Cholinesterase Inhibitory Potential and Anti-oxidant Activities of Leaf and Bark Extract of *Diospyros blancoi* Relevant for the Treatment of Alzheimer's Disease

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

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Dedicated To
My Parents

Certificate by the Chairperson

This is to certify that the thesis entitled "Cholinesterase Inhibitory Potential and Anti-oxidant Activities of Leaf and Bark Extract of *Diospyros blancoi* Relevant for the Treatment of Alzheimer's Disease" 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 Taposhi Sultana Mou, ID: 2013-1-70-012, during the period 2016 of her research in the Department of Pharmacy, East West University.

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Certificate by the Supervisor

This is to certify that the thesis entitled "Cholinesterase Inhibitory Potential and Anti-oxidant Activities of Leaf and Bark Extract of *Diospyros blancoi* Relevant for the Treatment of Alzheimer's Disease" 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 Taposhi Sultana Mou, ID: 2013-1-70-012, 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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Declaration by the Candidate

I, Taposhi Sultana Mou, hereby declare that the dissertation entitled "Cholinesterase Inhibitory Potential and Anti-oxidant Activities of Leaf and Bark Extract of *Diospyros blancoi* Relevant for the Treatment of Alzheimer's Disease" 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 Kushal Biswas, 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.

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Taposhi Sultana Mou

Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviated form	Full form
AD	Alzheimer's Disease
MCI	Mild Cognitive Impairment
APP	Amyloid Precursor Protein
sAPPb	soluble N-terminal Portion of Amyloid Precursor Protein
CTFb	Membrane-bound C-terminal Portion
apoE4	Apolipoprotein E4
MAP	Microtubule Associated Protein
LTP	Long Term Potentiation
D. blancoi	*Diospyros blancoi*
MIC	Minimum Inhibitory Concentration
CME	Crude Methanoic Extract
FCR	Folin–Ciocalteu Reagent
DPPH	1, 1-diphenyl-2-picrylhydrazyl radical
RBC	Red Blood Cell
AchE	Acetylcholinesterase Enzyme
DTNB	Dithiobisnitro Benzoic Acid
BChE	Butyrylcholinesterase Enzyme
BTCI	Butyrylthiocholine Iodide
PET	Petroleuom Ether Fraction
CLF	Chloroform Fraction
GAE	Gallic Acid Equivalent
CE	Catechin Equivalent

Abstract

Oxidative stress and decreased level of neurotransmitter (especially acetylcholine) are main characteristics of Alzheimer's disease (AD), a progressive neurodegenerative disease. Increasing the function of acetylcholine by inhibiting acetylcholinesterase or butyrylcholinesterase enzyme (both are responsible for the breakdown of Acetylcholine) and reducing oxidative stress with antioxidants are most effective treatment therapy of AD. Beside this atherothrombosis is a major cause of global life threatening cerebral diseases. Traditionally *Diospyros blancoi* has widely known for its medicinal values. Our aim is to investigate the cholinesterase inhibitory activities as well as thrombolytic activities of the bark and leaves crude methanolic extracts (CME) and other fractions in the treatment of AD and clotting disorder. The crude methanol extract was prepared by cold extraction method and were assessed for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activity by the Ellman's method. For thrombolytic activity clot lysis method were applied. The results revealed that the CME of both bark and leaf have Cholinesterase inhibitory activities as well as thrombolytic activities which may be useful in the treatment of AD as well as clotting disorder.

Chapter 1: Introduction

1.1 Alzheimer's disease

Alzheimer's disease (AD) is a non-reversible, growing syndrome of brain that gradually terminates memorial and intellectual abilities and ultimately the capability to do the simplest tasks. In maximum people with Alzheimer's, signs first seem in their mid-60s. Evaluations may differ, but experts recommend that 5 million Americans may suffer from this disease.

At present Alzheimer's disease is rated as the sixth major reason of death in the United States, but current assessments suggest that the disease may rank third, after heart disease and cancer.

This disease is the most renowned reason of dementia among older peoples. Dementia is the decrease in cognitive functioning—intellectual, recalling, and reasoning—and communication capabilities that it affects a person's daily life and work activities.

The reasons behind dementia can fluctuate, which depends on the forms of brain changes that may be happening. Other dementias are termed as Lewy body dementia, frontotemporal disorders, and vascular dementia. It is common to have combined dementia—a mixture of two or more disorders, here minimum one disease is dementia.

