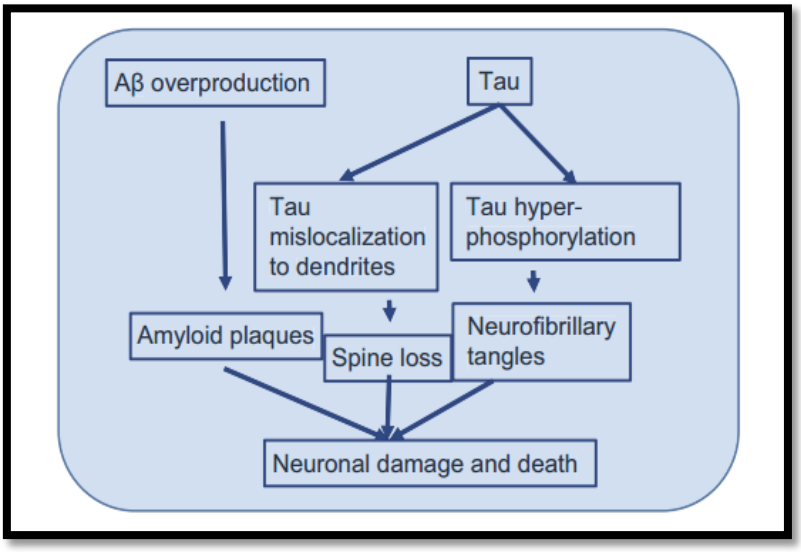
Advanced glycation end products, a post-translational modification of proteins created when the amino group of proteins interacts non-enzymatically with monosaccharides, can also be produced by protein oxidation. In addition, brain

oxidation can have an impact on DNA, leading to base modifications, sister chromatid exchange, strand breakage, and DNA-protein cross linking. Overproduction of ROS can cause oxidative stress, which can have negative effects and play a significant role in cell structure destruction, disease states, and ageing. [68, 69, 70]

3.5. C Hyperphosphorylation of τ and AD

The microtubule-binding domain of τ-protein assembles with tubulin to form matured microtubules. τ –protein can stabilize microtubules and construct linking between nearby microtubules to make a stable network of and hold them together. Neurofibrillary tangles (NFTs) are formed due to the microtubule-associated Tau (τ) protein hyperphosphorylation which is progressive feature diagnosed in AD. The profuse of beta-amyloid results in the release of kinases which get linked with τ – protein results in hyperphosphorylation. Due to this microtubules become unstable and chunks of T-filaments aggregate to form NFT's and these deposit in cytoplasm of neurons as insoluble patches leads to communication loss between signaling process and neurons. [71] as mentioned in Figure 5

Figure 5: Protein Tau in Alzheimer's disease.



3.5. 4. Genetics

Astrocytes and microglial cells secret Apolipoproteins especially ApoE which has a crucial role in transportation of lipid in CNS for repairing neuronal injuries, maintaining synaptic connections, toxin-scavenging and lipid homeostasis. The alleles viz, ApoE2, ApoE3 and ApoE4 are encoded by ApoE. ApoE2 is considered as neuroprotective however ApoE4 allele is identified as one of the major risk-factors in sporadic AD and linked to cognitive deficit. [72]

3.5. 5. Role of oxidative stress in memory impairment

Oxygen is required for the survival of all living things and many biological processes. In the mitochondria, cells break down oxygen and create adenosine triphosphate (ATP). During this process, free radicals are produced as by products. These free radicals are useful at low quantities, but at greater concentrations, they can cause tissue damage. The buildup of reactive oxygen species (ROS) can cause oxidative stress, which leads to brain damage by disrupting the cellular pro-oxidant – antioxidant equilibrium. [73]

An atom or molecule that has an unpaired electron in its outer orbit is said to be a free radical. This condition renders the atom or molecule very unstable and reactive. Free radical damage occurs within living cells when the production of reactive oxygen species surpasses the capacity of the intrinsic antioxidant. Free radicals are produced during normal metabolism. Additionally known as oxidative or oxidant stress, this condition. Because of its high energy needs, high oxygen consumption rate, high concentration of peroxidizable fatty acids, high concentration of transition metals, which may catalyse the formation of the reactive hydroxyl radical, and relative lack of antioxidant defences compared to other organs, the brain

may be particularly vulnerable to oxidative damage. [74] An increasing unit of research indicates that oxidative damage has a role in the aetiology of dementia. The free radical theory of ageing, which contends that the build-up of reactive oxygen species with ageing causes damage to important cell components, is where this idea first originated.[49 75] Because of its high lipid content and extremely high concentration of polyunsaturated fatty acids, which are particularly vulnerable to oxidation, the central nervous system is particularly sensitive to oxidative stress, which mostly appears as lipid peroxidation. This might then encourage the production of more reactive oxygen species, which would increase the oxidative damage to proteins and DNA. Superoxide dismutase activity has recently been found to rise with age in the cerebrospinal fluid (CSF), suggesting a potential reactive compensatory response as a result of this increasing oxidant stress with time. Superoxide dismutase activity has recently been found to rise with age in the cerebrospinal fluid (CSF), suggesting a potential reactive compensatory response as a result of this increasing oxidant stress with time. If free radical damage plays a role in the development or progression of AD, then treatment to lessen oxidative damage and boost endogenous antioxidant defences may prevent, postpone, or improve the disease process and lessen its negative effects on people and society. Treatment with antioxidant medicines may be helpful in neurological illnesses, including AD, according to evidence from both in vitro and animal trials. [76]

3.5. 7. Excitotoxicity

Olney explained the term 'excitotoxicity' due to elevated excitatory Glutamate NT's in the brain because, N-methyl-D-aspartate (NMDA) receptor is activated by excessive glutamate content which allows Ca2+ influx; promoting proteases, lipases – membrane damage, activation of nitric oxide synthase (NOS), elevated arachidonic

acid release .and leads to neuronal injury with increase in the free radical production. [77]

3.5. 8. Environmental triggers

The origin of neurodegenerative illnesses has been linked to infectious agents, environmental pollutants and acquired brain damage. Traumatic brain damage has been proposed as a cause of neurodegenerative illnesses, and there is some evidence to support this theory in the case of AD. When blood flow to the brain is disrupted, a series of neuronal processes occur, leading to subsequent repercussions such as cerebral oedema and inflammation, all of which can contribute to brain damage. Following reperfusion, further damage might occur due to the generation of reactive oxygen species when oxygenation is restored. These secondary processes might take hours to emerge, giving you the opportunity to improve the process early on.[78, 79]

3.6. Importance of herbal medicine in treatment

Medicinal herbs are becoming increasingly important for cognitive improvement. Ayurvedic, homoeopathic, Unani and Siddha systems of medicine are among the most important ancient therapeutic modalities. Traditional medicine is primarily preventative, nutritive, protective and curative in nature. Traditionally ancient remedies are therefore safe and innocuous, and they treat patients with minimal/no negative effects. [80]

Medicinal plants provide a variety of phytocompounds that could be extracted or used as raw material in various scientific studies. Secondary metabolites of plants have commercial importance used in the pharmaceutical industry with recently achieved widespread acceptability as a result of their less adverse effects as compared

to manufactured medications and the need to address the medical needs of an ever-increasing human population. However, several issues such as wide geographical location, climatic changes, cultural customs, labour cost, selection of better plant stock, and overexploitation by pharmaceutical businesses make a consistent supply of source material challenging. [18]

Herbal medicine is native to India, and Ayurveda has produced different medicinal formulations for various treatments. Medicinal herbs have been demonstrated to be helpful in ensuring improvement in nervous system function in a number of scientific studies. Phytochemical study indicates the existence of different bioactive chemicals in medicinal plants, including lignans, tannins, flavonoids, polyphenols, sterols, alkaloids and triterpenes which have anti-cholinesterase, anti-amyloidogenic, hypolipidemic anti-inflammatory, and antioxidant properties. [81]

3.7. Details of *Cynodon dactylon L.*

Indian system of Medicine has variety of medicinal species in *C.dactylon* has a prominent role belongs to 'Poaceae' family. Regional names of *Cynodon dactylon* are known as Bermuda grass, Dog's tooth grass (English), Dhoob, Dobri (Hindi), Garikehullu, Ambatehullu, Balligarike (Kannada), Niladurva, Ananta, Saddata (Sanskrit), Durba (Bengali), Karuka (Malayalum).

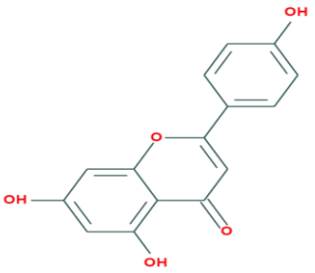
3.7.1 . Traditional uses

The herb was traditionally used to cure diarrhoea, dysentery, hyperdipsia and haemorrhage. Freshly prepared plant juice was used to treat anasarca, catarrhal ophthalmia, dropsy, secondary syphilis, chronic diarrhoea and was also used as a demulcent and astringent. The grass's fresh expressed juice has been used to treat

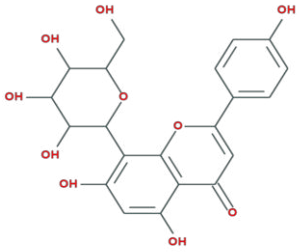
vomiting, and catarrhal ophthalmia. It may also be used to treat cuts and wounds, as well as chronic diarrhoea and dysentery. Root decoction was used to treat vesicles calculus and secondary syphilis, as well as piles and urinary organ irritation. [82]

3.7.2. Reported Bioactives

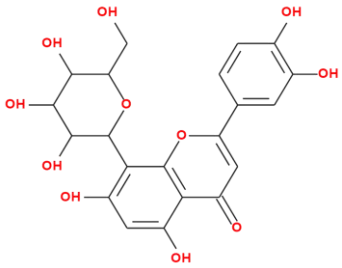
As per documents retrieved 'Gas chromatography-mass spectrometry' revealed *C. dactylon* phytocompounds and scientists isolated few bioactives such as **alkanes:** tricosane, 1, 2-propanediol, 3- benzyloxy-1, **flavoinoids**: Apigenin, Vitexin, Orientin, luteolin, quercetin, Kaempferol, catechin, beta-carotene. Hydroalcoholic extract was found to contain 22 compounds predominantly hexadecanoic acid, ethyl ester, D-mannose and linolenic acid, ethyl ester. Few compounds are sketched below;



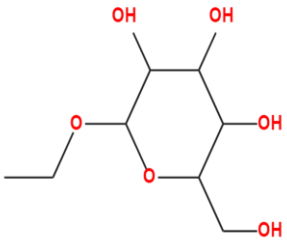
Apigenin
MF: $C_{15}H_{10}O_5$
MW: 270.24g/mol



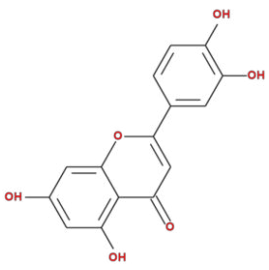
Vitexin
MF: $C_{21}H_{20}O_{10}$
MW: 432.4g/mol



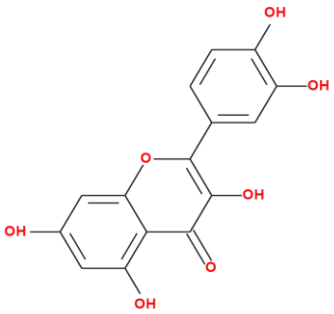
Orientin
MF: $C_{21}H_{20}O_{11}$
MW:448.4g/mol



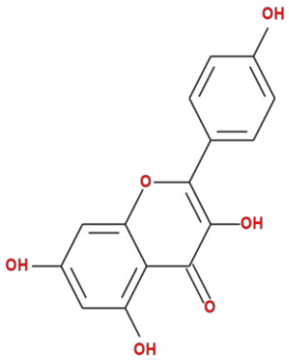
ethyl α-D- glucopyranoside
MF: $C_8H_{16}O_6$
MW:208.21g/mol



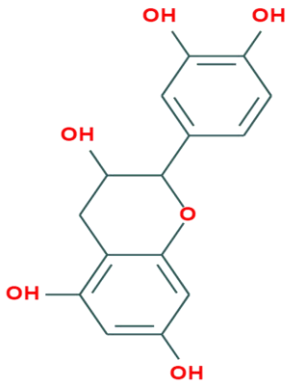
Luteolin
MF: $C_{15}H_{10}O_6$
MW: 286.24g/mol



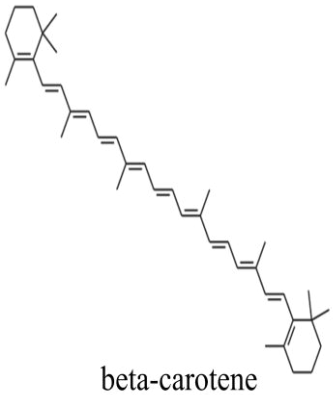
Quercetin
MF: $C_{15}H_{10}O_7$
MW: 302.23g/mol



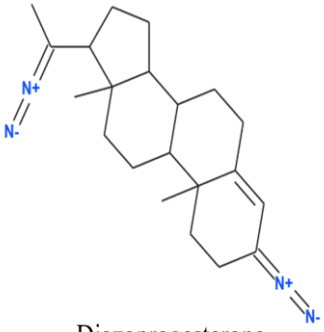
Kaempferol
MF: $C_{15}H_{10}O_6$
MW: 286.24g/mol



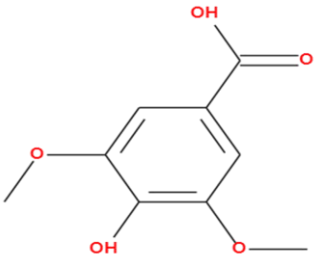
Catechin
MF: $C_{15}H_{14}O_6$
MW: 290.27g/mol



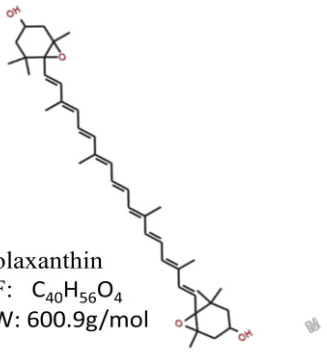
beta-carotene
MF: $C_{40}H_{56}$
MW: 536.9g/mol



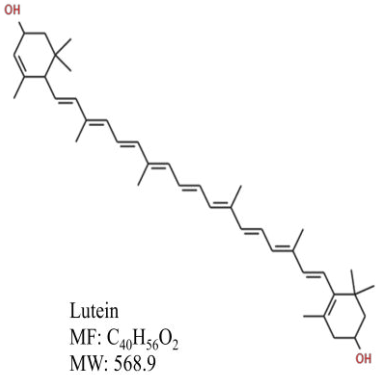
Diazoprogesterone
MF: $C_{21}H_{28}N_2O_2$
MW: 340.5g/mol



Syringic acid
MF: $C_9H_{10}O_5$
MW: 198.17



Violaxanthin
MF: $C_{40}H_{56}O_4$
MW: 600.9g/mol



Lutein
MF: $C_{40}H_{56}O_2$
MW: 568.9

3.7.3. Pharmacological action of C. dactylon:

Secondary metabolites of *C dactylon* possess multiple pharmacological spectra including antidiabetic: C. dactylon (aqueous extract) showed significant decrease in pre-prandial glucose level in streptozotocin induced diabetic rats. [83] Immunomodulatory: fresh juice of aerial parts C. dactylon showed protective effect against the diethyl nitrosamine induced mice model. [84, 85] Wound healing: aqua ethanolic extract of *C.dactylon* (15% ointment) regenerates epithelial tissues in experimental rats. [86] Hypolipidemic: 70% ethanolic extract of (C. dactylon rhizomes) ameliorates high cholesterol diet and avoids atherosclerotic changes in aorta and heart vessels. [87, 88]It was also proven to be protected against experimentally induced nephrolithiasis[89, 90]

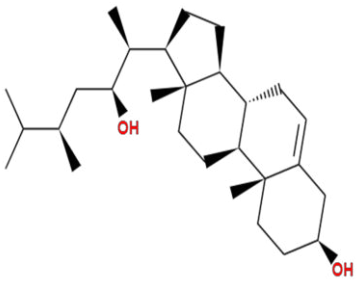
Anti-oxidant and neuroprotective: *C. dactylon*displayed defensive role in aluminum and carbofuran induced neurotoxicity in rats by lowering lipid peroxidation levels [91] and also decreased the cataleptic behavior in rotenone treated Sprague Dawley rats [92] The oral administration of aerial parts of *Cynodon dactylon* has also shown to overcome stress-induced sexual dysfunction [93]Antiviral: Isolated flavonoids from *C. dactylon* viz, luteolin and apigenin rich ethanolic fraction were effective against chikungunya[94] Hepatoprotective: Wistar rats administered with *C. dactylon* (ethanolic extract) showed significant decline in serum bilirubin and cholesterol level in CCl4 induced hepatotoxicity and maintained integrity of hepatocytes. [95] Isolated bioactives of *C. dactylon* (methanolic root extract) activated apoptotic pathways, increases anti-oxidant levels against *in-vitro* cancer cell lines (COLO320DM, AGS, MCF-7, and A549) and *in-vivo* studies also supported by protection against carcinogenic model in rats. [96, 97] Other pharmacological actions of

C. dactylon such as analgesic, antipyretic [98], diuretic [99], treatment of epilepsy [100] were addressed by researchers.

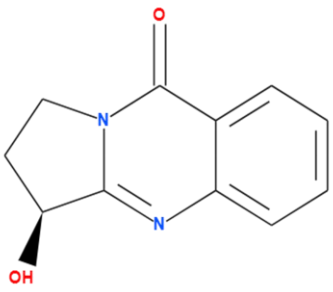
3.8 Details of *S. rhombifolia L.*

Genus: Sida, Family: Malvaceae, Vernacular names of *S. rhombifolia* are known as Janglimedhi (Hindi), Mahabala, Bala (Sanskrit), Atibata (Kannada), Jagli methi (Marathi).

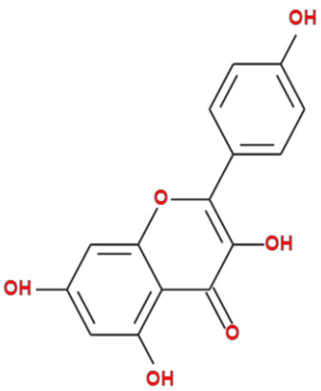
3.8.1. Bioactives: *S. rhombifolia* reported to have rich in multiple secondary metabolites as per ChEBI database includes flavonoids, alkaloids: ß-phenethylamine, Methyl ß- phenethylamine, Ephedrine, Ψ- ephedrine; Quinazoline: Vasicine, Vasicinol, Vasicinone; Choline, Betaine, Hypaphorine, Hypaphorine methyl ester, Cryptolepine, Phytosterols: Stigmasterol, Campesterol, β-Sitosterol, Spinasterol, Cholesterol, Ecdysteroids, Ecdysone, 20-hydroxy Ecdysone,2-deoxy-20-hydroxy ecdysone3-O-β-D-glucopyranoside, 20-hydroxy ecdysone-3-O-β-D-glucopyranoside. [101-104] Out of these, few compounds are sketched below.



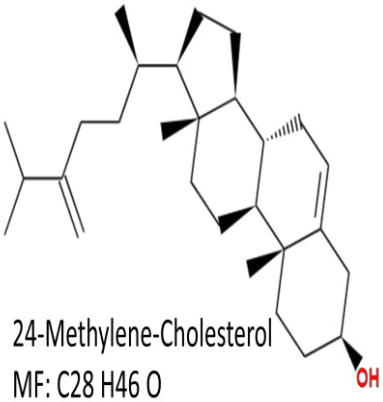
22-Diydro-Campesterol
MF: C28 H48 O2
MW: 416.37g/mol



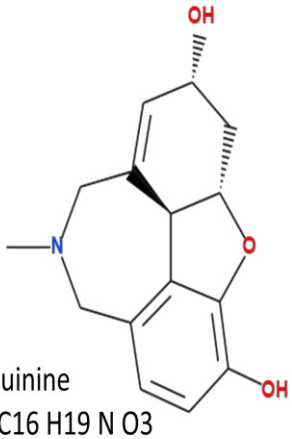
Vasicinone
MF: C11 H10 N2 O2
MW: 202.07g/mol



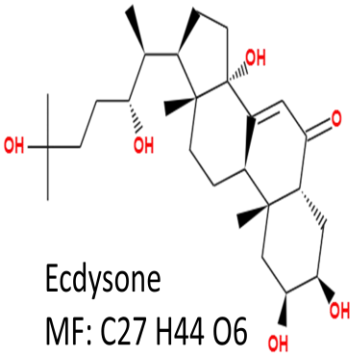
Kaempferol
MF: C15 H10 O6
MW: 286.05g/mol



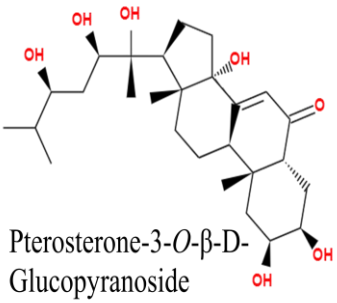
24-Methylene-Cholesterol
MF: C28 H46 O
MW: 398.35g/mol



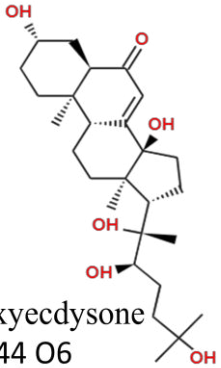
Sanguinine
MF: C16 H19 N O3
MW: 273.14g/mol



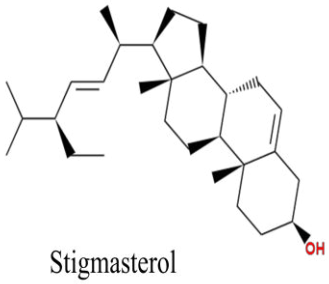
Ecdysone
MF: C27 H44 O6
MW: 464.31g/mol



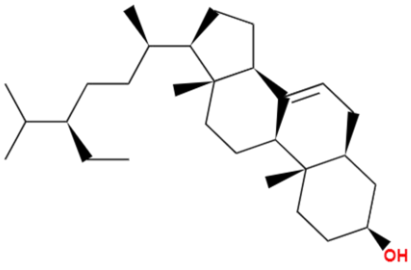
Pterosterone-3-*O*-β-D-Glucopyranoside
MF: C27 H44 O7
MW: 480.31g/mol



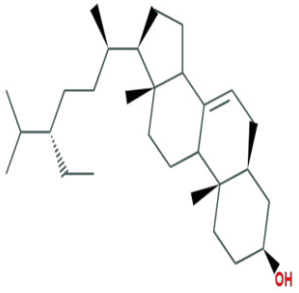
2D-Hydroxyecdysone
MF: C27 H44 O6
MW: 464.31 g/mol



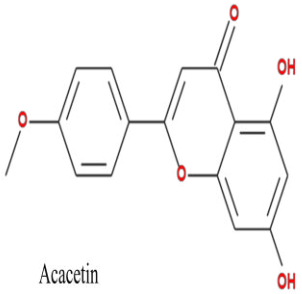
Stigmasterol
MF: C29 H48 O
MW: 412.37g/mol



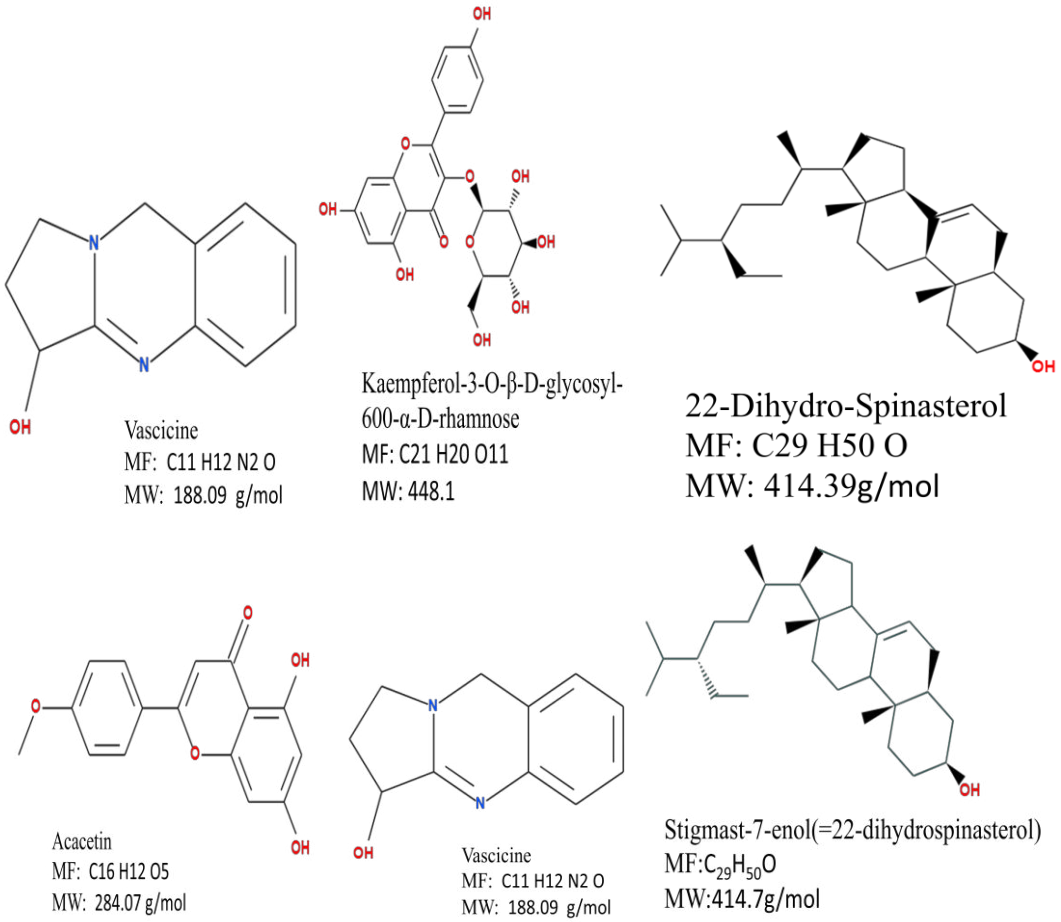
22-Dihydro-Spinasterol
MF: C29 H50 O
MW: 414.39g/mol



Stigmast-7-enol(=22-dihydrospinasterol)
MF:$C_{29}H_{50}O$
MW:414.7g/mol



Acacetin
MF: C16 H12 O5
MW: 284.07 g/mol



***3.8.2. Traditional Uses*:** With respect to "Ayurveda" *S. rhombifolia* useful in the management of heart disease, fever, different forms of inflammations, eliminates 'tridoshas'. In combination with other drugs it acts as antidote for snake and scorpion bites. *S. rhombifolia* has widely accepted for treatment of rheumatism and pulmonary tuberculosis. The stem part acts as demulcent, juice of leaves used for treatment of spermatorrhea and in menstrual pain. Hot aqueous extract of *S. rhombifolia* used as abortifacient in pregnant women, aphrodisiac and for relief of cough. Infused preparation applied for skin infections.[105]

3.8.3 Ethnopharmacological importance of S. rhombifolia

Some of the extensively investigated pharmacological properties of *Sida rhombifolia* include **vasorelaxant:** alkaloids (Crytolepinone) isolated relaxes blood vessels, [102] Antibacterial: β-Sitosterol &Stigmasterol isolated reported as bactericidal,[106] alkaloid fraction (aerial parts) possessed anti-yeast, anti-fungal activites against various microbes. [107] **Anti-inflammatory**: ethanolic and ethyl acetate extract exhibited anti-nociceptive activity against EPP induced rat oedema. [108]**Cytotoxic effect**: leaf extract demonstrated cytotoxicity against various cell lines (MCF-7, CA-HS-578T, breast cancer cell lines-MDA-MB-231, human leukemia cell lines CCRFCEM, Leuk-SR, Leuk-K562, human colon cancer cell line HCC-2998) [109]**Anti-oxidant** : root extract reported to inhibit lipid peroxidation in the rat liver and brain homogenate. [110]**Anticholinesterase:** isolates from n-hexane fraction (whole plant) reported activity on enzyme inhibiton 'AchE'. [111] **Anti-arthritic:** ethanol and methanol extracts (stem, root and aerial parts) as anti-arthritic agent against adjuvant induced arthritic rats. [112]**Hepatoprotective:** methanolic extract alleviates high fat diet induced non-alcoholic liver disease in rats. [113] **Nephroprotective:** leaf extract ameliorates genatmicin induced nephrotoxic rats. [114] **Anti-diarrhoea**: methanolic extract (roots) possess activity against castor oil induced diarrhoea in rats. [115] **Hypoglycemia**: arial parts exhibit anti-hyperglycemic effect . [116]

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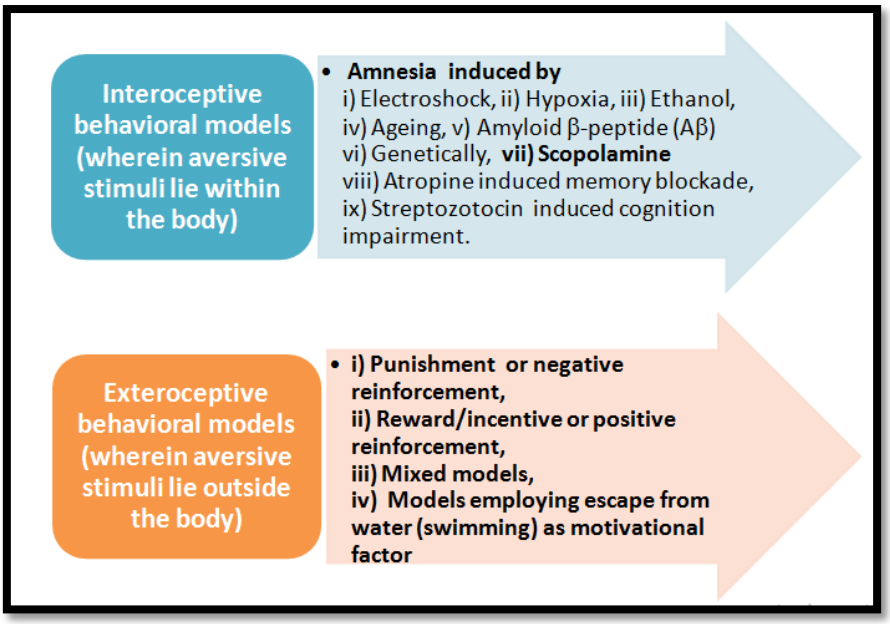
3.9. Animal Models for cognitive impairment.

