A Comparative Study of Cholinesterase Inhibitory, Thrombolytic and Antioxidant Activities of Three Medicinal Plants (*Basella alba, Blumea balsamifera,* and *Curculigo orchioides*) Available in Bangladesh for the Treatment of Neurodegenerative Disorders and Clotting Disorders

A research paper is submitted to the Department of Pharmacy, East West University in conformity with the requirements for the degree of Bachelor of Pharmacy.

Submitted by

Farzana Khan Sristy

Id: 2013-1-70-080

Department of Pharmacy

East West University

Submitted to

Tirtha Nandi

Lecturer

Department of Pharmacy

East West University

East West University

Dedicated To My Beloved Parents Without Whom I Could Not Be Here……

Certificate by the Chairperson

This is to certify that the thesis entitled "A Comparative Study of Cholinesterase Inhibitory, Thrombolytic and Antioxidant Activities of Three Medicinal Plant (*Basella alba, Blumea balsamifera, and Curculigo orchioides*) Available in Bangladesh for the Treatment of Neurodegenerative Disorders and Clotting Disorders" submitted to the Department of Pharmacy, East West University for the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy, was carried out by Farzana Khan Sristy, Id: 2013-1-70-080, during the period 2016 of her research in the Department of Pharmacy, East West University.

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Dr. Shamsun Nahar Khan

Associate Professor & Chairperson

Department of Pharmacy

East West University, Dhaka

Certificate by the Supervisor

This is to certify that the thesis entitled "A Comparative Study of Cholinesterase Inhibitory, Thrombolytic and Antioxidant Activities of Three Medicinal Plant (*Basella alba*, *Blumea balsamifera*, and *Curculigo orchioides*) Available in Bangladesh for the Treatment of Neurodegenerative Disorders and Clotting Disorders" submitted to the Department of Pharmacy, East West University for the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy, was carried out by Farzana Khan Sristy, Id: 2013-1-70-080, during the period 2016 of her research in the Department of Pharmacy, East West University, under the supervision and guidance of me. The thesis has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

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Tirtha Nandi

Lecturer

Department of Pharmacy

East West University, Dhaka.

Certificate by the Co-Supervisor

This is to certify that the thesis entitled "A Comparative Study of Cholinesterase Inhibitory, Thrombolytic and Antioxidant Activities of Three Medicinal Plant (*Basella alba*, *Blumea balsamifera*, and *Curculigo orchioides*) Available in Bangladesh for the Treatment of Neurodegenerative Disorders and Clotting Disorders" submitted to the Department of Pharmacy, East West University for the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy, was carried out by Farzana Khan Sristy, Id: 2013-1-70-080, during the period 2016 of her research in the Department of Pharmacy, East West University, under the supervision and guidance of me. The thesis has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

_____

Kushal Biswas

Lecturer

Department of Pharmacy

East West University, Dhaka.

Declaration by the Candidate

I, Farzana Khan Sristy, hereby declare that the dissertation entitled "A Comparative Study of Cholinesterase Inhibitory, Thrombolytic and Antioxidant Activities of Three Medicinal Plant (*Basella alba*, *Blumea balsamifera*, and *Curculigo orchioides*) Available in Bangladesh for the Treatment of Neurodegenerative Disorders and Clotting Disorders" submitted by me to the Department of Pharmacy, East West University and in the partial fulfillment of the requirement for the award of the degree Bachelor of Pharmacy, work carried out by me during the period 2016 of my research in the Department of Pharmacy, East West University, under the supervision and guidance of Tirtha Nandi, Lecturer, Department of Pharmacy, East West University. The thesis paper has not formed the basis for the award of any other degree/diploma/fellowship or other similar title to any candidate of any university.

Farzana Khan Sristy

Id: 2013-1-70-080

Department of Pharmacy

East West University, Dhaka.

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Farzana Khan Sristy
Id: 2013-1-70-080
Department of Pharmacy
East West University, Dhaka.

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Abbreviations

APOE- Apolipoprotein E

MHC-Major histocompatibility complex

NGR-Nerve growth factor

MCI-Mild cognitive impairment

CDC-Centers for Disease Control and Prevention

CHAP-Chicago Health and Aging Project

NMDA- N-methyl-D-aspartate

AMPA- α-Amino-3-hydroxy-5-methyl-4- isoxazole-propionate

CSF-Cerebrospinal fluid

NSAIDs-Non-steroidal anti-inflammatory drugs

AchE-Acetylcholinesterase

HMF Co-A-3-hydroxy-3-methyl-glutaryl-coenzyme A

DTH-Delayed type hypersensitivity

FCR-Folin-Ciocalteu Reagent

GAE- Gallic acid equivalent

CE- Catechin equivalent

DTNB-5, 5´-dithio-bis-(2-nitro) benzoic acid

ATCI-Acetylthiocholine iodide

BTCI-Butyrylthiocholine iodide

TF-Total Flavonoids

DPPH-1, 1-diphenyl-2-picrylhydrazyl

µg/µl- Microgram per micro litter

Abstract

Among the pathologic hypotheses of Alzheimer's disease (AD), cholinergic deficit and oxidative stress have been implicated as major hallmarks. Therefore, inhibition of cholinesterase and oxidation are the two promising strategies in the development of a drug for AD. *Basella alba*, *Blumea balsamifera* and *Curculigo orchioides* are three available plants with numerous medicinal value. In this study these plants are used in this research to investigate its anticholinesterase, antioxidant and thrombolytic potentials. Anticholinesterase activity was measured by modified Ellman method. Antioxidant potentials were evaluated by the assay of reducing power, radical scavenging and ferrous reducing capability. Thrombolytic assessment is carried out by clot lysis method. The Methanolic extract showed strong anticholinesterase effect. Additionally, the extract exhibited pronounced reducing capacity, radical scavenging ability, and thrombolytic activity. Phytochemical screening of the extract revealed the presence of significant amount of total phenolics and flavonoids. The tested sample reflects potential antioxidative and anticholinesterase inhibitory effect which may warrant its effectiveness in the treatment of AD as well as clotting disorders.

CHAPTER 1: INTRODUCTION

Chapter 1: Introduction:

Alzheimer's disease was first described by a German doctor named Alois Alzheimer in 1906.[1] It is the most common form of dementia in older people [2] and an irreversible progressive neurological disorder [3] in which brain cells of hippocampus are damaged causing memory loss; reduce ability of thinking and carrying out of daily life. [4]

In this disease protein structures called 'plaques' and 'tangles' are build up in the brain that lead to loss of connection between nerve cells. [5] The mechanisms of 'plaques' and 'tangles' formation will describe later.

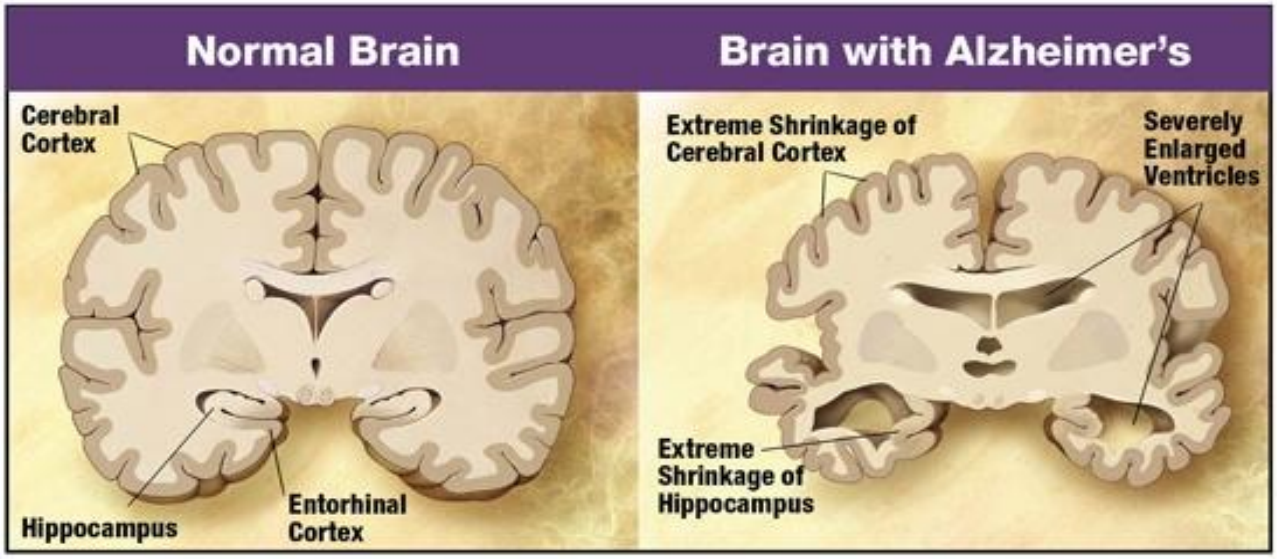


Figure1.1: Anatomical View of Brain in Alzheimer's Disease

1.2. Disease Progration:

There are some hypothesis for the development and disease progration which are discussed as follows:

1. Mutation of Gene Related to Early Onset and Late Onset

Early-onset of Alzheimer's disease occurs before 65 years of age. It account for less than 10% of Alzheimer's disease. Early onset related to family link is called familial Alzheimer's disease.

This is most often related to Autosomal Dominant Inherited Gene Mutation:

a. Amyloid precursor protein
b. Presenilin 1
c. Presenilin 2

Alzheimer's disease related to these genes is referred as Autosomal-Dominant Alzheimer's disease. Aβ production is facilitated by mutation in the Amyloid precursor protein whereas Presenilin 1 and 2 increases Aβ production by γ-secratase.

Late-onset Alzheimer's disease occurs after 65 years of age and is known as sporadic Alzheimer's disease. It accounts for 85%-95% of Alzheimer's disease. The largest known genetic risk factor for late onset is APOE gene. This gene is the product of a single gene on chromosome 19 and produced by astrocytes and microglia in the brain. Also it is involved in the transportation and metabolism of triglycerides and cholesterol.

Three APOE isoforms have been identified as contributing to the disease with the following population prevalence: APOE3 (77%–78%) > APOE4 (14%–16%) > APOE2 (7%–8%)

The APOE gene exists as three different alleles in humans as ε2, ε3 and ε4 allele is recognized as a major risk factor for late onset Alzheimer's disease. It increases the risk of developing disease by three fold in heterozygotes and by fifteen fold in homozygotes.

The primary cause of late onset is indicated the Cholesterol metabolism and immune response. [6]

2. Amyloid Plaques:

In neural development and neurogenesis Amyloid precursor protein plays an important role. Amyloid protein is cleaved by the action of γ-secretase at the N-terminal of Aβ sequence and forms a 99 amino acid fragment C99.

This Aβ sequence further cleaved into an Aβ fragment and APP intracellular domain of 36 to 43 amino acids, respectively. An extracellular fragment of APP interacts and binds to death receptor 6 and initiates the degeneration of cell bodies. [6]

- ✓ Increase production and accumulation of Aβ protein occurs by the missense mutation of the Amyloid precursor protein, Presenilin 1, Presenilin 2 genes.
- ✓ Then Aβ protein oligomer formation takes place and these oligomers deposit as diffuse plaque.
- ✓ Oligomers of Aβ proteins have minimal effect on synapses and initiate microglial and astrocyclic activation.
- ✓ Then progressive synaptic and neuritic injury occurs that leads to altered neuronal ionic homeostasis and oxidative injury.
- ✓ The next effect is altered kinase or phosphatase activity that may lead to tangles. Widespread neuronal dysfunction and cell death with transmission difficulties lead to dementia. [7]

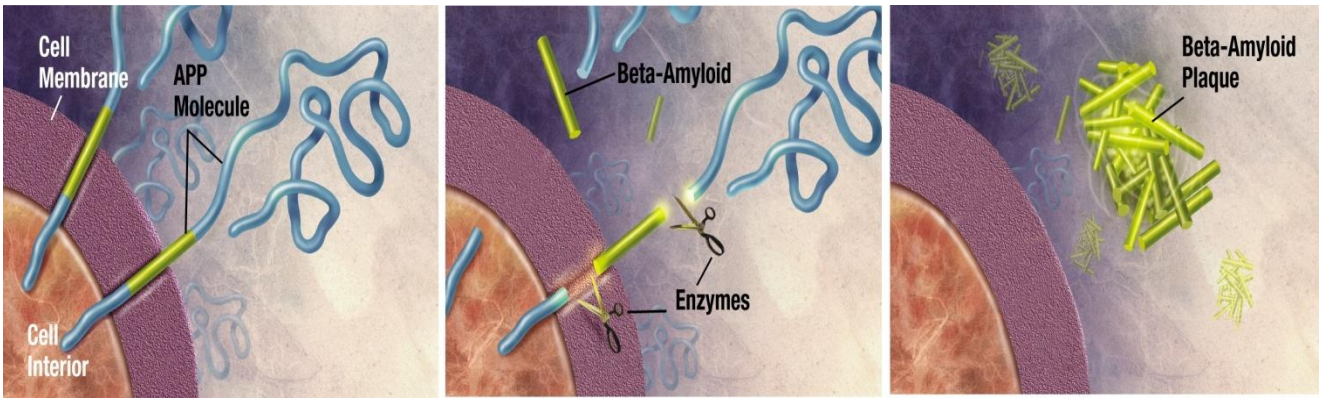


Figure 1.2: Formation of Amyloid Plaque

3. Tau Protein and Formation of Neurofibrillary Tangle:

For the stabilization of microtubules and neurite tau protein is required which is mainly a microtubule associated protein. Tau protein interacts with tubulin then facilitates the arrangement of microtubules and stabilizes their structure. For appropriate neurite growth phosphorylation of tau protein within microtubule is necessary. In the adult brain the ratio of 3R and 4R tau isomer is 1:1. Hyperphosphorylated tau accumulates and promotes their dissociation from microtubules which results in their destabilization and disruption of neuronal transport. The extent of disease progression in Alzheimer's is correlated by the number of neurofibrillary tangles. [6]

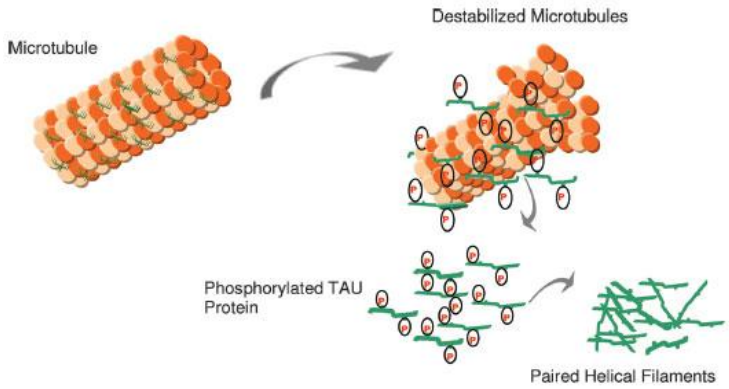


Figure 1.3: Formation of Neurofibrillary Tangle

4. Inflammation induced Alzheimer's Disease:

In the senile plaque of Alzheimer's disease Aβ and other proteins are present. These are potent activators of innate immune response. Chronic activation of innate immune system activates microglia and astrocytes which then kill adjacent neurons by releasing highly toxic products as nitric oxide, reactive oxygen intermediates, proteolytic enzyme, inflammatory cytokines, and excitatory amino acids, complement factors, etc.

In the innate immune system of brain Microglial cells are the most important cells. They act as cerebral macrophages, recruit and stimulate astrocytes. Microglial cells are activated by brain trauma, ischemia or neurodegeneration. After activation Microglial cells express Major histocompatibility complex (MHC) I and II, integrins and Fc receptors. Activation of Microglial cells associates with chemotatctic responses which lead to cluster of activated microglia at the Aβ deposition site. Endocytosis and degradation facilitates with the opsonization of Aβ and fibrils with carrier protein.

On the other hand, astrocytes remove and degrade Aβ protein without mediators or stimulations. Activated astrocytes can release cytokines and growth factors like Microglial cells; however, they also produce trophic substances as nerve growth factor (NGF). Excitatory amino acids as glutamate are regulated by astrocytes which contribute to central nervous system homeostasis.

Metabolism of excitatory amino acids or in the antioxidant pathway by neurotrophic factors, may help to protect neurons and other brain cells from damage by controlling the production of potentially toxic substances.