Alzheimer's disease is termed as the name of Dr. Alois Alzheimer. In 1906, he observed alterations in the brain tissue of a woman. She was died because of a mental sickness. Her signs and symptoms were loss of memory, language complications and erratic activities. After her death, he inspected her brain and found various clumps which were not normal (now these are termed as amyloid plaques) and tangled clusters of fibers (at present which is named neurofibrillary or tau, tangles).

These plaques and tangles are till now said as some of the main characteristics of Alzheimer's disease. Another factor is the destructions of links between neurons in the brain. Neurons spread signals between various regions of the brain as well as from the brain to muscles and other vital organs in the human body.

1.1.1 Changes in the Brain due to Alzheimer's Disease

Cross sections of the brain represent degeneration or decrease of brain tissue caused by this disease.

Scientists continue to undo the complex brain changes associated with the beginning and development of Alzheimer's disease. It appears likely that destruction of the brain occurs a decade before remembrance and another cognitive difficulties appear. During this pre stage of this disease, individuals seem to be healthy and symptom-free, but deadly changes are occurring in the brain. Unusual deposits of proteins is the reason behind forming of amyloid plaques and tau tangles all over the brain and if fit neurons discontinue functioning, they fail to remain connected with other neurons and the result is death of neurons.

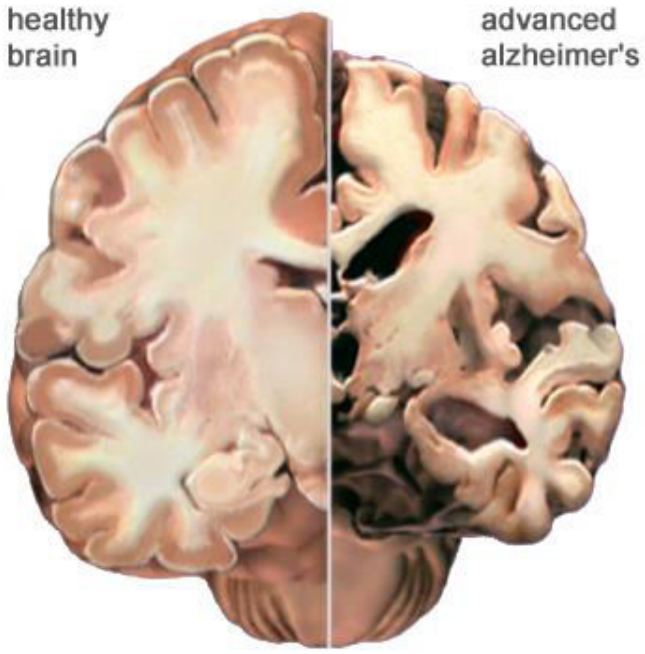

Figure 1.1: Comparison between healthy and diseased brain

The destruction at first occurs in the hippocampus which is the vital part of the brain in generating memories. As death of more neurons occur, other parts of the brain are affected and they start to squeeze. By the terminal stage, destruction is extensive and brain tissue has shrunk meaningfully.

1.1.2 Signs and Symptoms of Alzheimer's Disease

Problems of remembering anything are typically the initial sign of cognitive problem associated with Alzheimer's disease. A number of people with cognitive difficulties have a disorder termed as mild cognitive impairment (MCI). In this situation,

individuals have additional memory complications than typical for their age, but their signs do not affect their daily lives. Movement problems and difficulties with the sense of odor have also been associated with MCI. Older adults with MCI are at large danger of developing Alzheimer's.

The initial symptoms of Alzheimer's differ among individuals. For various numbers of people, decrease in non-memory features of cognition such as finding of word, visualization and reduced reasoning can be the signal of very early step of Alzheimer's disease. Scientists are studying biomarkers to find initial changes in the brains of people having MCI and in normal people who can be at larger risk for Alzheimer's.

1.1.3 Stages of Alzheimer's Disease

There can be three stages of the diseases which includes:

- **Mild Alzheimer's Disease**

As the disease develops, individuals experiences more memory loss and other cognitive problems. Difficulties can consist of wandering and becoming lost, problem in controlling money and giving bills, repeating questions, taking long times to do usual daily jobs and character and manner changes. People are often evaluated in this phase.