Animal models are used to assess cognitive capabilities, both directly as a main screening tool that might aid in the drug development process and indirectly through their essential role in the exploration of cognition's neurochemical foundation. Drugs' effects on cognitive processes must be distinguished from their effects on psychiatric processes, particularly sensory and motor function, in animal models developed for cognition enhancement. Physical activity and cognitive training are equally sensitive to the hippocampus and spatial learning capacities, according to animal study. Wheel running and hippocampus-dependent learning activities have been found in rats to increase neurogenesis and neurotrophin production, both of which improve spatial memory. Animal model development is difficult because no single animal model can clarify all behavioural, biochemical and histopathological defects. A superlative animal model would replicate human illnesses in rats while also reflecting the complexity of human behaviour. Rodent models are important because mice do not naturally generate plaques and tangles, but they may be forced to do so in specific brain areas. Dementia models have previously been developed using monkeys, rodents, aged rhesus, flies, and worms. To screen newer drugs which influence ‘memory and learning skill’ processes, a number of experimental models are now available and have been divided as follows. [117, 118]



3.9.1. Interoceptive behavioral models (wherein stimuli lie within the body):

i) Scopolamine Induced Amnesia

Scopolamine (hyoscine), is an alkaloid isolated from belladonna plant. It is a nonselective muscarinic antagonist agent used by physicians of many centuries. In the discipline of neuropsychopharmacology, scopolamine is used as a standard or reference agent to cause cognitive abnormalities in healthy individuals and animals linked to ageing and dementia. The use of scopolamine as a pharmacological model of 'cholinergic amnesia' most likely causes blockade of cholinergic signalling in a healthy brain. This is utilized as a model for cognitive deficiency associated with aging and AD. Effects of scopolamine are known to be biphasic on the central nervous system i.e. at a given dose may cause drowsiness or excitement, fatigue or restlessness. Higher dosages (3 mg/kg) may result in deficiencies in both cognitive and non-cognitive abilities. Several behavioural functions, including aversion, anxiety, taste, short-term memory, and attention, have been linked to scopolamine

intracerebro-ventricular (ICV) injections. It is determined that scopolamine at larger dosages results in impaired learning and memory function. [119]

Scopolamine induced cognitive defect is a gold standard model to validate drugs because of its ability to induce impairment in a wide range behavioural tasks. It also has the benefit of offering a rapid and easy method for assessing novel medication's ability to improve cognition. Although it can inhibit nicotinic receptors at high doses, scopolamine has a strong selectivity for the muscarinic receptor. If a substance can successfully reverse the cognitive deficiencies brought on by scopolamine in animals, it may also be able to enhance cognitive performance in healthy volunteers or persons with neuropsychiatric disorders. [120]

3.9.2. Exteroceptive models

i) ***Passive avoidance task or Negative reinforcement model***

It is a well-known method for evaluating learning ability. Because animals in this paradigm learn to avoid an unpleasant occurrence by repressing a particular response, the term "passive avoidance" is typically used to characterise this kind of experiment. More frequently, the phrase "inhibitory avoidance" is employed. Passive avoidance models include 2-way, shuttle box active, run-way avoidance tasks.

ii) Positive reinforcement models or Reward (Incentive) models:

As a motivating component, these models use various types of incentives and rewards, such as food pellets or water. It helps in assessing working memory but these types of models are tedious, time consuming, e.g. Elevated plus maze, radial arm maze, stone T-maze, Y-maze, three panel runway are reward/incentive tasks.

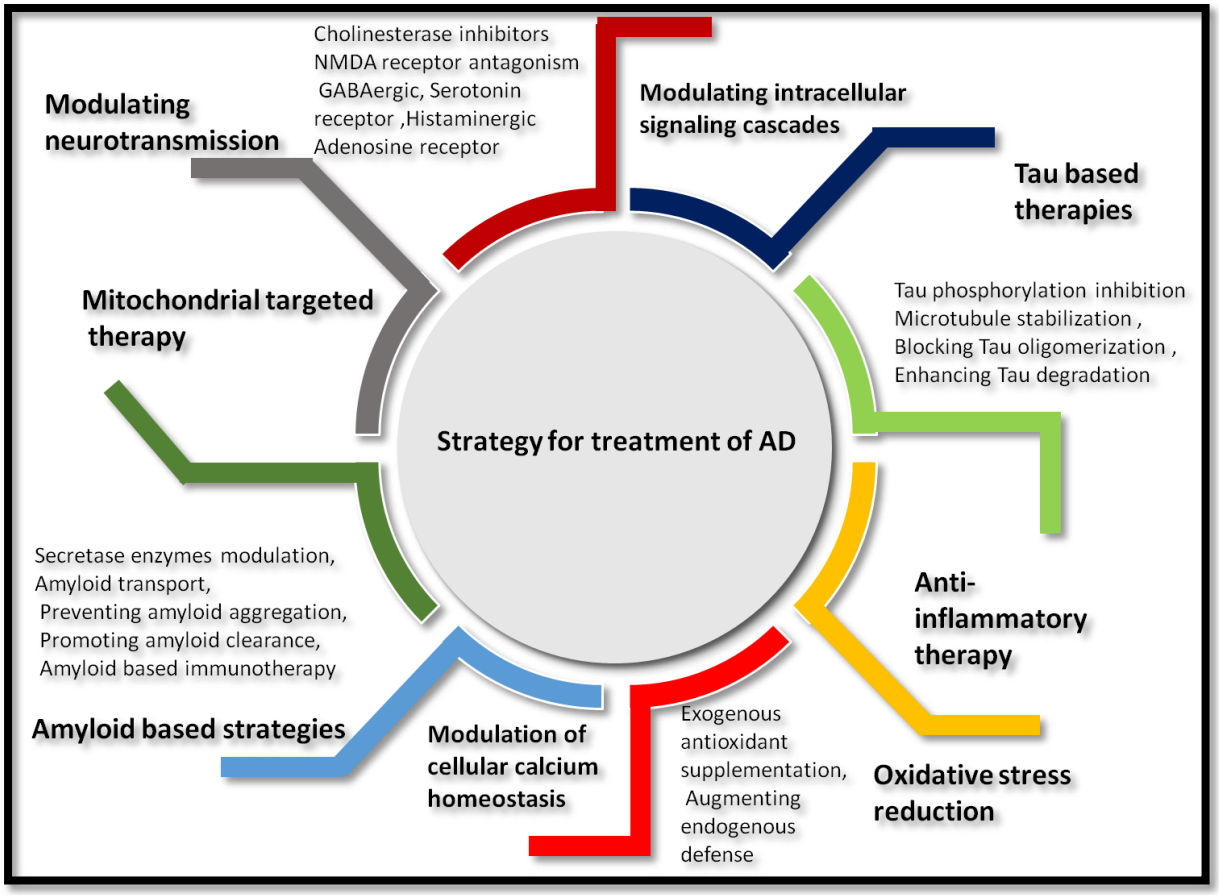
iii) Mixed models :

The T-maze and the olfactory box paradigm are examples of mixed models that incorporate both punishment and reward. They are also used to assess both spatial working and reference learning.

iv) Swimming model as motivational factor:

These models help to evaluate "spatial working and reference memory". It does not require food/water to interrupt the study e.g. Radial water maze and Morris water maze.

Figure 6: Current treatment approaches for the management of AD



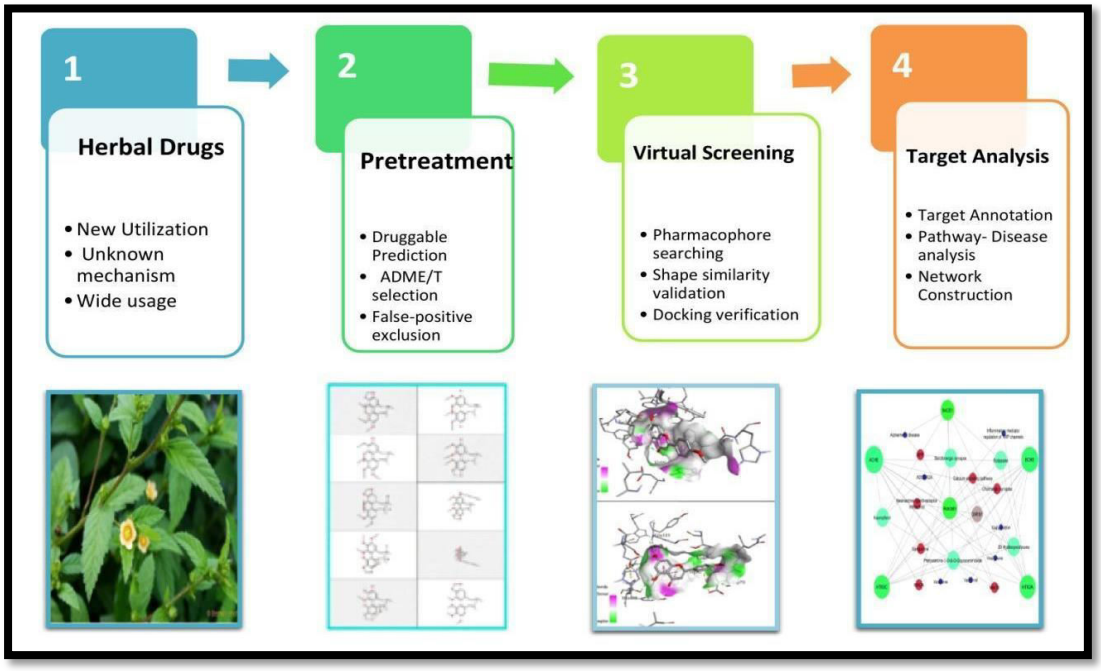
3.10. Role of *in-silico* models

The objective of updating the use of traditional medicinal herbs is to identify and forecast on the basis of pharmacological activity. Since medicinal plants chemical ingredients are diverse and variable, determining the particular chemical components and their key biological activities is a challenging feat.

A variety of innovative technologies as well as approaches are currently used for the production of enriched phytochemicals from medicinal plants in recent years, with an improving knowledge of the structure and function of phytochemicals.

In the era of advanced computer technology the *in-silico* strategy can figure out combinations of simulations with specific targets precisely. Furthermore, the quick elucidation of complicated interconnections between substances and their different activity targets has been made possible by the advent of network pharmacology technology. [121]

Figure 7: Steps involved in *in-silico* approach to predict molecular mechanism



To determine the pharmacological properties of medicinal plants the complete information is essential mainly retrieved by several resources:

1. Segregation and compound purification by laboratories,
1. Previous literature reports,
2. Compound databases.

The chemical extraction by regional laboratory is a direct and suitable approach among these three information-gathering processes and it can supply samples for further laboratory research. When a chemical is purified by herbs, all pertinent details must be documented, including "Recording number, CAS number, name, source plant, extractive fraction and structural information such as the SMILES code/InChiKey". Exceptional research objects are undoubtedly the chemicals acquired in the laboratory; yet, compounds are frequently either very simple or impossible to collect integral constituents of a specific plant. As a result, the literature and other databases may provide a quick approach to gather data on a number of distinct chemicals found in our herb of interest. Specific information: name, structure, categorization, and source of the plant must be documented in order to utilize these resources. The online database search engines for large-scale information has become a faster and more expedient option as virtual screening technology improves.

3.10.1. Phytocompound pre-treatment

Chemicals extracted through medicinal plants is enormous, although the vast majority are not pharmacologically active. The first stage in improving screening efficiency is to eliminate non-promising chemicals and filter the compounds that remain.

3.10.2 .Druggability prediction

Drug-like properties are a 'qualitative' notion utilized in drug development to describe compound's value in terms of parameters like bioavailability, which is calculated using molecular structural features. [122]

Pharmacologists are interested in the drug's qualities, which include, but are not limited to: Structural, physicochemical, biochemical, pharmacokinetics, and toxicity properties. Molecules appropriate for development as a medicine, according to Lipinski's suggestion which have Role of 5 (RO5). [123] Small compounds that meet the RO5 requirements have a better bioavailability in the organism's metabolic process, making them more likely to be used as oral medicines.

3.10.3. Selection of "ADME/T"

The "Absorption, Distribution, Metabolism, Excretion, and Toxicity" (ADME/T) qualities have a significant role for drug filtering when drug-likeness is determined by analyzing the physio-chemical properties & structural aspects of current drugs. As a result, we used the ADME/T selection subsequently to evaluate other features of drug-likeness. During the creation of a pharmacological molecule, it is necessary to forecast the position as well as movement of the medication in the human body. The ADME/T characteristics of medicines are assessed throughout the early phases of drug development. [124]

The key to passing clinical trials is having appropriate pharmacokinetic parameters and minimal toxicity during the ADME/T process. Prior to drug design, predicting and screening ADME/T characteristics of lead drug molecules could lower the cost of lead drug development and increase the favourable outcome of the entire

process. On the basis of monotonous description of the underlying biophysical-processes, reasonable prediction models for ADME/T characteristics have been established. Simulations with "ADME/T predictor PK-Map" and "Discovery Studio from Accerlary" firm are examples of software that can do ADME/T. [125]

Virtual screening

This can be described by selecting active lead compounds using computer technology and professional softwares, and further testing the activity empirically, depending on theories of drug design and novel drug screening.

3.10.4. Molecular Docking

Ligand-receptor binding mechanism is used to accomplish drug design. Molecular docking uses the features relied on lock and key principle by interaction pattern of ligand and its the receptor (drug target/protein/enzyme).[126]

3.10.5. Pharmacophore model for virtual screening

Paul Ehrlich initially proposed the notion of a pharmacophore in 1909. The energetic and geometrical matching processes flanked by drug and their receptor are required for binding relationship to take place. While comparable chemical features have the same or similar pharmacological actions, differing group configurations have varied impacts on the activity.[127] The pharmacophore does not depict a specific particle or a single functional region. It could contribute to a large number of active drugs with comparable pharmacological characteristics or it can refer to a group of compounds with important interaction evidence on the same receptor class. Building a pharmacophore model may be used to virtually screen a small chemical library, searching for better skeletal active molecules, predict compound activity and enhance

and modify compounds. This may be used to measure the structure–activity correlations of drugs and to uncover their selectivity mechanisms. [128]

Target Annotations

Researchers must scrutinize the necessary information on the targets after gathering druggable targets through virtual screening: protein structure, binding pocket shape, target type, related pathway, and corresponding illnesses. Normally, the Universal Protein Database programme (UniProt ID) is used to nominate the targets. The largest database containing the most beneficial data and protein structures is this ID, which also contains the TrEMBL, PIR-PSD databases and Swiss-Prot databases.[129] Detailed information on the target protein, with related molecular activities and biological processes, may be found on the "UniProt" website (http://www.uniprot.org/) using UniProt ID. By searching for PDB IDs, KEGG IDs, and other information, databases can bridge to other databases. Many additional databases and software systems, in addition to UniProt, can scrutinize the category and potential targets functions, as well as the illnesses they cause.

3.10.6. Network Pharmacology

The notion of systems biology underpins network pharmacology. It constructs a map of topological networks of their intricate interactions by treating each medication, gene, target, route, and disease as a distinct "signal node" and an edge is considered for each action model**.** [130]The connections of topological network maps may be used to understand the multi-component-target-multi-pathway processes of herbal drugs, creating traditional plants open for advanced study and innovation. Currently, software tools & web resources could analyze pertinent data on target sets,

linked pathways, and illnesses. QIAGEN Bioinformatics' Ingenuity Pathway Analysis (IPA) software, KEGG pathway database[131] and MetaCore provides few examples. [132]

By analyzing the findings of the virtual screening and target analysis, we may gain a lot of resources on a herb, chemical, target-pathway, or illness, as well as its interrelationships, using the processes outlined above. The relationships between these connected nodes may be shown using a variety of network visualization tools. The most widely used open access software is Cytoscape which is extensively compatible and has good graphical effects compared with other software applications. This allows users to create a 2-D topological network map within particular node using edges and further analyze the pharmacological foundation and mechanism of medicinal plants in a brief and straightforward manner. Every type of node (protein, chemical/illness), as well as the strength of their relationships, may be changed and analyzed independently. [133, 134]

4. MATERIALS AND METHODS

4.1. Drugs and Reagents.

Analytical grade reagents/kits and drugs were used for the study and procured as stated in below Table 1 and 2.

Table 1: List of Chemical and experimental kits

Sr. No	Material Name	Manufacturer
1.	5,5'-dithio-bis[2- nitrobenzoic acid (DTNB)	Hi media, India
2.	Acetylcholinesterase enzyme	Sigma Aldrich, USA
3.	Acetylthiocholine iodide	Sigma Aldrich, USA
4.	Ascorbic acid	Sigma Aldrich, USA
5.	Donepezil	Apotex Research Laboratory Pvt Ltd., Bangalore.
6	Disodium hydrogen phosphate	Hi-Media Laboratories Pvt. Ltd., Mumbai, India.
7.	Sodium dihydrogen orthophosphate	Hi-Media Laboratories Pvt. Ltd., Mumbai, India.
8.	DPPH	Hi-Media Laboratories Pvt. Ltd., Mumbai, India.
9.	ELISA kit (Rat-Amyloid $_{1-42}$ Peptide)	Synergy scientific services Pvt, ltd. Chennai,
10.	Gallic acid	Merk., USA
11.	Quercetin	Hi-Media Laboratories Pvt. Ltd., Mumbai, India.
12.	Scopolamine	Vital Laboratories Ptv Ltd., Gujarat

Table 2: List of Instruments and Equipments

Sr. No	Instrument/ Equipment	Manufacturer
1.	Electronic Weighing Balance (SE-391 Capacity:300 g)	Scientech
2.	UV-VIS Spectrophotometer (2600i)	Shimadzu, China
3.	ELISA Plate reader (96- well plate)	Thermo Scientific Multiskan GO, India
4.	Incubator (Memmert Type acterological Incubator)	Bio Technics
5.	Heating Mantle (KT_HeatingMantle220Volt)	WKM, India
6.	Rota evaporator (RV 10 auto pro V)	IKA RV 10
7.	Tissue homogenizer (RQ-127A/D)	Remi
8.	Centrifuge (Lab X)	Tomy Micro one

The following methods were approached to evaluate the effect of *C. dactylon* and *S. rhombifolia* on cognitive dysfunction by using *in-silico*, *in-vitro*, *in-vivo* studies,

4.2. *In-silico* pharmacology of *C. dactylon* and *S. rhombifolia* against AD.

Network Pharmacology

4.2.1. Mining and drug-likeness property of Phytocompounds [102, 103, 135-137]

The earlier scientific reports, Duke's database and Chemical Entities of Biological Interest (ChEBI) were used to retrieve the phytoconstituents information of the selected plants using keywords '*C. dactylon'* and *'S. rhombifolia'*. Molecular formula, canonical SMILES, molecular weight, hydrogen bond acceptors and donors of each phytochemical was derived from chemical database 'Pubchem'. Further the use of model 'Molsoft', each compound's "drug likeness property" was predicted with the support of "Lipinski rule of five".

4.2.2. Target identification

To collect AD- related targets "Therapeutic Target Database" website https://db.idrblab.org/ttd/ was availed; Uniprot was used to collect each protein and gene ID with 'Homo sapiens' as standard reference. Phytocompounds having druggable features have canonical SMILES and these were submitted to "Find my compound Targets" present in web server Binding DB for identification of target at percentile score of ≥70%. After prediction of targets of compounds, these were correlated with approved therapeutic proteins deposited in TTD and were obtained. [138]

4.2.3. Gene-set enrichment & network analysis

The pathogenesis of AD is due to the alterations in protein targets and these will be modulated by selected phytocompounds; using "STRING" (https://string-db.org/) these combinations were speculated. Further KEGG database was used to collect enriched pathways. Cytoscape 3.6.1 software was used to construct a "compound-gene-pathway" network and this was analyzed by giving command 'Network Analyzer' and treated 'direct'. Gene-compound interactions were revealed by edge count. [139]

4.3. Docking study

AutoDock Vina was utilized to study the docking scores of phytocompounds present in *C.dactylon* and *S. rhombifolia*. 3D-structure of compounds were retrieved as .sdf format from pubchem. Further these were converted to .pdb using Discovery studio 2019 software. X-ray crystallographic structures of protein molecules were obtained from RCSB PDB as .pdb format. Macromolecules were imported and converted to pdbqt format using PyRx 0.8v. Default grid box, exhaustiveness was

kept at 8 for docking. The lowest binding energy of the protein-ligand complex was visualized in Discovery studio visualizer. 2019. [140]

4.4. Taxonomic Details of *C. dactylon L.* and *S. rhombifolia L.*[141, 142]

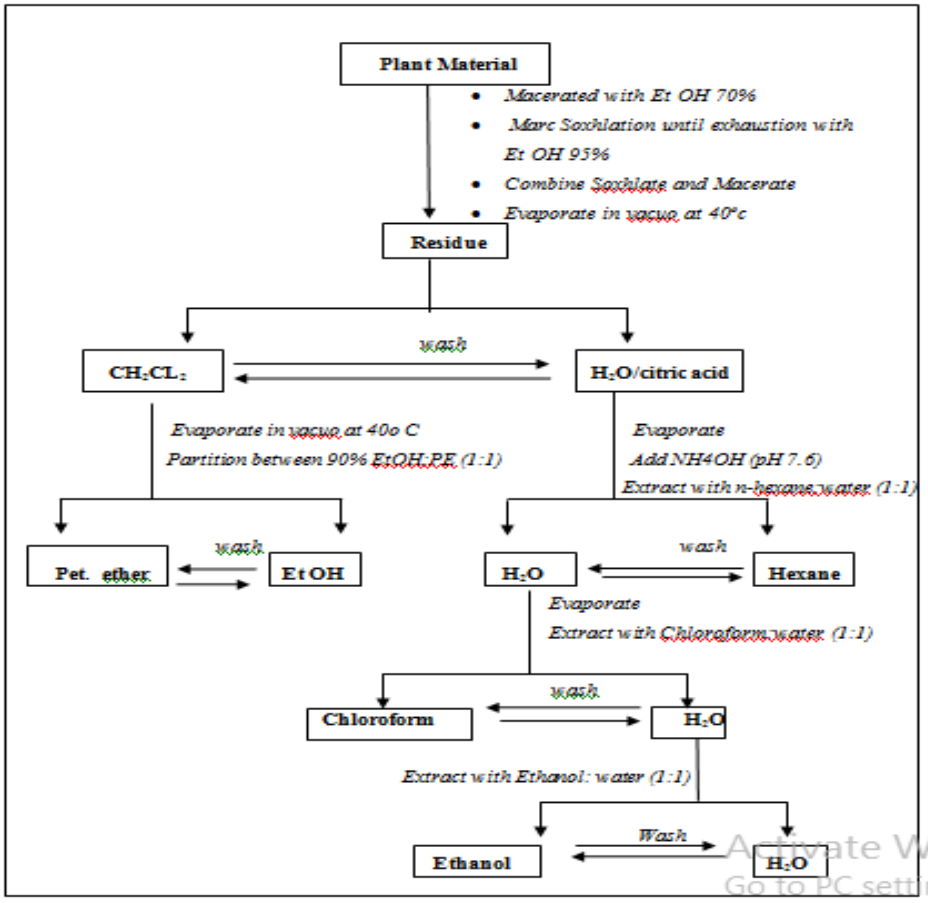
Table 3: Taxonomical details of *Cynodon dactylon* and *sida rhombifolia*

Description/plant	***C. dactylon***	***S.rhombifolia***
Location	Local areas of Belagavi city, Karnataka.	Botanical Garden, Karnataka University Dharwad. Dharwad, Karnataka.
Identification	Hardy, perennial grass, rooting at nodes, forming a dense tuft on the surface of the soil, leaves 2.5-20 cm long, 2-6 mm broad, flat or sometimes folded or convolute; inflorescence on culms 15 cm to 1 m tall consisting of 2-12 spikes arranged star-like at apex of stem; spikes 2.5-10 cm long with numerous spikelets, arranged in 2 rows on one side of spike; spikelets flat, 2-2.5 mm long, awnless, with 1 floret; glumes unequal, the upper longer and one-third to three-fourths length of floret.	Erect branched up to 1 m high. Stem cinereous with stellate hairs. Leaves obovate to cuneate, often more or less rhomboid, ovoid, or lanceolate, apically serrate to crenate, entire towards base, Leaf blades ca 6× 3cm, Flowers axillary, solitary, sometimes in apparent racemes; flowers ca 1.5 cm diameter. Calyx 5 × 6 mm, campanulate, pubescent. Corolla paleyellow or creamy-white, ca 10 × 7 mm, mericarps 8–10, apex with a pair of short divergent awns of unequal length. Seeds brown or black Flowering and Fruiting: August to January. Distribution: Common along roadsides, wastelands, moist places and hills slopes.
Authentication- Botanist at ICMR-National Institute of Traditional Medicine, Belagavi	Voucher specimen-1391 deposited at herbarium of ICMR-NITM Belagavi.	Voucher specimen-**1399** was deposited at herbarium of ICMR-NITM Belagavi.
Image		

4.5. Extraction, preliminary phytochemical test, and LC-MS analysis.

The proposed plants were collected and washed thoroughly in running water, dried for 15 days at room temperature. The dried plant was powdered using a mechanical blender. The maceration of 200 g of plant powder with 1 L of a mixture of ethanol-water (70:30) each time for 12 h. and residue was refluxed at 50°C in a soxhlet apparatus for 8-12hr to prepare hydro-ethanolic extract. The liquid extract was cooled and concentrated by evaporation. The extract was kept in sterile containers in refrigerated conditions, until further use. Fractionation of *C. dactylon* and *S. rhombifolia* extract were carried out as per Cos *et al*., with minor modifications [Figure 8] [84 143]

Figure 8: Schematic chart for crude fractionation of extract



After extraction, crude extract was considered for preliminary qualitative tests [144, 145] as per explained in below table 4.

Table 4: Preliminary Qualitative Analysis

Test	Procedure	Inference
Alkaloids	**Mayer's Test:** To a few ml of plant sample extract, two drops of Mayer's reagent are added along the sides of test tube.	Appearance of white creamy precipitate
Glycoside	50 mg of extract is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to Legal's test 50 mg of extract is dissolved in pyridine, sodium nitroprusside solution is added and made alkaline using 10% NaOH.	pink colour.
Phenolic compounds and Tannins	**Ferric Chloride test** The extract (50 mg) is dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution are added.	Dark green colour indicates the presence of phenolic compound.
Flavonoid	**Alkaline reagent test** An aqueous solution of the extract is treated with 10% ammonium hydroxide solution.	Yellow fluorescence indicates the presence of flavonoids.
Saponins	The extract (50 mg) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 minutes.	A two cm layer of foam

4.5.1. Total Ash value: The dried plant extract (3gms) was placed in a silica crucible and in the furnace incineration was carried out at a temperature of 400^{o}C. Later +the crucible was cooled. Calculated % of total ash.

4.5.2. Extractive Values

4.5.2.1 Water soluble extractive value

The 5gm of powdered drug was added to 100 ml of chloroform water and with the help of an electric shaker, the mixture was shaken for 6 hours continuously.

Further placed overnight for maceration and filter was evaporated to dryness. Percentage was calculated and recorded as explained by Kokate C. K. [146]

4.5.2.2 Alcohol soluble extractive value

The 90% alcohol (100ml) was added to a 5gm powdered plant, placed in a conical flask. It is shaken for 6 hours using an electric shaker and set aside overnight for maceration purposes. Later carefully filter and evaporate to dryness. The extractive was weighed and percentage was calculated as per the protocol described by Kokate CK. [146]

4.6. Determination of the Total Flavonoid content.

Briefly 1ml of different concentrations of standard Quercetin was reacted with 2% methanolic aluminium trichloride (1ml) and placed in a dark area for 10 min. Alongside as blank 2% methanolic $AlCl_3$ was used at 367 nm absorbance was measured using a double beam spectrophotometer. Later the absorbance of extracts were determined and compared to standard curve obtained. Total flavonoid were expressed as mg Quercetin equivalent per gram of sample.[147]

4.7. Determination of Total Phenolic content.

Hydro-ethanolic extract plants were tested for total phenolic content; expressed as Tannic acid equivalent (mg/g) using Folin- Ciocalteu reagent. Standard calibration curve of tannic acid was considered. Concentration (1mg/ml) of plant extract was prepared & added “Folin- Ciocalteu " reagent (2.5ml), 7.5% sodium carbonate (2ml). Absorbance recorded at 760 nm after 30 min of incubation[148]

4.8. LC_MS analysis of compounds

LC_MS 2010A (Shimadzu Japan) was used to perform LC-MS. Column C18 was selected as stationary phase and methanol: water (90:10v/v) was chosen as mobile phase. The dissolved sample (5 µl) was injected and at 254 nm of wavelength absorbance was recorded. Electrospray ionization peaks were used to detect phytocompounds present in samples.[149]

4.9 Evaluation of *in-vitro* antioxidant activity of CDE and SRE

4.9.1. DPPH assay

This assay was performed according to Teh et al method. Sample and control were prepared in triplicates. The DPPH (5mg/2mL) solution was prepared in ethanol. Series of plant extract concentrations (10, 20, 40, 80, 160µg/mL) with ethanol were prepared. In each 96-well plate, an aliquot of plant extract sample was added with DPPH (20µl). To prepare negative control, 200 µl of ethanol and DPPH (20µl) were added. Ascorbic acid was used as standard control. Later in the dark region the microplate was incubated for 30min and at 517 nm using microplate reader, absorbance of each plate was recorded to attain "half maximal effective concentration" (EC50) values.[149]

4.9.2. H_2O_2 Scavenging activity

According to the method explained by Ruch et al, minor modification was followed to estimate the ability of plant extracts to scavenge H_2O_2. 43mM of H_2O_2 solution was prepared using phosphate-buffer (pH 7.4). Sample at different concentrations of 10-100 µg/ml were mixed with 0.6ml of H_2O_2 solution. 10 min

later, absorbance at 230 nm was obtained against a blank solution having a phosphate buffer without H_2O_2. Later percentage of inhibition was calculated.[150]

4.9.3. In-vitro Acetylcholinesterase Enzyme assay

The "Ellman's Method" is used to detect AchE by the spectrophotometer method. 50 mM Tris–HCl (1710 µL) was added to 250 µL samples ranging from 10–160 µg/ mL. To this 10 mM of DTNB (20 µL) and 6.67UmL^{-1} AChE (10 µL) were maintained with pH 8.0. Standard drug solution was prepared in serial concentration similarly. Further incubated at 37^{o}C for 15 min. Acetylthiocholine iodide (200 mM; 10 µL) was added, absorbance at 412 nm noted for 3 minutes.