As astrocytes greatly outnumber microglia in the brain, these cells can perform a more critical role in the Alzheimer's disease development. [8]

1.3. Signs and Symptoms of Alzheimer's Disease:

Initially the signs and symptoms of patient with Alzheimer's disease are mild but gradually they become worse to interfere with daily life. [5] Most common symptoms of this disease are memory problems. In some cases, patients have condition known as mild cognitive impairment (MCI) where problems with memory loss are more than normal with age but do not interfere with daily works. [4] Due to loss of memory a person may face:

- Difficulties to find the appropriate word during conversation
- Forgetting about keys, glasses, etc.
- Forgetting appointments
- Losing road direction
- Forgetting about recent events or conversation

With the development of disease problems with thinking, reasoning, perception and communication occur. They are associated with:

- Disability to follow a conversation or repetition of same sentence
- Difficulties in judging objects in three dimensions
- Problems in taking decisions, solving problems or completing tasks
- Confusion about dates [5]

1.4. Stages of Alzheimer's Disease:

There are seven stages of Alzheimer's disease according to the Alzheimer's Association Fact Sheet, 2004. They are:

Stage 1: No Cognitive Impairment:

In this stage, person does not experience memory problem and during a medical interview it is not a not an evident to physician.

Stage 2: Very Mild Decline:

Person feels that he or she has memory defects such as forgetting about names, words, keys, glasses, etc. but during medical examination these are not the evident to health care professionals.

Stage 3: Mild Cognitive Decline:

Person's family, friends and co-workers can notice deficiencies in memory and concentration.

The problems can be measured in clinical testing or medical interview. Common problems are:

- Reduce ability to remember names of people
- Problems with word or name finding
- Difficulties to carry out performance in social or work settings
- Difficulties in reading a passage and retaining material
- Misplacing or losing valuable objects
- Reduce ability to plan or organize

Stage 4: Moderate Cognitive Decline (Early Stage of Alzheimer's Disease)

- Medical interviews detect the deficiencies and they are as follows:
- Reduce ability to perform challenging mental arithmetic as counting backward from 100 by 7seconds
- Reduced knowledge of recent occasion or events
- Decreased capacity to perform complex tasks such as planning dinner for guest, paying bills, managing finance and marketing
- Forgetting about personal history
- Person may feel mitigated and withdrawn in society

Stage 5: Moderate Severe Cognitive Decline (Moderate or Mild-Stage Alzheimer's Disease)

Major loss of memory and deficiency in cognitive function can occur in this stage with the difficulties of carrying out day-to-day activities. Person may

- Unable to recall important details such as current address, telephone number, name of the collage or high school of graduation in medical interview.
- Confusion about where they are or about the date day of the week or season
- Trouble with less challenging mental arithmetic as counting from 40 by 4 seconds
- Difficulties to choose proper clothing according to season or occasion
- Retain substantial knowledge about themselves and their own name and name of spouse or children
- Require no assistance with eating or using toilet

Stage 6: Severe Cognitive Decline (Moderately Severe or Mild Stage Alzheimer's Disease)

Memory defects become worsen with significant personality disorder occurs in this stage and patient needs help for daily activities.

Symptoms include:

- Lose of awareness of recent experiences, events and also their surroundings
- Difficulties in recollecting their personal history but can remember their name
- Forgetting the name of spouse or children
- Need help for getting dressed due to making error as wearing shows on wrong feet
- Frequent disruption of their normal sleep-waking cycle
- Increase urinary or fecal incontinence
- Behavioral symptoms as suspiciousness and delusion, hallucination; compulsive, repetitive behavior as hand wringing or tissue shredding

Stage 7: Very Severe Cognitive Decline (Severe or Late-Stage Alzheimer's Disease)

- Patient loss the ability to response to their surrounding environment, ability to speak and control movement and this is the final stage of the disease. In this stage:
- Patient frequently loss their capacity for recognizable speech
- Need help for eating and toileting and there is general incontinence of urine
- Lose the ability to walk without assistance, sit without support; ability to smile and to hold their head up
- Abnormal reflexes and rigid muscle [9]

1.5. Current Trend of Alzheimer's Disease:

The determination of death rate for Alzheimer's disease each year is difficult to determine. Though some statistical data can help to estimate the rate of death and many other parameters related to Alzheimer's disease.

According to National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC), in 2013 84767 people died from Alzheimer's disease.

Recent study from the data of the Rush Memory and Aging Project and the Religious Orders Study estimated that 500000 deaths of people with the age of 75 or older could be attributed to Alzheimer's Disease in the United States in 2010.

Chicago Health and Aging Project (CHAP) have estimated that 600000 people with the age of 65 and older in the USA had Alzheimer's disease when they died in 2010.

Medicare data states that about one-third of all older who died in 2010 had Alzheimer's or other types of dementia. CHAP data has estimated that in 2016, 700000 people in United States in the age of 65 and older will have Alzheimer's disease when they die. [10]

Death Statistics for Alzheimer's Disease:

Death rate of Alzheimer's disease is increasing while death rate due to the number one disease as heart disease is decreasing. Between 2000 and 2013 death rates due to Alzheimer's disease has increased 71%.

In 2013, the mortality rate in United States for Alzheimer's disease was 27 per 100000 people.

Alzheimer's disease is least prevalent in Sub-Saharan Africa. [10]

Disease Duration from Diagnosis to Death:

People with 65 years or older can survive about 4-8 years after diagnosis of Alzheimer's disease; however, some may live as long as 20 years with Alzheimer's disease.

In the most sever stage of Alzheimer's disease; it will spend about 40% of the total number of year of the patient in dementia.

Caring in the nursing home by age 80 is expected for 75% of people who have Alzheimer's disease compared to the general population. [11]

Economic Hazards for Alzheimer's Disease:

Alzheimer's disease is a leading cause of death of elderly people in the United States and one of the top diseases that cannot be cured or prevented.

In 2012, America had spent $200 billion for patient with Alzheimer's disease and approximately $140 to Medicare and Medicaid.

Medicare costs per person with Alzheimer's and other times of dementias are three times higher than patients without Alzheimer's. However, Medicaid costs per senior patient in nineteen times higher.

Patients with Alzheimer's disease are not only the sufferers, their family, friends or caregivers suffer as well. Statistical data shows that in 2011, 15.2 million patient's family members and friends provided care which in unpaid able; but if valued it could be over $210 billion.

The emotional stress of care giving has been rated high or very high by more than 60% of Alzheimer's and dementia caregivers. The additional health cost for Alzheimer's and dementia caregivers had $8.7 billion in 2011. [12]

Annual costs of caring patients with Alzheimer's disease have been estimated at $80-100 billion in United States. About 93% patients had at least one comorbid condition but 61% had more than three. A study conducted by Medicare reported that comorbid condition in each patient was associated with high cost which could be $10435 compared to patient without Alzheimer's disease. [13]

1.6. Treatment Hypothesis of Alzheimer's Disease:

Alzheimer's disease is the chronic neurodegenerative disorder and is the most common cause of dementia in the elderly population. According to statistical data, the rate of having Alzheimer's disease in the elder people is increasing and for giving people relief from this disease for the time being some medication are used. Medications that have passed the FDA approval are used in this purpose currently. Acetylcholinesterase inhibitors are used for the mild to moderate stages of Alzheimer's. For the treatment of moderate to severe Alzheimer dementia Memantine and N-methyl-D-aspartate receptor antagonist (NMDA) are used. Medications available for the disease give symptomatic improvement to the patients but are not able to completely cure or stop the disease progression. Thus, development of novel therapeutic medications is expected to cure the disease. [14] Some hypothesis based targets are used to develop new medication with strong disease modifying properties:

➢ Cholinergic Hypothesis:

The neurotransmitter acetylcholine is rapidly degraded after its release into the synaptic cleft by the hydrolytic activity of cholinesterase enzyme. Acetyl cholinesterase is the most prominent enzyme to hydrolyze acetylcholine. But recent studies states that butyryl cholinesterase also has capability of hydrolyzing acetylcholine in the brain which may play role in the transmission of cholinergic transmitters. In the basal forebrain the destruction of cholinergic neurons occur which results in lack of cholinergic transmission and contribute to cognitive and non-cognitive symptoms in patients. In the Alzheimer's disease this phenomena is observed more and referred as a selective destruction of cholinergic neurons. On the basis of these hypothesis acetyl cholinesterase inhibitors for

Alzheimer's disease has been developed. Other alternative approaches for improving the cholinergic neurotransmission; for example acetylcholine precursors administration, presynaptic acetylcholine release stimulation and muscarinergic agonists were not successful because of insufficient efficacy and large range of side effects.

An increase in the acetylcholine concentration in the synaptic cleft results by inhibiting these enzymes; which is expected to enhance cholinergic transmission and improve cholinergic activity. Galantamine, Donepezil and Rivastigmine are three different cholinesterase inhibitors used to treat Alzheimer's disease. [14]

➢ Glutamate-Mediated Neurotoxicity:

Excessive activation of N-methyl-D-aspartate receptors causes Glutamate excitotoxicity which play an important role in the death of neurons and other neurodegenerative conditions.

The main excitatory neurotransmitter in the central nervous system is Glutamate and for normal brain function glutamate receptor activity in the physiological level is required. Glutamate receptors are divided into two classes as:

- ✓ Metabotropic glutamate receptors coupled to G-proteins
- ✓ Ionotropic receptors that are mainly ligand gated ion channels

Ionotropic receptors are classified as NMDA, α-amino-3-hydroxy-5-methyl-4- isoxazole-propionate (AMPA) and kainate receptors on the basis of their sensitivity to synthetic agonists.

Excessive activation of NMDA receptors in the Alzheimer's disease increases intracellular Ca2+ and triggers downstream events which finally lead to neurodegeneration.

On the other hand, a therapeutic potential of NMDA-receptor antagonists can protect neurons from glutamate-mediated neurotoxicity. Phencyclidine, a potent NMDA receptor

antagonist has reported to produce psychotomimetic side effects may be due to interfere with the physiological functions of NMDA glutamate receptor.

A non-competitive NMDA-receptor antagonist Memantine has moderate affinity and able to protect neurons. [14]

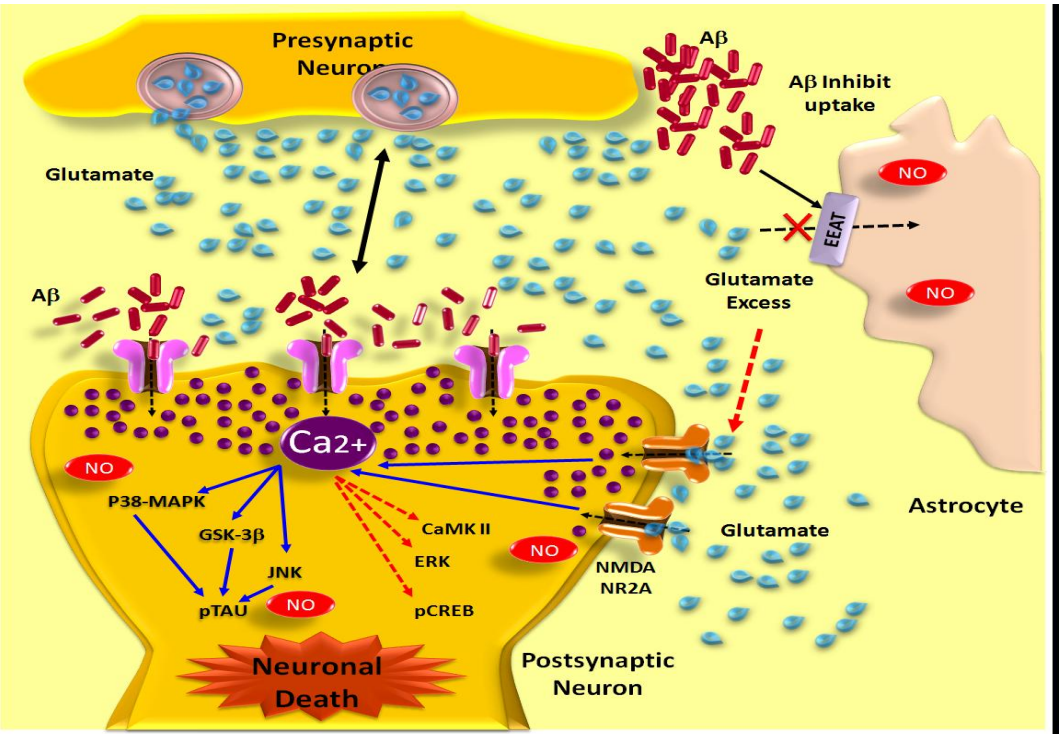


Figure 1.4: Glutamate Mediated Neurotoxicity

- **Amyloid Hypothesis:**

As in the first section Amyloid mechanism for developing Alzheimer's disease has been discussed therapeutic approach on the basis of this hypothesis will be covered here.

a. Modulation of Aβ Production

Recently a γ- secretase inhibiting compound has tested in a Phase II trial for six week. It was reported to reduce Aβ levels in blood plasma but the same concentration does not reduce Aβ in cerebrospinal fluid (CSF).

The generation of highly amyloidogenic Aβ is reduced by some non-steroidal anti-inflammatory drugs (NSAIDs) and proves the presence of γ-secretase modulating

mechanism as a useful drug target. This may allow the lowering of Aβ levels without initiating potential side effects.

Stimulation can be made by muscarinic acetylcholine-receptor agonists to cleave Amyloid Precursor Protein and this also reduces Aβ generation in cell culture. M1 muscarinic acetylcholine-receptor agonists are potentially useful not only for symptomatic relief but also for casual therapy to some limited extent. [14]

b. Inhibition of Aβ-aggregation

Preventing the formation aggregates of Aβ by small molecules represents another promising approach for the development of novel and causal therapeutics for Alzheimer's disease.

A Phase II clinical trial of glycosaminoglycan mimetic drug has been completed by Neurochem Inc., a Canadian company. This drug has been designed to bind to Aβ peptides and so inhibits their aggregation. Also they are planning for Phase III trial.

The Aβ aggregation can be mediated by metals as Cu^{2+} and Zn^{2+}. In the APP transgenic mice a significant decrease in Aβ aggregation in brain has been observed after nine week treatment with Clioquinol. Clioquinol is an antibiotic and metal chelator that can cross the blood brain barrier. [14]

➢ Tau Hypothesis:

Tau hypothesis has been discussed in the first section but target on the basis of tau hypothesis is described here.

a. Activation of Phosphatases

The activation of kinases and phosphatases results from phosphorylation of phosphoproteins. An imbalance of kinase and phosphatase may lead to abnormal hyperohosohorylation in the Alzheimer's disease. In Alzheimer's disease reduced activities of tau phosphatase has been observed. Some phosphatase proteins can

dephosporylate tau protein in vitro and also can involved in tau protein phosphorylation in vivo.

For this reason, kinase inhibitors are suggested to use with restoration of tau phosphatase activities which may be potential for inhibiting abnormal tau hyperphosohorylation. [14]

b. Inhibition of Tau Aggregation

A phenothanine compound, methylene blue has currently approved for treating methemoglobinemia and as antiseptic. Methylene blue inhibits tau protein and Aβ protein aggregation in vivo. It has significantly improved cognitive function in Phase II clinical trial and slowed the progression of Alzheimer's disease by one year.

Compounds like anthraquinones and cyaninedyes are being under examination for their ability to disrupt tau aggregates in vitro and incellcultures. [15]

CHAPTER 2: PLANT and LITERATURE REVIEW

Chapter 2: Plant and Literature Review

2.1. Plant used for the Treatment of Alzheimer's disease:

1. *Ginkgo biloba* :

In China, *Ginkgo biloba* (Coniferae) has been used for respiratory disorders and in Iran it is used to improve cognitive loss associated with abnormal blood circulation.