- **Moderate Alzheimer's disease**

In this phase, destruction takes place in zones of the brain that regulate language, judgment, sensory processing and sensible thought. Loss of memory and misperception become worse and individuals started to have difficulties identifying family and friends. They may be incapable to study new topics, carry out complex jobs such as getting dressed or deal with different circumstances. In addition, individuals at this phase may have illusions, misunderstandings and fear and may behave thoughtlessly.

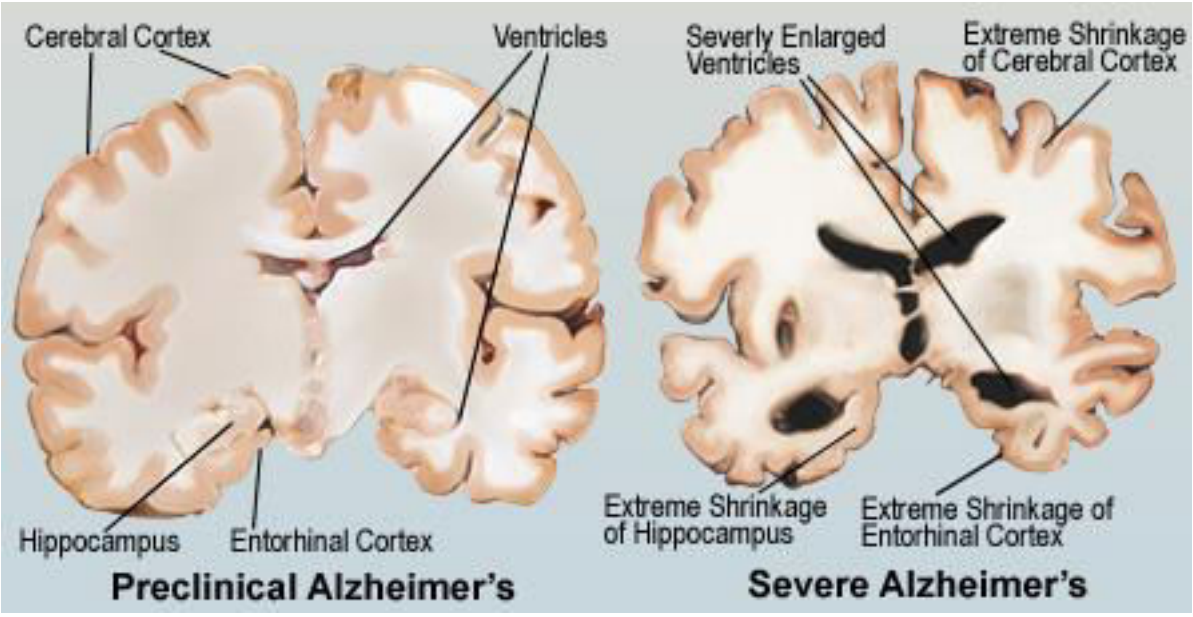

Figure 1.2: Different stages of Alzheimer's disease

- **Severe Alzheimer's disease**

Eventually, plaques and tangles spread all over the brain and tissues of shrinks meaningfully. Persons with severe Alzheimer's cannot talk properly and are totally reliant on others for their care. At the final stage, the patient may be in bed all of the time as the body cannot function.

1.1.4 Causes of Alzheimer's Disease

Researchers don't yet fully know what causes Alzheimer's disease in most of the people. In persons with early-onset Alzheimer's, mutation in the genetic factors are typically the causes. Late-onset Alzheimer's rises from a multifaceted sequence of brain changes that take place over decades. The reasons possibly include a mixture of genetic, ecological and lifestyle factors. The significance of these factors in developing Alzheimer's disease can vary person to person. Alzheimer's is the result of brain cell death. (Alzheimer's Disease education and referral center, 2016)

It is a neurodegenerative disorder, which means there is advanced brain cell death that occurs over a passage of time. The total brain size decreases with Alzheimer's - the tissue has increasingly fewer nerve cells and connections. In Alzheimer's disease,

plaques are originated among the dying cells in the brain. It is the result of the build-up of a protein called beta-amyloid. The tangles remain within the brain neurons. These are result of fragmentation of another protein, called tau. (DeFina et al, 2016)

1.2 Recent data of Alzheimer's Disease

1.2.1 Alzheimer's Worldwide Statistics of morbidity

Globally, approximately 44 million persons have Alzheimer's or a correlated dementia. Only 1-in-4 individuals with these symptoms have been identified. Prevalence of Alzheimer's is most common in Western Europe and North America. Alzheimer's is least dominant in Sub-Saharan Africa. Alzheimer's is the main cause for debilities in older people.