The test was run in triplicate, and using the Microsoft Excel programme, a linear regression analysis between the percentages of inhibition and the concentration of the extract was run to determine the concentrations of the test extract (IC_{50}) that prevent the hydrolysis of acetylcholine substrate by 50%. [151]

4.10. *In-vivo* pharmacology of CDE and SRE against amnesia

The CDE and SRE effect on cognitive functions were evaluated in a scopolamine induced amnesia experimental model in rats after receiving animal ethical approval from IAEC by 'KLE College of Pharmacy, Belagavi (KLECOP/CPCSEA- reg no-221/Po/Re/S/2000/CPCSEA, Res.25-13/10/2018, either sex rats); maintained pathogen free until study completed'.

4.10.1. Animals

Adult Wistar either sex rats (200-300gm) were selected for study. Polypropylene transparent cages were used to house the experimental rats and

maintained at 25 ± 2ºC by providing pellets (Amrut Laboratories) with water *ad libitum* in a 12/12 hour light-dark cycle. After 7 days of acclimatization, rats were randomized into following different groups.

4.10.2. Acute oral toxicity studies

To find the safe dose of plant extracts *C. dactylon* and *S. rhombifolia*, "acute oral toxicity" test performed according with 'OECD-guidelines 423' to find out observable adverse effects. Three groups having six albino *Wistar rats* were used for experiment. Distilled water was used as a vehicle in the control group. Suspension of prepared extract in distilled water was administered to group II, III respectively with a single dose of 2000 mg/kg through orally using an oral feeding needle. At consecutive hours (i.e, 1, 2, 4, 6 and 24 hours) symptoms of toxicity were recorded if any. The rats were kept under observation for mobility, sensitivity to pain, sound, aggressiveness, and respiration movements for 14 days. Later on the mortality basis LD_{50} was expressed.[152]

4.10.3. Induction of amnesia

Scopolamine induced in aged-rats (22-24 months old) could non-selectively bind to cholinergic receptors and block the acetylcholine transmission in the brain via, enhancing the AchE activity, and increases oxidative stress which induces amnesia. [153, 154] Alongside, based on the previous reports, a combination of age-related factor, administered with scopolamine depicts the neuropathology, oxidative stress and memory impairment.

Scopolamine favours the central cholinergic system's barrier in the animal model and causes informational and new recognition abnormalities. This is depicted

as memory and learning deficit confirmed through *in-vivo* studies with the models of "spatial learning task", "contextual and cued fear conditioning" as well as "inhibitory avoidance" as per the previous reports of Xian et al.[155]

Hence, scopolamine-induced amnesia in aged rats was selected for present study. By administration of scopolamine 1 mg/kg, through intraperitoneal route for 30days to rats as an inducing agent since, it provokes cognitive impairment in animals. [156]

4.10.4. Grouping of experimental animals and treatment

Total 54 *wistar* rats were randomly categorized into 9 groups having six in each as shown in table 5. The scopolamine dissolved in normal saline (1mg/kg, i.p) was induced for 30 days (From day 1 to day 30) to disease and test control groups to provoke amnesia. Test controls such as Donepezil; 3mg/kg, p.o, and hydro-ethanolic extract of *C. dactylon* and *S. rhombifolia* (reconstituted in CMC 0.5%) [157, 158] were administered to the respective groups at the therapeutic doses (100, 200, and 400mg/kg, p.o) for 15 days (16th - 30th day of experiment) as represented in schematic diagram 9. Further, behavioural models (MWM, EPM, PAT) were used to assess cognition of rats. Additionally later, rats were euthanized and decapitated; brain was isolated for assessment of neuronal bio-markers.

Figure 9: Study Design for Behavioural models

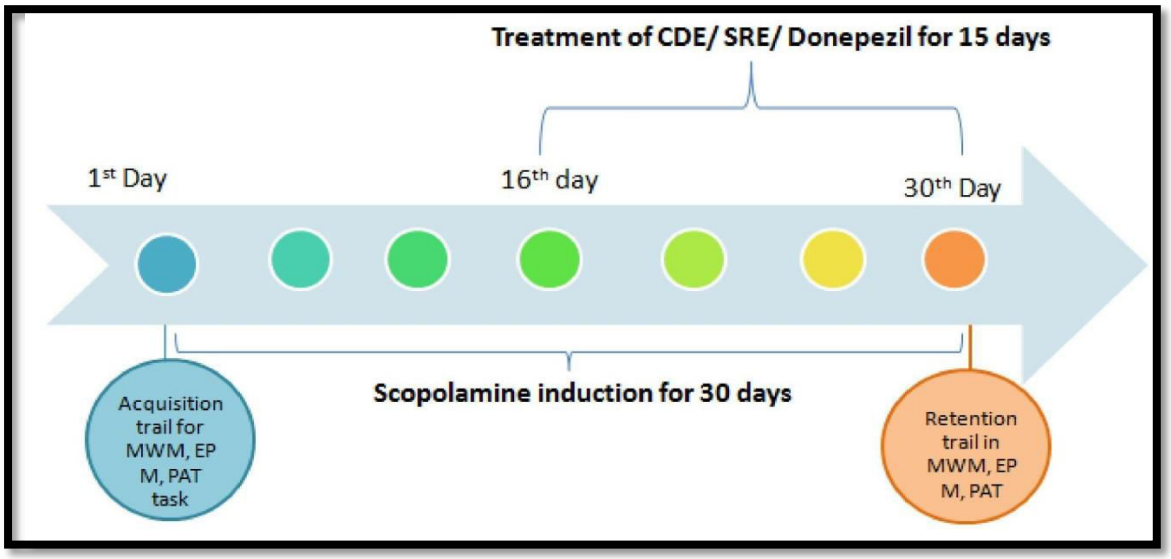


Table 5: Grouping of animals for cognitive study

Group (n=6)	**Treatment**
I	Normal Control (Normal Saline p.o for 30 days)
II	Disease Control (Scopolamine Hydrobromide 1mg/kg, i.p)
III	Standard control (SCO + Donepezil 3 mg/kg p.o for 15 days)
IV	SCO + CDE 100 mg/kg p.o for 15 days
V	SCO + CDE 200 mg/kg p.o. for 15 days
VI	SCO + CDE 400 mg/kg, p.o. for 15 days
VII	SCO + SRE 100mg/kg, p.o for 15 days
VIII	SCO + SRE 200mg/kg; p.o for 15 days
IX	SCO + SRE 400mg/kg; p.o for 15 days
Note: Scopolamine 1 mg/kg was administered intra-peritoneally to groups II-IX for 30 day to provoke cognitive impairment. **CDE**: *C. dactylon* hydro-ethanolic extract, **SRE:** *S. rhombifolia* hydro-ethanolic extract, **p.o:** orally	

4.11. Behavioural models

4.11.1. Morris water maze

This model is used to evaluate "spatial memory" of experimental animals. By acting as its own motivation, getting out of the water eliminates the need for other inducements like hunger and thirst. Water creates a constant environment and eliminates disturbance caused by olfactory signals. [159] The water-filled circular pool with a platform of 10 cm × 10 cm immersed 2 cm under the water surface. During each training day, the starting place was randomized but kept the same for all the rats in each experiment; in each trial, rats' head pointed at the sidewall when allowed to swim for 90 s to check the missing platform. Before the test, rats were habituated for training trials and were allowed to swim. If it is not habituated, then the rat was placed manually for 30 s suppose if rats do not reach the platform in 120 s.

Acquisition trail- The initial location for each day of the trial was randomized, but it remained the same for all rats during experiment. At each trial, the rat was released into the pool with its head oriented towards the walls of the tank and given 90 seconds to find a concealed platform.

Retention trail- The time spent in search of missing platform is known as probe trial which was referred as memory retrieval. [160]

4.11.2. Elevated Plus Maze

This apparatus consists of two open arms (50 x 10cm) and two closed arms (50 x 10 x 40cm) with an open roof and elevated to 50cm height from ground level. To note the acquisition memory; rats were placed on day 1 of the treatment

individually on either end of the open arm. Time taken for the rat to move to the closed arm was considered as transfer latency (TL). Later on day 30 of treatment retention of the memory was determined. The animal moved quickly from the open arms to the closed ones, keeping the 60-second cutoff time as the maximum amount of time permitted. [161]

4.11.3. Passive Avoidance Test:

As part of the acquisition trail, each rat was placed in the illuminated compartment, and ten seconds later the guillotine door was raised. Rat entered the dark box and was shocked for two seconds (50 hertz/1 mA) of continuous current. Each rat had to be retrained in the apparatus, and if it failed to enter the dark room within 120 seconds, it was shocked on the foot. After the rat had been in the light chamber for 120 seconds, the acquisition trail for the first day came to an end. On the thirty-day mark, the animal was put into the light compartment, and the latency of the animal to enter the dark room was noted. This escape delay was used to assess the retention efficiency of the step-through avoidance reaction. [162]

4.12. Euthanasia and brain sample collection

For euthanasia; the method 'Cervical dislocation' was used for sacrificing experimental animals. Further rat brains was removed. Brain homogenate 10% w/v was prepared by placing in ice-chilled 0.1M phosphate buffer maintained at pH 8.0. To perform neurochemical estimations the supernatant layer was collected by centrifugation at 3000rpm for 10min.

4.13. Acetylcholinesterase enzyme estimation

The Ellman et al. procedure was followed for AchE enzyme estimation. The rat brain homogenate 0.4ml was mixed with 2.6ml; 0.1M phosphate buffer (pH 8). The 100 µl of Ellman's reagent [(5,5-dithio-bis-(2-nitrobenzoic acid)] , followed by Acetylthiocholine iodide (20 µl) reagent was added. The absorbance change per minute was noted at 412 nm upto 5min in terms of moles of substrate hydrolyzed per min per gram was recorded. [163]

4.14. Amyloid beta $_{1-42}$ estimation

Enzyme Linked Immunosorbent Assay (ELISA) method was performed to test the β amyloid content in brain homogenate. The sandwich method is used for accurate quantitative detection of Rat amyloid beta peptide $_{1-42}$ in plasma, cell lysates, tissue homogenates and serum samples.

Procedure:

Sample Preparation: The brain tissue was rinsed with Phosphate Buffer Solution (PBS) pH 7.4 to remove excess blood and weighed before homogenization. On the ice bath tissue was homogenized with PBS (pH 7.4) in glass homogenizer. Later thaw at 2-8^{o} C and centrifuged at 3000 RPM for 20min approximately.

Standard stock solution: 120µl of standard diluent was added to 120µl of standard (2400pg/ml) to make 1200pg/ml as stock solution. Further serial dilution was prepared in 1:2 ratio to make 600, 300, 150, and 75 pg/ml solutions.

Blank well: contains anti Aβ1-42 antibody (biotin labelled), streptavidin-HRP, reagent chromogen A and B, & stop solution. **Standard well**: Add 50µl of standard

and streptomycin–HRP 50 µl. **Sample well**: Fill with 40 µl of sample and 10 µl Aβ1-42 antibody, 50 µl streptomycin–HRP. Seal the plate and incubate at 37^0 C for 60 minutes. Take out the sealer and wash using the wash buffer. Every well was soaked with 0.35 ml of wash buffer for 30 sec. This step is repeated 5 times later paper is blotted. Chromogen A (50 µl) was added to each well followed by Chromogen B (50 µl) for color development. This mixture was incubated for 10 min in a dark place. Add 50µl 'Stop solution' to each well, the blue to yellow colour changes immediately. Absorbance measured within 10 min, after addition of stop solution. Using multi-scan spectrophotometer at OD 450 nm range absorbance was measured. The readings were made triplicate.

4.15. Assay of Lipid Peroxidase activity

Using the Ohkawa et al. approach, the quantity of TBARS found in the hippocampus is used to evaluate lipid peroxidation. µBriefly, 1.5 ml of 20% acetic acid was added after 100 µl of whole brain homogenate in phosphate buffer (0.1 M, pH 7.4) was incubated with 200µl of sodium dodecyl sulphate (10%, w/v) for 10 min. 1.5 ml of thio-barbituric acid (0.8%) was added to their action mixture and incubated there for 1 hour in a water bath that was boiling. The hue of the solution changes to pink. This was read at 532 nm, and the quantity of TBARS was determined using a 1.56 105 m/cm molar extinction coefficient. Nano moles of MDA/mg of protein were used to express the TBARS value. [164]

4.16. Estimation of Glutathione (reduced)

Ellman et al method was followed to measure Glutathione content. Briefly to the 2.5ml of sodium phosphate buffer and 50µl of Ellman's reagent, 250 µl of brain

homogenate was added.Mixed well and incubated and within 15-min at 412 nm absorbance was noted using UV spectrophotometer to express GSH in µmoles/mg of sample tissue. [165]

4.17. Histopathology

The brain tissues of rats were collected from different groups and fixed in 10 % Neutral buffered formalin. 4µm 'paraffin-embedded' sections were processed. Reagent Hematoxylin and Eosin was used to stain different sections and focused under microscope 40X. [166]

4.18. Statistical analysis

In the *in-silico* pharmacology Gene count and false discovery rate interprets protein-protein interactions regulated by KEGG pathway. Based on the edge count phytocompound- protein-pathway interactions were evaluated. For the Molecular docking study binding energy (kcal/mol) and H-bond interaction/s interprets lead hits specific activity. All the experimental data were presented in mean ± SEM wherever applicable. "One-way/ two way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparison test" were adopted to evaluate quantitative data using GraphPad Prism (Version 5: Graph Pad Software Cooperation, San Diego California, USA).

5. RESULTS

In-silico, *in-vitro*, *in-vivo* pharmacology of *C. dactylon* against cognitive impairment.

5.1. *In-silico* pharmacology of *C. dactylon*

5.1.1. Mining and drug-likeness property of C. dactylon phytocompounds and targets prediction

78 Phytocompounds from the *C. dactylon* were identified from phytochemical interaction database, Dr. Dukes database, Chemical Entities of Biological Interest (ChEBI) and scientific reports. Among 78 compounds, 8 showed positive drug likeness score and Catechin scored highest i.e. 0.64 (Table 6). These compounds were predicted to target 122 protein molecules. The peer-interpretation identified 3 compounds to target 5 therapeutic protein molecules associated with AD (Table 7).

Table 6: Drug likeness property of *C. dactylon* L. phytocompounds

Phyto constituents	PubchemID	MF	MW	HBD	HBA	LogP	DLS
Apigenin	5280443	C15 H15 N O3	257.11	1	3	2.68	0.29
Vitexin	5280441	C21 H20 O10	432.11	7	10	0.77	0.6
Orientin	5281675	C21 H20 O11	448.1	8	11	0.33	0.59
Luteolin	5280445	C15 H10 O6	286.05	4	6	2.78	0.38
ethyl α-D-glucopyranoside	11127487	C8 H16 O6	208.09	4	6	-1.79	0.01
Quercetin	5280343	C15H10O7	302.23	5	7	1.19	0.52
Kaempferol	5280863	C15H10O6	286.24	4	6	1.61	0.5
Catechin	9064	C15H14O6	290.27	5	6	0.53	0.64

Table 7: Probable targets involved in AD modulated by *C. dactylon* L. phytocompounds

Compound	Compound type	Gene ID (compound – target probability score)
Apigenin	Flavonoid	ACHE (0.93), BCHE (0.86), ADORA2A (0.86), MAOA (0.97), MAOB(0.97),
Kaempferol	Flavonoid Amine oxidase (flavin-containing) A	ACHE (0.97), BCHE (0.97), ADORA2A (0.97), MAOA (0.97), MAOB (0.86), BACE1 (0.7) MAOA(0.97)
Luteolin	Flavanoid	MAOA (0.9), MAOB (0.9).
Quercetin	Flavonoid	MAOA (1), MAOA Amine oxidase (flavin-containing) A (0.92)

5.1.2. Gene set enrichment pathway

The gene set enrichment analysis showed 6 molecular pathways modulated by the 3 phytocompounds. These pathways were potentially involved in AD pathogenesis i.e. metabolic pathways, serotonergic synapse, arginine and proline metabolism, Tyrosine metabolism, Tryptophan metabolism, Phenylalanine metabolism and Histidine metabolism (Table 8). The network analysis showed Apigenin, Luteolin, Quercetin and Kaempferol to score highest edge count and these compounds were identified as Flavonoids to target MAOA and MAOB. (Figure 10)

Table 8: Enrichment analysis of protein targets involved in AD

KEGG ID/Gene Ontology ID	Pathway description	Gene count	False Discovery Rate	Protein targets within the network
hsa01100	Metabolic pathways	30	1.16E-08	HSD17B2,ALOX12,NT5E,ARG2,TYR,MECR,SI,AKR1B1,CBR1,NOS3,FASN,GAA,ALOX12B,COX5A,GANAB,MAOA,AKR1B10,PTGS1,PNLIP,ALOX5,ALPL,MAOB,XDH,CYP1A1,ALOX15B,CYP19A1,TST,MGAM,ALOX15,HSD17B1
hsa04726	Serotonergic synapse	8	1.04E-05	ALOX12,ALOX12B,MAOA,PTGS1,ALOX5,MAOB,ALOX15B,ALOX15
hsa00330	Arginine and proline metabolism	4	0.002	ARG2,NOS3,MAOA,MAOB
hsa00350	Tyrosine metabolism	3	0.0083	TYR,MAOA,MAOB
hsa00380	Tryptophan metabolism	3	0.0108	MAOA,MAOB,CYP1A1
hsa00360	Phenylalanine metabolism	2	0.0234	MAOA,MAOB
hsa00340	Histidine metabolism	2	0.0317	MAOA,MAOB

Figure 10: Network representation of *C.dactylon* Phytocompounds-target proteins-pathways

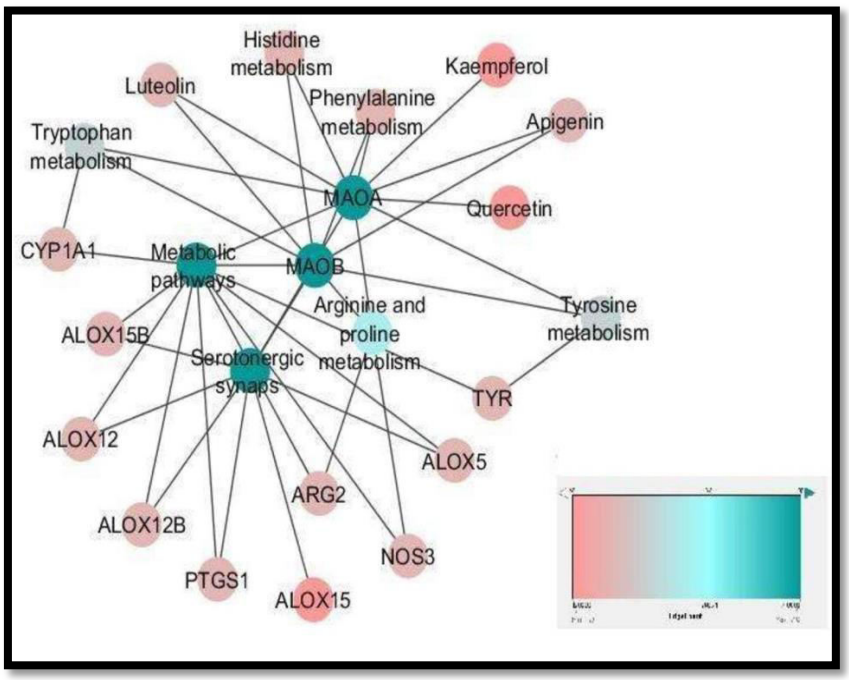
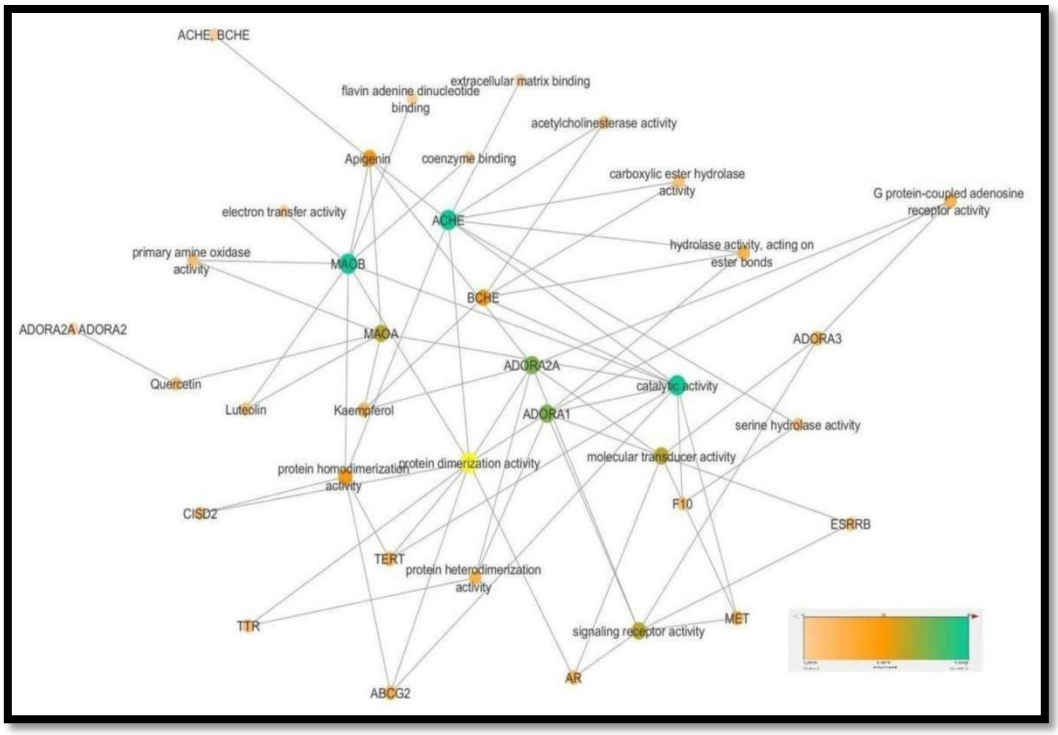


Figure 11: Gene ontology presentation of *C.dactylon* Phytocompounds-target proteins-pathways



5.1.3. Gene ontology analysis

The 4 functional pathways i.e, identical protein binding, acetylcholinesterase activity, protein dimerization activity, serine hydrolase activity were identified to target the proteins of interest present in AD pathogenesis. The apigenin, luteolin, Kaempferol and quercetin showed highest edge count score for ACHE, BCHE, ADORA2A targets. (Figure 11)

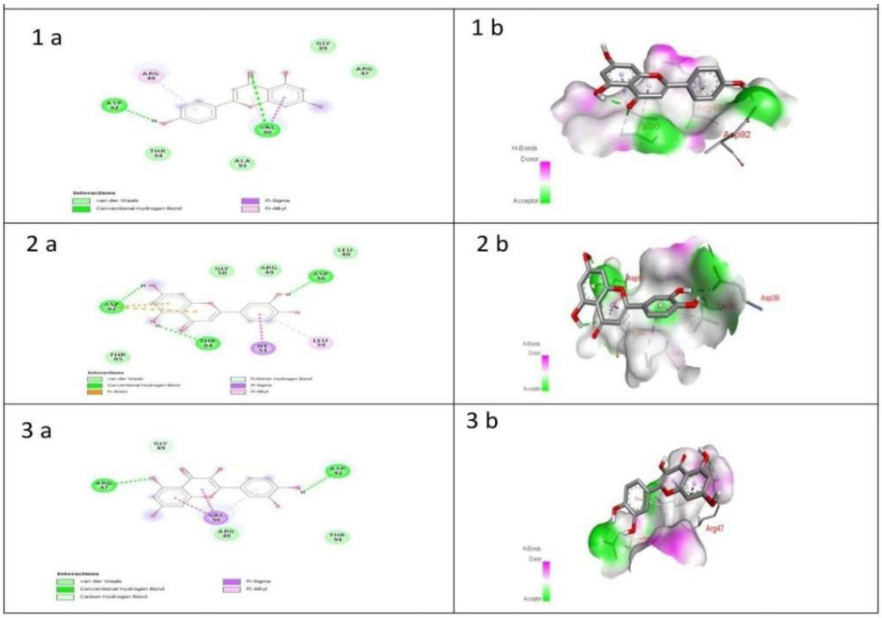
5.1.4. Docking Score

Among the three compounds, Quercetin scored least binding energy (-4.04 kcal/mol) whereas luteolin scored least binding energy (-3.65 kcal/mol). The binding affinity, Inhibitory Constant and Hydrogen bond interaction is represented in Table 9. The interaction of each phytoconstituents with AChE is shown in Figure 12.

Table 9: Binding Affinity of bioactives with AchE

Compounds	**Binding energy (Kcal/Mol)**	**Inhibitory constant (mM)**	**H bond interaction**	**Number of Hydrogen Bond interactions**
Quercetin	-4.04	1.08	ARG47, ASP92	2
Apigenin	-3.82	1.59	ASP92, VAL90	2
Luteolin	-3.65	2.11	ASP36,THR94, ASP92	3

Figure 12: Docking Score of *Cynodon dactylon L.* Phytoconstituents with AChE



Binding analysis Apigenin, Luteolin, Quercetin of with AChE 1) Interaction of Apigenin with AChE a) 2D representation b) Apigenin within AChE binding pocket. 2) Interaction of Luteolin with AChE a) 2D representation b) Luteolin within AChE binding pocket 3)) Interaction of Quercetin with AChE a) 2D representation b) Quercetin within AChE binding pocket

5.2. Preliminary Phytoconstituents tests.

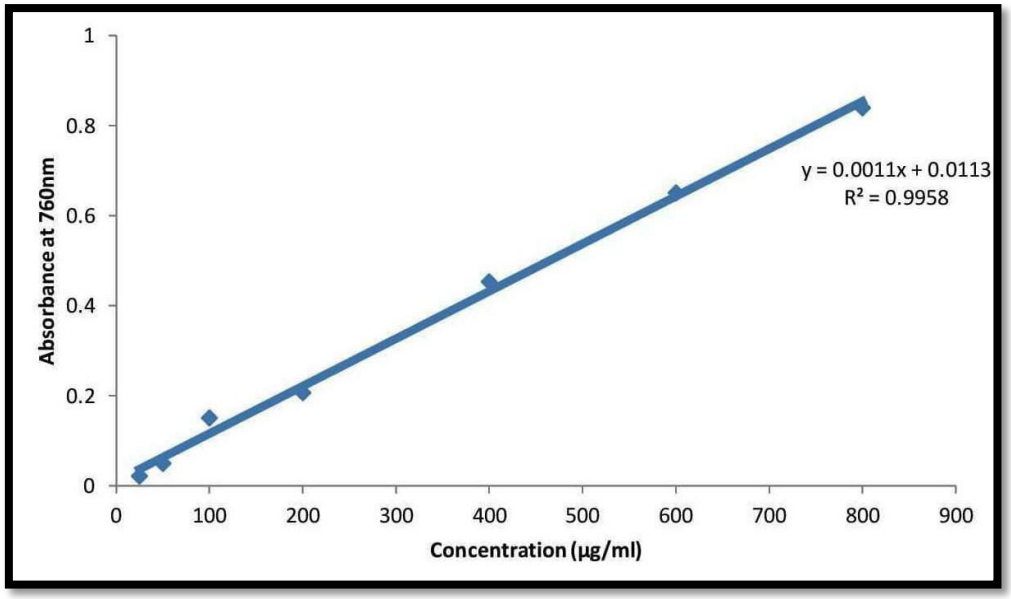
Table 10: Preliminary phytochemical tests of CDE

Physico-chemical Properties	***C. dactylon* extract**
% yield (W/W)	9.47%
Phytoconstituents	Phenols, flavonoids, tannins, saponins, and steroids
Total ash value	9.5%
acid insoluble ash	6.0%
water-soluble extract (W/W)	10.2%
alcohol-soluble extract (W/W)	4.2%
Total phenolic content (GAE/g)	**71.07ug/ml**
Total flavonoid content (QE/g)	**106 ug/ml**

5.2.1. Total Phenolic content

Total phenolic content was estimated by using Gallic acid as standard and expressed as mg of Gallic Acid Equivalent (GAE). The extract having considerable amounts of phenolic content present in *C. dactylon* hydroethanolic extract was 71.07 µg GAE /mg as shown in Figure 13.