Numerous investigations has done on this herb for it potentiality in cognitive disorders. Especially EGb761 derived from the leaves can be successfully used for the improvement of cognitive and memory impairment as herbal drug. *Ginkgo biloba* also has favanoids and terpinic lactones that have medicinal value for brain disorders. It also has antioxidant activities. The ability to reduce oxidative stress in lymphocytes and brain can be beneficial to the treatment of Alzheimer's disease. [16]

Figure 2.1: *Ginkgo biloba*

2. Galantamine

Galantamine is derived from snowdrop or *Galanthus alpines* and is an alkaloid form members of the family Amaryllidaceae. The local use of it in a remote part of Europe has become the reason of developing medicine for Alzheimer's disease. It has the capability to slow down the neurological degeneration process in Alzheimer's disease. It is also used in poliomyelitis. [16]

Figure 2.2: *Galanthus alpinus*

3. Cannabinoid

Cannabinoid therapy has been discussed in scientific literature which indicates that it can provide symptomatic relief to Alzheimer's disease patients as well as slows the progression of disease. Cognitive impairment prevention and reduction of neurotoxicity rate with the prevention of β-Amyloid peptide in rat can be achieved by intra-cerebro ventricular administration of synthetic Cannabinoid. It is derived from *Cannabinoid cannabidiol.*

A study has reported that Cannabinoid can provide neuroprotection and reduce neuroinflammation as well as can repair brain's intrinsic mechanism. [16]

Figure2.3: *Cannabinoid cannabidiol*

4. Curcumin

Curcumin is responsible for the yellow color of turmeric and is a polyphenol compound. Is is derived from the root of *Curcuma longa* and decrease β-Amyloid plaques, slower the degradation of neurons, capability of chelating with metals as well as have anti-inflammatory and antioxidant properties. It can also decrease microglia formation and improve memory in patients with Alzheimer's disease. Curcumin can clear the Amyloid plaque by helping macrophages. [16]

Figure 2.4: *Curcuma longa*

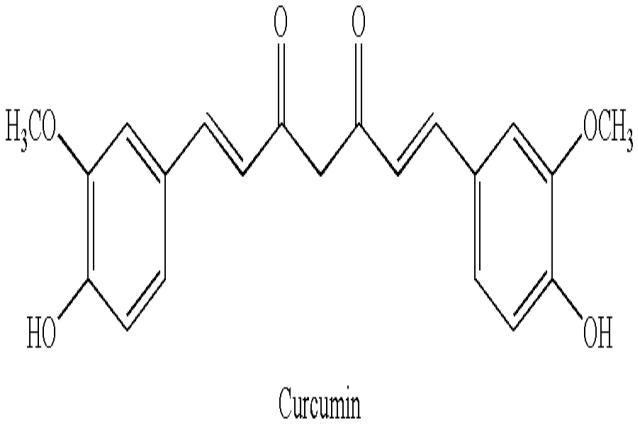
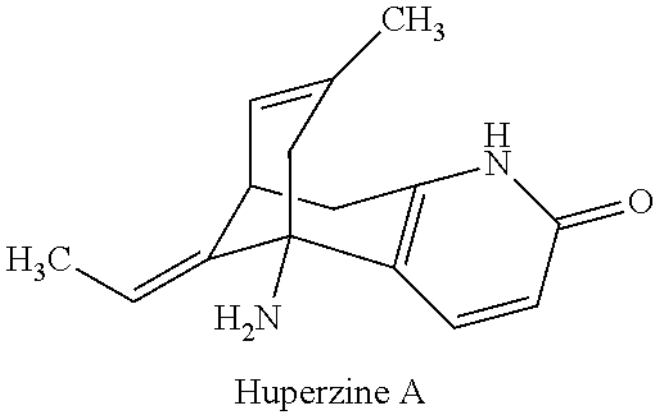


Figure 2.5: Curcumin

5. Huperzine A (Qian Ceng Ta)

Huperzine A enhances memory and protects cognitive functions; also improve cognition in Alzheimer's disease. Alzheimer's disease patients who take Huperzine A have improved cognitive function and reduced behavioral disturbance. It also has memory enhancing properties and effective for memory and learning. It is a neutral compound derived from Qian Ceng Ta, an ancient Chinese remedy. Qian Ceng Ta is prepared from *Huperzia serrata* which is used in the treatment of fever, inflammation, irregular menstruation and also as a diuretic. Researchers discovered that Huperzine A can reversibly inhibit acetyl cholinesterase (AChE) and can readily cross the blood brain barrier to prevent breakdown of acetylcholine. [16]



Huperzine A

Figure 2.6: Huperzine A

Figure2.7: *Huperzia serrata*

2.2. Plants of Evaluation:

2.2.1. *Basella alba*

Basella alba is a cool season vegetable and cultivated in a climbing growth habit. It is branched, has smooth and herbaceous stem of purple or green color. Leaves of *Basella alba* are fresh 5-12 cm long, ovate or heart shaped, stalked and tapering to a pointed tip. Fruit is greenish when unripe and purple when properly ripen stalk less. [17]

Taxonomy of the plant

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Caryophyllales

Family: Basellaceae

Genus: *Basella*

Species: *alba* [18]

Chemical constituents of *Basella alba*:

Per 100 gm dried leaves of *Basella alba* contains 20% protein, 3.5% fat, 54% carbohydrate, 9% fiber, and 19% ash. Leaves are rich in vitamins as A, C, E, K, B9, riboflavin, niacin, thiamine and also contain high level of calcium, magnesium, iron. [19]

Other than these components leaves contain betacyanin, flavonoid as accretion, oxalic acid, 4, 7-dihydroxy kempferol, phenolic content as vanilla, syringic and ferulic acid. [18]

Figure 2.8: *Basella alba*

Figure 2.9: *Basella alba* fruit

Traditional Use of *Basella alba*:

Basella alba has diuretic, demulcent and emollient properties. In the Chinese medicine the entire plant is used to reduce fever and neutralize poison. Leaves are used to treat ulcers and to prevent maturation of abscesses. Leaves also have laxative properties to treat constipation in children and pregnant women. Plant extract mixed with rosa-sinensis is also a good laxative. The plant jucie is used as dye and food coloring. [19]

Medicinal Use of *Basella alba*:

Basella alba has various medicinal uses in studied by researchers. They are as follows;

1. **Wound healing activity:**

Leaf extract of *Basella alba* has maximum wound healing activity with the capacity of wound closure.

2. **Antimicrobial activity:**

Ethanol extract of *Basella alba* has antimicrobial effect against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans.* But it does not have capability to inhibit *Candida albicans.*

3. **Antiviral activity:**

Seed of *Basella alba* has ribosome inactivating proteins that has antiviral activity and can inhibit AMVC induced infection of *Nicotiana benthamiana.*

4. **Anti-inflammatory activity:**

500mg/kg and 100mg/kg aqueous extract of *Basella alba* can reduce inflammation significantly. Both aqueous and methanol extract of *Basella alba* leaves can exhibit membrane stabilization properties.

5. **Anti-ulcer activity:**

Due to improved gastric cytoprotection and capability of inhibiting gastric acid secretion *Basella alba* has gastroprotective activity and significant antiulcerogenic activity. [20]

2.2.2.*Blumea balsamifera*

The local name of *Blumea balsamifera* is sambong or kukur sunga. It is one of the popular medicinal plants that are being used in herbal medicines for thousands of years in South-East Asia, especially in India, China, Malaysia, Thailand, Vietnam and Philippines.

Blumea balsamifera grows on riverbeds, forest edges, valleys and grasses. It is commonly called "Ainaxiang" and "Dafeng'ai" in China and used as aroma producing resin as it has high level of essential oils. The whole plant or the leaf is used as traditional medicine in China to treat eczema, dermatitis, rheumatism, beriberi, skin injury and as insecticide. [21]

Taxonomy of the Plant

Kingdom: Plantae

Order: Asterales

Family: Asteraceae

Subfamily: Asteroideae

Genus: *Blumea*

Species: *balsamifera* [22]

Chemical Constituents:

Blumea balsamifera contains essential oil, monoterpenes, triterpenes, sesquiterpines and steroids, aliphatic hydrocarbons, sesquiterpines. Other than that the plant also contains, diester of coniferyl alcohol, xanthoxylin, some polyacetylene and thiophane derivatives, eranthin, flavonoids, etc. [22]

Figure 2.10: *Blumea balsamifera*

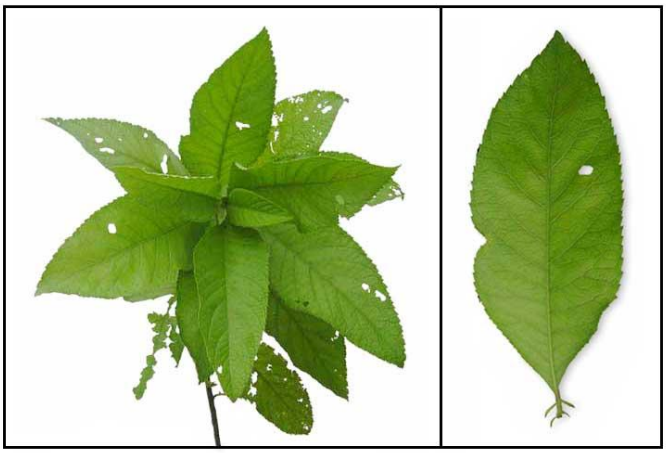


Figure2.11: *Blumea balsamifera* leaf

Plant Description:

Blumea balsamifera is a strongly aromatic shrub, half woody, densely and softly hairy. It is about 1-4m in height and steams of this plant grow up to 2.5cm in diameter.

It has simple, alternate, elliptic leaves which are about 7-20cm long toothed at the margins, pointed at the tip. Flowers are stalked yellow and numerous about 6-7 mm long. Flowers are two types such as peripheral flowers are tiny with tubular corolla; central flowers are large with campanulate corolla. Fruits are dry achenes, one seeded, hairy at top. [23]

Traditional Use of *Blumea balsamifera*:

Blumea balsamifera leaves are used to relieve headache. For woman during childbirth it can be used as an infusion. The leaf and root juice are used for fever and stomach pain. It is also used as diuretic in hypertension and for dissolution of kidney stones.

The leaves are also prescribed to treat fever, coryza, influenza, cough and dyspepsia. Leaves are also reported for good antibacterial, antifungal and antofebrile properties.

Aqueous extract of this plant is a source of tannin and used for food and drinks; for perfume also. [24]

Medicinal Uses of *Blumea balsamifera*:

- **Antitumor Activity:**

Dihydroflavonol is extracted from *Blumea balsamifera* which showed the most significant synergism with tumor related apoptosis inducing ligand (TRAIL). The level of TRAIL-R2 promoter activity enhances and promotes the expression of surface proteins that destroy tumor cells.

- **Hepatoprotective Activity:**

Blumea balsamifera exhibited a significant protective activity against the liver injury caused by prednisolone and paracetamol.

- **Superoxide Radical Scavenging Activity:**

The methanol concentrates of *Blumea balsamifera* leaves demonstrated a higher radical searching property than the chloroform extracts. Nonetheless, the pet-ether extracts had less activity against nonenzymatically produced superoxide radicals.

- **Antioxidant Activity:**

The methanol extracts of *Blumea balsamifera* exhibits strong xanthine oxidase inhibitory activity with an IC50 value of 6.0 μg/mL.

- **Antiplasmodial Activities:**

Traditional use for relieving fever has made choice for investigating of methanol extract of *Blumea balsamifera* for antiplasmodial activity. The root and stem extracts exhibit activity against plasmodium falciparum.

- **Antityrosinase Activities:**

The ethylacetate extract were isolated from the leaves of Blumea *balsamifera* consisting of nine flavonoids that has anti-tyrosinase activity. [21]

2.2.3.*Curculigo orchioides*

Curculigo orchioides in known as ‘kalamusil’ in Hindi and ‘golden eye grass’ or ‘black musal’ in English and is a perennial herb having long cylindrical rhizomes. (Asif, M., 2012)In Bengali it is known as ‘talamuli’ or ‘tallur’. (Soni, N., Lal, V.K., Agrawal, S. and Verma, H., 2012)

Distribution of *Curculigo orchioides*:

Curculigo orchioides generally grows in shady or hilly forest of Asian region. It is a small herb that has been distributed in Himalayas region, Bengal, Assam, Konkan and southern portion of Asia. In Japan, Sri Lanka, Malaysia and Australia Curculigo *orchioides* distribution has been observed. [25]

Taxonomy of *Curculigo orchioides:*

Kingdom - Plantae

Subkingdom - Tracheobionta

Superdivision - Spermatophyta

Division - Magnoliophyta

Class - Monocotyledon

Subclass - Liliidae

Order - Liliales

Family - Amaryllidacae

Genus - *Curculigo*

Species –*orchiodes*

[25]

Figure2.12: *Curculigo orchioides*

Figure 2.13: *C. orchioides* Root

Plant Description:

Curculigo orchioides is a perennial herb of 30cm in height. It has elongated root stock with several fleshy and lateral root that are cream internally and blackish brown externally.

- ✓ **Rhizome:**

Rhizomes are of 2.5-5cm long, straight to slightly curved, cylindrical, cut surface of 1.0 to 4.5 cm in diameter. It is mainly with a few rootlets and root scars, mucilaginous and slightly bitter.

- ✓ **Leaf:**

Leaves are 15-45 cm long simple and crowded on the short stem.

- ✓ **Flower:**

Flowers of *Curculigo orchioides* are brightly yellow. Male flowers are smaller in size and female ones are bigger.

- ✓ **Fruit:**

Fruits of Curculigo *orchioides* are capsule shaped with slender beak and spongy septa. Frits are about 1.5-2 cm long and 8mm broad; however, it is 8 seeded that are blockish in color. [25]

Chemical Constituents:

Curculigo orchioides contains saponines as curculigenin, curculigosaponins, etc; Glycosides as curculigoside and curculigoside. The plant also contains aliphatic compounds such as 4-methylheptadecanoic acid, 21-hydroxytetracontane-20-one, 27-hydroxytriacontan-6-one and 23-hydroxytriacontane-2-one. Fatty acid contents are linoleic, arachidic palmitic, oleic, and behenic acid. Other than that, alkaloid as lycorine is also present. Many steroids, triterpine alcohol and metal element is also present. [25]

Therapeutic activity:

- ✓ **Oxytocic activity:**

Curculigo orchioides has an oxytocic activity of a flavone glycoside.

- ✓ **Hepatoprotective activity:**

Curculigo orchioides has the anti-inflammatory and hepatoprotective activities it has showed a hepatoprotective activity against rifampicin-induced Hepatotoxicities.

- ✓ **Antioxidant activity:**

Methanol extract of *Curculigo orchioides* has showed anti-oxidant activity in rats.

- ✓ **Hearing Loss:**

One of the most common causes of hearing loss is noise exposure. Primarily the damage of sensory hair cells occurs in noise-induced hearing loss (NIHL). Research is on going for the therapeutic effect of *Curculigo orchioides*.

- ✓ **Immunomodulatory Activity:**

In purified glycoside-rich fraction isolated from the ethyl acetate extract, *Curculigo orchioides* has significant immuno-stimulant activity.

- ✓ **Aphrodisiac Activity:**

Curculigo orchioides rhizome ethanol extract at a dose of 100 mg/kg improved sexual behavior in male rats.

- ✓ **Spermatogenic Activity:**

Curculigo orchioides ethanolic extract of rhizomes has effect on orientation behavior and spermatogenesis in rats.

- ✓ **Antidiabetic Activity:**

Ethanolic and aqueous extracts of *Curculigo orchioides* possess antihyperglycemic activity

- ✓ **Estrogenic Activity:**

Ethanolic extract of *Curculigo orchioides* rhizome possesses estrogenic activity due tom increase in percentage of vaginal cornification.

- ✓ **Antibacterial Activity:**

Curculigo orchioides root oil possesses significant antimicrobial activity against various bacteria strains such as *Bacillus subtilis, Salmonella pyllorum ,Bacillus anthracis,* and *Staphyllococcus aureus* and fungi stains such as *Aspergillus flavus* and *Cladosporium.*

- ✓ **Analgesic Activity:**

Curculigo orchioides aqueous and alcoholic extracts of the roots exhibit analgesic activity using.

- ✓ **Antihistaminic Activity:**

The alcoholic extract of Curculigo orchioides has stabilization potential against mast cell degranulation.

- ✓ **Antitumor Activity:**

Curculigo orchioides roots, fractionated with different solvents screened for their antimicrobial and antitumor activity. [25]

2.3. Literature Review:

2.3.1. Pharmacological Investigational Reviews of *Basella alba*:

Antioxidant Potential:

From Olaniyi, T.A., it has became related to search for antioxidants in various plants fractions since they limit the oxidation of macro molecule by free radical and it leads to cellular oxidative damage concerned in numerous chronic human diseases. To discover the antioxidant and inhibitory potentials of lipid peroxidation by using ethyl acetate and chloroform extracts of *Basella alba* leaves the study was undertaken. As related to the disease hypothesis we have shown interest for this work. [26]

Hematological and Biochemical:

From Bamidele, O.,etal in Wistar strain albino rats the property of the aqueous leaf extract of Basella alba on hematological and biochemical parameters were considered. Administration of the extract was done orally and at the end of the treatment period, hematological parameters and biochemical parameters were determined. A significant increase in white blood cell count, red blood cell count, packed cell volume, hemoglobin concentration and platelet count is observed in *B.alba*. [27]

Hypocholesterolemic and Antiatherosclerotic Potential

From Baskaran, G., one of the major risk factor that leads to atherosclerosis is Hypercholesterolemia. Hypocholesterolemic and anti-atherosclerotic effects of *B. alba* was studied using hypercholesterolemia induced rabbits. Treatment with *B. alba* extract also significantly concealed the aortic plaque development and reduced the intima. [28]

HMG-CoA Reductase Inhibitory Activity:

From Baskaran, G., the key enzyme of the mevalonate pathway that produces cholesterol is 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase. Resistance of HMG-CoA reductase abates cholesterol biosynthesis in the liver.