1-in-9 people of America over age of 65 has Alzheimer's disease. The time when the initial wave of babies reaches age about 85 (in 2031), it is estimated or assumed that more than 3 million person will suffer from Alzheimer's disease. 1 in 3 Americans over age 85 are suffering with the sickness. 5.3 million Americans are existing with Alzheimer's disease. If no treatment is established, above 16 million Americans will suffer from the disease by 2050. Alzheimer's disease is the 6th top reason of death in America. 1-in-3 older people's death occur by Alzheimer's disease. Usual life probability after an Alzheimer's identification is 4-to-8 years. In 2016, the individuals above age 85 contains about 2 million persons with this disease or 40% of total population with Alzheimer's age above 65. By 2050, the number could be 7 million people age 85 and above with Alzheimer's disease. Percentage of Individuals with Alzheimer's disease in the America by Age: 85+ years is 38%, 75-84 years is 44%, 65-74 years is 15%, <65 years is 4%.

Table 1.1: The percentage of prevalence of Alzheimer's disease among different nations

Age (years)	Percentage
65-75	2.9% Caucasian 9.1% African American 7.5% Hispanic

75-84	10.9% Caucasian 19.9% African American 27.9% Hispanic
>85	30.2% Caucasian 58.6% African American 62.9% Hispanic

Table 1.2: Assumed number of Americans might have this disease

Year	**Number of people (Ages 65+)**
2010	4.7 Million
2020	5.8 Million
2030	8.4 Million
2040	11.6 Million
2050	13.8 Million

Another informations of this disease include:

- 2 of 3 individuals with Alzheimer's are females.
- African Americans as well as Hispanic Americans are more expected to grow Alzheimer's compared to Caucasian Americans. (Alzheimer's Association)
- North Dakota has a greater rate of Alzheimer's mortality compared to any other state. (54 patients die a year per 100,000 inhabitants)
- Alzheimer's mortality rate is lowermost in Nevada. (11 patients die a year per each 100,000 inhabitants)
- 30% of individuals with Alzheimer's also have cardiovascular disease and 29% suffer from diabetes.

1.2.2 The expenditure of Care of peoples having Alzheimer's disease

- The expenditure of caring for patients with this disease in the America is expected to be $236 billion dollars in 2016.
- The worldwide expenditure of Alzheimer's is assumed to be $605 billion dollars which is equal to 1% of the total world's native product.

- Medicare and Medicaid are anticipated to fee $154 billion in 2015 for persons with Alzheimer's disease.
- Combined expenditure of Care for American patients of Age 65 and Older are Medicare $113 Billion, Medicaid $41 Billion, Out of pocket $44 Billion, Other $29 Billion. (Alzheimer's.net, 2016)

1.2.3 Mortality rate caused by Alzheimer's disease

Table 1.3: Mortality rate caused by Alzheimer's disease

Rank	Name of country	Mortality rate per 1000000 people
1	Finland	53.77
2	America	45.58
3	Canada	35.50
4	Iceland	34.08
5	Sweden	32.41
6	Switzerland	32.25
7	Norway	30.24
8	Denmark	29.53
9	Netherlands	29.32
10	Belgium	27.23
11	Spain	26.90
12	Australia	25.91
13	France	25.62
14	England	24.35
15	Cuba	22.38
16	Chile	21.03
17	Uruguay	20.74
18	Israel	19.90
19	New zealand	19.02
20	Ireland	17.70
21	Italy	16.96

22	Hungary	15.23
23	Malta	14.92
24	Luxembourg	14.17
25	Germany	13.39
26	Brazil	12.56
27	South korea	12.32
28	Cyprus	10.40
29	Costa rica	9.98
30	Iran	7.75
31	South Africa	7.67
32	Austria	7.41
33	Serbia	7.39
34	Trinidad	7.25
35	Bahamas	6.79
36	Maldives	6.71

(World health rankings, 2016)

Among individuals having age 70, 61 percent of people with Alzheimer's are anticipated to die before the age of 80 in comparison with 30 percent of individuals without Alzheimer's. The death rate is double.