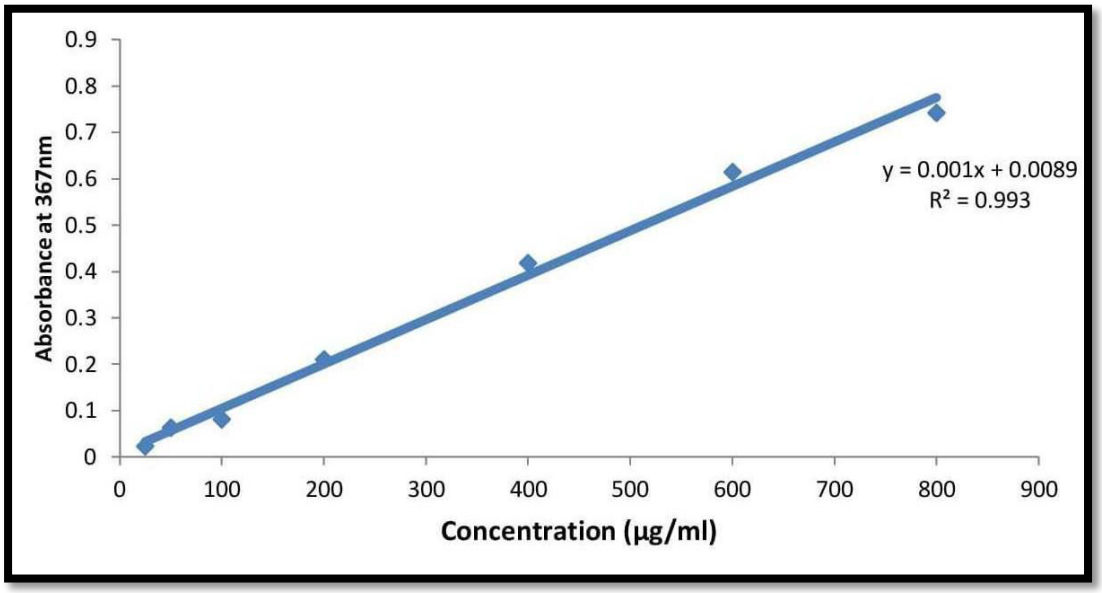
Figure 13: Standard Calibration of Gallic acid



5.2.2. Determination of flavonoid-content

'Quercetin' considered as the most acceptable standard for estimation of total flavonoid-content in sample drugs and expressed as milligrams of Quercetin Equivalent (QE). Hydro-ethanolic extract of *C. dactylon* showed the presence of flavonoid content as 106.27 µg QE/mg as shown in Figure 14

Figure 14: Standard Calibration of Quercetin



5.2.3. LC-MS studies to identify the phytochemicals from C. dactylon Figure 15A, 15B

Figure 15A: LC-MS chromatogram of *C.dactylon* Aqueous Extract

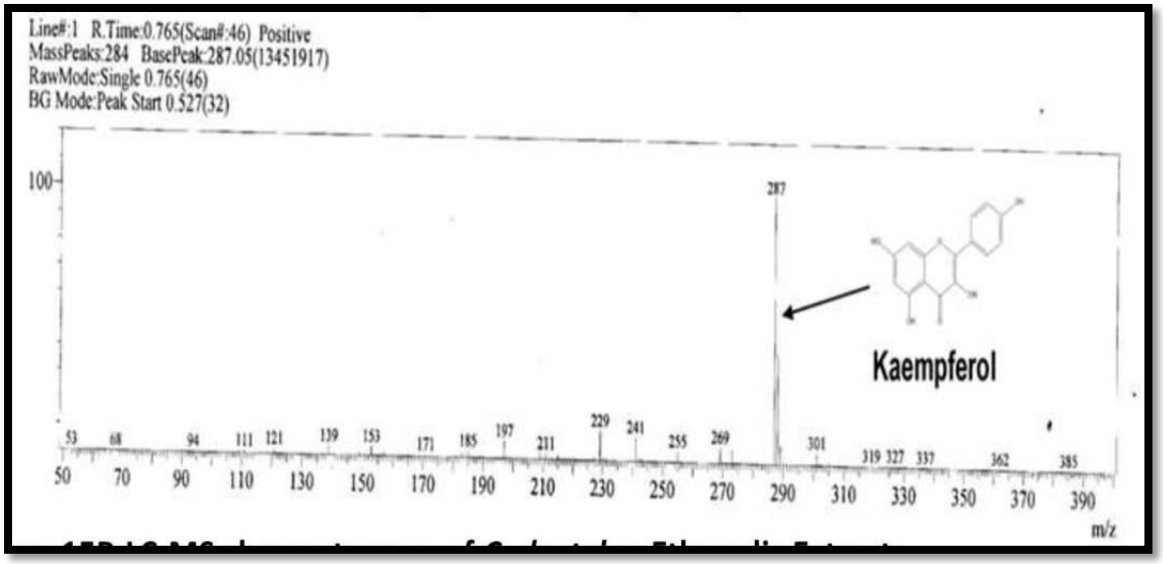
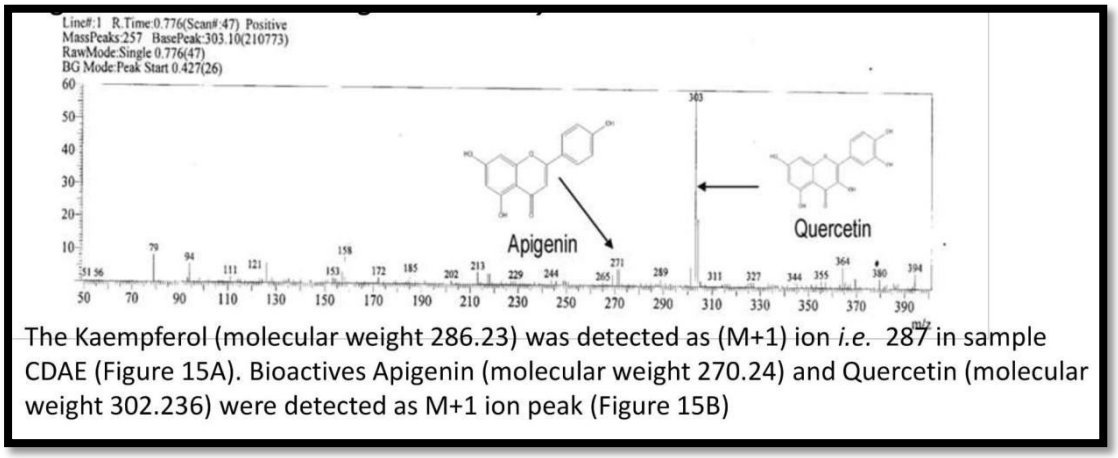


Figure 15B: LC-MS chromatogram of *C.dactylon* Ethanolic Extract

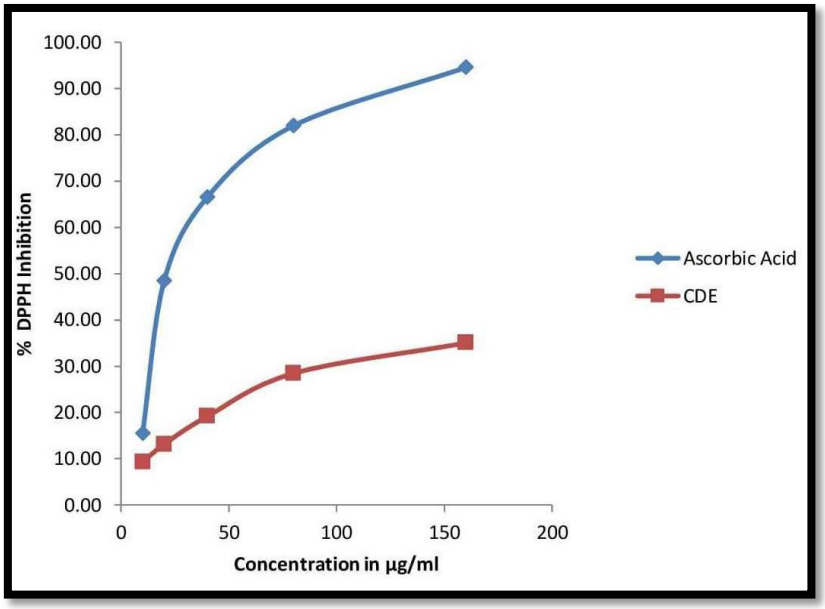


The Kaempferol (molecular weight 286.23) was detected as (M+1) ion *i.e.* 287 in sample CDAE (Figure 15A). Bioactives Apigenin (molecular weight 270.24) and Quercetin (molecular weight 302.236) were detected as M+1 ion peak (Figure 15B)

5.3. *In-vitro* antioxidant screening

To understand the antioxidant potential of extract, DPPH radical scavenging property is the most commonly utilized method based on evaluating IC_{50} parameter and % DPPH inhibition in specific time which was measured by using Ascorbic acid as standard. The *C. dactylon* extract inhibits DPPH at IC_{50} value at 255.6 ± 17.54 µg/ml as compared to ascorbic acid IC_{50} at 35.73 ± 0.85µg/ml. The extract has dose-dependent activity by increasing DPPH scavenging as shown in Figure 16.

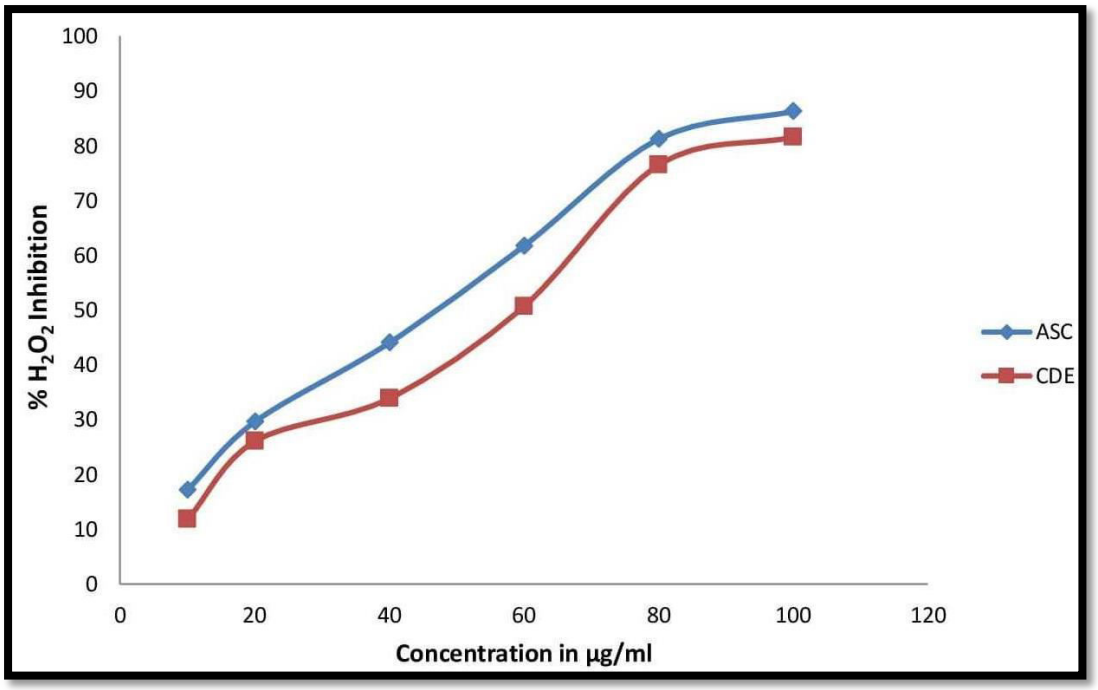
Figure 16: DPPH scavenging activity by CDE



5.3.1 H_2O_2 scavenging activity of CDE

H_2O_2 modifies lipids, proteins and DNA which results in loss of synaptic function considered as one of the characteristic features of AD. Hence the current study validates percentage inhibition of H_2O_2 by the selected plant extract using *in-vitro* method. H2O2 was inhibited by CDE at IC_{50} at 56.94 ± 0.594µg/ml as compared to ascorbic acid 47.81 ± 0.6639µg/ml indicated antioxidant potency as shown in Figure 17.

Figure 17: H_2O_2 Inhibition by CDE



5.4. In vitro estimation of Ach E enzyme inhibition by CDE

Ellaman's method is widely used to estimate acetylcholinesterase enzymes. The IC_{50} value indicates the amount of sample concentration required to inhibit 50% of AchE enzyme. The smaller IC_{50} value higher the inhibition. We identified that Hydro-ethanolic extracts of *C. dactylon* inhibited Acetylcholinesterase enzyme significantly as shown Figure 18 and 19.

Figure 18: IC_{50} values for AchE enzyme inhibition by *in-vitro* method

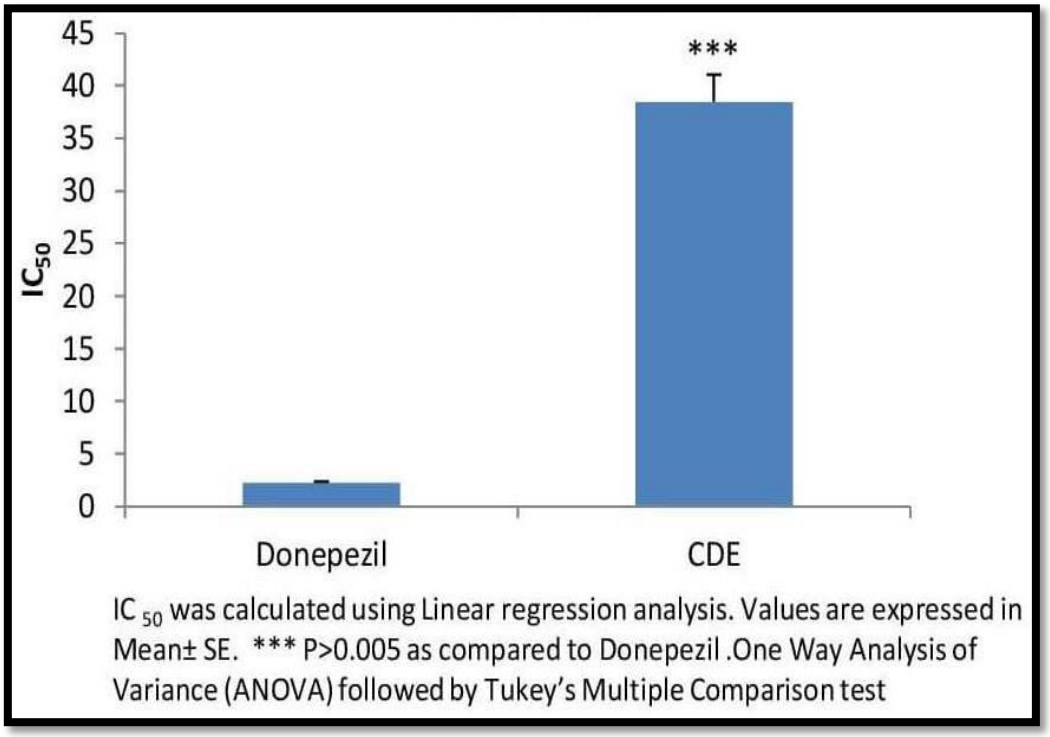
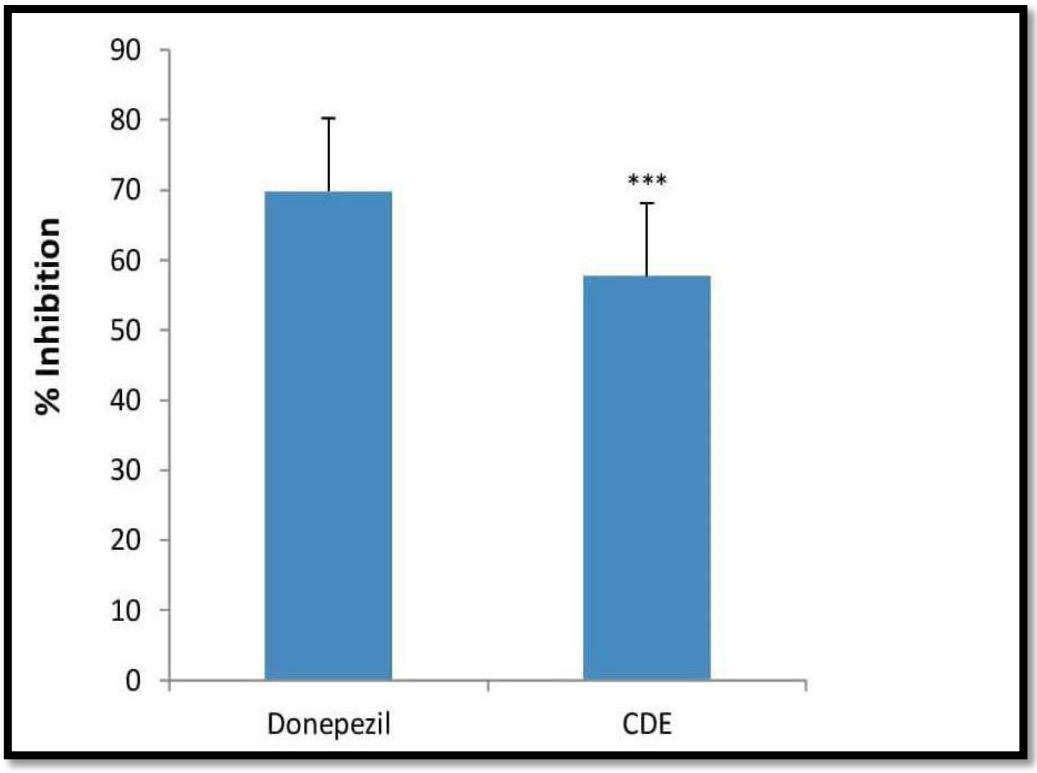


Figure 19: % inhibition AchE enzyme by CDE



5.5. *In-vitro* AchE inhibition by fractions

The *C. dactylon* fraction rich in flavonoids showed significant inhibition of AChE (8.32±045 µg/ml) as compared to fraction rich in alkaloids, phytosterols, terpenoids in the *in-vitro* assay as describe Table 11.

Table 11: *In-vitro* AchE inhibition by *C. dactylon* crude fractions

Plant extract/ Crude fraction (% yield) Fraction rich in	AChE inhibition IC_{50} (µg/ml)
Flavanoids (0.6)	8 .32± 045*
Alkaloids (0.35)	12.47± 1.72*
Phytosterols (0.4)	18.74± 1.92*
Terpenoids (0.5)	29.54± 0.41*
C. dactylon extract (9.47)	38.47 ± 2.56 *
Donepezil	2.23 ± 0.141

IC_{50} values were expressed as Mean±SEM, *P <0.05 as compared to Donepezil

5.6. Effect of *C. dactylon* extract on behavioral models

5.6.1. Spatial memory improvement by C. dactylon extract

Morris water maze task evaluates spatial memory restoration. Figure 20a represents 7 days training for each rat to train the rats and escape latency was remarkably decreased on 7th day compared to 1st day. Figure 20b reports intervention by *C. dactylon* and Donepezil treatment and showed decreased escape latency compared to scopolamine induced rats from day 16 to day 30. Figure 20c expressed the significant elevation in escape latency ($P < 0.001$) compared to the normal control group on retention trial. However, treatment with Donepezil and *C. dactylon* extract

showed decreased escape latency significantly ($P < 0.001$) compared to scopolamine given rats. Figure 20d noticed that the scopolamine induced rats spent less time in the target quadrant as compared to normal control rats; but treatment of donepezil and *C. dactylon* extract to rats showed more time spent in the target quadrant ($P < 0.001$) compared to scopolamine-induced rats significantly.

Figure 20: Effect of CDE on spatial memory in MWM

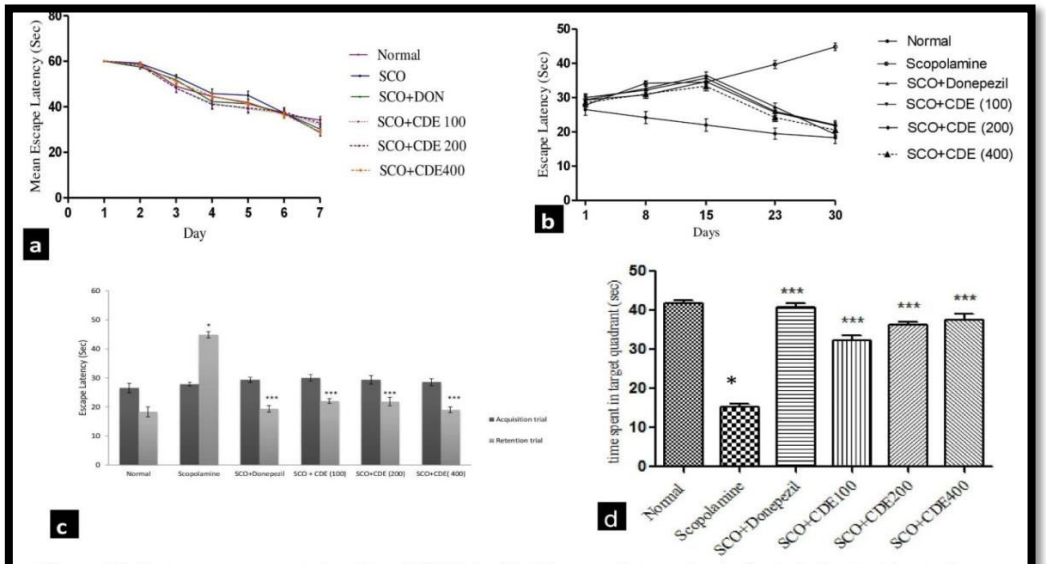


Figure 20: Data were represented as Mean± SEM (n=6), (a) escape latency (sec) of rats during training trails, (b) escape latency expressed by rats for every 7 days of treatment, (c) Probe trial (time spent to reach target quadrant) compared to acquisition trial, (d) time spent in target quadrant by rats on probe trial. * As compared to normal control group. ***Compared to scopolamine control group. $P < 0.001$ was considered as statistically significant. SEM= Standard error of mean

5.6.2. Effect of C. dactylon extract on short term memory and fear aggravated task

Following 5 minutes of exploration in an elevated plus maze, Figure 21a depicts the influence of CDE on the TL, whereas Figure 21b depicts the proportion of time spent in open arms. In comparison to the control group, the scopolamine-induced rats had a higher TL. When compared to scopolamine-induced rats, donepezil (3 mg/kg) and *C. dactylon* extract at 100, 200, and 400 mg/kg dosages resulted in a substantial reduction in TL. When compared to normal control rats, scopolamine-induced rats spent considerably less time at open arms ($P < 0.001$). In comparison to

scopolamine-induced rats, donepezil and CDE at specific therapeutic dosages demonstrated increased time spent percentage in open arms. This suggests that the *C. dactylon* extract retains short-term memory.

On the day 30 i.e, retention phase, rats given SCO had a significantly lower ($P<0.001$) step through latency, which might imply enhanced fear exacerbated behavior. However, as demonstrated in Figure 16c, treatment groups treated with donepezil (3 mg/kg), CDE 100, 200, and 400 mg/kg doses showed enhanced step through latency, which implies better non declarative memory.

Figure 21: Effect of CDE on short term memory and fear aggravated task

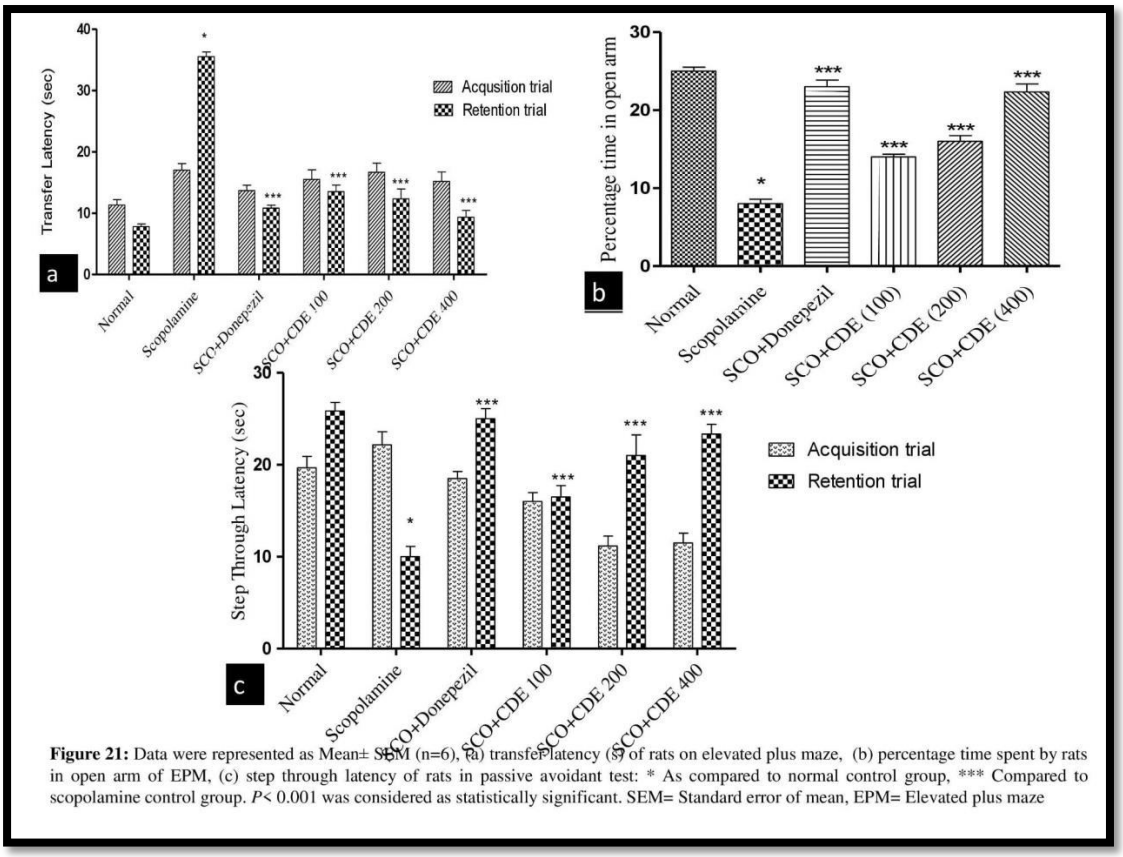


Figure 21: Data were represented as Mean± SEM (n=6), (a) transfer latency (s) of rats on elevated plus maze, (b) percentage time spent by rats in open arm of EPM, (c) step through latency of rats in passive avoidant test: * As compared to normal control group, *** Compared to scopolamine control group. *P*< 0.001 was considered as statistically significant. SEM= Standard error of mean, EPM= Elevated plus maze

5.7. Neurochemical studies

5.7.1. AchE Inhibition level

In comparison to normal group rats (7.263±0.4508), scopolamine significantly enhanced AChE enzyme activity expressed as micromoles hydrolyzed/min/g (24.71 ± 0.9231; P< 0.001). When compared to SCO provoked animals, the donepezil treated group showed a reduced AChE level of (8.099±0.75; P< 0.001). When compared to scopolamine-induced animals, rats treated with *C. dactylon* at 100 mg/kg, 200 mg/kg, and 400 mg/kg significantly inhibited AChE levels (P < 0.001; 14.04 ±1.77, 11.34 ±0.49, 9.326± 0.33). As a result, the *C. dactylon* extract may improve cholinergic transmission by lowering the level of the acetylcholinesterase enzyme which is illustrated in Figure 22.

Figure 22: Effect of CDE on AchE level in brain homogenate

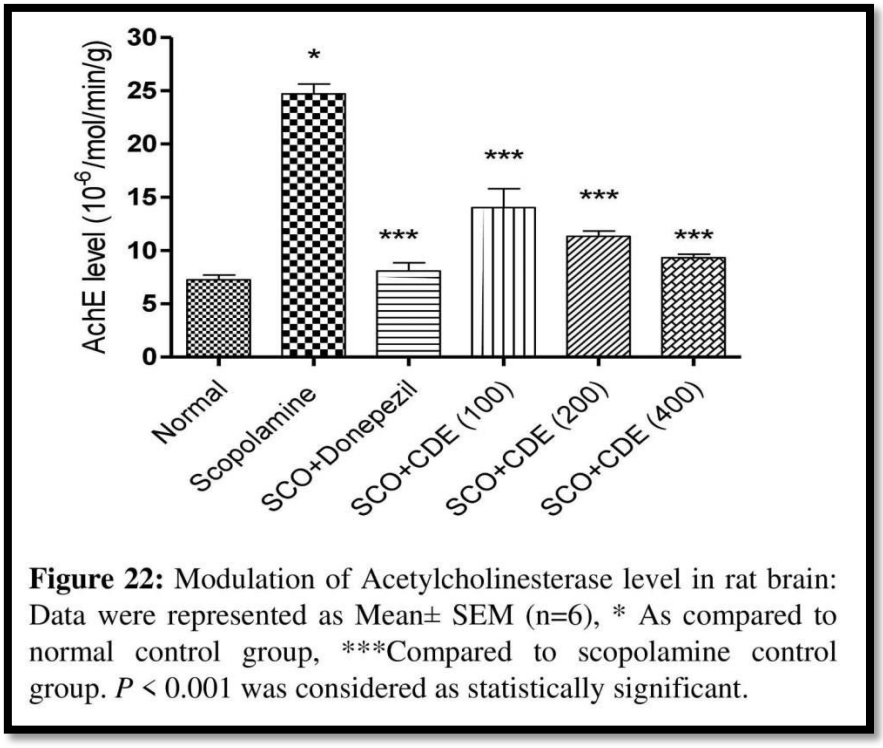
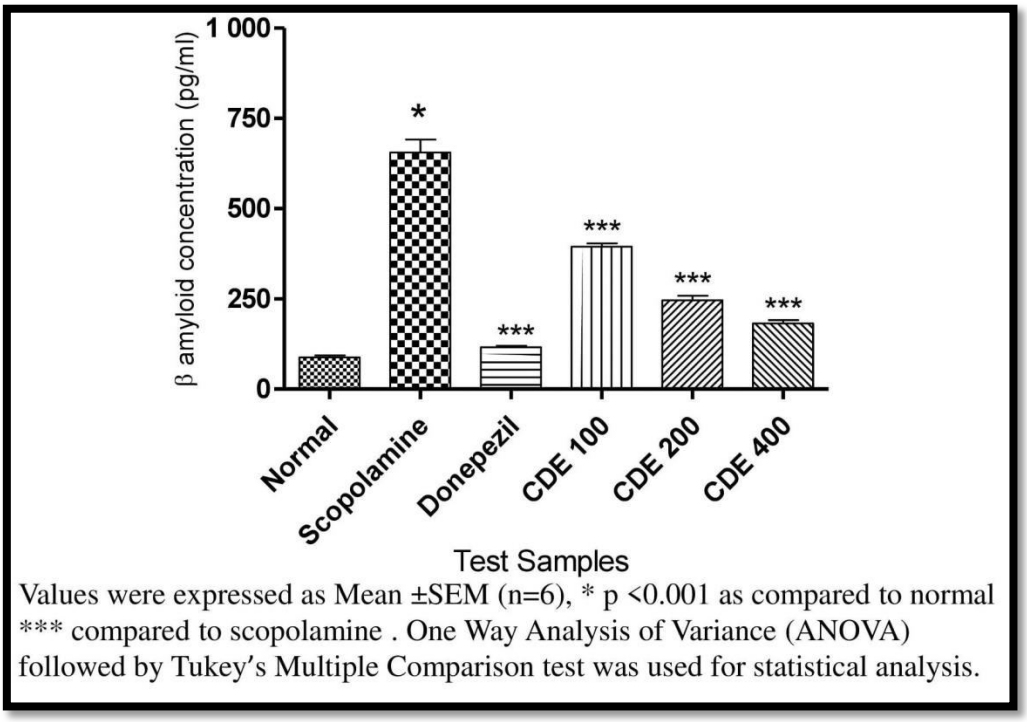


Figure 22: Modulation of Acetylcholinesterase level in rat brain: Data were represented as Mean± SEM (n=6), * As compared to normal control group, ***Compared to scopolamine control group. $P < 0.001$ was considered as statistically significant.

5.7.2. Effect of C. dactylon extract on Beta amyloid $_{1-42}$ level in brain homogenate.

Beta amyloid content is one the hallmark of AD to assess this sandwich method suitable for rat brain homogenate. In comparison to normal control rats (88.27pg/ml), scopolamine-induced animals had higher beta amyloid levels (P< 0.001; 655.7pg/ml). Donepezil, C.dactylon, considerably reduced beta amyloid quantities in rat brain homogenate as compared to scopolamine-induced rats, as seen in Figure 23.

Figure 23: Effect of CDE on Aβ$_{1-42}$ level in rat brain



Values were expressed as Mean ±SEM (n=6), * p <0.001 as compared to normal *** compared to scopolamine . One Way Analysis of Variance (ANOVA) followed by Tukey's Multiple Comparison test was used for statistical analysis.