By inhibiting the HMG-CoA reductase activity *B. alba* extract is capable of lowering cholesterol levels. Also, the compounds of *B. alba* extract have been reported to have advantageous effects in treating hypercholesterolemia and its associated diseases. [29] As relation to the treatment approach with Alzheimer's disease we have shown interest for this work.

Antimicrobial Activities:

The antimicrobial property of ethanolic extract of *B. alba* against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa,* and *Candida albican* was determined using the agar cup plate method. The consequence of this study indicates that the ethanolic extracts of *B. alba* could be suitable for the treatment of diseases caused by *Pseudomonas aeruginosa, Staphylococcus aureus, and Escherichia coli* except *Candida albicans.* [30]

Anti-inflammatory Property:

Inflammation is the multifaceted biological reaction of vascular tissue to injurious stimuli such as damaged cells, pathogens, or irritants. The methanolic extract of *B. alba* have interesting anti-inflammatory activity. [31] As relation to treatment approach we have shown interest for this work.

Effects on Steroid Production:

Methanolic extract of *B. alba* directly stimulated estradiol, testosterone and aromatase mRNA levels in isolated Leydig cells. The traditional application of *B. alba* in the treatment of male infertility and sexual asthenia could be due to its ability to stimulate not only androgens production, but also estrogens. [32]

Hepatotoxicity Reducing Effects:

From Bamidele, O.,etal, *Basella alba* was claimed to be used as an antidote for poisons but had not been scientific adequately investigated. Study had investigated the effect of ethanolic extract of *B. alba* on lead- induced hepatotoxicity in male rats. [27]

Metabolic Engineering Approaches:

From Kumar, B.R., *Basella* is an important leafy vegetable and a good source of naturally occurring bioactive compounds with high medicinal value. To identify the effects of pathway engineering in particular plant species which leads to significant changes in composition of natural products on an industrial scale, metabolic engineering is an important method. In pharmaceutical and nutraceutical industries, progress of various metabolic engineering strategies obtainable for flavonoid metabolism in *Basella* and the accumulated compounds may give out as an important precursor of therapeutic drugs and food products. [33]

Learning and Memory Enhancing Activity:

From Pavani, K., the aqueous and ethanolic extracts of above ground parts of *B. alba* were considered for the learning and memory enhancing properties. As the memory enhancing property is related to Alzheime's disease we have shown interest for this work. [34]

2.3.2. Pharmacological Investigational Reviews of *Blumea balsamifera*:

Superoxide Radical Scavenging Properties:

From Fazilatun, N., Nornisah, M. and Zhari, I., The pharmacological significance in flavonoids, the superoxide radical scavenging capacity of pet-ether, chloroform, and methanol extracts and flavonoids of *Blumea balsamifera* leaves on nonenzymatically yielded superoxide radicals were evaluated. In the Alzheimer's disease free radical

scavenging activity is important to treatment hypothesis we have shown interest for working on this plant. [35]

Healing of Burn Injuries:

From Fan, Z.W.,etal *Blumea balsamifera* oil is an extract obtained from Blumea balsamifera leaves, which are widely used as a conventional medicine by the Miao and Li Nations to endorse skin trauma or burn injury healing. The plant was initiated to explore the healing efficacy in deep second-degree burn model in rats. [36]

Gastroprotective Effects:

Study was done to investigate the protective effect of the combination of *Curcuma domestica*, *Amomum compactum* and *Blumea balsamifera* on gastric mucosa in aspirin-induced gastric ulcer model rats.

The groups of patients receiving herbal infuse combination displayed less number and smaller area of gastric ulcers and smaller score of mucosal damage compared to patients treated with Aspirin. [37]

Antimicrobial Activities:

Antimicrobial activity of the *Blumea* essential oils beside two Gram-positive bacteria, two Gram-negative bacteria, and yeast was evaluated using the Standard agar diffusion method and the MIC method. [38]

Analgesic, Antipyretic, Antidiarrheal and Anxiolytic Activity:

Study was conducted on the basis of traditional uses of *Blumea* and to assess in-vivo analgesic, antipyretic, antidiarrheal and anxiolytic activities. Analgesic effect was evaluated using Eddy's hot plate models and antipyretic activity was calculated by Brewer's yeast-induced pyrexia in mice. The antidiarrheal effect was considered in mice against castor oil induced diarrhea. The ethanolic extract of *Blumea* reduces the number

of evacuation. By open field test, hole cross test and swing test the anxiolytic activity was evaluated. [39]

Effect on Hepatotoxicity

From Pang, Y.X., the volatile oil from *B. balsamifera* showed that the plant is safe for using as a treatment of liver toxicity. But there is a suspect that higher amount of the volatile oil may cause mild liver injury. [40]

Antimicrobial Activity:

B. balsamifera leaves are used in conventional Thai and Chinese medicine for the healing of septic wounds and other infections. Study has found that, the essential oil, dichloromethane, hexane, and methanol extracts of these leaves were examined for antibacterial and antifungal activities. [41]

Phototoxic Activity:

Ethanolic extracts of forty-four viable herbal drugs and two fresh plants used in folk medicine were examined for their phototoxic activity against *Saccharomyces cereuisiae, Escherichia coli, and Candida albicans. B. balsamifera* has phototoxic activity against *Saccharomyces cereuisiae.* [42]

Xanthine Oxidase Inhibitory Activities:

From Nessa, F., Ismail, Z. and Mohamed, N., in the treatment of various diseases related to urolithiasis in southeast Asia Blumea balsamifera leaves have been suggested for use as a folk medicine. Study was carried out to establish the xanthine oxidase (XO) inhibitory and enzymatically produced superoxide radical scavenging activity of different organic extracts one of which was *B. balsamifera*. As relation to Alzheimer's disease we have shown interest for this work. [43]

2.3.3. Pharmacological Investigational Reviews of *Curculigo orchioides*:

Anti-oxidant Activity:

From Anandakirouchenane, E. etal the methanolic extract of *Curculigo orchioides* enhanced the antioxidant protection against reactive oxygen species formed under hyperglycemic conditions. As in Alzheimer's disease, the antioxidant capacity is related for the development of disease we have shown interest for this work. [44]

Anti-asthmatic Activity:

From Pandit, P, etal by using various in vitro and in vivo animal models, the ethanol extract of *C. orchioides* was evaluated for anti-asthmatic activity. The extract is useful against histamine-induced contraction. [45]

Immune-stimulatory Effect:

From Bafna, A.R. and Mishra, S.H., the methanol extract of *C. orchioides* has potential as a protective agent against cytotoxic drugs. When studied the extract on humoral and cell mediated immunity and cyclophosphamide-induced immunosuppressed mice initiated an increase in delayed type hypersensitivity (DTH), humoral antibody titre, and levels of WBC in a dose dependent manner. As Alzheimer's disease has a hypothesis related to inflammation mediated by immune system we have shown interest for this work. [46]

Antibacterial Activity

The rhizome extracts of *C. orchioides* were examined for antibacterial activity against pathogenic strains of Gram-positive as *Staphylococcus epidermidis* and *Staphylococcus aureus*; Gram-negative as *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium* bacteria. barely the *S. aureus* had showed more sensitivity towards water extracts. [47]

Hepatoprotective Effect

From Venukumar, M.R., Latha, M.S., study was conducted to evaluate the hepatoprotective effect of *C. orchioides* methanolic extract of rhizomes in rats treated with carbon tetrachloride. The study proves the hepatoprotective potential of methanolic extracts of crude extract. [48]

Antihyperglycemic Activity

From Chauhan, N.S. and Dixit, V.K., study was carried out to evaluate the effects of C. orchioides on blood glucose level. The ethanolic extracts of the C. orchioides rhizome was applied to diabetic rats and a potential anti-diabetic agent was found. [49]

Estrogenic Activity:

From Vijayanarayana, K., etal, a study was conducted for the comparative study of estrogenic activity of alcoholic extract of *C. orchioides* with diethylstilbestrol in bilaterally ovariectomized young albino rats. By taking percentage vaginal cornification, uterine glycogen content, uterine wet weight, and uterine histology as parameters of assessment estrogenic activity was assessed. [50]

Ameliorative Effects:

From Wu, X.Y., etal the ameliorating effects of curculigoside from *Curculigo orchioides* on learning and memory in aged rats was evaluated. The effects of curculigoside were determined through animal performance studies. The potential mechanisms were explored by assessment of the activity of acetylcholinesterase (AchE). Alzheimer's the availability of the acetylcholinesterase is important we have shown interest to work on this plant. [51]

CHAPTER 3: MATERIALS and METHODS

Chapter 3: Materials and Methods:

3.1. Collection of Plant Materials:

a. *Basella alba* collection:

Basella alba is a seasonal vegetable and it was collected from Rampura, Dhaka district of Bangladesh, in March, 2016 and identified by taxonomist.

b. *Blumera balsamifera* collection:

Blumera balsamifera is a shrub found in forest areas of Asian countries. The whole plant was collected from Natore, Rajshahi, Chapainobabganj and Mymensingh districts of Bangladesh, in March, 2016 and also identified by an expert taxonomist. The herbarium of the Department of Botany, Rajshahi University had a voucher specimen of this plant.

c. *Curculigo orchioides* collection:

Curculigo orchioides is generally available in hilly areas of South Asian countries and it is an herb. From Chittagong, Rangamati and Mymensingh district of Bangladesh, in March 2016. The plant was identified by taxonomic expert. The herbarium of the Department of Pharmacy, East West University had a voucher specimen of this plant.

3.2. Preparation of Plant Material:

a. *Basella alba* preparation:

The collected plants were first washed with water to clean and remove adhering dirt and then shade dried for several days with little sun drying. For better grinding these plants then dried for 24 hours at considerably low temperature in an oven. By a grinding machine in the Department of Pharmacy, East West University, the dried plants were ground into coarse powder.

Figure 3.1: *Basella alba* powder

b. Blumera balsamifera preparation:

The collected plants were first washed with water to clean and remove adhering dirt and then shade dried for several days with little sun drying. For better grinding these plants then dried for 24 hours at considerably low temperature in an oven. By a grinding machine in the Department of Pharmacy, East West University, the dried plants were ground into coarse powder.

c. Curculigo orchioides preparation:

The collected plants were first washed with water to clean and remove adhering dirt and then shade dried for several days with little sun drying. For better grinding these plants then dried for 24 hours at considerably low temperature in an oven. By a grinding machine in the Department of Pharmacy, East West University, the dried plants were ground into coarse powder.

3.3. Cold extraction of the plant materials:

a. Cold extraction of *Basella alba*:

About 500mg powdered plant materials (*Basella alba*) were taken in an amber colored reagent bottle and soaked in 1.5 liter of methanol. With occasional shaking and stirring the bottle with its contents were sealed and kept for a period of about 7 days. through cotton and then through Whatman No.1 filters paper the whole mixture was then filtered and concentrated with a rotary evaporator to afford crude methanolic extract (CME) under reduced pressure at 50°C temperature.

Figure 3.2: Filtration of *Basella alba* extract

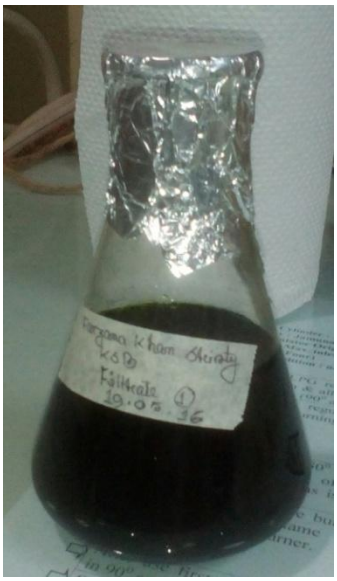


Figure 3.3: Filtrate of *Basella alba*

b. Cold extraction of *Blumera balsamifera:*

About 500mg powdered plant materials (*Blumera balsamifera*) were taken in an amber colored reagent bottle and soaked in 1.5 liter of methanol. With occasional shaking and stirring the bottle with its contents were sealed and kept for a period of about 7 days. through cotton and then through Whatman No.1 filters paper the whole mixture was then filtered and concentrated with a rotary evaporator to afford crude methanolic extract (CME) under reduced pressure at 50°C temperature.

c. Cold extraction of *Curculigo orchioides*:

About 500mg powdered plant materials (*Curculigo orchioides*) were taken in an amber colored reagent bottle and soaked in 1.5 liter of methanol. With occasional shaking and stirring the bottle with its contents were sealed and kept for a period of about 7 days. through cotton and then through Whatman No.1 filters paper the whole mixture was then filtered and concentrated with a rotary evaporator to afford crude methanolic extract (CME) under reduced pressure at 50°C temperature.

3.4. Determination of Total Phenolics Test:

The determination of total phenolic content of the different extractives of samples were done by employing the method as described by Singleton in 1965 which involves catechin as standard Folin-Ciocalteu reagent as oxidizing agent.

Principle:

By Folin–Ciocalteu Reagent (FCR) the content of total phenolic compounds of different fractions in the plant was determined. A sample's reducing capacity is measured by FCR. Though it is believed to contain hetero polyphosphotungstates–molybdates, the exact chemical nature of the FC reagent is not known. One or two reversible electron reduction reactions sequences, lead to blue species, possibly $(PMoW_{11}O_{40})_4$. In summary, it is believed that the reduction of molybdenum is easier in the complex and electron-transfer reaction occurs between reductants and Mo (VI):

$$\mathbf{Mo\ (VI) + e^- \rightarrow Mo\ (V)}$$

Materials:

- Sodium carbonate (Sigma chemical company, USA),
- Folin – ciocalteu reagent (Sigma chemical company, USA),
- Gallic acid (Wako pure chemicals Ltd., Japan),
- Methanol (Sigma chemical company, USA),
- Micropipette (10-100 µl),
- Pipette (1-10 ml),
- UV-spectrophotometer (Shimadzu, USA).

Experimental Procedure:

By using the Folin-Ciocalteu procedure the amount of total phenolics in extract was determined. Samples (100µl and 300µl) were introduced into test tubes. Then Folin-Ciocalteu reagent about 2.5mL and 2 ml of sodium carbonate (7.5%) were added. The tubes were mixed and let to stand for 2 hours. At 760 nm absorbance was measured. As calculated from standard Gallic acid graph by the following formula, the total phenolic content was expressed as Gallic acid equivalents (GAE) in milligrams per gram extract.

$$C = (c \times V)/m$$

Where,

C = total content of phenolic compounds, mg/g plant extract, in GAE;

V = the volume of extract, ml;

c = the concentration of gallic acid established from the calibration curve, mg/ml;

m = the weight of different pure plant extracts, gm.

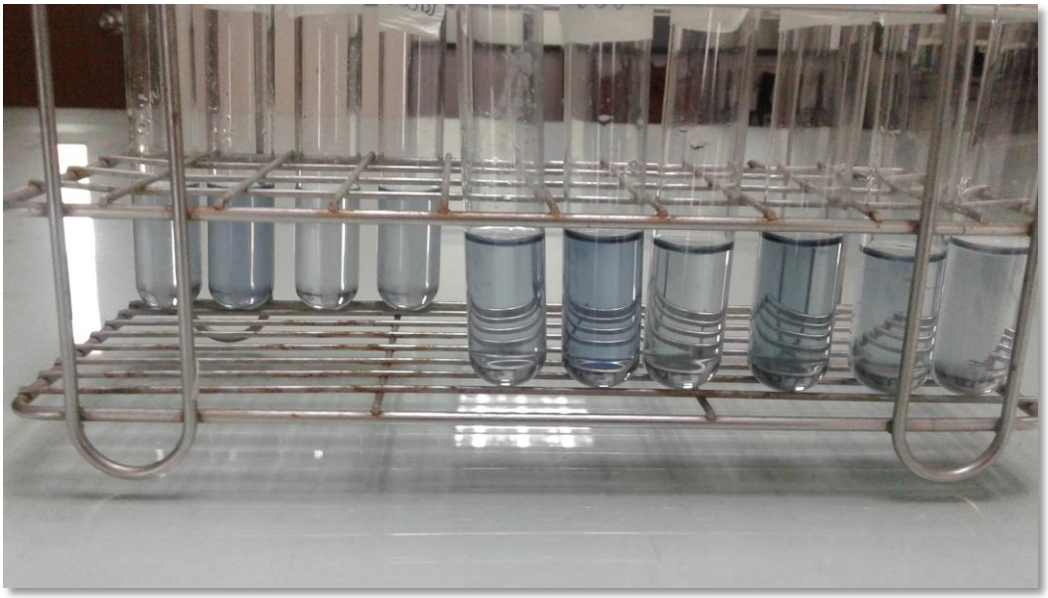


Figure 3.4: Total Phenolic Test

3.5. Determination of Total Flavonoids (TF) Test:

The determination of total flavonoid content of sample is done by aluminum chloride colorimetric method. As a standard, Catechin was used and the flavonoid content of the extractives was expressed as mg of catechin equivalent/gm of dried extract.