Alzheimer's disease is authentically registered as the sixth-leading reason of lethality in the United States. It is the fifth major reason of death for persons age 65 and above. As the inhabitants of the United States become older, Alzheimer's is becoming a further cause of lethality. Although number of people died from other main reasons have reduced meaningfully in the last era, deaths from Alzheimer's disease have enlarged significantly, about 71 percent. In the year 2013, over 84,000 Americans died from Alzheimer's. However, in 2016 it is projected that 700,000 persons with Alzheimer's will be dead.

Alzheimer's is the only disorder among the top 10 reasons of lethality in America that cannot be prohibited, healed or even reduced. (Alz.org, 2016)

1.3 Different type of hypothesis in the mechanism of Alzheimer's disease

1.3.1 Formation of beta amyloid plaques

Alzheimer's affected tissue has fewer number of nerve cells and synapses compared to a healthy brain. Plaques, abnormal clusters made of protein fragments form between nerve cells. Plaques form when protein pieces termed as beta-amyloid cluster together. Beta-amyloid originates from a larger sized protein originated in the fatty membrane adjoining nerve cells. Beta-amyloid is chemically sticky in nature and progressively builds up into plaques. The most destructive form of beta-amyloid may be clusters of a few pieces than the plaques themselves. The minor clusters may inhibit cell-to-cell signaling process at synapses. They may also trigger immune system cells that activate inflammation and overwhelm disabled cells. (Alz.org,2011)

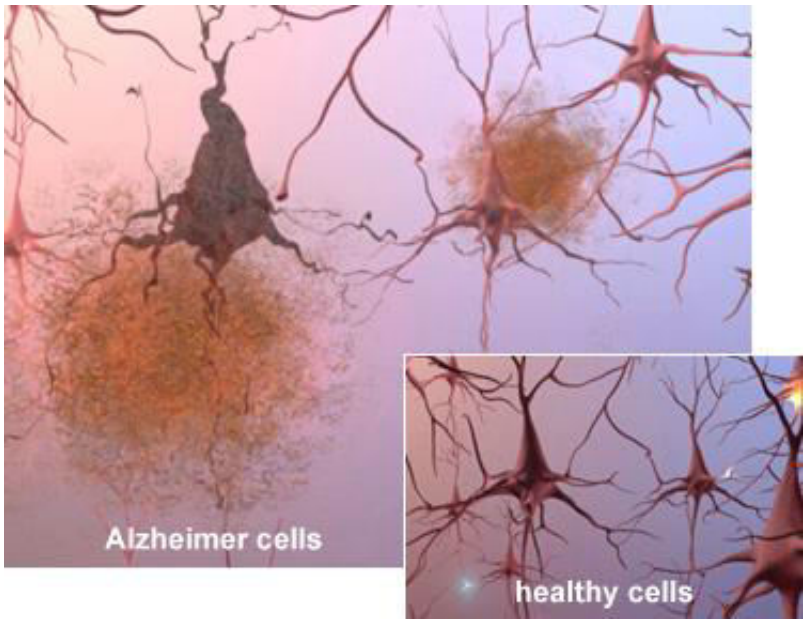

Figure 1.3: Formation of amyloid plaque

In the plaque-forming passageway, Amyloid precursor protein (APP) is cut by a dissimilar enzyme, beta-secretase which is a transmembrane aspartic protease, resulting in a soluble N-terminal portion (sAPPb) and a membrane-bound C-terminal portion (CTFb). This portion is brought closer to the N-terminal end of APP than with a-secretase, resulting in CTFb longer than CTFa. CTFb is then performed by gamma-secretase, resulting in a membrane-bound C-terminal fragment the same as before, and a soluble N-terminal fragment termed as beta amyloid. Though beta amyloid is

necessary for neuronal function, it can gather in the extracellular space of the brain, where it can be deposited to form amyloid plaques. Beta amyloid can exert poisonous effects on neuronal and synaptic function, eventually causing neuronal cell death. In addition, beta amyloid is supposed to do oxidative damage to cells. The body is unable to break these clusters and dissolve them, so they gather in the brain. (Alz.org, 2011)

The apoE4 (apolipoprotein E4) gene which is a genetic defect has been involved in Alzheimer's disease, may be associated in the formation of amyloid plaques. The gene may yield a protein that latches on the lethal beta amyloid and makes it difficult for the body to dissolve. As a consequence, the beta amyloid gathers as plaques in the brain. Molecules termed as free radicals may also show a role. Usually, free radicals show vital roles in the body, such as boosting the immune system and fight with disease. But, too many free radicals may initiate to upset the desired balance within a neuron. Nerve cells generating beta amyloid appear to make more free radicals. May be the free radicals stimulate beta amyloid formation. (Catherine, 2006)

1.3.2 Tau hypothesis

Tau is the key microtubule associated protein (MAP) of a mature neuron. The principle biological activity of tau is to boost association of microtubule and to stabilize structure microtubule. This biological function of tau is controlled by its degree of phosphorylation.