5.7.3. Antioxidant potential of C. dactylon extract on glutathione and malondialdehyde levels.

In comparison to normal group rats (1.39±0.05), animals given with scopolamine had significantly lower glutathione levels (P< 0.001; 0.53± 0.05). As

revealed in Figure 24a, animals given donepezil (1.23 ±0.115; P < 0.001) and *C. dactylon* extract significantly raised GSH levels (0.87 ±0.15; 1.04 ±0.06; 1.18± 0.036: P< 0.001) as compared to SCO-induced rats.

Compared to normal rats (12.8 ±22.86), the animals induced with scopolamine showed a significant rise in MDA levels (P<0.001; 39.53 ±3.05). In contrast to scopolamine-induced rats, *C. dactylon* extract and donepezil supplied at therapeutic dosages to rats significantly lowered MDA levels, (P<0.001; 19.23±4.05, 17.09±3.16, 16.03±2.75, 11.75±1.97) indicating decreased LPO in the brain, as shown in Figure 24b.

Figure 24a: Effect of CDE on GSH level in rat brain

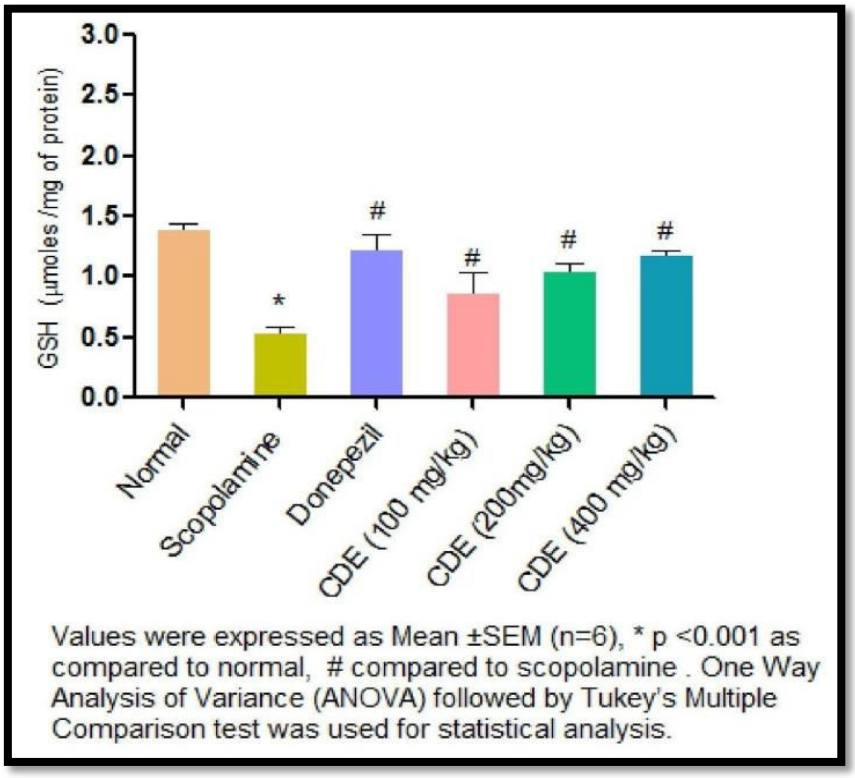
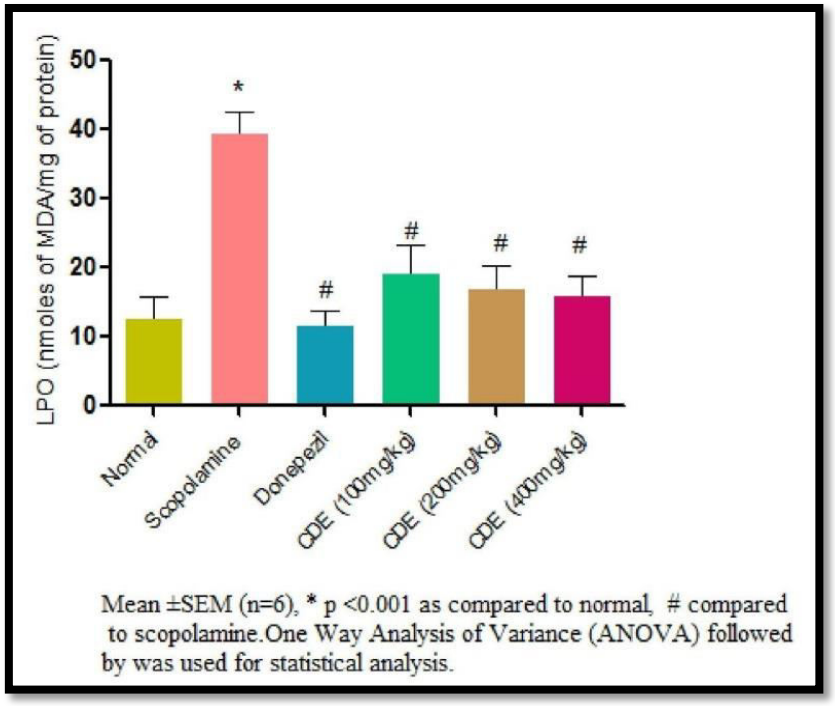


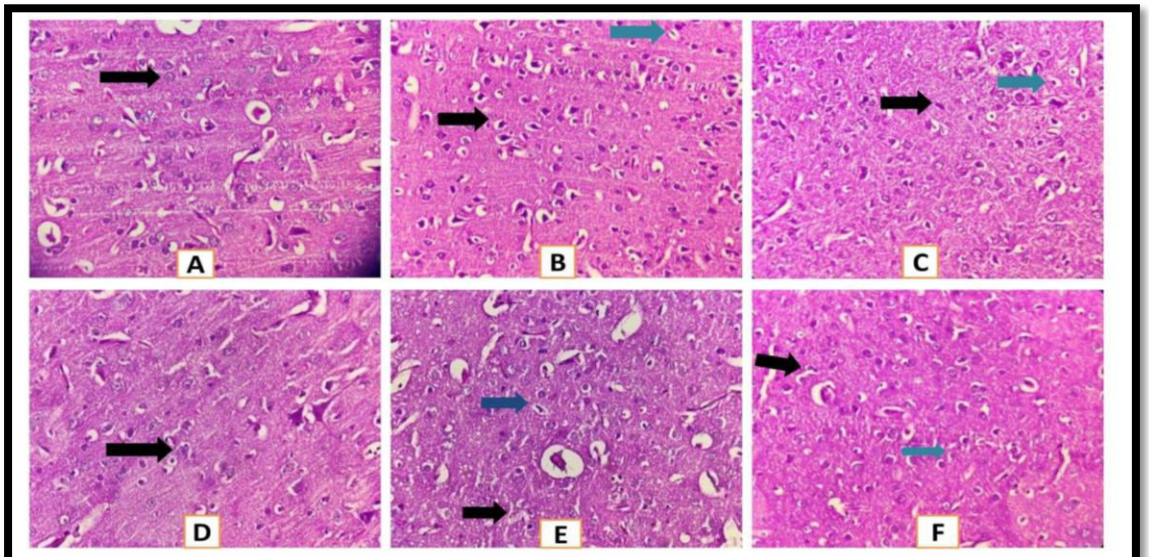
Figure 24b: Effect of CDE on LPO level in rat brain



5.7.4. Histopathology of rat Brian

In the cortex of normal control rats, H and E stained brain tissue slices revealed normal, healthy neurons. Gliosis and neuronophagia were seen in the scopolamine-induced rats, indicating serious neuronal injury. As demonstrated in Figure 25, animals given *C. dactylon* extract showed modest neuronal injury with vacuolated degeneration. The donepezil-treated control group had modest neuronal injury and vacuolated degeneration. In normal control rats, the hippocampal area was histologically normal, but scopolamine induction resulted in significant neuronal injury and perivascular edoema. When compared to scopolamine-induced rats' brain histology, donepezil-treated rats showed substantial pyramidal cell destruction with perivascular edema, whereas *C. dactylon* extract-treated rats showed minor damage to neurons with few pyknotic cells and mild edema as shown in Figure 26.

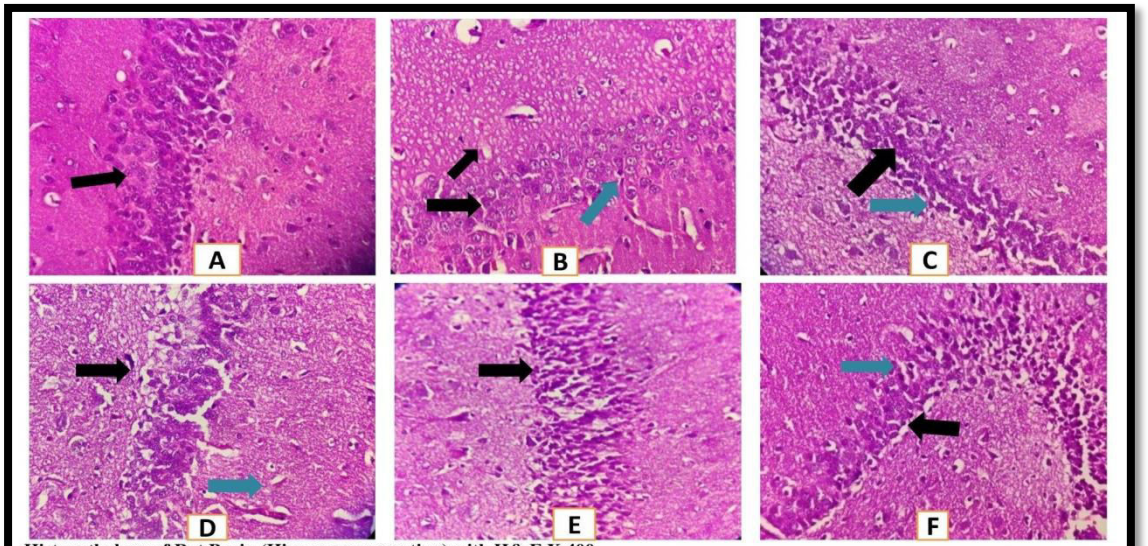
Figure 25: Histopathological examination of modulation in cerebral cortex by CDE



Histopathology of Rat Brain (Cerebral Cortex section) with H& E X 400

A. Normal control group showing normal histological finding. (Black arrow - Normal Neuron).
B. SCO (1mg/kg) (Black arrow – severe neuronal degeneration, neuronophagia and blue arrow pyknotic nucleus)
C. CDE (100 mg/kg) + SCO group showing moderate neuronal degeneration with neurophagia with congestion of blood vessels. (Black arrow Moderate edema and Blue arrow shows moderate neurodegeneration)
D. CDE (200 mg/kg)+ SCO group showing neuronal degeneration.(Black arrow – Mild Neuronal degeneration)
E. CDE (400 mg/kg)+ SCO group (Black arrow shows mild congestion and blue arrow shows mild neurodegeneration)
F. Donepezil (3mg/kg)+ SCO group showing minimal neuronal degeneration. (Black arrow – Mild congestion , Blue arrow minimal neurodegeneration).
***CDE: *Cynodon dactylon* hydroethanolic extract; SCO: Scopolamine.**

Figure 26: Histopathological examination of modulation in hippocampus by CDE



Histopathology of Rat Brain (Hippocampus section) with H& E X 400

A. Normal control group showing normal histological finding healthy normochromic neurons with well-outlined cell bodies and absence of intercellular spaces.(Black arrow – Normal pyramidal cell)
B. SCO induced group showing marked and severe neuronal degeneration with congested blood vessels, perivascular edema, neuronophagia, pyknotic cells and gliosis. (Black arrow shows edema and blue arrow shows pyknotic cell)
C. CDE (100 mg/kg) + SCO showing moderate neuronal degeneration with neurophagia with congestion of blood vessels with edema.(Blue arrow shows intercellular edema)
D. CDE (200 mg/kg)+ SCO showing mild perivascular edema with mild congestion and neuronal degeneration. (Black arrow shows pyramidal cell degeneration and blue arrow shows edema)
E. CDE (400 mg/kg)+ SCO showing mild perivascular edema and pyknosis. (Black arrow shows pyknosis)
F. Donepezil (10 mg/kg)+ SCO group showing mild neuronal degeneration & neuronophagia.(Black arrow shows mild edema and blue arrow shows few cells showing degeneration)
*** CDE: *Cynodon dactylon* hydroethanolic extract; SCO: Scopolamine**

5.8. *In-silico, in-vitro, in-vivo* pharmacology of *S. rhombifolia* against cognitive impairment.

5.8.1. Mining and Drug likeness property of *Sida rhombifolia* L. Phytocompounds

From the phytochemical interaction database, Dr. Duke's database, Chemical Entities of Biological Interest (ChEBI), and scientific studies, phytocompounds from *S. rhombifolia* were identified. 26 of the 35 compounds had a positive drug-likeness score, with 2D-Hydroxyecdysone scoring the highest at 1.39 (Table 12). 23 of the 26 compounds were projected to target 167 protein molecules, whereas three compounds were unable to identify the targets due to a lack of structural similarity in the database. Furthermore, the peer-review identified nine compounds to target ten therapeutic protein molecules linked to Alzheimer's disease (Table 13).

Table 12: Drug likeness property of *Sida rhombifolia L. Phytocompounds*

Compounds	PubChem CID	Molecular Formula	Molecular Weight(g/mol)	HBD	HBA	LogP	DLS
2D-Hydroxyecdysone	9912297	$C_{27}H_{44}O_6$	464.60	5	6	1.81	1.39
Acacetin	5280442	$C_{16}H_{12}O_5$	284.26	2	5	3.74	0.2
Ecdysone	19212	$C_{27}H_{44}O_6$	464.6	5	6	0.92	1.06
Kaempferol	5280863	$C_{15}H_{10}O_6$	286.24	4	6	1.61	0.5
Pterosterone-3-O-β-D-Glucopyranoside	441836	$C_{27}H_{44}O_7$	480.6	6	7	0.74	1.1
Sanguinine	443722	$C_{16}H_{19}NO_3$	273.33	2	4	1.09	0.77
Vascicine	72610	$C_{11}H_{12}N_2O$	188.23	1	2	1.08	0.33
Vasicinol	442934	$C_{11}H_{12}N_2O_2$	204.22	2	3	0.62	0.61
Vasicinone	442935	$C_{11}H_{10}N_2O_2$	202.21	1	3	0.35	0.38

Table 13: Probable targets involved in AD modulated by *Sida rhombifolia* L. Phytocompounds

Compound	Compound type	Gene ID (compound – target probability score)
2D-Hydroxyecdysone	Steroid	HTR2A (0.73), HTR2C (0.73), ACHE (0.7), BACE1 (0.76), BCHE (0.98)
Acacetin	Flavonoid	ACHE (0.7), ADORA2A (0.84), MAOA (0.84), MAOB (1.0), APP (0.79), BACE1 (0.7), BCHE (0.7)
Ecdysone	Steroid	HTR2A (0.71), HTR2C (0.71), ACHE (0.7), BACE1 (0.74), BCHE (0.7)1
Kaempferol	Flavonoid	ACHE (0.7), ADORA2A (0.97), MAOA (0.97), MAOB (0.86), BACE1 (0.7)
Pterosterone-3-O-β-D-Glucopyranoside	Steroid	HTR2A (0.7), HTR2C (0.7), ACHE (0.7), BACE1 (0.73), BCHE (0.7)
Sanguinine	Alkaloid	ACHE (0.98), CHRM1 (0.7), BCHE (0.98)
Vascicine	Alkaloid	ACHE (0.7), BCHE (0.7)
Vasicinol	Alkaloid	ACHE (0.76), BCHE (0.76)
Vasicinone	Alkaloid	ACHE (0.7), BCHE (0.71)

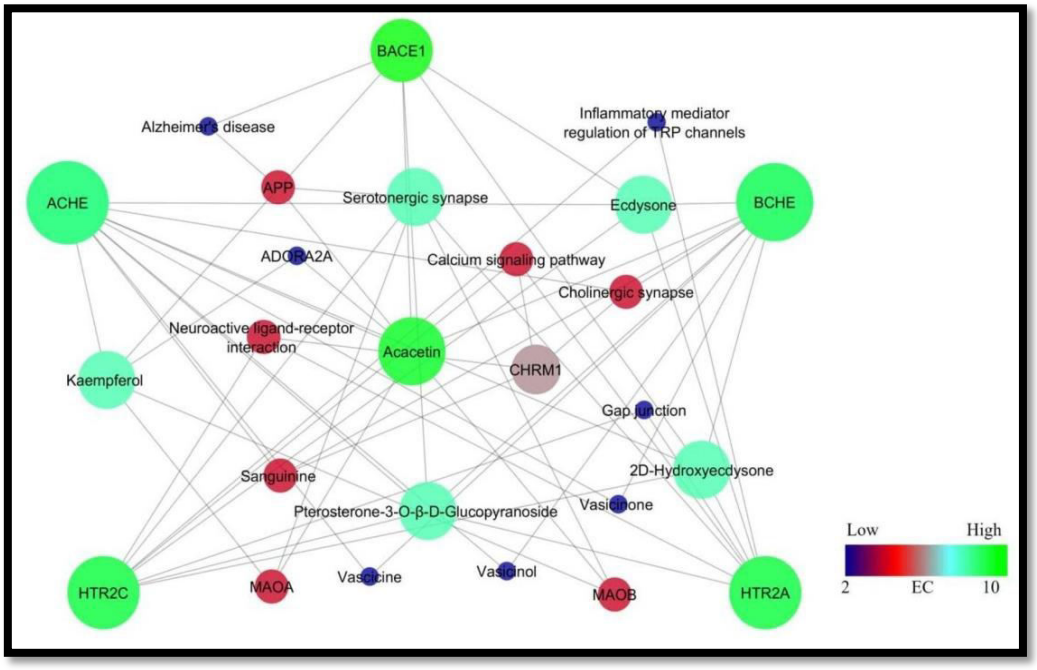
5.8.1. Prediction of Gene set enrichment and network analysis

The 9 phytocompounds altered 18 molecular pathways, according to the gene set enrichment analysis. Table 14 lists seven pathways that may be implicated in AD pathogenesis, and network analysis using cytoscape revealed that flavonoids, alkaloids, and steroids from *S. rhombifolia* had an effect on AD targets as shown in Figure 27.

Table 14: Enrichment analysis of protein targets involved in AD

KEGG ID	Pathway description	Gene count	False Discovery Rate	Protein targets within the network
has04726	Serotonergic synapse	5	3.96E-08	APP, HTR2A, HTR2C, MAOA, MAOB
hsa04750	Inflammatory mediator regulation of TRP channels	2	0.0016	HTR2A, HTR2C
hsa04080	Neuroactive ligand-receptor interaction	3	0.00082	CHRM1, HTR2A, HTR2C
hsa04020	Calcium signaling pathway	3	0.00054	CHRM1, HTR2A, HTR2C
hsa04540	Gap junction	2	0.0016	HTR2A, HTR2C
hsa04725	Cholinergic synapse	3	0.0022	ACHE, BCHE, CHRM1
hsa04728	Dopaminergic synapse	2	0.0027	MAOA, MAOB
hsa05010	Alzheimer's disease	2	0.0041	APP, BACE1

Figure 27: Network representation *S. rhombifolia* L. phytocompounds-target proteins-pathways

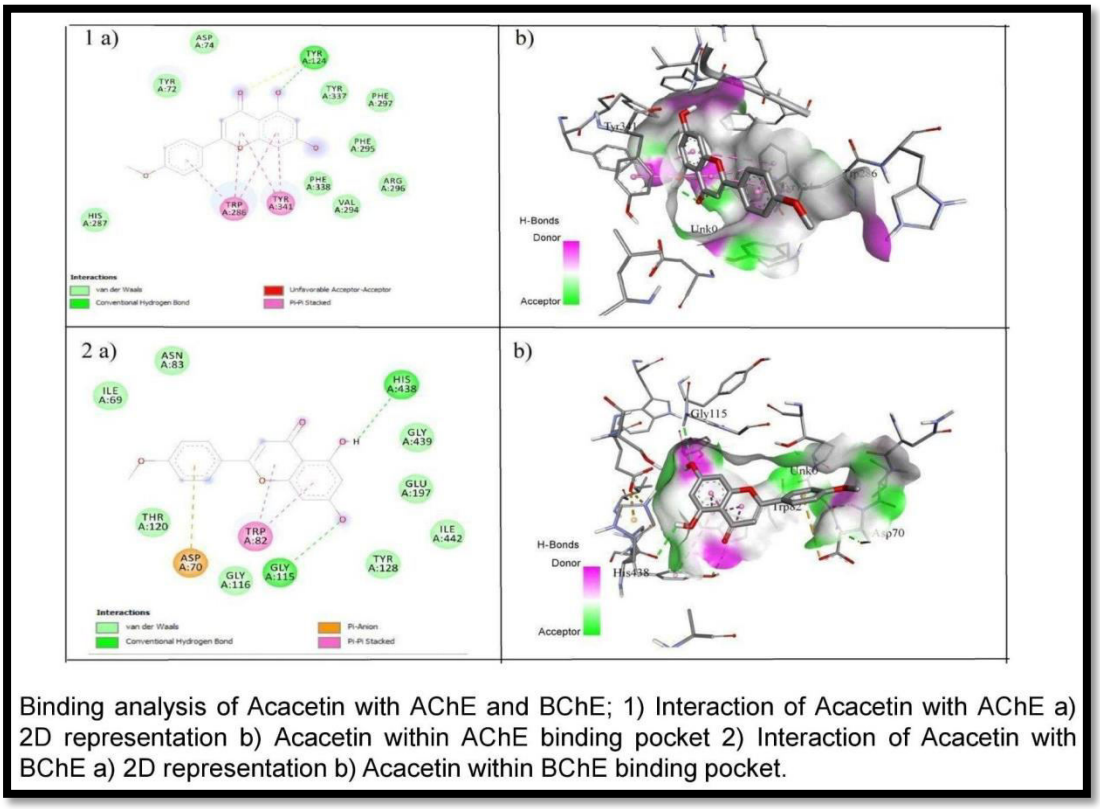


Among the compounds Acacetin scored lowest binding energy with ACHE (-8.9kcal/mol). The binding affinity, Inhibitory Constant and Hydrogen Bond interaction is represented in Table 15. The interaction of each phytoconstituents with AChE is shown in Figure 28.

Table 15: Docking score of *S. rhombifolia* phytoconstituents with AChE

Compounds	Binding energy (Kcal/Mol)	Inhibitory constant (mM)	H bond interaction	Number of Hydrogen Bond interactions
Acacetin	-8.9kcal/mol	1.59	Thr124	2

Figure 28: Binding analysis of Acacetin from *S. rhombifolia* L. by Docking study



Binding analysis of Acacetin with AChE and BChE; 1) Interaction of Acacetin with AChE a) 2D representation b) Acacetin within AChE binding pocket 2) Interaction of Acacetin with BChE a) 2D representation b) Acacetin within BChE binding pocket.

5.9. Preliminary phytochemical screening of *S. rhombifolia* extract

Table 16: Preliminary phytochemical tests of SRE

Physico-chemical Properties	*S. rhombifolia* extract
% yield (W/W)	10.5%
Phytoconstituents	Flavonoids, saponins, phenols, glycosides, tannins and steroids
Total ash value	5.9%
acid insoluble ash	1.5%
water-soluble extract (W/W)	3.8%
alcohol-soluble extract (W/W)	1.5%
Total phenolic content (GAE/g)	120.75 µg/mg
Total flavonoid content (QE/g)	87.78 ug/mg

5.9.1. Total Phenolic content

Total phenolic content was estimated by using Gallic acid as standard and expressed as mg of Gallic Acid Equivalent (GAE). The extract having considerable amounts of phenolic content present in *S. rhombifolia* hydroethanolic extracts was 120.75 µg GAE/mg.

5.9.2. Determination of Total flavonoid content

Quercetin was considered as the most acceptable standard for estimation of total flavonoid content in sample drugs and expressed as milligrams of Quercetin Equivalent (QE). Hydro-ethanolic extract *S. rhombifolia* showed the presence of flavonoid content as 87.78 µg QE/mg.

5.9.3. LC-MS studies to identify the phytochemicals from S. rhombifolia

Figure 29a: LC-MS chromatogram of *S. rhombifolia* aqueous extract

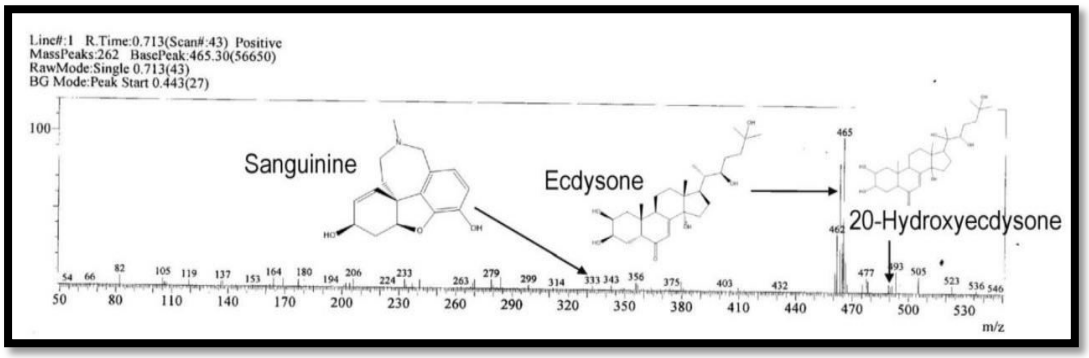
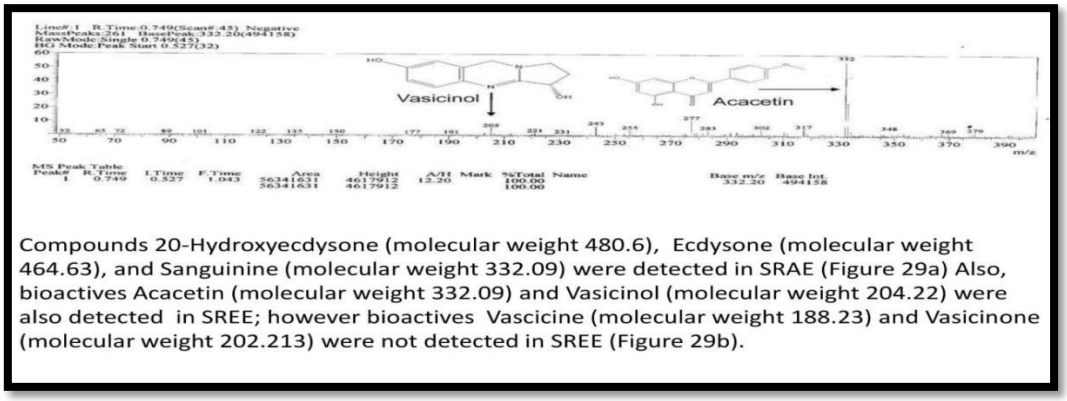


Figure 29b: LC-MS chromatogram of *S. rhombifolia* ethanolic extract

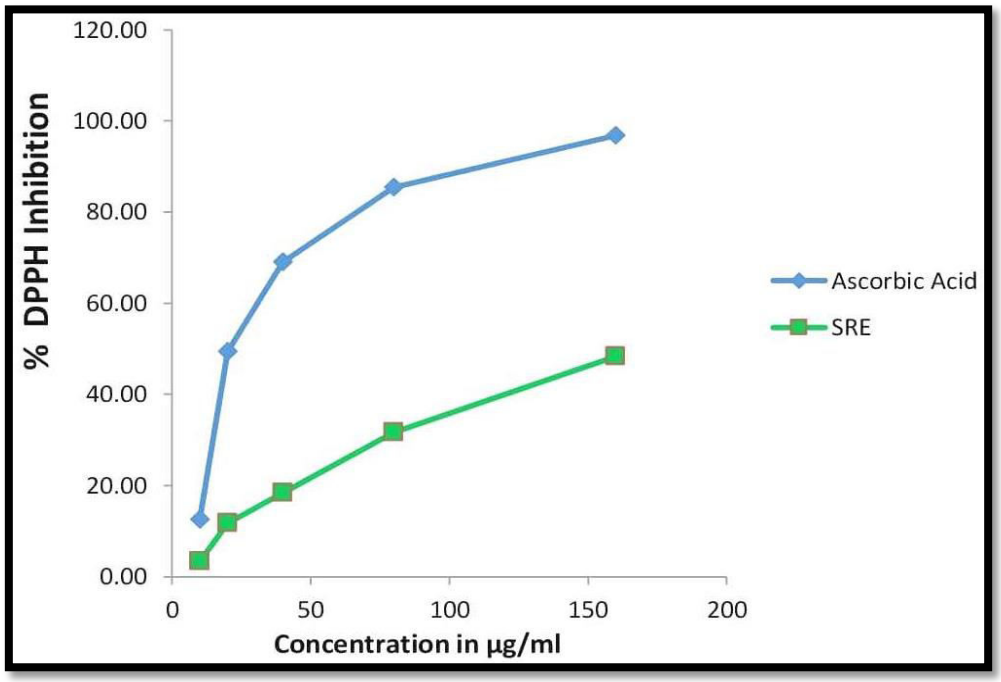


Compounds 20-Hydroxyecdysone (molecular weight 480.6), Ecdysone (molecular weight 464.63), and Sanguinine (molecular weight 332.09) were detected in SRAE (Figure 29a) Also, bioactives Acacetin (molecular weight 332.09) and Vasicinol (molecular weight 204.22) were also detected in SREE; however bioactives Vascicine (molecular weight 188.23) and Vasicinone (molecular weight 202.213) were not detected in SREE (Figure 29b).

5.10. *In-vitro* antioxidant screening

To understand the antioxidant potential of extract, DPPH radical scavenging property is the most commonly utilized method based on evaluating IC_{50} parameter and % DPPH inhibition in specific time which was measured by using Ascorbic acid as standard. The SRE inhibited DPPH at IC_{50} value of 175.74 ± 10.51 µg/ml, $p < 0.01$ compared to ascorbic acid. The extract has dose-dependent activity by increasing DPPH scavenging as shown in Figure 30.