Principle:

By the well-known aluminum chloride colorimetric method the content of total flavonoids in different extractives of plant extract was determined. In this method hydroxyl group of flavonoids present in the samples are formed complex with Aluminum chloride. The maximum absorbance of this complex has at 510 nm.

Materials:

- Aluminum Chloride (Sigma chemical company, USA)
- 1 mMNaOH
- 5% $NaNO_2$
- Catechin (Wako pure chemicals Ltd., Japan)
- Methanol (Sigma chemical company, USA)
- Micropipette (10-100 µl)
- Pipette (1-10 ml)
- UV-spectrophotometer (Shimadzu, Japan)

Experimental Procedure:

The procedure by Dewanto, Wu, Adom, and Liu, (2002) was used to determine total flavonoid (TF). In a 10 mL volumetric flask one milliliter of extract containing 0.1 g/mL of dry matter was placed and then 500µl of distilled water added. After that f 0.15mL of 5% $NaNO_2$ was added. After 5 min of incubation, 0.3 mL of 10% $AlCl_3$ was added. After another 5 min of incubation 1 mL of 1M NaOH was added and then volume made up with distilled water. The solution was mixed and absorbance measured at 510 nm. Total Flavonoid amounts were expressed as catechin equivalents per dry matter. By analyzing thrice all the samples results were averaged.

In plant extracts in catechin equivalents the total content of flavonoid compounds were calculated by the following formula equation

$$C = (c \times V)/m$$

Where,

C = total content of flavonoid compounds, mg/g plant extract, in catechin equivalent (GAE);

c = the concentration of catechin established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant extracts, gm.

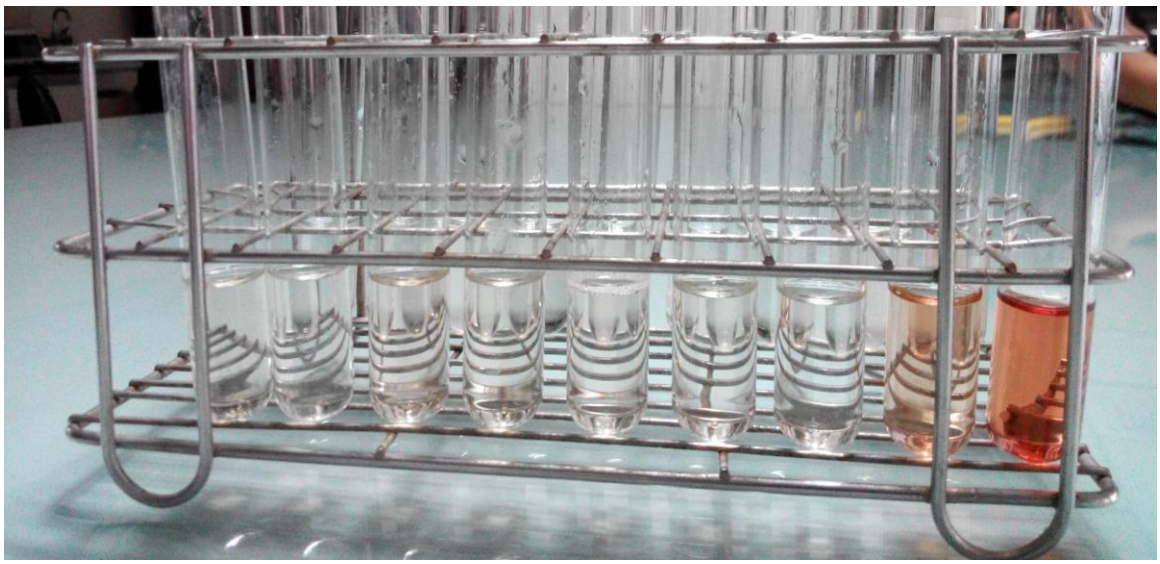


Figure 3.5: Total Flavonoid Test

3.6. Determination of Total Flavanol Test:

Total Flavanol content of the methanol extract of samples were determined by aluminum chloride colorimetric method. Gallic acid was used as standard and the flavanol content of the extractives was expressed as mg of Gallic acid equivalent/gm of dried extract.

Principle:

The content of total flavanols in methanolic extract of samples were resolute by the well-known aluminum chloride colorimetric method. In this method aluminum chloride forms complex with hydroxyl groups of flavanols present in the samples. This complex has the maximum absorbance at 440 nm.

Materials:

- Aluminum Chloride 2% solution (Sigma chemical company, USA)
- Sodium acetate 5% solution
- Gallic acid
- Micropipette (10-100 µl)
- Pipette (1-10 ml)
- UV-spectrophotometer (Shimadzu, Japan)

Experimental Procedure:

Total flavanol was determined using aluminum chloride and as a standard Gallic acid was utilized. One milliliter of extract containing 1µg/µl of dry matter was placed in a 10 mL testube in a volume of 100µl and 300µl. Then methanol was added up to 1 ml. after that 1 ml of aluminum chloride solution (2%) is added in the previous solution. 5% solution of sodium acetate was added in the testube which is then incubated at room temperature for two and half hours. The solution was mixed and absorbance measured at 440 nm. Total Flavanol amounts were expressed as Gallic acid equivalents per dry matter. All samples were analyzed thrice and result averaged.

The total content of flavonoid compounds in plant extracts in Gallic acid equivalents was calculated by the following formula equation

$$C = (c \times V)/m$$

Where,

C = total content of flavonoid compounds, mg/g plant extract, in catechin equivalent (GAE);

c = the concentration of catechin established from the calibration curve, mg/ml;

V = the volume of extract, ml;

m = the weight of pure plant extracts, gm.

3.7. DPPH (1, 1-diphenyl-2-picrylhydrazyl) Free Radical Scavenging Assay:

To evaluate the free radical scavenging activity of various fractions isolated pure compounds and column subtractions DPPH was used.

Principle:

To evaluate the free radical scavenging capacity of antioxidants the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been widely used. When it reacts with hydrogen donors DPPH free radical is reduced to the corresponding hydrazine. In aqueous or methanol solution DPPH can make stable free radicals. It was possible to determine the antiradical power of an antioxidant activity with this method by measurement of the decrease in the absorbance of DPPH at 517 nm. When the DPPH was scavenged by an antioxidant, resulting from a color change from purple to yellow the absorbance decreased through donation of hydrogen to form a stable DPPH molecule. In the radical form this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule.

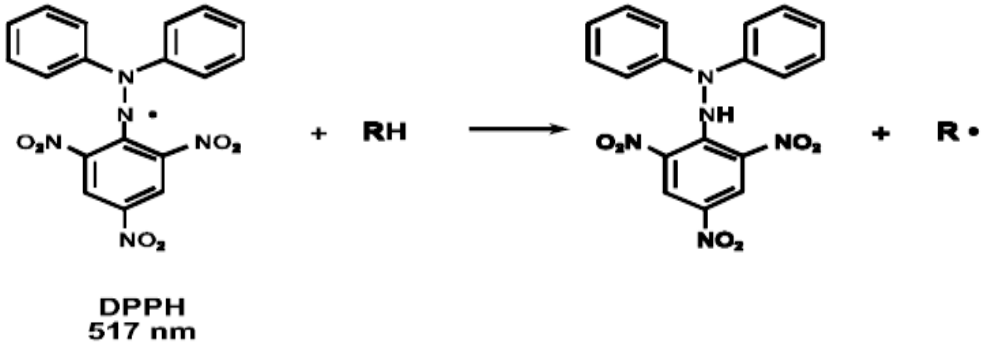


Figure 3.6: Mechanism of DPPH˙ with an antioxidant having transferable hydrogen radical.

Materials:

- DPPH (Sigma chemical company, USA)
- Catechin
- Methanol (Sigma chemical company, USA)
- Pipette (1-10 ml)
- UV spectrophotometer (Shimadzu, Japan)

Experimental Procedure:

Based on the method described by Braca et al the free radical scavenging activity of the extracts, different sub-column fractions and isolated compounds of samples were detected. To 3ml of a 0.004% methanol solution of DPPH sample (2.5 ubml) will be added. After 30 minutes absorbance at 517 nm will be determined and the percentage inhibition activity was calculated from

$$I\% = [(A_0 - A_1)/A_0] \times 100,$$

Where,

I% is the percentage of scavenging activity

A_0 is the absorbance of the control, and

A_1 is the absorbance of the extract/standard.

Then % inhibitions were plotted against concentration and from the graph IC_{50} was calculated.

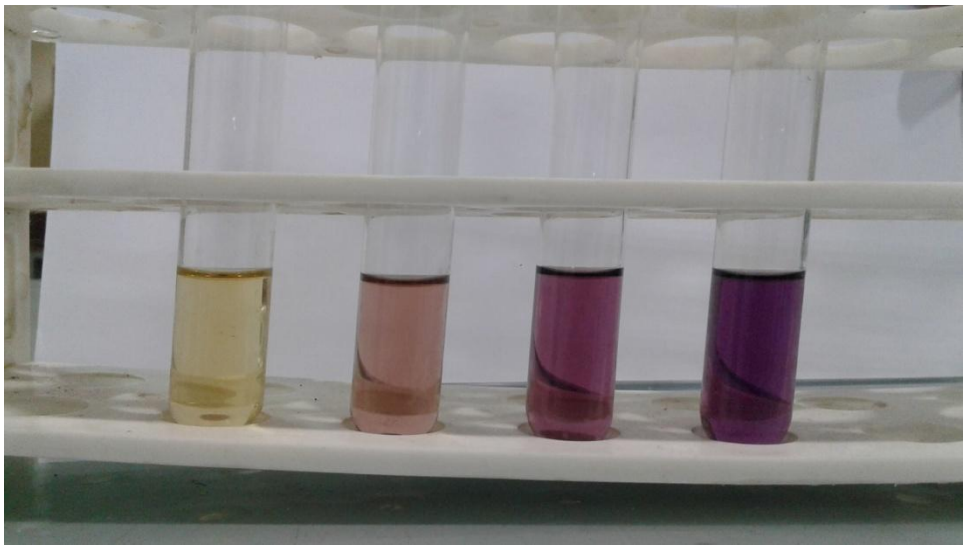


Figure 3.7: DPPH test

3.8. Reducing Power Capacity Assessment:

The reducing powers of the methanolic extracts of samples were evaluated by the method of Oyaizu (1986).

Principle:

In this assay, depending on the reducing power of antioxidant samples the yellow color of the test solution changes to various shades of green and blue. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidant substances in the samples causes the reduction of the Fe^{3+}-ferricyanide complex to the ferrous form by donating an electron. The amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

$$\textbf{Fe}^{3+}\textbf{-ferricyanide} + e^{-} \longrightarrow \textbf{Fe}^{2+}\textbf{-ferricyanide}$$

Materials:

- Potassium ferricyanide (Merck, Germany)
- Ferric Chloride (Sigma chemical company, USA)
- Trichloro Acetic acid (Merck, Germany)
- Phosphate buffer (Sigma-Aldrich, USA)
- Ascorbic acid (Sigma chemical company, USA)
- Water bath
- Centrifuge machine
- Pipette (1-10 ml)
- UV spectrophotometer (Shimadzu, USA)

Experimental Procedure:

Using the method developed by Oyaizu (1986) reducing power was investigated. A 2.5 mL fraction of sample was mixed with 2.5 mL of phosphate buffer (200mM, p_H 6.6) and 2.5 mL 1% potassium ferricyanide. In a water bath for 20 minutes the mixture was placed at 50^0C. The resulting solution was cooled rapidly, mixed with 2.5 mL of 10% trichloroacetic acid. A 2.5 mL fraction from the supernatant was mixed with 2.5mL of distilled water and 1mL of ferric chloride. Absorbance of the resultant mixture was measured at 700 nm after 10 min. The higher the absorbance value the stronger the reducing power.

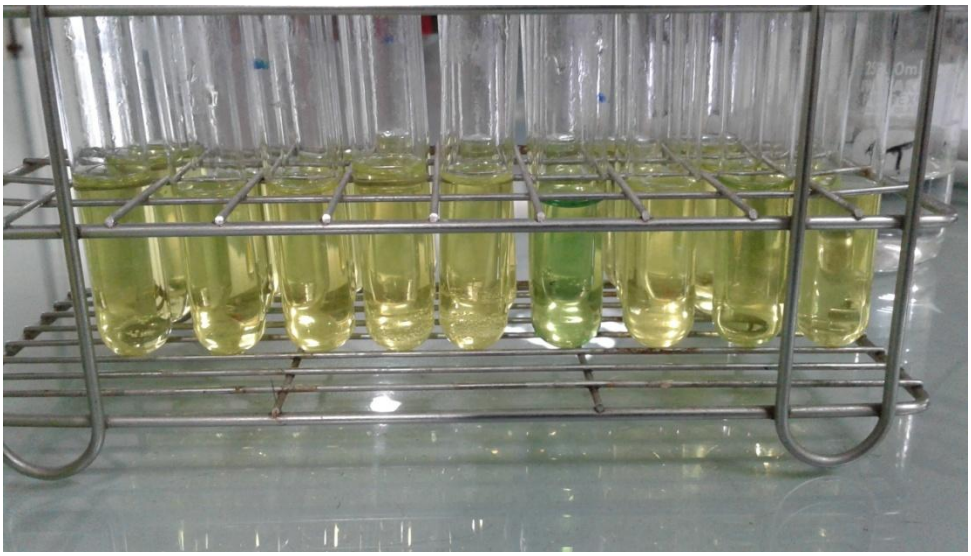


Figure 3.8: Reducing Power Capacity Assessment

3.9. Purification of acetyl cholinesterase enzyme:

The major source of acetyl cholinesterase enzyme is brain, muscle and the RBC. Among all brains neuronal junctions are enriched with acetyl cholinesterase. But this source of enzyme needs 4 step of purification for ready to use.

Materials:

- Bovine/Rat brain,
- Wash buffer,
- Extraction buffer,
- Dilution buffer,
- DTNB (Sigma chemical company, USA),
- ATCI (Sigma chemical company, USA),
- Ammonium Sulphate (Sigma chemical company, USA),
- Centrifuge Machine (Osaka, Japan)
- UV spectrophotometer (Shimadzu, Japan),
- Ice bath,
- Sephadex G-200 gel (Sigma chemical company, USA).

Formulation of Reagents:

- Wash buffer: 10mM Tris buffer.
- Extraction Buffer: 50mM Tris buffer + 10% Triton-X + 50mM $MgCl_2$ + 50mM NaCl.
- DTNB: 0.7mM solution.
- ATCI: 0.35mM solution.

Procedure:

A. Preparation of Crude Enzyme Extract:

The bovine brain (10gm) was weighted, cut into small pieces and grinned into a mortar and pestle with 50ml of homogenization buffer, pH 7.4. The temperature was maintained at 4^0C by putting ice in the outer chamber of the homogenizer. The suspension was filtered through double layer of muslin cloth in the cold room. The filtrate was collected and clarified further by centrifugation at 10000 rpm for 25 minutes at 4^0C. This clear supernatant was used as crude enzyme extract.

B. Precipitation with Ammonium Sulphate:

The crude extract was precipitated with super saturated ammonium sulphate salt. Because of low density, as compare to ammonium sulphate solution, the precipitate rose to the surface on standing. Centrifuge this mixture at 3000 rpm for 25 min. The bottom layer was withdrawn. Finally the precipitate dissolved in homogenization buffer and used as a one-step purified enzyme source

3.10. Preparation of Enzyme Source (Blood serum):

Butyryl cholinesterase is also known as pseudo cholinesterase or nonspecific cholinesterase, is a serine hydrolase and catalyzes the hydrolysis of esters of choline. It is made in the liver in humans, found mainly in blood plasma. It is very similar to the neuronal acetyl cholinesterase.