Usual mature human brain tau consists of 2–3 moles phosphate/mole of tau protein. Hyper phosphorylation of tau lowers the biological activity of tau. (kopke et al, 1993)

Phosphorylated tau is not capable to attach microtubules and as an alternative polymerizes with another tau molecules results in the formation of straight filaments which consequently become combined helical filaments resulting in the formation of neurofibrillary tangles. This can be the reason of the failure of neuronal transport that ultimately leads to death of cell. (Mi and Johnson, 2006, Wang et al, 2007)

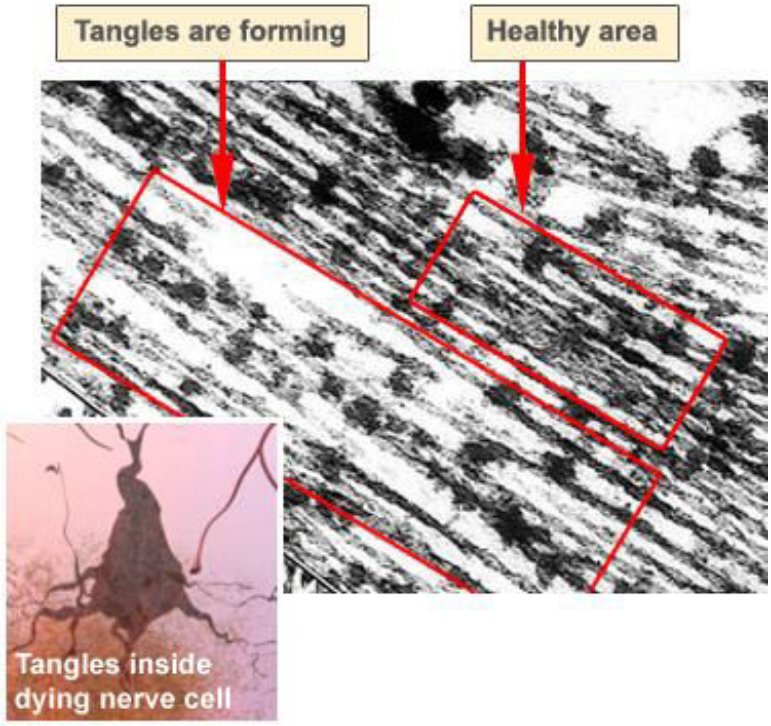

Figure1.4: Formation of tau tangles

In addition to irregular hyper phosphorylation, tau also go through various other abnormal post-translational modifications. (Gong et al, 2005)

Neurofibrillary tangles are a type of intracellular clusters of the hyper phosphorylated microtubule related protein, tau. In usual healthy neurons, tau steadies microtubules that is responsible for the formation of cytoskeleton of the cell by a procedure involving phosphorylation and dephosphorylation of the protein. (Combo et al, 2011)

Tangles are mainly abundant in the regions of the brain such as entorhinal cortex, hippocampus, amygdala, association cortices of the frontal, temporal and parietal lobes and certain subcortical nuclei that project into these areas. The quantity and circulation of cortical tangles associates positively with cognitive disorders and termed as a good marker of disease development. (Seelkoe and Schenk, 2003, Braak and Braak, 1998)

Tangles terminate a crucial cell transport system that is made of proteins. This electron microscope picture describes a cell having some healthy regions and other regions where tangles are developing. In healthy regions, the transport system is arranged in systematic parallel strands. Food particles, cell fragments and other key ingredients travel along the "tracks." A protein named tau supports the tracks to remain straight. In regions where tangles are developing, tau ends into abnormal and twisted strands

termed as tangles. The tracks cannot remain straight. They fall apart and split. Nutrients and other important supplies cannot travel through the cells, which ultimately results in death. (Alz.org, 2011)

1.3.3 Calcium channel hypothesis

Calcium plays an important part in neuronal physiology by extensive and small term regulation. Small or short term regulation of function is by neurotransmitter release originated from the presynaptic nerve terminals and the extensive or long term regulation is by long term potentiation (LTP) and long term suppression forms of synaptic plasticity in the regulation of memory. (Orsini and Maren, 2012, Nikoletopoulou and Tavernarakis, 2012)

The neurons associated with learning and memory are top susceptible regions for deterioration. The memory processing passageways such as long term potentiation as well as long term depression forms of synaptic plasticity are affected principally due to extreme release of calcium that leads to lethality of the cells by excitotoxicity. Calcium regulation is identified as the main significant pathway associated with the pathophysiology of Alzheimer's disease.