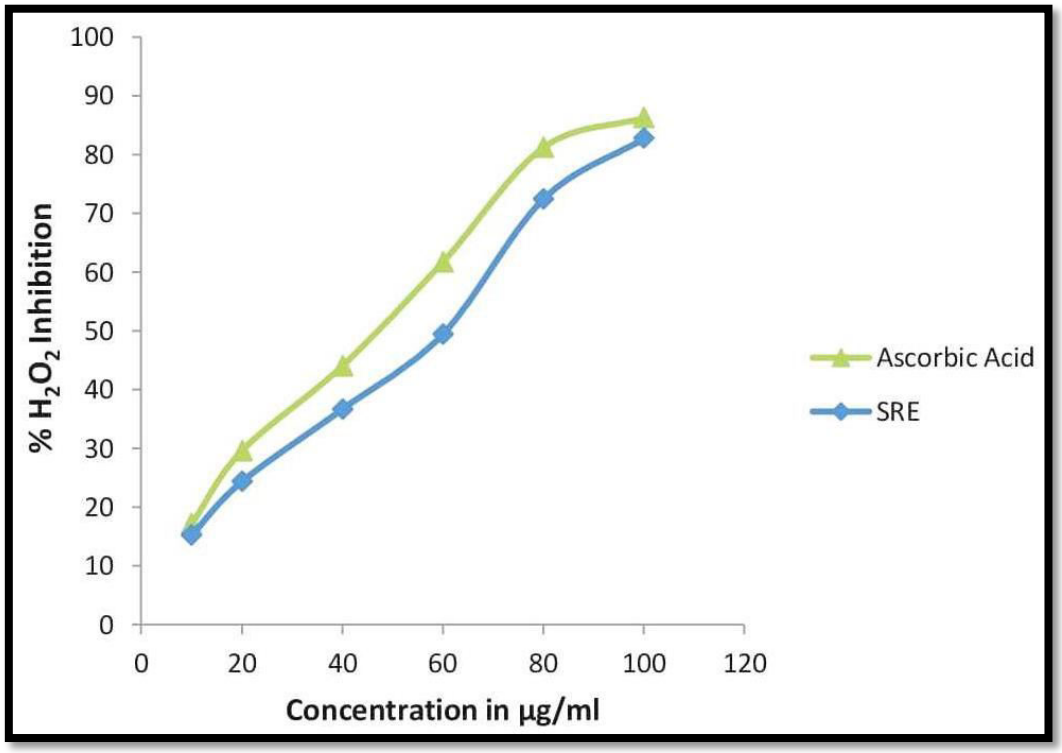
Figure 30: DPPH scavenging activity by SRE



5.10.1. H_2O_2 scavenging activity of SRE

H_2O_2 modifies lipids, proteins and DNA which results in loss of synaptic function considered as one of the characteristic features of AD. Hence the current study validates percentage inhibition of H_2O_2 by the selected plant extract using *in-vitro* method. *S. rhombifolia* extract also inhibited H_2O_2 at IC_{50} of 55.84 ± 0.636 µg/ml; $P < 0.01$ when compared with ascorbic acid as shown in Figure 31.

Figure 31: H_2O_2 inhibition by SRE



5.10.2. In vitro estimation of Ach E enzyme inhibition by SRE

Ellaman's method is widely used to estimate acetylcholinesterase enzyme. The IC_{50} value indicates the amount of sample concentration required to inhibit 50% of AchE enzyme. The smaller IC_{50} value higher the inhibition. We identified that Hydro-ethanolic extracts of *S. rhombifolia* inhibited Acetylcholinesterase enzyme significantly as shown Figure 32 and 33.

Figure 32: IC_{50} values for AchE enzyme inhibition by SRE using *in-vitro* method

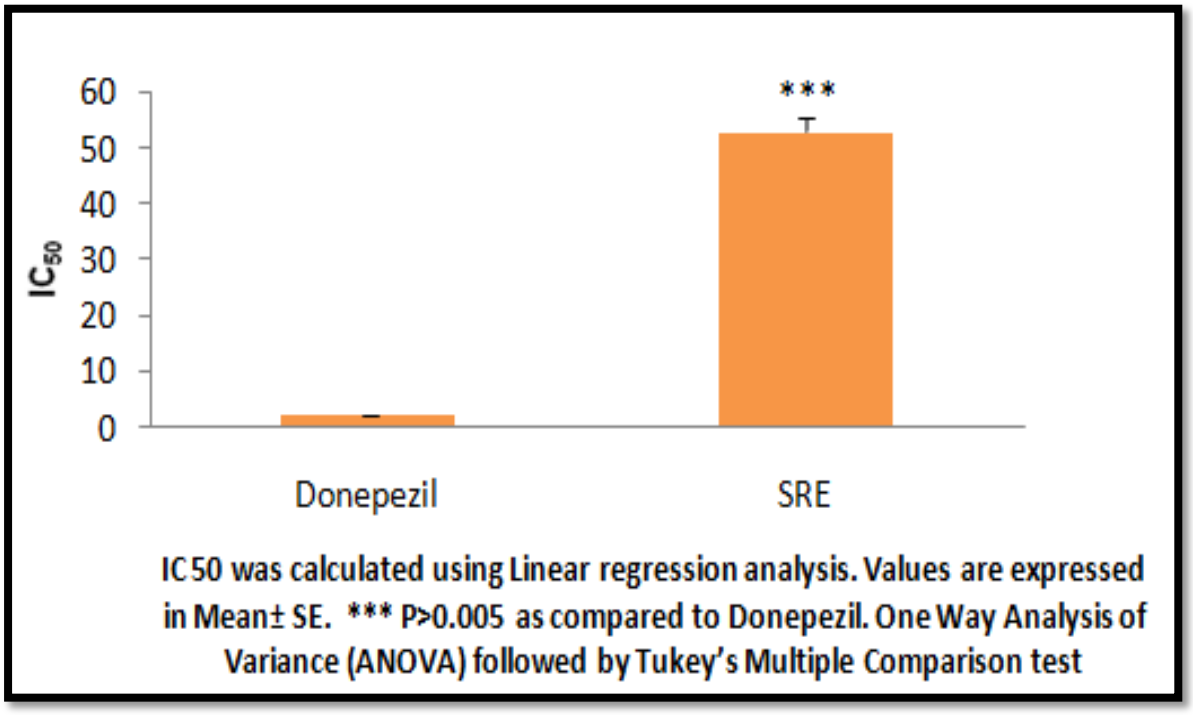
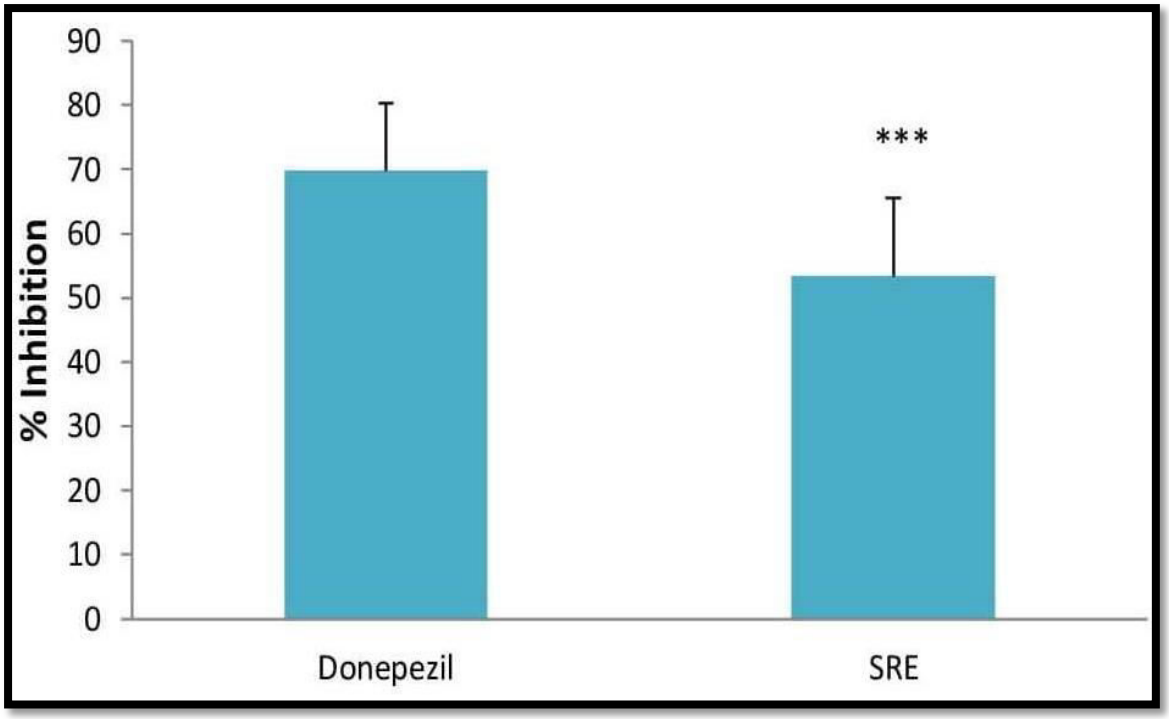


Figure 33: % inhibition of AchE enzyme by SRE



5.10.3. Acetylcholinesterase enzyme inhibition assay of S. rhombifolia fractions

The *S. rhombifolia* fraction rich in flavonoids and Alkaloids showed significant AChE inhibition (12.87±0.64; 18.96±0.96µg/ml respectively) as compared to fraction rich in phytosterols, saponins and *S. rhombifolia* extract in the *in-vitro* assay as described in Table 17.

Table 17: *In-vitro* AchE inhibition by *S. rhombifolia* crude fractions

Plant extract/ Crude fraction (% yield)	AChE inhibition IC_{50} (µg/ml)
Fraction rich in Flavanoids (0.6)	12.87± 0.64*
Fraction rich in Alkaloids (0.35)	18.96±0.96 *
Fraction rich in Phytosterols (0.4)	20.75±0.55*
Fraction rich in saponins (0.5)	30.47±1.10*
S. Rhombifolia extract (10.5)	53.11 ± 2.39
Donepezil	2.23 ± 0.141

IC_{50} values were expressed as Mean±SEM, *P <0.05 as compared to Donepezil

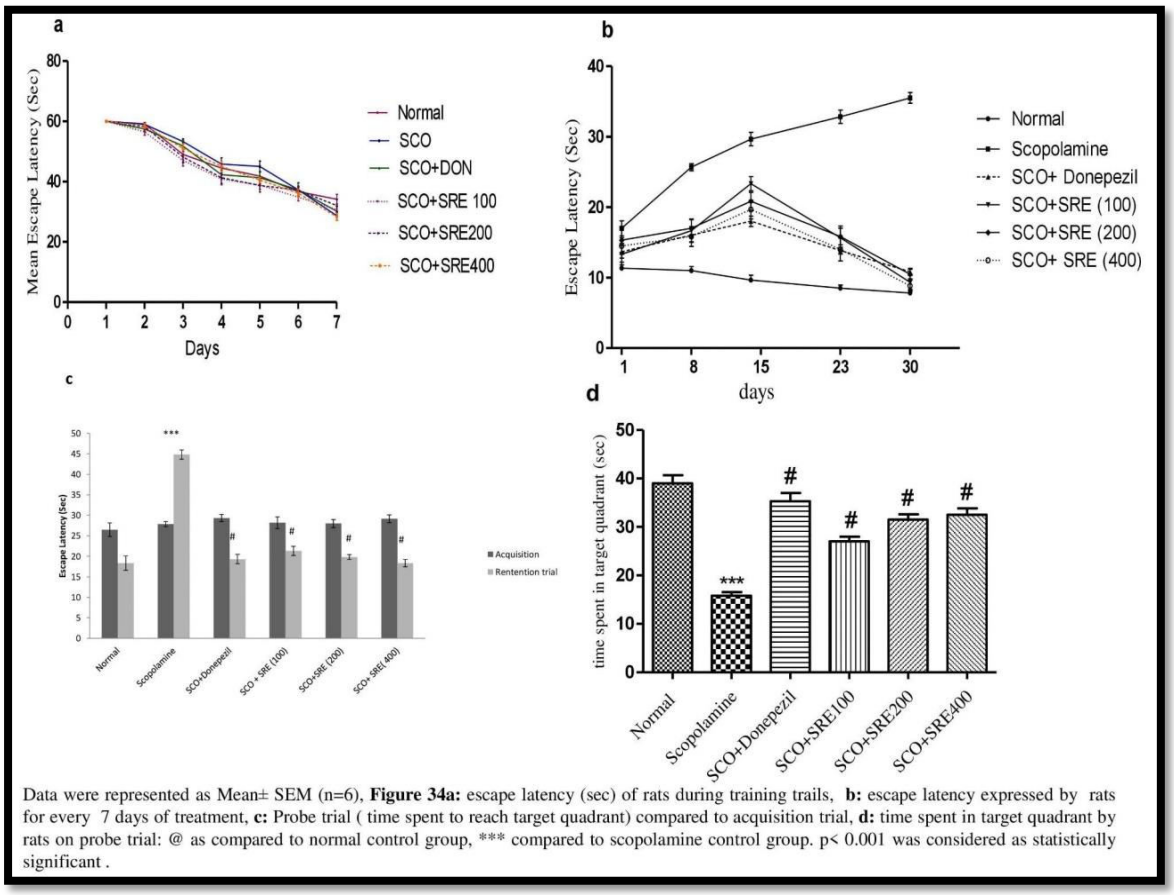
5.11. Effect of *S. rhombifolia* extract on behavioral models.

5.11.1. Impact of S. rhombifolia extract on spatial memory

Figure 34 illustrates escape delay in seconds using the Morris Water Maze test to assess spatial memory restoration. Figure 34a depicts 7-day training trials before starting therapy on rats, which showed a significant reduction in escape latency on day 7 compared to day 1 of the training experiment. Figure 34b demonstrates that when donepezil and SRE were given to the corresponding groups, the escape latency was significantly reduced as compared to the SCO-induced rats. Figure 34c indicates

that SCO induction significantly increases escape latency (P <0.001) when compared to normal control rats. In contrast, donepezil (3 mg/kg) and SRE at 100, 200 and 400 mg/kg therapy significantly lowered escape latency (P < 0.001) when compared to rats exposed to SCO. Figure 34d depicts a probe trial in which rats induced by SCO spent less time in the target quadrant than normal control rats, but donepezil and SRE-treated rats spent significantly more time in the target quadrant than SCO-induced rats (P < 0.001). These findings suggest that spatial memory has improved.

Figure 34: Effect of SRE on spatial memory



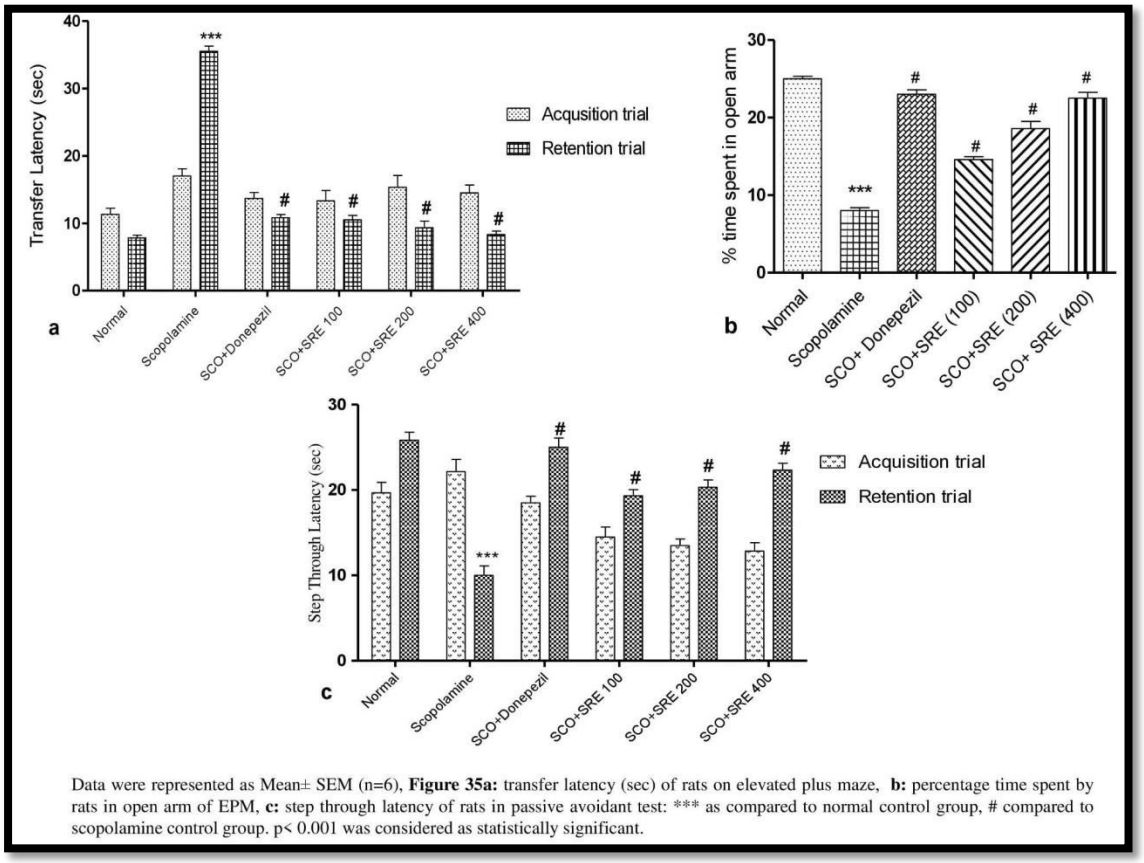
Data were represented as Mean± SEM (n=6), **Figure 34a:** escape latency (sec) of rats during training trails, **b:** escape latency expressed by rats for every 7 days of treatment, **c:** Probe trial (time spent to reach target quadrant) compared to acquisition trial, **d:** time spent in target quadrant by rats on probe trial: @ as compared to normal control group, *** compared to scopolamine control group. p< 0.001 was considered as statistically significant .

5.11.2. Effect of S. rhombifolia extract on transfer latency and step through latency

Figure 35a depicts the effect of SRE on the TL, whereas Figure 35b depicts the proportion of time spent in open arms after 5 minutes in an elevated plus-maze. When compared to the control group, the SCO induced rats had a higher TL. In comparison to SCO-induced animals, donepezil (3 mg/kg) and SRE therapy at 100, 200, and 400 mg/kg dosages resulted in a significant decrease in TL. When compared to normal control rats, SCO induced animals spent significantly less time in the open arms ($P < 0.001$). In comparison to SCO-induced rats, donepezil and SRE at 100, 200 and 400 mg/kg demonstrated increased time spent % in the open arms, indicating preservation of short term memory.

On day 30, the SCO-treated rats showed a significant ($P < 0.001$) decrease in step-through latency, implying an increase in the fear-aggravated behavioral task. However, as demonstrated in Figure 35c, treatment groups containing donepezil (3 mg/kg), SRE-100, 200 and 400 mg/kg doses showed enhanced step-through latency, indicating better non-declarative memory.

Figure 35: Effect of SRE on short term memory and fear aggravated task

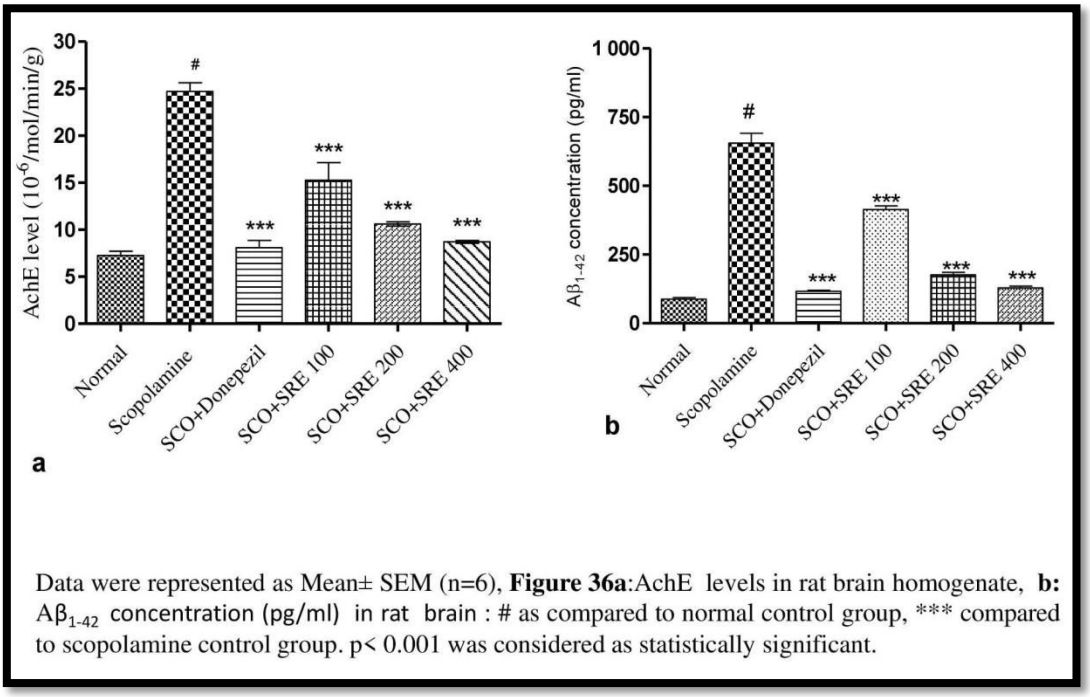


Data were represented as Mean± SEM (n=6), **Figure 35a:** transfer latency (sec) of rats on elevated plus maze, **b:** percentage time spent by rats in open arm of EPM, **c:** step through latency of rats in passive avoidant test: *** as compared to normal control group, # compared to scopolamine control group. p< 0.001 was considered as statistically significant.

5.11.3. Effect of S. rhombifolia extract on AchE and Beta amyloid in the brain homogenate

SRE at 100, 200 and 400 mg/kg significantly suppressed Acetylcholinesterase levels (15.27 ±1.87, 10.62 ± 0.22, and 8.71 ± 0.16; P < 0.001), respectively, while Donepezil treated rats likewise had lower AChE levels (8.09± 0.75; P < 0.001) as compared to amnesia caused animals (24.71± 0.92). As demonstrated in Figure 36a, SCO-induced rats had significantly higher AChE enzyme activity (24.71 ± 0.92; P <0.001) than normal rats (7.26± 0.45), and amyloid $_{1–42}$ levels were significantly higher than in normal controls (655.7 ±35.68 pg/ml; P <0.001). Although, as shown in Figure 36b, there was a significant decrease in beta-amyloid $_{1–42}$ content in rats treated with SRE and donepezil (P < 0.001) compared to SCO-induced animals.

Figure 36: Effect of SRE on AchE enzyme and Beta amyloid $_{1-42}$ in the brain homogenate



Data were represented as Mean± SEM (n=6), **Figure 36a**:AchE levels in rat brain homogenate, **b:** $A\beta_{1-42}$ concentration (pg/ml) in rat brain : # as compared to normal control group, *** compared to scopolamine control group. p< 0.001 was considered as statistically significant.

5.11.4. Antioxidant effect of S. rhombifolia on GSH and LPO content

Malondialdehyde levels in SCO-induced rats were significantly higher (39.53 ± 3.05; P < 0.001) than in control rats (12.8 ±2.86). In comparison to SCO-induced rats, animals given SRE (14.96 ±.16, 13.89 ±3.05, 12.29 ± 2.41; P < 0.001) and Donepezil (11.75 ± 1.97; P < 0.001) had significantly lower malondialdehyde (MDA) levels, indicating decreased lipid peroxidation in the rat brain (Table 18).

As compared to normal control rats (1.39 ±0.05), SCO-treated rats had significantly lower GSH levels (0.53 ± 0.05; P < 0.001). As demonstrated in Table 20, rats treated with Donepezil (1.23 ± 0.11; P< 0.001) had significantly higher GSH levels (1.07±0.12, 1.20 ±0.07, 1.31 ±0.05: P < 0.001) than SCO-induced animals.

Table 18: Anti-oxidant activity of *S. rhombifolia* extract on GSH and LPO content

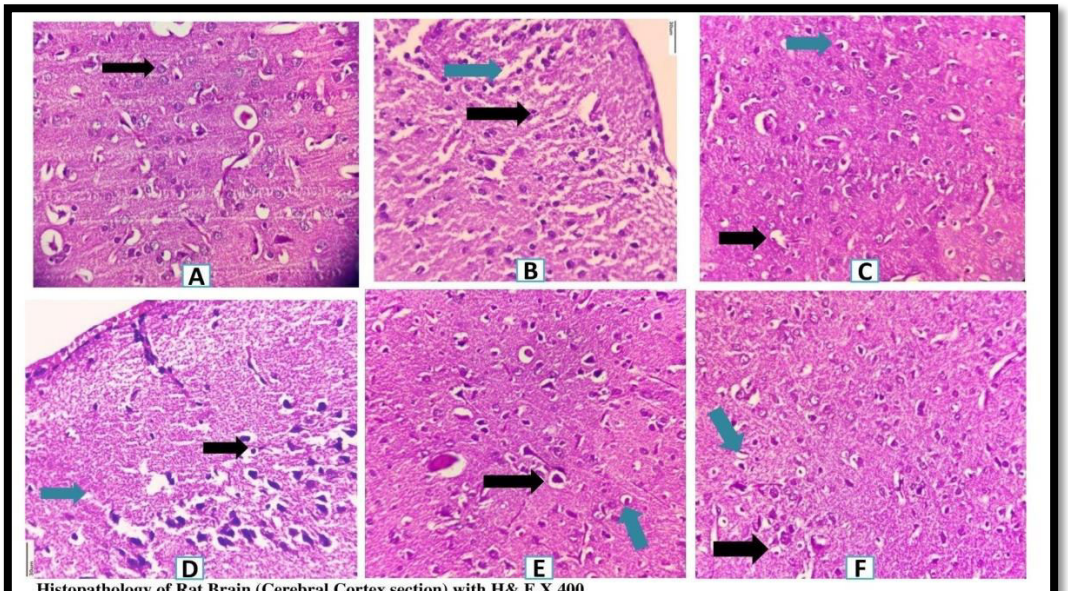
Group	GSH reduced (mmoles /mg of protein)	LPO level (nmoles of MDA/mg of protein)
Normal control	1.39±0.05182	12.82±2.867
SCO (1mg/kg)	0.53±0.05763 *	39.53±3.059 *
SCO+ Donepezil (3mg/kg)	1.23± 0.1158 #	11.75±1.970 #
SCO+SRE (100mg/kg)	1.077±0.124#	14.96±3.16#
SCO+SRE (200mg/kg)	1.209±0.077#	13.89±3.05#
SCO+SRE (400mg/kg)	1.318±0.050#	12.29±2.41#

Mean±SEM, @ as compared to normal control and # as compared to scopolamine control group. p< 0.001 was considered as statistically significant.

5.11.5. Histopathological changes in the rat brain

At a magnification of 40X, SCO-induced animals showed severe neurodegeneration in rat brain; cortex and hippocampus regions, with perivascular edema, neuronophagia and pyknotic nucleus. In comparison to the illness group, groups treated with donepezil and SRE protected neurons and showed mild congestion as seen in Figures 37 and 38.

Figure 37: Histopathological changes in Cerebral cortex by SRE



Histopathology of Rat Brain (Cerebral Cortex section) with H& E X 400

A. Normal control group showing normal histological finding. (Black arrow - Normal Neuron).

B. SCO (1mg/kg) (Black arrow – severe neuronal degeneration, neuronophagia and blue arrow pyknotic nucleus)

C. SCO+ Donepezil (3mg/kg) group showing minimal neuronal degeneration. (Black arrow – Mild congestion, Blue arrow minimal neurodegeneration).

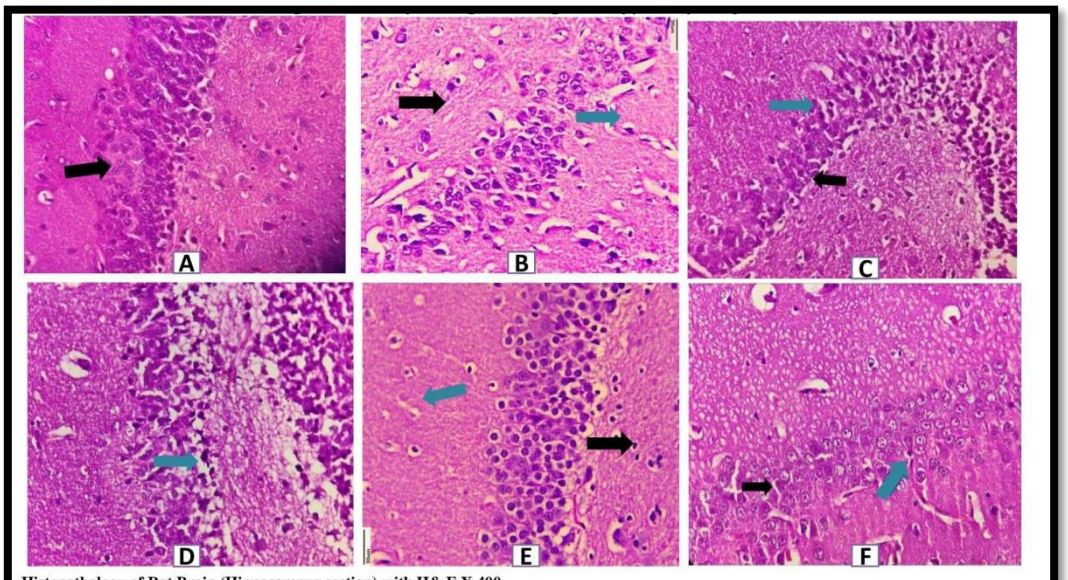
D. SCO+SRE (100 mg/kg) group showing moderate neuronal degeneration with neurophagia with congestion of blood vessels. (Black arrow Moderate edema and Blue arrow shows moderate neurodegeneration)

E. SCO+ SRE (200 mg/kg) group showing neuronal degeneration.(Black arrow – Mild edema, Blue arrow shows mild neurodegeneration)

F. SCO+SRE (400 mg/kg) group (Black arrow shows mild congestion and blue arrow shows mild neurodegeneration)

***SRE: *Sida rhombifolia* hydroethanolic extract; SCO: Scopolamine.**

Figure 38: Histopathological changes in Hippocampus by SRE



Histopathology of Rat Brain (Hippocampus section) with H& E X 400

A. Normal control group showing normal healthy normochromic neurons with well-outlined cell bodies and absence of intercellular spaces.(Black arrow – Normal pyramidal cell)

B. SCO (1mg/kg) induced group showing marked and severe neuronal degeneration with congested blood vessels, perivascular edema, neuronophagia, pyknotic cells and gliosis. (Black arrow - edema and blue arrow shows pyknotic cell).

C. SCO+ Donepezil (3mg/kg)+ SCO group showing mild neuronal degeneration & neuronophagia.(Black arrow -mild edema and blue arrow -few cells showing degeneration).