Materials:

1. Screw cap testube
2. Syringe
3. Ethanol
4. EDTA (Ethylene diamine tetraacetic acid)
5. Cotton
6. Centrifugation machine

Formulation of Reagents:

- ✓ 1% EDTA solution

1gm EDTA solution is mixed with 100ml distilled water.

Procedure:

By using syringe blood is collected from healthy human volunteer. In 10 ml of blood about 3-4 ml of EDTA solution is added. The prepared solution is then centrifuged in 3000rpm for 10 minutes. The clear serum portion is collected as butyryl cholinesterase enzyme source.

Figure 3.9: Preparation of Butyryl cholinesterase enzyme

3.11. In-Vitro Acetyl Cholinesterase Inhibitory Studies:

Principle:

The acetyl cholinesterase inhibitory activity of sample extract was determined by Ellman's method. This method estimates AchE using acetylcholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). By the yellow color compound produced by thiocholine when it reacts with dithiobisnitro Benzoate ion the enzymatic activity was measured.

$$\text{Acetylcholine} \xrightarrow{\text{enzyme}} \text{Thiocholine + Acetate}$$

$$\text{Thiocholine + dithiobisnitro Benzoate} \longrightarrow \text{yellow color}$$

The color intensity can be measured on a spectrophotometer and the enzyme activity expressed as the rate of reaction per minute.

Materials:

- 5, 5´-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan)
- Acetylthiocholine iodide (Sigma-Aldrich, Japan)
- Tris-Hcl buffer (Merck, Germany)
- Rat brain homogenate (Crude enzyme)
- Triton X-100 (Sigma chemical company, USA)
- BCA kit (bicinchoninic acid; Sigma Co., USA)
- Bovine serum albumin (Merck, India)
- Donepezil (Sigma-Aldrich, Japan)
- Micropipette (100-1000 µl)
- UV spectrophotometer (Shimadzu, USA)

Experimental Procedure:

The acetyl cholinesterase (AChE) inhibitory assay was performed according to the colorimetric method of Ellmanusing acetylthiocholine iodide as a substrate. For the enzyme source, the rat brains were homogenized in a homogenizer with 5 volumes of a homogenation buffer [10 mMTris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM $MgCl_2$ and 1% Triton X-100] and centrifuged at 10,000 rpm for 30 min. The resulting supernatant was used as an enzyme source. All of the extraction steps were carried out at 4°C. Protein concentration was determined using the BCA kit (bicinchoninic acid; Sigma Co., USA) with bovine serum albumin (BSA) as a protein standard. The rates of

hydrolysis by acetyl cholinesterase were monitored spectrophotometrically. Each sample or standard (20µl, 50µl, 100µl, 200µl) was mixed with an enzyme solution (200 µl) and incubated at 37°C for 10 min. Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture which is combination of 200µl DTNB and 400µl ATCI to the above reaction mixture. Reading was taken after 10 minutes incubation to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme.

3.12. In- Vitro Butyryl cholinesterase Inhibitory Studies:

Principle:

The butyryl cholinesterase inhibitory activity of sample was determined by Ellman's method. This method estimates Butyryl Cholinesterase using butyryl choline iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

The color intensity can be measured on a spectrophotometer and the enzyme activity expressed as the rate of reaction per minute.

Materials:

- 5, 5´-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan),
- Butyrylthiocholine iodide (Sigma-Aldrich, Japan),
- Tris-Hcl buffer (Merck, Germany),
- Human blood plasma (Crude enzyme),
- Triton X-100 (Sigma chemical company, USA),
- BCA kit (bicinchoninic acid; Sigma Co., USA),
- Bovine serum albumin (Merck, India),
- Donepezil (Sigma-Aldrich, Japan),
- Micropipette (100-1000 µl),
- UV spectrophotometer (Shimadzu, Japan),

Experimental Procedure:

The butyryl cholinesterase (BuChE) inhibitory assay was performed according to the colorimetric method of Ellman using butyryl thiocholine iodide as a substrate. All of the extraction steps were carried out at 4°C. Then 50 µl enzyme, extraction buffer (up to 3ml) and plants extracts (20 µl, 50µl, 100µl, 200µl) are incubated for 20 minutes at room temperature. The rates of hydrolysis by butyryl cholinesterase were monitored spectrophotometrically. After 20 minutes 200 µl DTNB (0.7mM) and 400 µl BTCI (0.35mM) added respectively. Heat this for 15 minutes at 37^0C. For measuring the background BTCI was avoided. Reading was taken at 412nm. From the difference between BTCI positive and negative data the activity of extract was measured. The blank reaction was measured by substituting saline for the enzyme.

3.13. Thrombolytic Activity Test:

Thrombolytic activity of the methanol extract of sample was determined by using human blood by taking streptokinase as standard.

Principle:

Thrombosis is the clotting of blood in circulatory system. Blood clot in the brain can be contributed to the development of Alzheimer's disease.

The thrombolytic activity of methanolic extract of sample was calculated by the following equation

$$\textbf{\% of Thrombolysis} = \frac{(\textbf{weight of clot before treatment} - \textbf{weight of clot after treatment})}{\textbf{weight of clot before treatment}} \times 100$$

Materials:

- Human blood
- Ependorf tube

Experimental Procedure:

Blood was collected from healthy human volunteer. An empty ependorf tube was measured and weight was written as document. 1 ml of blood was dropped in the tube and incubated for 1 hour at room temperature. Then the serum was discarded and weight of blood clot is measured by subtracting the weight of empty ependorf tube. After that 100µl plant extract was added. Then it was incubated for 90 minutes. Then liquid part was discarded and again weights the clot. Finally from the weight difference percentage of thrombolytic can be determined by the equation.

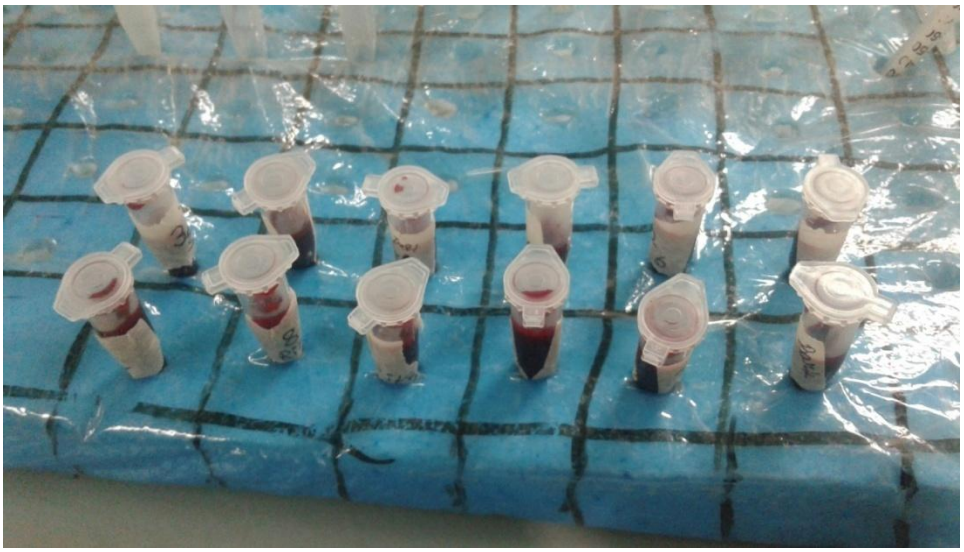


Figure 3.10: Thrombolytic Activity Test

CHAPTER 4: RESULTS

Chapter 4: Results:

4.1. Preparation of Crude Methanolic Extract:

The plants of *Basella alba, Blumea balsamifera and Curculigo orchioides* were dried under shade and pulverized in a mechanical grinder. The coarse powder was extracted with methanol and the resulting solution was filtered by cotton and then by filter paper (Whatman No.1) to get the pure extract. The filtrate was then concentrated with a rotary evaporator under reduced pressure to achieve crude methanol extract.

Table 4.1: Different fractions with amount obtained from the methanol extract of plants

Name of the fractions	Weight of the fractions (gm)
Basella alba	20.4 gm
Blumea balsamifera	19.7 gm
Curculigo orchioides	19.3 gm

4.2. Determination of Total Phenolics:

Phenolic content of the crude methanolic extract and chloroform fraction were determined using Folin-Ciocalteu reagent. Phenolic content of the samples were calculated on the basis of the standard curve for Gallic acid as shown in Table 3.2 and in figure 3.1. The results were expressed as mg of Gallic acid equivalent (GAE)/gm of dried extractives.

Table 4.2: Absorbance of Gallic acid at different concentrations after treatment with Folin-Ciocalteu reagent

Concentration (µg/ml)	Absorbance			Mean ±STD
	A	b	c	
1	0.098	0.103	0.096	0.099 ± 0.003606
2	0.176	0.179	0.182	0.179 ± 0.003
4	0.403	0.411	0.401	0.405 ± 0.005292
8	0.785	0.789	0.792	0.789 ± 0.003512
16	1.452	1.456	1.432	1.447 ± 0.012858
32	2.654	2.664	2.659	2.659 ± 0.005

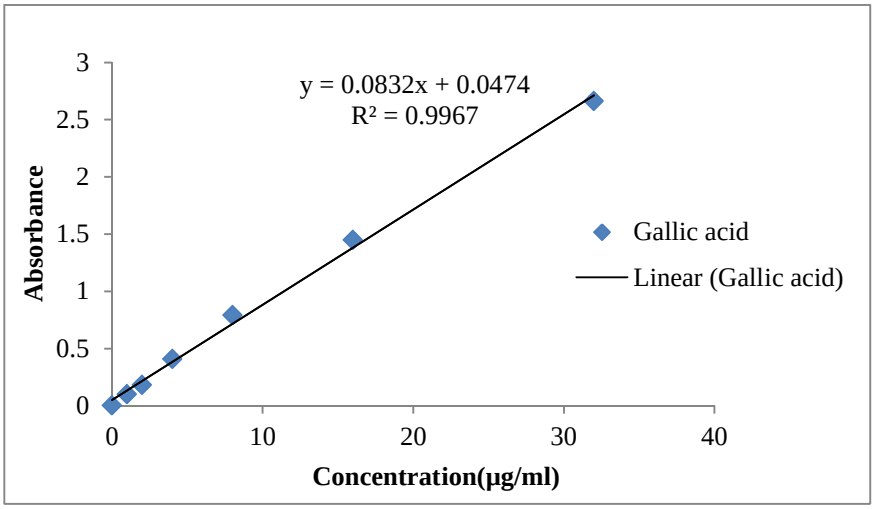
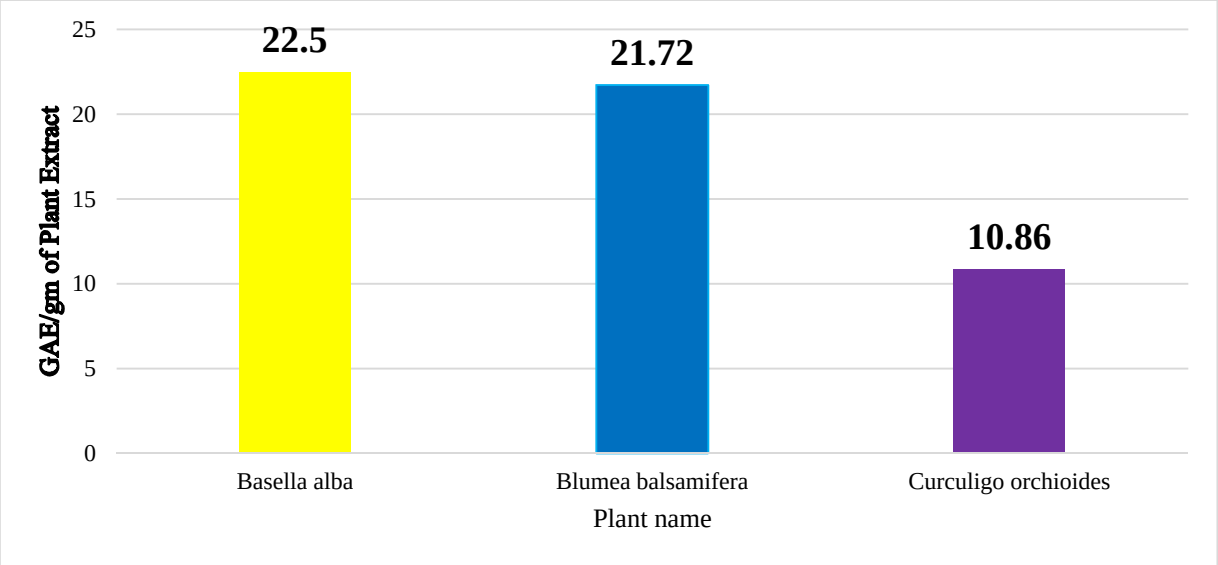


Figure 4.1: Standard curve of Gallic acid for the determination of total phenolics.

Table 4.3: Determination of total phenolic content of the crude methanol extracts (CME)

Plant Name	Sample	Conc. (µg/µl)	Absorbance	GAE/gm of dried sample
Basella alba	**CME**	300	0.375	22.50
Blumea balsamifera	**CME**	300	0.362	21.72
Curculigo orchioides	**CME**	300	0.181	10.86

Figure 4.2: Total phenol content (mg/gm plant extract in Gallic acid equivalent) of crude methanol extract



4.3. Determination of total flavonoids of crude methanol extracts (CME):

Total flavonoids content of crude methanol extract (CME) were determined using much known aluminum chloride colorimetric method. Flavonoid content of the samples was calculated on the basis of the standard curve for catechin as shown in Table and in Fig. The results were expressed as mg of catechin equivalent (CE)/gm of dried sample.

Table 4.4: Absorbance of catechin at different concentrations for quantitative determination of total flavonoids

Concentration (µg/µl)	Absorbance			Absorbance Mean ± STD
	a	b	c	
31.25	0.241	0.238	0.244	0.241 ± 0.003
62.5	0.380	0.378	0.382	0.38 ± 0.002
125	0.726	0.720	0.732	0.726 ± 0.006
250	1.476	1.472	1.480	1.476 ± 0.004
500	2.667	2.657	2.677	2.667 ± 0.007

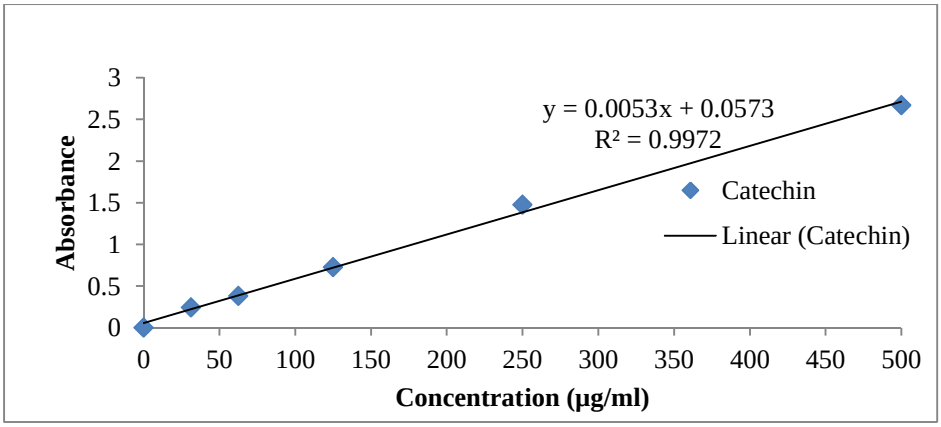
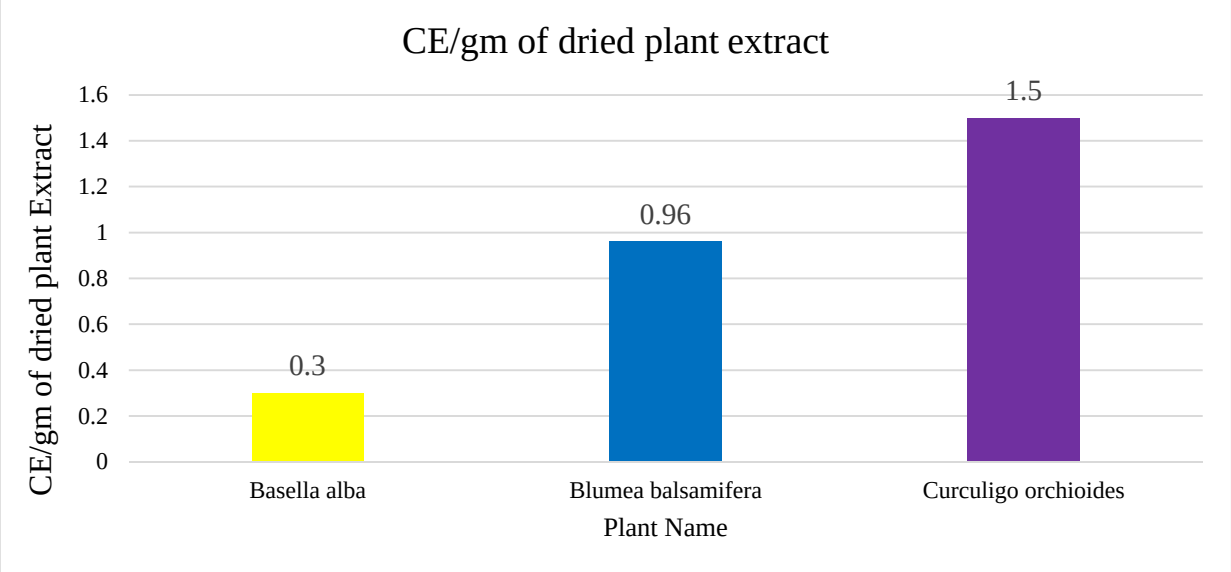


Figure 4.3: Standard curve of catechin for the determination of total flavonoids.

Table 4.5: Determination of total flavonoid content of the crude methanol extract

Plant Name	Sample	Conc. (µg/µl)	Absorbance	CE/gm of dried sample
Basella alba	**CME**	300	0.005	0.30
Blumea balsamifera	**CME**	300	0.016	0.96
Curculigo orchioides	**CME**	300	0.025	1.50

Figure 4.4: Total flavonoid content (mg/gm plant extract in catechin equivalent) of crude methanol extract (CME)



4.4. Determination of Total Flavanol:

Phenolic content of the crude methanolic extract was determined using reagents. The results were expressed as mg of Gallic acid equivalent (GAE)/gm of dried extractives.

Table 4.6: Absorbance of Gallic acid at different concentrations

Concentration (µg/ml)	Absorbance			Mean ±STD
	A	b	c	
1	0.098	0.103	0.096	0.099 ± 0.003606
2	0.176	0.179	0.182	0.179 ± 0.003
4	0.403	0.411	0.401	0.405 ± 0.005292
8	0.785	0.789	0.792	0.789 ± 0.003512
16	1.452	1.456	1.432	1.447 ± 0.012858
32	2.654	2.664	2.659	2.659 ± 0.005

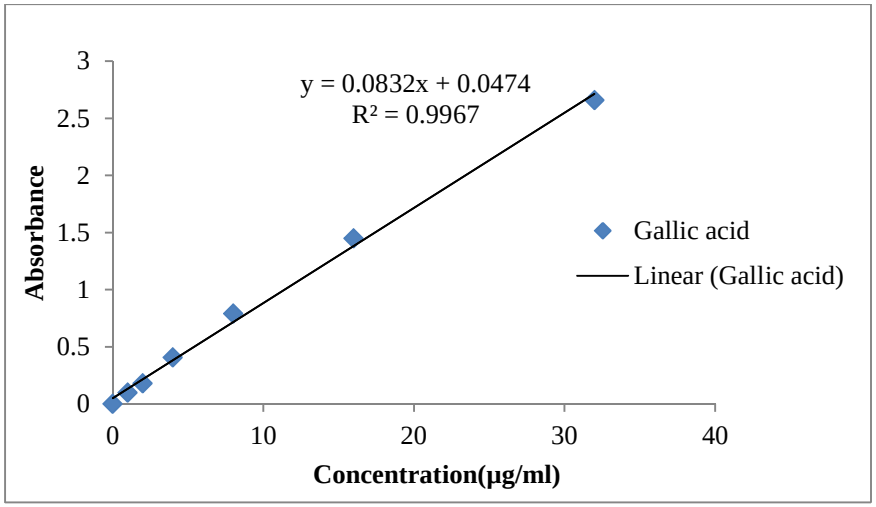
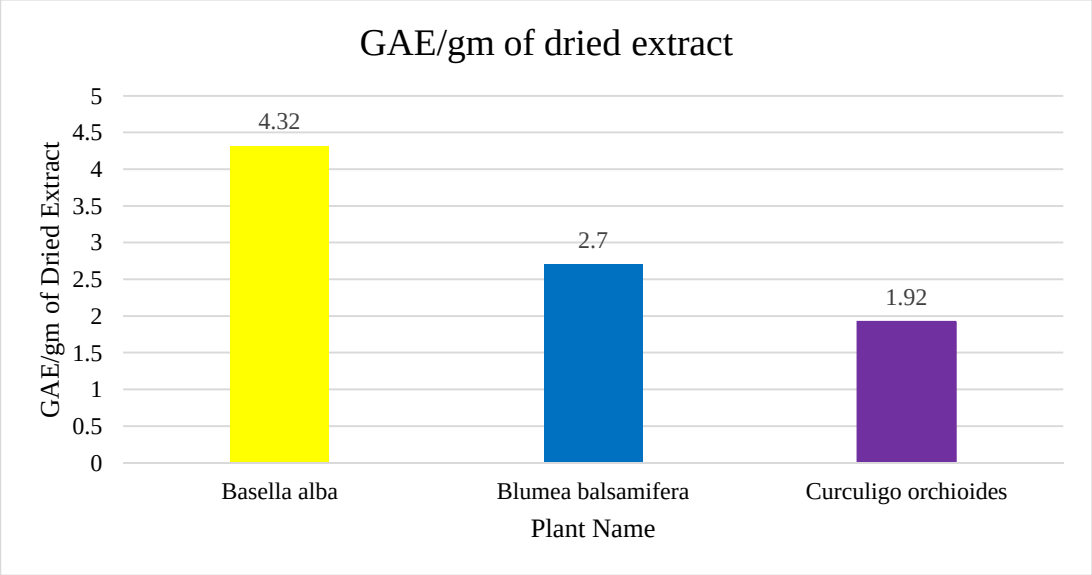


Figure 4.5: Standard curve of Gallic acid for the determination of total flavanols.

Table 4.7: Determination of total flavanol content of the crude methanol extract (CME)

Plant Name	Sample	Conc. (µg/µl)	Absorbance	GAE/gm of dried sample
Basella alba	**CME**	300	0.072	4.32
Blumea balsamifera	**CME**	300	0.045	2.70
Curculigo orchioides	**CME**	300	0.032	1.92

Figure 4.6: Total flavanol content (mg/gm plant extract in Gallic acid equivalent) of crude methanol extract



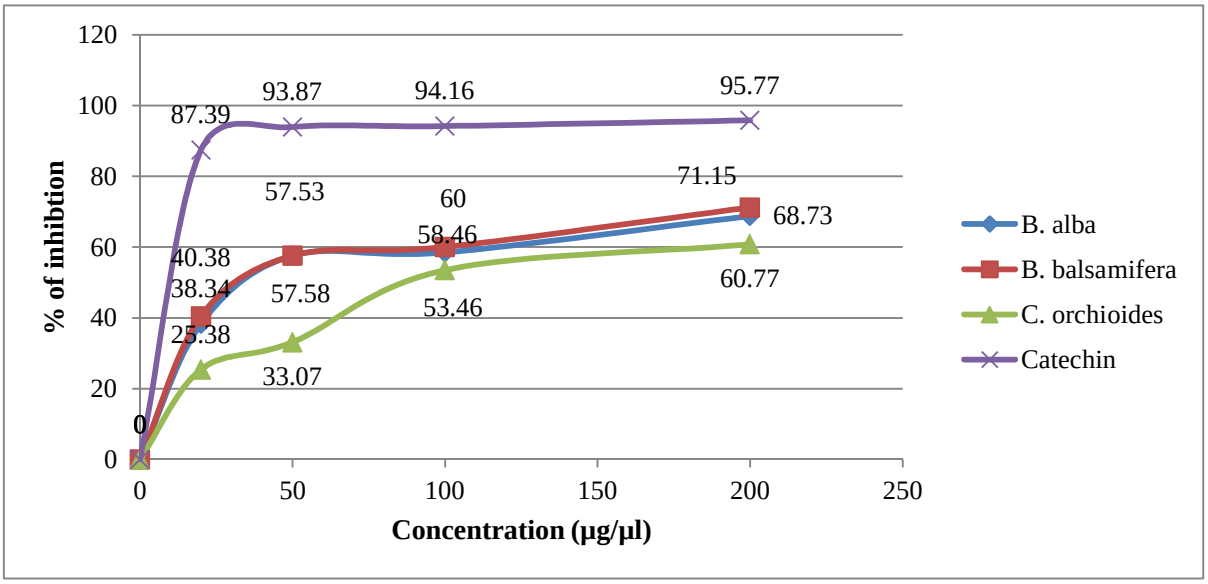
4.5. DPPH Radical Scavenging Activity:

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples including plant extracts. DPPH antioxidant assay is based on the ability of 1, 1 diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the change in absorbance and % of scavenging activity is calculated.

Table 4.8: Determination of DPPH Radical scavenging activity of the crude methanol extract

Name of Plant	Concentration (µg/µl)	Absorbance	% of Inhibition
Basella alba	20	1.59	38.84
	50	1.11	57.30
	100	1.08	58.46
	200	0.813	68.73
Blumea balsamifera	20	1.55	40.38
	50	1.104	57.53
	100	1.04	60.00
	200	0.75	71.15
Curculigo orchioides	20	1.94	25.38
	50	1.74	33.07
	100	1.21	53.46
	200	1.02	60.77
Catechin (Standard)	20	0.19	87.39
	50	0.14	93.87
	100	0.11	94.16
	200	0.09	95.77

Figure 4.7: DPPH Radical (mg/gm plant extract in Catechin equivalent) of crude methanol extract



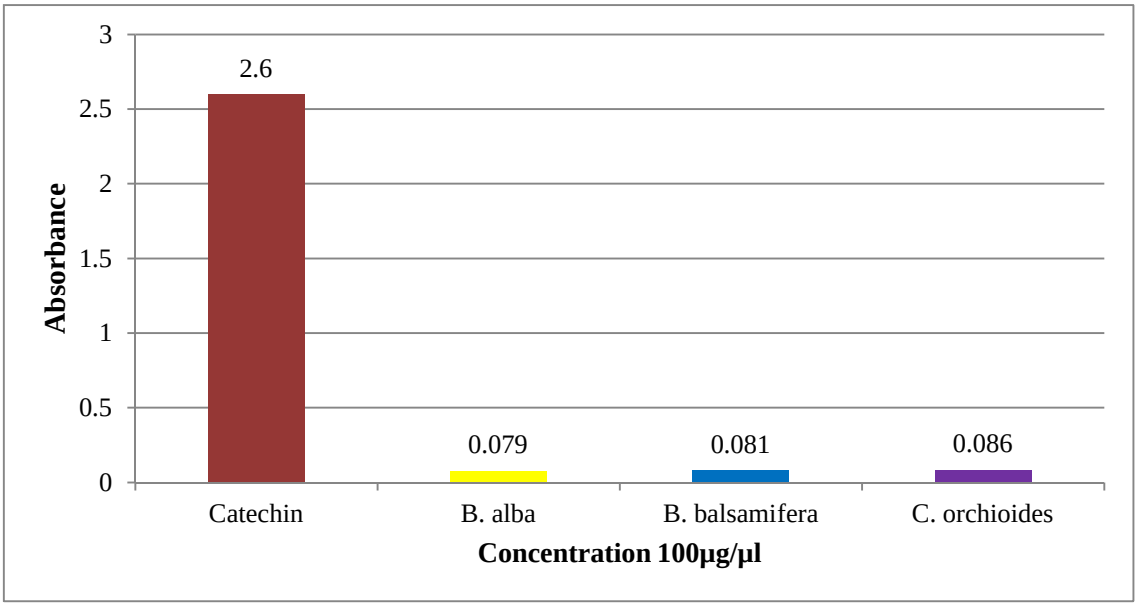
4.6. Reducing Power Capacity:

The Fe^{3+} reducing power of the crude methanolic extract (CME) was determined by the method of Oyaizu (1986) with slight modification. The reductive capabilities of crude methanol extract (CME) and its four fractions and the reference standard catechin are shown in Table.

Table 4.9: Determination of Reducing Power Capacity of the crude methanol extract

Name of Plant	Concentration (µg/µl)	Absorbance
Basella alba	100	0.079
Blumea balsamifera	100	0.081
Curculigo orchioides	100	0.086
Catechin (Standard)	100	2.660

Figure 4.8: Reducing Power Capacity (mg/gm plant extract in Catechin equivalent) of crude methanol extract



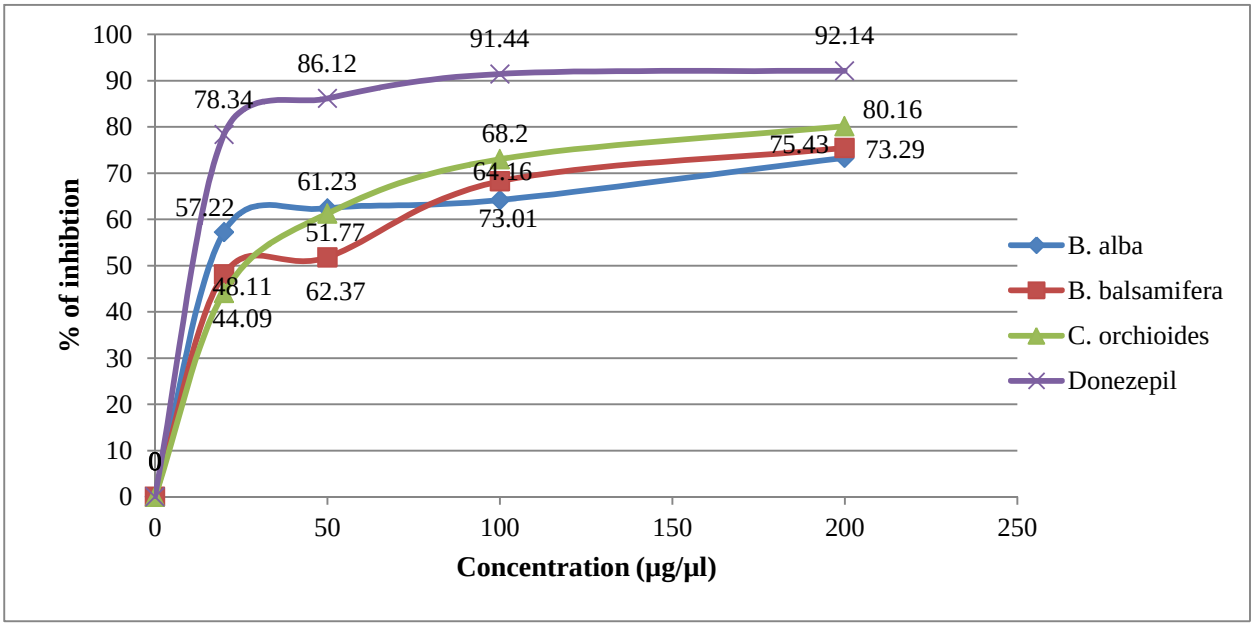
4.7. Acetylcholinesterase inhibitory activity assay

Inhibition of acetylcholinesterase, which enhances cholinergic transmission by reducing the enzymatic degradation of acetylcholine, is a widely accepted strategy for the development of AD drug. In this study, the acetylcholinesterase inhibitory activity of the crude methanol extract and its different fractions and the compounds was assessed by modified Ellman'smethod and compared with the reference standard donepezil. This method estimates acetylcholinesterase (AChE) using acetylcholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion.