D. SCO+SRE (100mg/kg) + SCO showing moderate neuronal degeneration with neuronophagia with congestion of blood vessels and edema.(Blue arrow shows intercellular edema)

E. SCO+ SRE (200mg/kg)+ SCO showing mild perivascular edema with mild congestion and neuronal degeneration. (Black arrow - pyramidal cell degeneration and blue arrow - edema)

F. SCO+ SRE (400mg/kg)+ SCO showing mild perivascular edema and minimal pyknosis. (Black arrow shows minimal pyknosis, Blue arrow shows mild edema)

6. DISCUSSION

Medicinal plants have great importance in the pharmaceutical industry due to the presence of rich secondary metabolite resources such as tannins, saponins, steroids, phenolic compounds, alkaloids and flavonoid contents. Hence, identifying phytochemicals have become diverse in the preliminary phase.[1]

The present study explored the anti-amnesic effect of *C. dactylon*and *S. rhombifolia* hydroethanolic extracts. The selected plants were subjected to prelude phytochemicals present in extract by qualitative tests. Later, converging insight on cognitive modulation by *in-silico, in-vitro, in-vivo* studies were performed.

In the era of drug discovery of new compounds traditional herbs have been approached with advanced pharmacology by newer techniques such as bioinformatics and polypharmacology which could give strategy to research affirmations. Because *in-silico* studies can claim various disease pathways modulated by numerous targets with their drug-like attributes with use of tools to assess the therapeutic and adverse effects of drugs.

Based on the drug likeness properties, we discovered different connections between the proteins and the compounds when the phytoconstituents of the plant were made to interact with different proteins and were used for further analysis; thus, we predicted the probable targets of phytoconstituents using TTD (Therapeutic Target Database).

6.1. Effect of *C. dactylon* on dementia

6.1.1. In-silico studies of C. dactylon

We found that 5 compounds namely, apigenin, luteolin, kaempferol, quercetin and tyrosine modulating 10 therapeutic protein targets associated with Alzheimer's disease i.e., Monoamine Oxidase (MAO-A, MAO-B), Cytochrome P450 1A1 (CYP1A1), Arachidonate lipoxygenases (ALOX5, ALOX12, ALOX12B, ALOX15, ALOX15B), Arginase (ARG2) and Nitric Oxide Synthase (NOS3). Further, metabolic pathways, tryptophan metabolism, histidine metabolism, phenylalanine metabolism, tyrosine metabolism, serotonergic pathways, arginine and proline metabolism were found to be the prominent pathways in Alzheimer's disease.

As we know according to literature, Monoamine Oxidase is an enzyme that catalyses the oxidative deamination of various amines. It has two isomers namely MAO-A and MAO-B. The substrates for MAO-A are serotonin, dopamine, nor-adrenaline and adrenaline and the substrates for MAO-B are tyramine and phenylalanine. When the actions of MAO increases, the oxidation of neurotransmitters will also increase thereby decreasing the concentration of neurotransmitters, leading to neurodegenerative disorder such as Alzheimer's Disease and Parkinsonism. [167]

Apigenin, which is available in many herbs, is a "flavone" present as a 'aglycone' of various naturally occurring glycosides. A computer study discovered a probable molecular interaction between isoform of Apigenin i.e., 6-prenyl apigenin, MAO-A active site and MAO-A binding affinity was also found to be quite high

thereby inhibiting the enzyme; Therefore, it could be a suitable lead molecule for the synthesis of new MAO-A inhibitor.[168]

As per the reports of Kim Y et al., Apigenin improved cognition by regulating BDNF/TrKB, amyloidogenesis and apoptosis signaling pathways. Researchers also reported that treatment of synthetic Apigenin at 100 & 200mg/kg/day promoted cognition confirmed through behavioural tasks [T maze, MWM, & novel objective recognition task]. Another report suggests docking ability of apigenin with acetylcholinesterase within the binding pocket.[169] According to literature study, Tryptophan is the precursor for the synthesis of Serotonin (5-HT); when Tryptophan metabolism takes place, some part of it is directed for the synthesis of serotonin (serotonergic pathway) and a major portion of Tryptophan is directed for the synthesis of kynurenic acid and quinolinic acid (kynurenic pathway).[170]

MAO enzymes are responsible for the degradation of serotonin. Tryptophan metabolism pathway can be a suitable target for inhibiting the actions of MAO-A and MAO-B, thereby increasing the serotonin synthesis for neuroprotection. Flavonoids have been shown to have the ability to pass the blood–brain barrier (BBB), making them potential agents in the prevention of neurodegenerative diseases like AD. Quercetin is a polyphenol that belongs to the flavanols family of flavonoids. In Alzheimer's disease, because of an increase in Amyloid-β proteins, oxidative stress is common and it plays a role in neurodegeneration. Aggregation of Aβ is triggered by the production of ROS. Quercetin is an antioxidant that inhibits the fibril/ senile plaque production of amyloid-proteins, preventing cell damage and inflammatory processes. This neuroprotective action of Quercetin has already been studied *in-vivo*

and *in-vitro*. Thus, Quercetin can be a probable target for the inhibition of MAO-A enzyme in the pathogenesis of AD. [171]

In the present network image there is a direct connection between Kaempferol and MAO-A. *In-vitro* studies have shown that Kaempferol acts as a MAO Inhibitor where MAO-A inhibition was greater than MAO-B inhibition. Therefore, Kaempferol can be targeted to inhibit the effects of MAO-A in the pathogenesis of AD.

Luteolin is another flavonoid compound directly connected to MAO-A and MAO-B, and has shown MAO-A inhibitory actions. [172, 173] In animal models, luteolin protects against the development of Alzheimer's disease, as it can cross the BBB, thereby reducing Aβ levels by inhibiting β-secretase and γ-secretase which produces amyloidogenic Aβ. Luteolin also reduces the phosphorylation of tau protein and formation of neurofibrillary tangles, as well as decreases the formation of Aβ deposits. Luteolin also improves brain insulin sensitivity and decreases neuroinflammation; Thus, in animal models, luteolin administration can thereby protect against the development of Alzheimer's disease. [174] Therefore, Luteolin can be targeted to inhibit MAO-A and MAO-B to produce neuroprotection in AD. Yoo DY et al., findings imply that the treatment with luteolin enhances the SCO-induced suppression of neuroblast differentiation and cell proliferation in the dentate gyrus. Increases in BDNF, acetylcholine, and lipid peroxidation may be connected to the mechanism by which luteolin ameliorates SCO-induced amnesia. [175]

According to literature we know that tyrosine is the precursor for producing catecholamines such as 'Dopamine, adrenaline/epinephrine and noradrenaline/ norepinephrine'. Because of loss of noradrenergic neurons and also because of the metabolism by MAO-A and MAO-B enzymes, the levels of catecholamines are

reduced in Alzheimer's disease. So therefore, tyrosine can be targeted to cause MAO inhibition through tyrosine metabolism.[167]

In a docking study it was found that Apigenin has anti– AChE properties and was found to inhibit the activity of the AChE enzyme. Therefore, Apigenin can be a promising compound to target and inhibit the AChE enzyme thereby increasing the levels of Acetylcholine (ACh)in Alzheimer's Disease pathogenesis.[176]

Adenosine receptors ADORA2A and ADORA2 are highly expressed in the basal ganglia of the brain. Studies have shown that they are abundantly expressed in Alzheimer's disease and play a key role in the AD progression. [177] As a result, blocking ADORA2A and ADORA2 expression with a particular antagonist can prevent memory loss as well as the development of amyloidogenesis. [178]

Thus, Quercetin and Apigenin can be a probable target to reduce the expression of the receptors ADORA2A and ADORA2.

Arachidonate lipoxygenase enzymes namely, ALOX12, ALOX12B, ALOX15 and ALOX15B are important sources of oxidative stress. The expression of the ALOX enzyme as well as the metabolic products of the ALOX enzyme i.e, hydroxy derivatives of eicosatetraenoic acid were found to be enhanced in Alzheimer's patients. Because the increase in ALOX expression causes lipid peroxidation, genetic deletion of the ALOX enzyme is a likely target for lowering cellular oxidative stress in AD brains. As a result, inhibiting the ALOX metabolic pathway (which causes neurodegeneration) can help to correct the oxidative imbalance in Alzheimer's brain. [179]

Nitric oxide (NO)-induced oxidative stress in the brain has been suggested as a pathogenic mechanism in Alzheimer's disease. NO Synthase (NOS) converts L-arginine to NO in endothelial cells. Reactive oxygen species react with NO to form per-oxynitrate, which can promote lipid peroxidation and hastening degenerative processes such as those that lead to Alzheimer's disease. The NOS3 gene is responsible for encoding Endothelial NO synthase (ecNOS). Furthermore, there is a structural polymorphism in the NOS3 gene at position 298 i.e., Glu298Asp polymorphism. According to biological functional and genetic association studies this Glu298Asp polymorphism of the ecNOS (NOS3) gene may be a genetic risk factor for late-onset Alzheimer's disease (LOAD). Therefore, by reducing the expression of NOS3 gene can be a probable target for AD.[180]

Additionally, Kaempferol showed substantial increases in brain levels of the antioxidant enzymes glutathione and superoxide dismutase while lowering levels of tumour necrosis factor- and malondialdehyde and findings show that Kaempferol can ameliorate STZ-induced memory impairment in ovariectomized rats, most likely via increasing endogenous glutathione and superoxide dismutase levels in the hippocampus and lowering neuroinflammation. According to this study, Kaempferol may have neuroprotective properties against cognitive deficits in AD.[181]

Based on the network pharmacology the phytocompounds Apigenin, luteolin, kaempferol, quercetin and tyrosine may modulate the scopolamine induced acetylcholinesterase, oxidative stress, neuroinflammation, and monoamine oxidase pathways.

Qualitative investigation of the freshly prepared *C. dactylon* and *S. rhombifolia* extracts showed the presence of alkaloids, tannins, flavonoids,

glycosides, saponins, phenolic compounds, proteins, carbohydrates, phytosterols etc. which complies with Kumar JM et al,. earlier reports. [182, 183]

6.1.2. An in-vitro antioxidant and anti-acetylcholinesterase activity of CDE

DPPH assay is the most widely used tool for scrutinizing radical scavenging properties of various medicinal plants. Scavenging of free radicals plays an important role in prevention of harmful effects of free radicals in AD. The scavenging of DPPH radicals (stable) is a frequently used model approach for evaluating the potential of diverse substances, including plant extracts. [184] Plant phenolic compounds are now widely regarded as one of the most effective natural antioxidant sources and these are considered as a special kind of secondary metabolite that protects tissues from oxidative stress caused by free radicals. Hence, preventing various diseases including Parkinson's and Alzheimer's diseases. [185] Given the direct link for *C dactylon,* it is believed that hydro-ethanolic extract of plant showed presence of phenolic compounds which may be responsible for scavenging DPPH.

A neutral molecule Hydrogen peroxide formed during the process of superoxide dismutation by enzyme superoxide dismutase diffuses externally free to the hydrophobic layer of the cell and targets other bioactive molecules. Another proposed hypothesis is based on elevated Aβ levels generating H_2O_2 which interacts with metal ions especially iron and copper and this is considered as "oxidative stress associated AD". [186]

However polyphenols such as flavonoids prevent and reverse the damage caused by various oxidative stress factors including H_2O_2. According to preliminary studies *C. dactylon* extract does confirm the presence of polyphenols which may be

attributed to show promising anti-oxidant action by scavenging H_2O_2 which is confirmed by *in-vitro* method. [187]

This research further directed us to evaluate *in-vitro* Acetylcholinesterase enzyme inhibition assay to know the effect of *C.dactylon* extract on memory enhancing property. Since we selected "Cholinergic hypothesis" as basis for treatment of dementia in this study. Selected plant extract prominently reduced Acetylcholinesterase enzyme which points for further study.

Phytoconstituents from *C.dactylon* help to prevent a variety of neurological illnesses. Carotenoids, saponins, flavonoids, alkaloids, phytosterols, and proteins are among the active elements in plants. Several flavonoids have been studied for their efficacy in treating Alzheimer's disease. As we screened the aqueous and ethanolic extract of *C. dactylon*, it was found that kaempferol, Acacetin and Quercetin were detected. This was correlated with Muthukrishnan et al. [189] who reported and confirmed the important components of C.dactylon such as flavonoids (Kaempferol 48 µg/g ; quercetin 164.7 µg/g; rutin 18.4 µg/g; myricetin 5.7 µg/g; catechin 12.1 µg/g). Alongside carotenoids especially (violaxanthin 5.8mg/100g; zeaxanthin 4.2 mg/100 g; lutein 17mg/100g; β-carotene 35.2 mg/100 g) were reported. The current study revealed the presence of Quercetin, Kaempferol and Apigenin with M+1 ion peaks . Principally, β-carotene, luteolin and zeaxanthin were also recognized in LC-MS analysis depicting flavonoid rich contents of plants.

6.1.3. *In-vivo* studies

In-vivo studies were performed to assess the behavioral (Learning and Memory) effect of *C. dactylon* extract using scopolamine induced amnesia in rats.

6.1.3. 1. Scopolamine induced dementia

Scopolamine hydrobromide intrinsically binds non-selectively to parasympathetic (cholinergic) receptors which interrupt Acetylcholine transmission in hippocampal region along with oxidative stress initiates to witness the memory loss. [190]Because SCO injection increases AChE function, it has been chosen as an amnesia-inducing drug in the current study. This is because AChE may then metabolise a significant quantity of ACh at synapses, which can indicate a cognitive imbalance. According to prior Rahimzadegan findings, administration of SCO at several doses exacerbates the AD disease. [191]

6.1.3. 2. Effect of CDE on behavioural models

Scopolamine induced rats elicit learning and memory loss features by elevating step-through latency (STL), and escape latency in morris water maze tasks alongside decreased transfer latency in elevated plus maze compared to normal control rats in the current experiment. However, the animals administered with *C. dactylon*at (100, 200, 400mg/kg) doses, Donepezil (3 mg/kg) swam faster and spent more time in target quadrant in water maze as compared to scopolamine induced rats with significantly decreased escape latency.

The number of times rats moved from closed arm to open arm of EPM signifies transfer latency; which were significantly reduced in *C. dactylon* extract and donepezil treated groups compared with scopolamine induced rats. The percentage time spent by rats in open arms administered with CDE at 100, 200, 400mg/kg and donepezil 3 mg/kg were increased as compared to scopolamine induced rats, which demonstrates CDE impacts on short term memory modulation. These conclusions

were correlated with Poojary et al.[192] who also reported treatment of CDE showed cognitive improvement in radiation induced dementia in experimental animals.

Multiple reports suggest that 'Stress' is an unavoidable vital factor which could change the homeostasis of neuronal functions discovered in rodents and humans which has been reported to accelerate onset and severe progressive impairment of cognition with negative reinforcement. The most commonly accepted model to create stress in rodent's learning and memory is the Passive Avoidance Test (PAT). This model depicts acquisition/ or retention of memory by negative reinforcement on the brain leads to disturb the neurotransmitters release especially Acetylcholine. [193]

Present study confirmed that scopolamine administration leads to antimuscarinic action at post-synaptic nerves and disrupts learning and memory by increased step through latency (enhanced negative reinforcement) significantly as compared to normal control rats. These results are pertinent with Svoboda J et al. [194] explanations. However the CDE and Donepezil at selected therapeutic doses showed aversive stimuli by modulating neurotransmitter actions and reports to indicate better cognitive improvement by increasing step through latency addressing the memory enhancement property of CDE to give emphasis on traditional support.

6.1.3. 3. CDE regulated acetylcholinesterase enzyme and β- amyloid $_{1-42}$ levels

Scopolamine, known to block muscarinic cholinergic receptor [mAchR] antagonist non-selectively with increasing AchE action, leads to neuronal injury. In this due course synaptic cleft will be insufficient if ACh misleads the signal transductions followed by memory acquisition failure. Hence, conventionally

scopolamine is extensively employed as a learning and memory impairment model to evaluate various new drugs.[195] Present study also showed that scopolamine induced groups expressed decline in memory by increased levels of AChE enzyme, thus indicating signs of dementia. Conversely, the rats administered by CDE at 100, 200 and 400 mg/kg showed significant decrease in AChE levels in the hippocampus and allows ACh to bind with cholinergic receptors which validates the protection of cholinergic neurons affected by scopolamine- induced amnesia in rats. These current observations were pertinent as per the previous reports of Poojary *et al*. and Rai *et al*. [192, 196] An additional support of aqueous extract of *C. dactylon* ameliorates cognition in zebra-fish model by influencing the retention of memory using social interaction tests, inhibitory avoidance task, and exploratory assessment proposed by Yendapalli PR et al. [197] CDE inhibits the excessive beta amyloid 1-42 deposition significantly as compared to scopolamine induced rats.

Memory loss is a prevalent symptom among the elderly which is unavoidable; to illustrate this illness, older animals can be used as a natural model of dementia, since they have been proven to acquire neuropathology, oxidative stress and memory impairment in the same way that Alzheimer's patients do. [198] Brain consumes a high amount of oxygen for neuronal functions however the neuronal injury is vulnerable to produce reactive oxygen species especially hydrogen peroxide originated from neuronal mitochondria. Moreover neuronal membranes are susceptible to oxidative stress. Amyloid beta aggregation and deposition in lipid bilayer generates hydrogen peroxides. Substantia nigra a specific area in brain contains high level of Fe, Cu ions which are transit metal ions and these react with H_2O_2 generates hydroxyl residues by "Fentom Reaction" which results in lipid peroxidation. Lipid peroxidation known to disturb vital functions by modifying proteins/DNA. [199]

6.1.3.4. Impact of CDE on antioxidant biomarkers and modulates histopathological changes against scopolamine induced amnesia.

Aging process is natural and associated with various disorders including AD where the risk of reactive oxygen species are robust to imbalance of antioxidant pathways, especially glutathione. Many other reports do claim enhanced activity of LPO causes neurotoxicity results in misbalancing glutathione as explained by Rahimzadegan M et al. [176, 200] Scopolamine is also reported to cause oxidative stress by decreasing GSH (reduced) levels and significantly elevating lipid peroxidation which is ideal for neuronal damage in the current study. Meanwhile the groups treated with CDE showed elevated GSH levels and activity of lipid peroxidation were deprived which were pertinent with previous study by Rai et al. [196]

Sumathi T et al. proposed the nootropic action of *C. dactylon* aqueous extract by protecting brain areas through mechanism of enhancing enzyme activity of Mg2+ ATPase and Na+/K+ ATPase with LPO inhibition in aluminum-trichloride induced neurotoxicity. [201] The basal forebrain nuclei provide significant cholinergic input to both the hippocampus and cortical portions of the brain. As a result, lesions of these nuclei have been utilized to simulate cholinergic deafferentation in Alzheimer's disease and to determine the behavioral effects of cholinergic deafferentation. Following lesioning of cholinergic pathways leading to the hippocampus, the most severe and persistent effects of such cholinergic lesioning on cognition are observed. [202] Hippocampus and its subparts; entorhinal cortex, presubiculum, dentate gyrus, subiculum, parasubiculum primarily mediates memory function. Atrophy of hippocampal (frontal-temporal horn) and cerebral cortex regions are linked in AD. Another study reports that cornu ammonis (CA1) area of hippocampus was deposited

by neurofibrillary tangle and so neuronal atrophy are seen prominently. [203] In the histopathological study most adversely used stain based technique is Hematoxylin-Eosin dye to examine different brain regions which could confirm pathological/ pharmacological differences especially related to hippocampus and cortex parts of the brain. Present study gives intervention of scopolamine administered rats with severe scale of damage to neurons, notably it was observed that standard drug (donepezil) and CDE given rats revealed mild neuronal tissue changes which is incorrigible with Barai et al reports. [204] To discern the changes with respect to neuronal changes especially to confirm deposition of beta- amyloid plaques or neurofibrillary tangles unique staining are essential viz, thioflavin S, Congo red used along with advanced microscopic techniques. To diagnose dementia advanced methods such as antibody-based immunohistochemistry may be used in future study. [205]

6.2.1. In-silico study of S. rhombifolia against dementia

Binding DB proficiently forecasts drug-likeness properties of various phytoconstituents by favoring ≥0.7 as probable score to detect targets for AD. Biological response of ligands acting as agonist/antagonists could be influenced by the structures that are likely to target the specified proteins in the initial phase of target prediction. [206] In the view of AD's hallmarks, adenylate- cyclase mediated by G-protein has got importance as a pathological condition along with other signaling pathways. [207] Liu et al. explained that targeting receptors associated with G-protein considered as key therapeutic strategies for Alzheimer's disease.[208] Presently *S. rhombifolia* phytocompounds targeted; Aβ cascades (amyloid precursor protein, Aβ-degradation), HTR2A, CHRM1, CHRM3 receptors. So, the compounds which modulate selected pathways were scrutinized by gene set enrichment and present

study identified around 10 pathways of AD namely 5-hydroxytryptamine receptors such as (HTR2A ,HTR2C), AchE, BChE, CHRM1, MAOA,MAOB, APP, BACE1. The pathways associated with AD such as; TRP channels regulated by Inflammatory mediator, Neuroactive-receptor interaction, Calcium signaling pathway, Gap junction, Serotonergic synapse, and cholinergic synapse were considered.

Permanent alterations in the brain is due to multiple neuronal losses connected to memory storage, such as cholinergic, serotonin, GABA, and glutamine transmission with their receptors, are changed, particularly in functioning hippocampal areas dominated by astrocytes, which represent 'Transient Receptor Potential Ankyrin 1 (TRPA1)'. When the intracellular Ca+2 concentration is altered by oxidative free radicals, channels are activated. [209, 210]

The constituents of *S. rhombifolia* ; Pterosterone-3-O-β-D-Glucopyranoside, Ecdysone, 2D-Hydroxyecdysone steroidal molecules are speculated to interlink with serotonergic synaptic pathway, TRPA1 channels by targeting HTR2A, 2C proteins along with others (AChE, BChE, and BACE1) are responsible to show memory enhancing property.

Synaptic deficit due to the imbalance of Ca2+ homeostasis has a prominent role to enhance accumulation of Aβ-insoluble plaque with NFT's is ubiquitous. Along with this another hallmark of the gliosis process occurs by phenotype modulations of microglia, astrocytes which could imbalance the expression proteins creating problems associated with formation of gap junctions with connexins. Moreover it was found that Connexin protein had been expressed in most of the astrocytes resulting in plaque. Amyloid β content generated by proteolysis of APP with the help of BACE1.

Hence this *in-silico* study focused on germline destruction of gene BACE1 to block Ad β formation. [211-215]

Acacetin considered as an active flavonoid has many roles as neuroprotective, anticancer, antidiabetic, anti-inflammatory effects by promising its action against decreasing cyclooxygenase, xanthine oxidase enzyme, acetylcholinesterase and glutathione reductase enzymes as per earlier Semwal et al,. reports. [216, 217]

Flavonol Kaempferol naturally has an anti-oxidative effect through which it can interfere with neuronal mitochondria and cell membrane damage. [218] Interestingly we recognized kaempferol, acacetin showed inhibition of proteins (AChE, BACE1, A2A, ADORA2A, APP and BChE) which is correlated with estimation of AChE enzyme by *in-vitro* techniques for flavonoid rich fraction of *S. rhombifolia* .

In the progression of AD; it was observed that regulation of AChE is associated with increase in BChE with the presence of amyloid plaque and neurofibrillary tangles. [219] As per the earlier reports 'Acacetin' attenuates BACE1 action to decrease the Aβ production which was revealed by western blot analysis and PCR. [220]

PI3-kinase/Akt and ERK signaling pathways have been involved by targeting proteins such as AChE, BACE-1, BChE activities. However, many flavonoids inhibit their properties to protect the neurons and improve cognition by neurogenesis and increase perfusion rate at hippocampal regions. Out of alkaloids detected in *S. rhombifolia* Sanguanine has shown capacity to manage AD by CHRM1 inhibition using AChE, BChE targets. [221, 222] Another study suggest that *Narcissus* genus plants do have high content of Sanguinine has similar structural similarity of Galantamine

and Lycorine " currently used therapies for AD" So in the near future alkaloids of importance can be isolated as lead compound to manage neurodegenerative conditions. [223, 224]

Compelling validation of network pharmacology performed it was traced that 9 phyto actives viz; steroids: pterosterone-3-O-β-Dglucopyranoside, ecdysone, 2D-hydroxyecdysone, Flavonoids: Acacetin, kaempferol, alkaloid: vasicinol, vasicinone, vascicine, sanguinine have optimized AchE inhibition in AD.

Synaptic plasticity is essential for between two neurons to communicate which depends on volume of conversation and Adenosine Receptor Subtype A2a (ADORA2A) plays a pivotal role in controlling NMDA dependent transmission along with neurogenesis in the CA3 region. Through a p38 MAPK-dependent mechanism, ADORA2A receptor blockade can prevent A-induced synaptotoxicity and memory impairment, as well as microglial cell translation. [225, 226] Liquid chromatography-Mass Spectroscopy (LC-MS) technique was utilized for identifying major constituents present in *S. rhombifolia* . We found the presence of Sanguinine, Ecdysone, 20-hydroxyecdysone , Vasicinol and Acacetin which were recognised as alkaloids, flavonoids and steroidal groups of compounds.

To summarize Sanguinine, Ecdysone,20-hydroxyecdysone, Pterosterone-3-O-β-Dglucopyranoside, Vasicinol and Acacetin phytocompounds regulated the integral changes associated by scopolamine induction and promoting the behavioural changes via, regulating PI3-kinase/Akt and ERK signaling pathways, cholinergic synapse, calcium signalling pathway by inhibiting ACHE, APP, BACE1, A2A, ADORA2A, BchE targets.

We realized that flavonoid rich (Acacetin, Kaempferol) targets ADORA2A and fosters the importance of *S. rhombifolia* in the management of AD. Mah et al,. [180] claims flavonoid rich fraction having terpenoids, steroids and phytosterols advertised AchE inhibition by *in-vitro* Ellman's method and recently our published data signifies anticholinesterase and antioxidant effect of hydro-ethanolic extract of *S. rhombifolia* by *in-vivo* methods.

6.2.2An in-vitro antioxidant and anti-acetylcholinesterase activity of SRE

In the presence of antioxidants, a stable free radical DPPH receives electrons by anti-oxidant which results in decolorization of visible deep purple color, which could be measured quantitatively from absorbance change at 517 nm and % scavenging activity should be calculated. The selected plant CDE also scavenges free-radical DPPH which was supported by earlier reports by Naik et al. [227] The ethanolic extract of *S. rhombifolia* also scavenges DPPH followed by Oroian procedure and this was correlated with previous reports of Dhalwal et al.[228]

Antioxidant property of *S. rhombifolia* was noticed by hydrogen peroxide scavenging activity; this was also correlated with Chung et al. who explained phenolic content present in *S. rhombifolia* converse ROS actions.

Selected flavonoid rich fraction of *S. rhombifolia* prominently reduced Acetylcholinesterase enzyme as compared to other chosen fraction which is pertinent with Mah et al reports. [111]

6.2.3. *In-vivo* models

Current study explained the possibility of hydro-ethanolic extract of *S. rhombifolia* for cognitive enhancing properties revealed by various methods such as *in-vivo*, computational models.

6.2.3. 1. Effect of SRE on cognitive models

To recognize reliable modifications in the cholinergic neuronal pathway, Morris water maze gives important key evidence. As observed in the results, the scopolamine administered rats spent less time in target-quadrant which was in comparison with Kour et al. [229] former published work addressed the 7th 8th and 9th day of scopolamine induction caused increase in escape latency time as compared to normal rats. However, for the first time present study likely to witness SRE treatment at 100, 200, and 400mg/kg showed considerable learning and memory modulations caused by scopolamine.

To assess short term memory, depression-like behavior, anxiety and response of fear can be speculated by using the EPM model. In this study repeated doses of scopolamine for 30 days showed the disturbed memory and fearfulness in rats by locating themselves at the corner of closed arms with excess fecal excretion. This was corroborated by Aydin et al study. [230] Meanwhile, Donepezil and SRE administered rats significantly decreased the number of transfer latency and time spent by rats were prominently increased which proclaims exteroceptive cognition advancement.

Step through latency measures time spent in light and dark compartment in passive avoidance tasks. The hydro-ethanolic extract *S. rhombifolia* administered rats expressed significant increase in step-through latency. In the dark area they spent less time compared to the scopolamine group. These reports are relatively compared with ethanolic extract of *S. cordifolia* nootropic property by administering at doses - 50, 100 and 250mg/kg and giving evidence of passive avoidance task results explained by Khurana et al. [231]

6.2.3.2. SRE regulated acetylcholinesterase enzyme and β- amyloid 1-42 levels

The most important hallmarks of cognitive disparity are showcased by indirect cholinergic neuronal destructions (acetylcholinesterase increased activity). Extracellular deposition of insoluble Amyloid $_{\beta 1\text{-}42}$ especially has been widely accepted in the research field. The present study addressed that "AchE concentration was increased significantly in scopolamine given rats and elevated Amyloid $\beta_{1\text{-}42}$ levels". In the converse, SRE and donepezil treated rat brain samples decreased the AchE levels and enhanced the availability of Acetylcholine at synaptic cleft and improved memory along with restricting the deposition of $A_{\beta 1\text{-}42}$ content as per Pattanashetti et al. [232]

6.9.3.3. SRE restores neuronal antioxidant biomarkers and modulates histopathological changes against scopolamine induced amnesia.

We observed the impact of SRE as antioxidant which regulates by elevating glutathione (reduced) content in brain which was in interlinked with Narendhirakannan et al reports [233] who observed free- radical scavenging property of stem and root extract of *S. rhombifolia* in the management of arthritic rats. Oxidative free radical stress is indexed by calculating malondialdehyde content and is a sign of increase in lipid peroxidation. The dose dependent administration of SRE showed a remarkable decrease in MDA levels which was compared with earlier study of Bihaqi et al. and Dhalwal et al. favouring noticeable antioxidant actions of *S. rhombifolia* extract. [234, 235]

To claim the memory in the mammals dentate, Cornuammonis (CA fields) and subicular regions in the hippocampus are anatomical areas of the medial temporal lobe that play a significant role according to Squire, [236] Neuronal damage, neuro fibrillary tangles, hippocampal edema and pyknotic cells were seen in the SCO-induced group in histopathological rat brain investigations, but groups treated with SRE and donepezil revealed modest neuronal damage compared to SCO-induced neuronal damage. Converging the insights the earlier scientists witness possible action of SRE phytoconstituents viz, flavonoids, phenylethylamines, phenolic compounds, chlorophyll derivatives, alkaloids and steroids compounds, may contribute for neurocognition improvement by cholinesterase enzyme inhibition. [103, 237]

Summary on effect of CDE on cognition

- CDE could act over multiple proteins and pathways to deal with cognitive impairment which could be demonstrated by predictive and preclinical data.
- It was observed that CDE has potency to improve short term, long term memory and down-regulates the negative reinforcement.
- Further inhibit the acetylcholinesterase enzyme and beta amyloid $_{1\text{-}42}$ content
- Additionally, CDE promotes the antioxidant biomarkers against scopolamine induced dementia.
- CDE ameliorates the scopolamine induced histopathological changes.
- *In-silico* finding predicts that the apigenin, luteolin, Kaempferol and quercetin present in*C. dactylon*showed connectivity with ACHE, BCHE, ADORA2A targets by sharing pathways of acetylcholinesterase activity, protein dimerization activity, serine hydrolase activity concerned with dementia.