Table 4.10: Determination of Acetylcholinesterase inhibitory activity of the crude methanol extract

Name of sample	Conc. (µg/µl)	% of inhibition Mean
Donepezil (Std)	20	78.34
	50	86.12
	100	91.44
	200	92.14
Basella alba	20	57.22
	50	62.37
	100	64.16
	200	73.29
Blumea balsamifera	20	48.11
	50	51.77
	100	68.20
	200	75.43
Curculigo orchioides	20	44.09
	50	61.23
	100	73.01
	200	80.16

Figure 4.9: Acetylcholinesterase inhibitory activity (mg/gm plant extract in Donepezil equivalent) of crude methanol extract



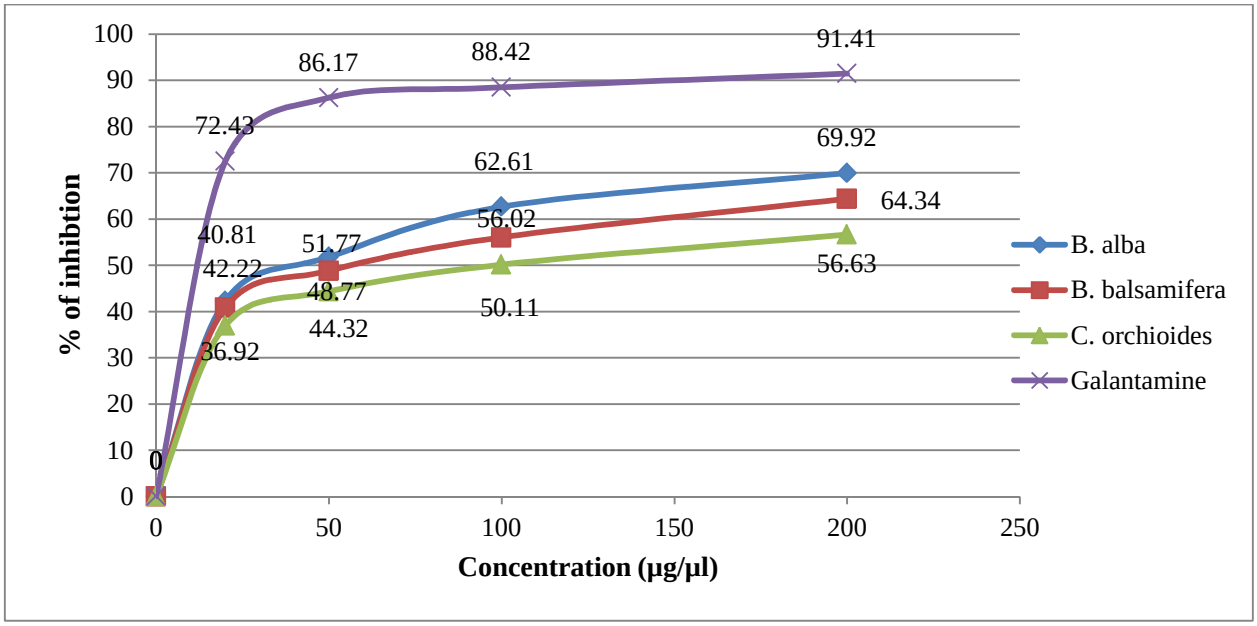
4.8. Butyrylcholinestserase inhibitory activity of enzymes:

Butyrylcholinestserase enhances cholinergic transmission by reducing the enzymatic degradation of both acetylcholine and butyrylcholine. Thus inhibition of Butyrylcholinestserase increases the neurotransmission not only in brain but also in other neuronal junctions. This strategy is a widely accepted most advance strategy for the development of AD drug. Butyrylcholinestserase inhibitors have synergistic activity of acetylcholinesterase inhibitory activity.

Table 4.11: Determination of Butyrylcholinestserase inhibitory activity of enzymes of the crude methanol extract

Name of sample	Conc. (µg/µl)	% of inhibition Mean
Galantamine (Std)	20	72.43
	50	86.17
	100	88.42
	200	91.41
Basella alba	20	42.22
	50	51.77
	100	62.61
	200	69.92
Blumea balsamifera	20	40.81
	50	48.77
	100	56.02
	200	64.34
Curculigo orchioides	20	36.92
	50	44.32
	100	50.11
	200	56.63

Figure 4.10: Butyrylcholinestserase inhibitory activity of enzymes (mg/gm plant extract in Galantamine equivalent) of crude methanol extract



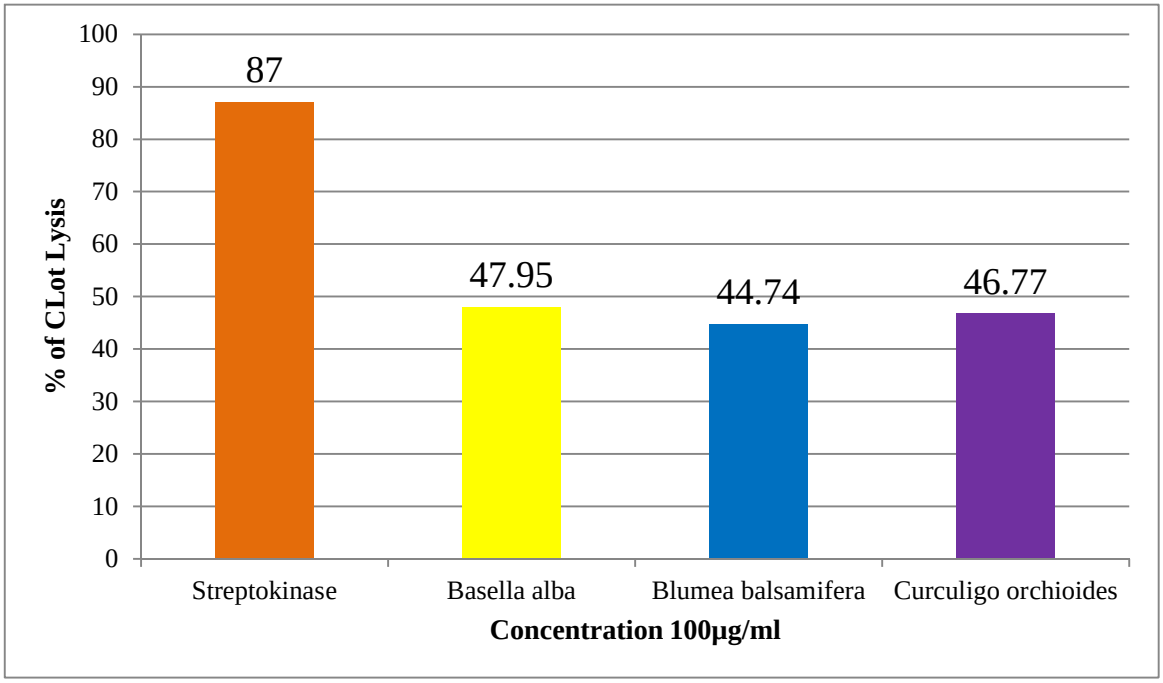
4.9. Thrombolytic Activity Test:

Thrombosis is a lethal disease and characterized by the formation of blood clots (thrombus) in the circulatory system due to the imbalance of homeostatic system of physiological procedures. This is a severe incident in the arterial diseases connected with acute coronary disorders such as pulmonary emboli, deep vein thrombosis, heart attacks, strokes and venous thromboembolic disorders that account for sudden morbidity and mortality. If in the brain thrombosis occurs the function of neurons will hampered. Thrombolytic activity will determine the efficiency of extracts to break down of clots.

Table 4.12: Determination of Thrombolytic Activity of the crude methanol extract

Plant name	Concentration (µg/µl)	% of clot lysis
Basella alba	100	47.95
Blumea balsamifera	100	44.74
Curculigo orchioides	100	46.77
Streptokinase	100	87.01

Figure 4.11: Thrombolytic Activity (mg/gm plant extract in Streptokinase equivalent) of crude methanol extract



CHAPTER 5: DISCUSSION and CONCLUSION

Chapter 5: Discussion and Conclusion

5.1. Discussion:

Determination of Total Phenolics:

Total phenolic content has been determined by using Folin-Ciocalteu reagent by using Ellman's method. The crude methanolic extracts of *Basella alba, Blumea balsamifera, and Curculigo orchioides* were used in this test. From the table of result, the crude methanolic extract of *B. alba* in concentration of 300 µg/µl is 0.375 and for which Gallic acid equivalent per gram (GAE/gm of dried sample) of dried sample is 22.50. *B. balsamifera* crude methanolic extract gives absorbance of 0.362 on the same concentration and GAE/gm of dried sample is 21.72. *C. orchioides* has given 10.86 GAE/gm of dried sample for the absorbance of 0.181. Dried extracts of fruit and shoot of *B. alba* is a prominent source of phenolic compounds as 300µg/µl CME contain 22.50 GAE/gm. Compare to this, other two plant contains less of it.

Determination of Total Flavonoids:

Total flavonoid contents were determined by using aluminum chloride colorimetric method. From the table of total flavonoid content it can be said that the crude methanolic extract of *B. alba* gives absorbance of 0.005 in a concentration of 300 µg/µl and the catechin equivalent/gm (CE/gm of dried sample) of dried sample is 0.30. Crude methanolic extract *Blumea balsamifera* has given absorbance of 0.016 and CE/gm of dried sample is 0.96. For *Curculigo orchioides* the absorbance is 0.025 in the same concentration and CE/gm of dried sample is calculated as 1.50. From the result, *B. alba* gives least GAE/gm of dried sample among three samples and *C. orchioides* gives the highest. That means dried extracts of fruit and shoot of *B. alba* is not a prominent source of flavonoid compounds as 300µg/µl CME contain 0.30 CE/gm of dried sample. While *B. balsamifera* has higher phenolic content than *B. alba* and *C. orchioides* is the most prominent source of flavonoids.

Determination of Total Flavanol:

Total flavanol content was determined by using aluminum chloride colorimetric method. From the table of total Flavanol content it is observed that *Basella alba* crude methanolic extract gives absorbance of 0.072 in concentration of 300 µg/µl for which Gallic acid equivalent /gm of dried sample (GAE/gm of dried sample) is 4.32. Crude methanolic extract of Blumea balsamifera gives absorbance of 0.045 and GAE/gm of dried sample is 2.70. While in the same concentration *Curculigo orchioides* gives absorbance of 0.032 with a calculated GAE/gm of dried sample 1.92. *B. alba* is representing that it has greater flavanol content then *B. balsamifera* and *C. orchioides*. Dried extracts of fruit and shoot of *B. alba* is a prominent source of flavanol compounds as 300µg/µl CME contain 4.32 GAE/gm of dried sample. Compare to this, other two plant contains less of it.

Determination of DPPH Radical Scavenging Activity:

The evaluation of free radical scavenging activities is done by using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH). From the table of DPPH radical scavenging activity test *Basella alba* gives absorbance of 1.59, 1.11, 1.08, 0.813 and percent of inhibition for the concentration is 38.84, 57.30, 58.46, 68.73 for the concentration of 20 µl, 50 µl, 100µl, 200 µl respectively. *Blumea balsamifera* in the same concentrations give absorbance of 1.55, 1.104, 1.04, 0.75 and percent of inhibition is 40.38, 57.53, 60.00, and 71.15 correspondingly. The absorbance of *Curculigo orchioides* is 1.94, 1.74, 1.21, and 1.02 for the above concentrations for which percent of inhibition is 25.38, 33.07, 53.46, and 60.77 respectively. Among the four different concentrations, 200 µg/µl concentrations of all the three samples give highest radical scavenging activity. However, *B. balsamifera* has given highest inhibition capacity. Compared to the percent of inhibition of Catechin standard in same concentration crude extract has less inhibitory activity. This is because the purity of standard is higher than crude extracts while crude extracts contain many other agonistic or antagonistic compounds.

Determination of Reducing Power Capacity

The evaluation of reducing power capacity was done by the method of Oyaizu (1986). Reducing power capacity table shows that *Basella alba* gives an absorbance of 0.079 in the concentration of 100µg/µl. *Blumea balsamifera* in the same concentration gives absorbance of 0.081 and *Curculigo orchioides* has an absorbance of 0.086. Among the three samples *C. orchioides* gives the highest reducing power capacity. However, comparing with Catechin standard the activity of the sample plants are much less because the purity of standard is higher than crude extracts because crude extracts may contain many other agonistic or antagonistic compounds. So, there is a chance to form more active molecules from those plants.

Determination of Acetylcholinesterase Inhibitory Activity:

The determination of acetylcholinesterase inhibitory activity is done by Ellman's method. Acetylcholinesterase inhibitory activity table presents that *Basella alba* gives percent of inhibition 57.22, 62.37, 64.16, 73.29 for the concentration of 20 µg/µl, 50 µg/µl, 100 µg/µl, 200 µg/µl respectively. For the same concentration *Blumea balsamifera* and *Curculigo orchioides* gives percent of inhibition 48.11, 51.77, 68.20, 75.43, and 44.09, 61.23, 73.01, 80.16 correspondingly. Donepezil (drug of choice) has used as standard in this test which has percent of inhibition values of 78.34, 86.12, 91.44, 92.14. Among all the three CME samples at a concentration of 200µg/µl *C. orchioides* gives the highest AchE inhibitory activity of 80.16% which is greater than *B. alba* and *B. balsamifera*. Compared to the percent of inhibition of Donepezil standard in same concentration crude extract has less inhibitory activity. This is because the purity of standard is higher than crude extracts while crude extracts contain many other agonistic or antagonistic compounds. So, there is a chance to form more active molecules from those plants.

Determination of Butyrylcholinestserase Inhibitory Activity:

The determination of Butyrylcholinestserase inhibitory activity is done by Ellman's method. The table of Butyrylcholinestserase inhibitory activity represents that *Basella alba* in the concentration of 20 µg/µl, 50 µg/µl, 100 µg/µl, 200µg/µl gives percent of

inhibition of 42.22, 51.77, 62.61, 69.92. *Blumea balsamifera* and *Curculigo orchioides* gives percent of inhibition 40.81, 48.77, 56.02, 64.34 and 36.92, 44.32, 50.11, 56.63 in the same concentration respectively. Galantamine has used as standard which has percent of inhibition of 72.43, 86.17, 88.42, 91.41. The three CME samples have Butyrylcholinestserase inhibitory activity and with the increase of concentration the inhibitory activity is also increasing. In the 200µg/µl of concentration *B. alba* gives the highest Butyrylcholinestserase inhibitory activity of 69.92% than the other two plant samples. Compared to the percent of inhibition of Galantamine (drug of choice) standard in same concentration crude extract has less inhibitory activity. This is because the purity of standard is higher than crude extracts while crude extracts contain many other agonistic or antagonistic compounds.

Determination of Thrombolytic Activity:

Thrombolytic activities were determined by using human blood and taking streptokinase as standard. From the table of thrombolytic activity test the percent of clot lysis for *Basella alba* is 47.95 in the concentration of 100 µg/µl. In the same concentration *Blumea balsamifera* and *Curculigo orchioides* gives 44.74 and 46.77 percent of clot lysis. Streptokinase has been used as standard which has clot lysis percentage of 87.01. Among the three CME samples *B. alba* has highest thrombolytic activity of 47.95% than *B. balsamifera* and *C. orchioides* Comparing with Streptokinase standard the activity of the sample plants are much less because the purity of standard is higher than crude extracts while crude extracts contain many other agonistic or antagonistic compounds. So, there is a chance to form more active molecules from those plants.

5.2. Conclusion

More than 600 disorders badly involve the nervous system. Neurodegenerative disorders are defined as heritable and intermittent conditions which are characterized by progressive nervous system dysfunction. These disorders are often associated with deterioration of the exaggerated central or peripheral structures of the nervous system. They include diseases such as Brain Cancer, Degenerative Nerve Diseases, Alzheimer's Disease and other dementias, etc. (Ec.europa.eu, 2016)

For determining cholinesterase inhibitory activity, thrombolytic activity and anti-oxidant activities we have used three crude methanolic extract of plants that are available in Bangladesh as well as south-Asian countries.

Under cholinesterase inhibitory activity we have considered acetylcholinesterase and butyrylcholinesterase inhibitory activity. *Curculigo orchioides* has 80.16% potential to inhibit AchE in the concentration of 200 µg/µl and it is highest among three extracts. *Basella alba* extracts has inhibited 69.92% of butyrylcholinesterase in the concentration of 200µg/µl.

Beneath of antioxidant activities test we have measured total phenolics, flavonoids, flavanol contents; ferrous reducing, and free radical scavenging activities. *B. alba* is a good source of phenol as it has specified 22.50 GAE/gm in the concentration of 300µg/µl. Flavonoids are highly obtainable in *C. orchioides* which has given 1.50 CE/gm of dried sample in 300µg/µl.

B. alba is an outstanding source of flavanol giving 4.32 GAE/gm of dried sample in 300µg/µl concentration. However, B. balsamifera has higher capacity to scavenge free radicals than two other samples which is 71.15% in 200µg/µl. C. orchioides has reducing power capacity but in the range which could be uncountable compared with the standard. So, we have not found a good radical scavenging activity from the concerned samples.

Thrombolytic activity is an important test for the approach to the treatment of AD which is prominently given by *B. alba* at 47.95%.

Chapter 5: Discussion and Conclusion

The work was done to find a new natural product for the treatment of Alzheimer's disease which has been dominating the diseases occurs in the elderly people.

CHAPTER 6: REFERENCING

Chapter 6: Referencing

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