Summary on effect of SRE on cognition

- SRE ameliorates the cognitive impairment caused by scopolamine administration and restores the memory by improving short-term and long term memory.
- SRE promotes the acetylcholine activity and decreases beta-amyloid level showing the neuroprotective effect against dementia.
- Further, SRE enhances neuronal antioxidant biomarkers and supports free radical scavenging properties.
- cholinergic synapse, Neuroactive ligand receptor interaction, Gap junction, Calcium signaling pathway were identified to directly connected against AD which were triggered by bio-actives of *S. rhombifolia* .

7. SUMMARY

The present thesis **"Evaluation of *Cynodon dactylon* L. and *Sida rhombifolia* L. on cognitive dysfunction in rats"** aimed to predict the molecular mechanism of the phytoconstituents responsible for memory enhancing action by *in-silico* methods and integrated to study the effect of hydro-ethanolic extract of *Cynodon dactylon* L. and *Sida rhombifolia* L. against cognitive dysfunction induced by scopolamine in rats. Considering the unavoidable aging process and other factors of pathogenesis, AD is progressive and incurable. Symptomatic treatments are prescribed for the management of dementia but drugs are associated with multiple side effects on long term use. Literature search gives clues regarding the neuroprotective effect of various medicinal plants hence, in this regard traditional claims of plants in the modern therapy are still in the pipeline. Traditional medications and therapies have inadequate scientific evidence on signal transduction mechanisms, effectiveness, and safety, as well as insufficient scientific documentation and validation, thus more research is needed to fully understand their potential in neurotherapeutics.

The plants *Cynodon dactylon* and *Sida rhombifolia* have been reported with a variety of pharmacological activities viz, haemorrhages, dysentery, hyperdipsia anti-inflammatory, wound healing, hepatoprotective etc. In the view of traditional reports of both plants used as brain and heart tonic, we planned to evaluate the efficacy in treatment of dementia Nevertheless, scientifically in preclinical literature its potential to treat cognitive impairment is limited.

The current anti-amnesic activity of CDE and SRE in the scopolamine induced rats were explored by behavioural modulations, effect on acetylcholinesterase enzyme, $A\beta_{1-42}$ content, and antioxidant systems.

Further, we observed the presence of flavonoids, alkaloids, phytosterols, saponins and other important chemical constituents by LC-MS analysis and current study dealt to reveal the memory enhancing efficacy of *C. dactylon* and *S. rhombifolia* extract by "network pharmacology" and "molecular docking" studies to confirm the modulation of hallmark in AD. The network pharmacology analysis depicted multiple molecular pathways that had links with specific proteins and targets in competence to modify the disease.

According to a current study, the flavonoid-rich fraction of *C. dactylon* and *S. rhombifolia* crude extracts may be able to prominently inhibit the acetylcholinesterase enzyme and scavenge free radicals by reducing DPPH and H_2O_2 activity which were confirmed by *in-vitro* methods.

The present study, declares scopolamine induction, confirmed evidence of amnesia by increasing acetylcholinesterase enzyme levels and oxidative stress, ultimately disrupting cholinergic pathways in rats that are important for learning and memory.

In the amnesia induced by scopolamine in rats, the hydro-ethanolic extract of *C. dactylon* provides evidence of cognitive modulation through the mechanism of decreased AChE enzyme levels and increased antioxidant levels, which may be attributed to the presence of phytoconstituents singly or in combinations in the extract.

In- silico studies of *C. dactylon* modulates AD pathways by regulating targets; ACHE, MAOB, ADORA2A, ADORA1 by compounds Apigenin, Kaempferol, Luteolin and Quercetin (Flavonoids) which was correlated with LC-MS analysis.

Four functional pathways i.e, identical protein binding, acetylcholinesterase activity, protein dimerization activity, serine hydrolase activity were identified to target the proteins of interest present in AD pathogenesis. The apigenin, luteolin, Kaempferol and quercetin present in *C. dactylon* showed connectivity with ACHE, BCHE, ADORA2A targets.

In SCO-induced rats, the efficacy of S. rhombifolia hydro-ethanolic extract was confirmed through the mechanism of AChE inhibition and antioxidant pathways. The flavonoids; acacetin, kaempferol, steroids; ecdyson, 2D-hydroxyecdysone, pterosterone-3-O-β-D-glucopyranoside, alkaloids; vasicinol, vascicine, sanguinine, vasicinone from *S. rhombifolia* modulates AD pathways by targeting BACE1, ACHE, MAO, ADORA2A, APP, BCHE, MAOB. This concludes synergistic action by multiple protein targets in the management of AD.

The network analysis revealed that kaempferol, acacetin, ecdysone, 2D-hydroxyecdysone, and pterosterone -3-O—D glucopyranoside, all found in *S. rhombifolia*, are predicted to modulate AD pathways: Inflammatory mediator regulation of TRP channels, Serotonergic synapse, cholinergic synapse, Neuroactive ligand receptor interaction, Gap junction, Calcium signaling pathway.

8. CONCLUSION

Anti-amnesic activity of *C. dactylon* is possible, may be by enhancing antioxidant action, acetylcholinesterase enzyme inhibition signaling pathway, and reducing insoluble beta amyloid $_{1-42}$ deposition by the mechanism of bioactives apigenin, kaempferol, luteolin and quercetin activities targeting ACHE, MAOB, ADORA2A, ADORA1 through identical protein binding, acetylcholinesterase activity, protein dimerization activity, serine hydrolase pathogenic pathways of AD.*S. rhombifolia* memory enhancing activity may be possible via, the mechanism of inhibition of acetylcholinesterase enzyme, antioxidant activity, and depleting beta amyloid $_{1-42}$ levels by phytocompounds acacetin, sanguinine, kaempferol, vasicinol modulating AD pathways; Inflammatory mediator regulation of TRP channels, Serotonergic synapse, Cholinergic synapse, Neuroactive ligand receptor interaction, Gap junction, Calcium signaling pathway targeting BACE1, ACHE, MAO, ADORA2A, APP, BCHE, MAOB.

This investigation validates evidence of *C. dactylon* and *S. rhombifolia* as a brain tonic stated in Unani and Ayurvedic systems of medicine. Further, in future bioactives from *C. dactylon* and *S. rhombifolia* plants may be isolated which, could modulate aforementioned pathways for the management of AD.

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ANNEXURE- A

Authentication letter of *Cynodon dactylon* L. whole plant

राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR-NATIONAL INSTITUTE OF TRADITIONAL MEDICINE
(भूतपूर्व क्षेत्रीय आयुर्विज्ञान अनुसंधान केन्द्र Formerly Regional Medical Research Centre)
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Dr. Harsha Hegde
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भारतीय आयुर्विज्ञान अनुसंधान परिषद
INDIAN COUNCIL OF MEDICAL RESEARCH
स्वास्थ्य अनुसंधान विभाग, स्वास्थ्य और परिवार कल्याण मंत्रालय, भारत सरकार
Department of Health Research,
Ministry of Health & Family Welfare, Govt. of India

Date: 22-05-2018

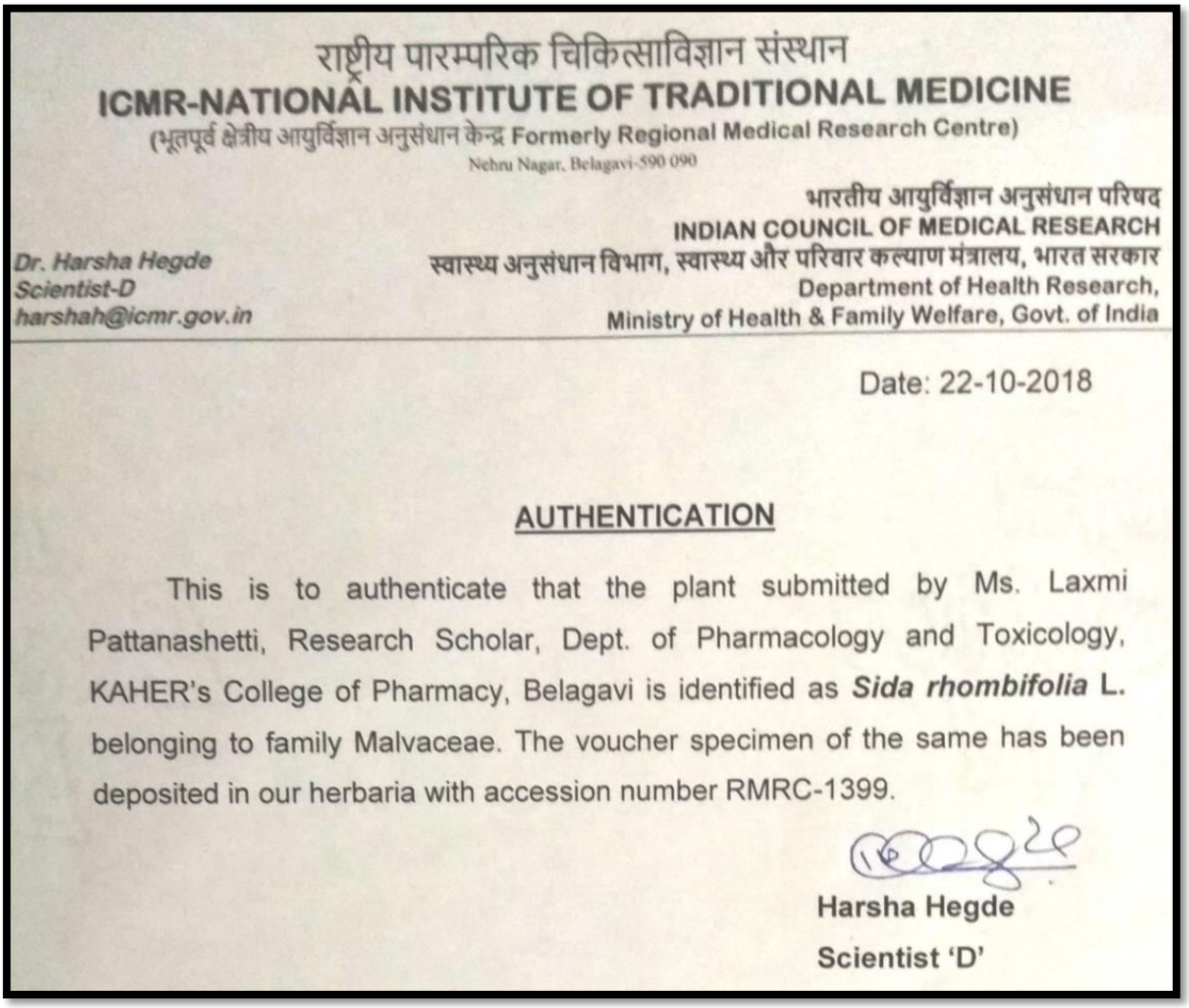
AUTHENTICATION

This is to authenticate that the plant submitted by Ms. Laxmi Pattanashetti, Research Scholar, Dept. of Pharmacology and Toxicology, KAHER's College of Pharmacy, Belagavi is identified as ***Cynodon dactylaon* (L.) Pers.** belonging to family Poaceae. The voucher specimen of the same has been deposited in our herbaria with accession number RMRC-1391.

Harsha Hegde
Scientist 'D'

ANNEXURE- B

Authentication letter of *Sida rhombifolia* L.whole plant



राष्ट्रीय पारम्परिक चिकित्साविज्ञान संस्थान
ICMR-NATIONAL INSTITUTE OF TRADITIONAL MEDICINE
(भूतपूर्व क्षेत्रीय आयुर्विज्ञान अनुसंधान केन्द्र Formerly Regional Medical Research Centre)
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भारतीय आयुर्विज्ञान अनुसंधान परिषद
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Department of Health Research,
Ministry of Health & Family Welfare, Govt. of India

Dr. Harsha Hegde
Scientist-D
harshah@icmr.gov.in

Date: 22-10-2018

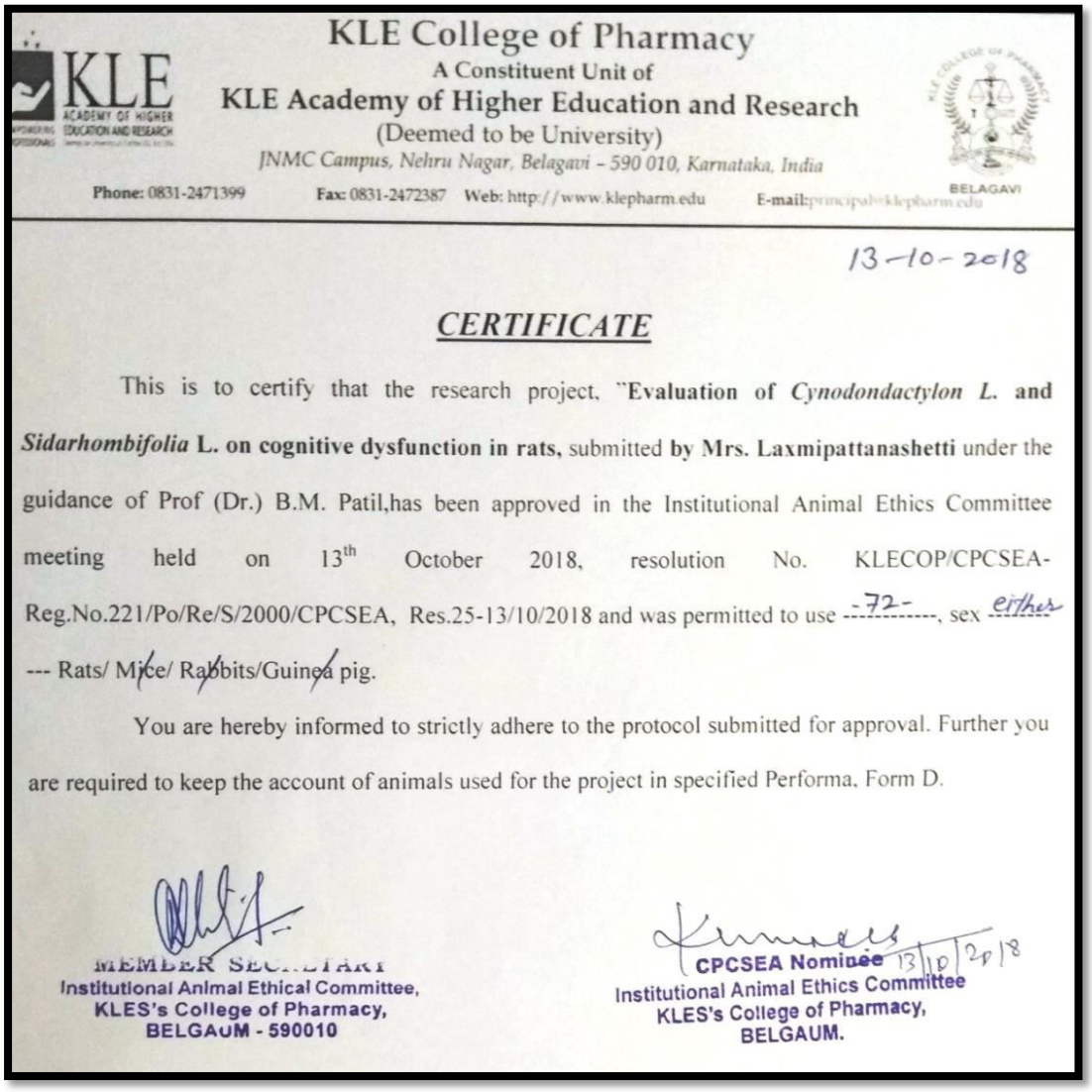
AUTHENTICATION

This is to authenticate that the plant submitted by Ms. Laxmi Pattanashetti, Research Scholar, Dept. of Pharmacology and Toxicology, KAHER's College of Pharmacy, Belagavi is identified as ***Sida rhombifolia*** L. belonging to family Malvaceae. The voucher specimen of the same has been deposited in our herbaria with accession number RMRC-1399.

Harsha Hegde
Scientist 'D'

ANNEXURE- C

Animal Ethical Approval certificate



KLE College of Pharmacy
A Constituent Unit of
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Phone: 0831-2471399 Fax: 0831-2472387 Web: http://www.klepharm.edu E-mail: principal@klepharm.edu
BELAGAVI

13-10-2018

CERTIFICATE

This is to certify that the research project, "**Evaluation of *Cynodondactylon L.* and *Sidarhombifolia* L. on cognitive dysfunction in rats,** submitted **by Mrs. Laxmipattanashetti** under the guidance of Prof (Dr.) B.M. Patil,has been approved in the Institutional Animal Ethics Committee meeting held on 13th October 2018, resolution No. KLECOP/CPCSEA-Reg.No.221/Po/Re/S/2000/CPCSEA, Res.25-13/10/2018 and was permitted to use -72-, sex either --- Rats/ Mice/ Rabbits/Guinea pig.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.

MEMBER SECRETARY
Institutional Animal Ethical Committee,
KLES's College of Pharmacy,
BELGAUM - 590010

CPCSEA Nominee 13/10/2018
Institutional Animal Ethics Committee
KLES's College of Pharmacy,
BELGAUM.

ANNEXURE- D

List of Publications/presentation

1. **Pattanashetti LA,** Patil BM, Hegde HV, Kangle RP. Potential ameliorative effect of *Cynodon dactylon* (L.) *pers* on scopolamine-induced amnesia in rats: Restoration of cholinergic and antioxidant pathways. *Indian J Pharmacol*. 2021;53(1):50-59. doi:10.4103/ijp.IJP_473_20. **Impact factor: 2.83, CiteScore: 2.7**

2. **Pattanashetti LA**, Patil BM, Hegde HV, Kangle R. Hydroethanolic extract of *Sida rhombifolia* L. ameliorates scopolamine-induced cognitive dysfunction in rats. J Appl Pharm Sci, 2021;11(05):118–126.**Impact factor:1.37, CiteScore: 2.3**

3. **Pattanashetti L**, Patil BM, Hegde HV. In silico and in vitro Approach To Identify Memory Enhancers from Sida rhombifolia L. J Young Pharm. 2021;13(4):363-9. **Impact factor: 0.18**

4. Presented Poster titled " ***Cynodon Dactylon* L. attenuates beta 1-42 Amyloid Content in Scopolamine Induced Amnesic Rats**"at KrupaPharmacon 2021; 3rd Global online conference and workshop on Drug Development 4.0: Emerging Technologies an event organized by Krupanidhi College of Pharmacy, Bengaluru, on 02-03 July 2021.

(Front page of articles/certificate are attached)

Note: The above mentioned impact factor and cite score of the journal are based on the Scopus and Web of Science respectively during the year of thesis submission (2022).

Research Article

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Website: www.ijp-online.com
DOI: 10.4103/ijp.IJP_473_20

Potential ameliorative effect of *Cynodon dactylon* (L.) *pers* on scopolamine-induced amnesia in rats: Restoration of cholinergic and antioxidant pathways

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Revised: 05-Oct-2020
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Published: 28-Apr-2021

Abstract:

AIM: The present study explored *Cynodon dactylon* hydro-ethanolic extract (CDE) effect on scopolamine-induced amnesic rats.

MATERIALS AND METHODS: *C. dactylon* extract was subjected to antioxidant (DPPH and H_2O_2) and acetylcholinesterase enzyme tests by *in vitro* methods. Scopolamine (1 mg/kg, i.p) was administered to rats except for normal control. Donepezil (3 mg/kg, p.o), CDE (100, 200, and 400 mg/kg p.o) were administered to treatment groups. Behavioral paradigm: Morris water maze (MWM), elevated plus maze (EPM), and passive avoidance test (PAT) were conducted. Later, rats were sacrificed and brain homogenate was tested for levels of acetylcholinesterase, glutathione, and lipid peroxidase. Histopathology examination of cortex and hippocampus of all the groups was done.

STATISTICAL METHOD: The statistical methods used were ANOVA and Tukey's *post hoc* test.

RESULTS: CDE antioxidant activity was demonstrated by decreasing DPPH and H_2O_2 levels confirmed through *in vitro* analysis. Treatment group rats reversed scopolamine induced amnesia by improvement in spatial memory, decreased transfer latency and increased step through latency significantly (P<0.001) in behavior models such as morris water maze, elevated plus maze and passive avoidance task respectively. CDE modulated acetylcholine transmission by decreased acetylcholinesterase enzyme level (P < 0.001) and scavenging scopolamine-induced oxidative stress by increased reduced glutathione levels and decreased lipid peroxidation levels in the rat brain. CDE and donepezil-treated rats showed mild neurodegeneration in comparison to scopolamine-induced severe neuronal damage on histopathology examination.

CONCLUSION: *C. dactylon* extract provides evidence of anti-amnesic activity by the mechanism of decreased acetylcholinesterase enzyme level and increased antioxidant levels in scopolamine-induced amnesia in rats.

Keywords:

Acetylcholinesterase, antioxidant, *Cynodon dactylon*, dementia, neuroprotection

Introduction

Dementia is an umbrella term that includes Alzheimer's disease (AD) expressing features of neurodegeneration that leads to memory impairment and behavioral changes that interferes with work, social activities, and impairs a person's ability to perform routine activities. According to Alzheimer's Disease

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Hydroethanolic extract of *Sida rhombifolia* L. ameliorates scopolamine-induced cognitive dysfunction in rats

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Key words:
Sida rhombifolia
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oxidative stress, β amyloid$_{1-42}$,
Alzheimer's disease.

ABSTRACT

Alzheimer's disease is a neurodegenerative condition that involves cholinergic neuronal dysfunction, oxidative stress, amyloid beta protein accumulation as a characteristic of neuropathology. *Sida rhombifolia* is traditionally known for the treatment of neuronal disorders. This study was designed to evaluate the effect of *S. rhombifolia* extract (SRE) on scopolamine (SCO) induced amnesia in rats. *Sida rhombifolia* hydroethanolic extract (SRE) was subjected to *in-vitro* tests. Furthermore, in rats amnesia was induced with SCO (1mg/kg. i.p) for 30 days and treated with 100, 200, and 400 mg/kg of SRE for 15 days. Antioxidant activity of SRE was demonstrated by decreasing DPPH and H_2O_2 levels. Treatment group rats reversed SCO induced amnesia by improvement in spatial memory, decreased transfer latency and increased step through latency significantly ($p < 0.001$) in behavior models such as Morris water maze, elevated plus maze, and passive avoidance task, respectively. SRE administration decreased acetylcholinesterase enzyme, β amyloid$_{1-42}$ levels significantly ($p < 0.001$), scavenges SCO induced oxidative stress by increased glutathione and decreasing lipid peroxidase levels. Histopathological studies revealed mild neuronal damage in treatment groups as compared to SCO-induced rats and is validated its implication for the treatment of cognitive impairment.

INTRODUCTION

The most prevalent form of dementia is Alzheimer's disease (AD) which expresses the features of memory loss and behavioral disturbance (Smith, 2017). Aging, especially people aged 65 years and above are at the risk of various diseases including AD. Every year nearly 1,275 new cases per 10,000 individuals are diagnosed with AD (Querfurth and LaFerla, 2010), and this has become challenging to nursing care facilities and financial burden for care givers.

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The increased acetylcholinesterase enzyme (AChE) activity, alteration of acetylcholine (ACh) receptors, or decreased production of ACh in central cholinergic system are all considered as significant hallmarks of AD (Anand *et al*., 2014). The treatment regimens were developed to target the pathogenesis of AD, but their use is restricted due to unavoidable side effects. The donepezil is being used as a first-line treatment drug; it has undesirable and unavoidable peripheral anticholinergic side effects. Hence, health care professionals are keen to find alternative therapies (Citron, 2010).

Many herbal drugs including extracts are focused to treat AD based on potential effects such as anti-oxidants, AChE enzyme inhibition, secretase enzymes responsible for producing Aβ, chelating heavy metals, or Aβ degradation mechanisms (Eckert, 2010).

The plant genus *Sida* L. is well documented in Ayurveda, an ancient Indian system of medicine. Around 17 species that occur in India are reported traditionally in *Rasayana* to treat various ailments including degenerative and musculoskeletal disease (Trikamji, 1984). *Sida rhombifolia* L. (*S. rhombifolia*) is a perennial plant that belongs to the family Malvaceae commonly known as arrowleaf

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Original Article

In silico and *in vitro* Approach to Identify Memory Enhancers from *Sida rhombifolia* L.

Laxmi Pattanashetti[1,*], BM Patil[1], Harsha V Hegde[2]
[1]Department of Pharmacology, KLE Academy of Higher Education and Research, College of Pharmacy, Belagavi, Karnataka, INDIA.
[2]Department of Ethnomedicine, Indian Council of Medical Research - National Institute of Traditional Medicine, Belagavi, Karnataka, INDIA.

ABSTRACT

Background: *Sida rhombifolia* L. is a well documented Ayurvedic medicine for the management of neurodegenerative diseases and to enhance cognitive function. Researchers demonstrated its activities under various animal model/s, but lack the probable molecular mechanism in the treatment of Alzheimer's disease. Current study was aimed to identify the acetylcholinesterase (AChE) inhibitory potency of phytocompounds and enriched fractions from *S.rhombifolia* using *in vitro* and network pharmacology approaches. **Methods:** Phytocompounds were retrieved from phytochemical databases, scientific reports and quired for druggability. Protein targets were predicted using BindingDB ($p \geq 0.7$). STRING database and KEGG pathway were utilized to perform gene set enrichment analysis and to identify the probable pathways modulated by the phytocompounds. Cytoscape v3.6.1 was used to construct a target-compound-pathway network. Docking was performed by PyRx 0.8v. Enriched fractions of *S. rhombifolia* were tested for *in vitro* AChE inhibitory potency using the AChE enzyme. **Results:** Among 35 compounds, 26 compounds showed positive drug likeness property. Out of 26 compounds, 9 compounds i.e. 2D-hydroxyecdysone, ecdysone, pterosterone-3-O-β-D-glucopyranoside, acacetin, kaempferol, sanguinine, vascicine, vasicinol, vasicinone were predicted to target AChE and other 9 therapeutic targets involved in Alzheimer's disease (AD). Acacetin scored lowest binding energy with AChE (-8.9kcal/mol). Among the selected enriched fractions, hexane fraction pertains highest AChE inhibition (IC_{50} 12.87µg/ml) compared to clinical approved drug Donepezil (IC_{50} 2.92µg/ml). **Conclusion:** The role of *S.rhombifolia* for the management of AD could be attributed due to the major effect of 2D-hydroxyecdysone, ecdysone, pterosterone-3-O-β-D-glucopyranoside, acacetin, kaempferol, sanguinine, vascicine, vasicinol, vasicinone on AChEand their action on multiple protein molecules associated with AD pathogenesis.

Key words: Alzheimer's disease, Acacetin, Acetylcholinesterase, *In silico* docking, Network pharmacology, *Sida rhombifolia*, Sangunine.

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DOI: 10.5530/jyp.2021.13.90

INTRODUCTION

The most commonly known neurodegenerative disorder with the age-related risk factor is Alzheimer's disease (AD). The specific regions of brain parts demonstrate loss of synaptic connections, including accumulation of abnormal proteins such as extracellular amyloid plaque, intracellular neurofibrillary tangles.[1] Another important characterized feature is the abnormal alteration of Acetylcholine (ACh) neurotransmission; due to lower amount of choline acetyltransferase and decreased ACh released leads to loss of cholinergic functions in neocortex and hippocampus regions of the brain and this phenomenon is common in AD patients.[2] As per Delphi study "24 million people suffering from dementia in 2001 worldwide and this Figure was estimated to double in 2020 and quadruple in 2040".[3] AD generates difficulty in the management of effective treatment due to its multiple mechanisms involved in pathogenesis.

Currently, US-FDA-approved drugs are cholinesterase inhibitors (Donepezil, Rivastigmine, Galantamine) and NMDA receptor modulator (Memantine) as first-line treatments but, these present limitations for the long term treatment due to unavoidable side effects mainly caused by peripheral cholinergic system activation.[4,5] Considerably, it is essential to explore new, effective, and safe drugs. Mankind has advantages from ancient days by the plants, which are an abundant source of phytoconstituents considered as a novel in the therapy of many ailments.[6] Because there is "eighty percent of the world population relies on medicinal plants to meet their primary health care" as per World Health Organization (WHO) reports.[7]

The genus *Sida*. L belongs to the family Malvaceae is well documented in Ayurveda; ancient Indian system of medicine and 17 species are reported to occur in India; used traditionally in *Rasayana* to treat various ailments including degenerative and musculoskeletal disease. Perennial plant *S. rhombifolia* L.is known as 'Mahabala' reported as an anti-inflammatory, anti-arthritic and hepatoprotective effect.[8] The hot aqueous extracts of dried leaf and root of the *S. rhombifolia* are used to treat nervous diseases, heart diseases, burning sensation of the body and as an aphrodisiac and tonic.[9] The recent report claim the effect *of S. rhombifolia* extract to decrease the beta-amyloid accumulation in rat brain.[10]

Presently drug discovery emerges advancement in the treatment of complex diseases like AD with the concept of multi-compound drug therapy using herbs targeting cluster of disease-associated proteins and pathways. Understanding of network pharmacology approach in a per view of the "Lock and Key" model to design ligand that acts on specific targets provides new insights to elucidate the multi-scale mechanisms of action of herbs. Moreover, it is well explained that the master key could "open multiple locks" by targeting many proteins instead of preferring a single target.[11]

However, there are no scientific reports to show the mechanism of *S. rhombifolia* fractions for the management of cognitive dysfunction

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***This is to certify that* Laxmi Pattanashetti**

of **KLE of College of Pharmacy** *has presented a poster titled* ***Cynodon Dactylon L.attenuates B1-42 Amyloid Content in Scopolamine Induced Amnesic Rats*** *co-authored with* ***B M Patil , Harsha V Hegde*** *In the*

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Convener & Principal
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