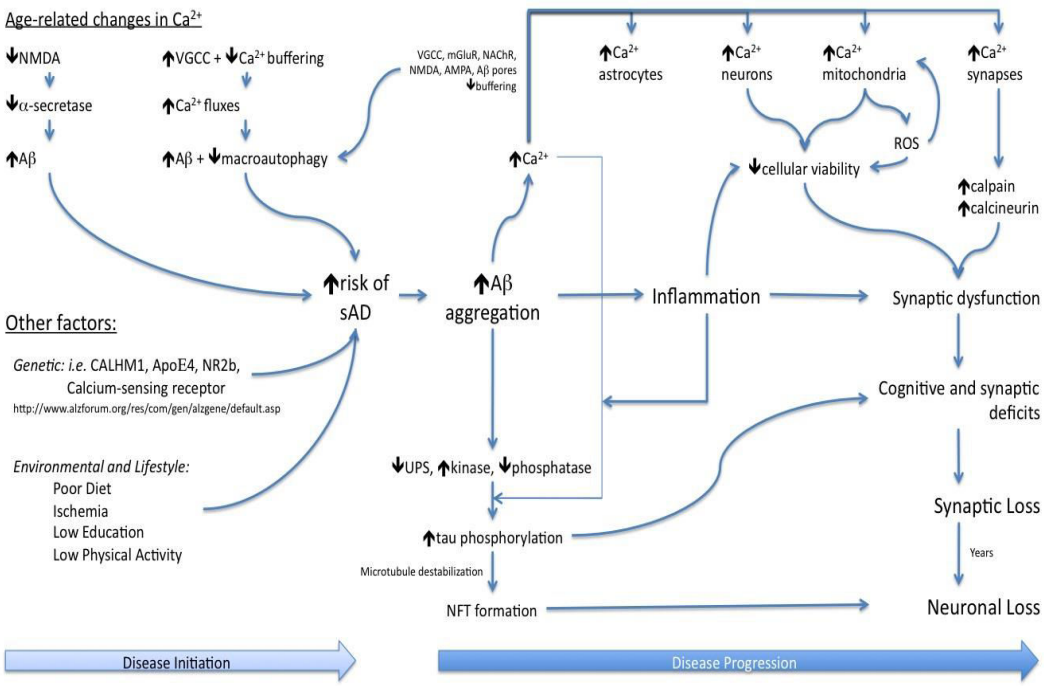

Figure 1.5: Calcium hypothesis

It has been stated that cytotoxicity to neurons was increased by hyper phosphorylated tau which was found in neurofibrillary tangles. Proofs indicate that tau phosphorylation is firmly regulated and appears to be calcium dependent. (Gonzalez et al, 2011)

It was also stated that total dephosphorylation or phosphorylation of tau is reliant on the rate or extent of calcium influx. (Stutzmann and Mattson, 2011)

This neuro degeneration prompted by beta amyloid or tau was regulated by the changes in calcium homeostasis. Beta amyloid interrupts with calcium homeostasis possibly by two main mechanisms. They consist of the formation of calcium conducting pores as well as formation of reactive oxygen through gathering of oxidative stress. It is also well known that beta amyloid can form a new calcium conducting pore in the lipid bilayers which provides a passage by which uncontrolled calcium uptake may take place in the strongly regulated intracellular calcium environment. (Yu et al, 2009)

1.3.4 Acetyl cholinesterase hypothesis

Signals which form memories and opinions pass by a single neuron as a small electrical charge. Neurons connect to each other at synapses. When a charge goes to a synapse, it may activate release of small clusters of chemicals termed as neurotransmitters. The neurotransmitters go through the synapse which carry signals to other cells. Researchers have recognized dozens of neurotransmitters. Alzheimer's disease interrupts with both the method by which electrical charges move within cells and the function of neurotransmitters. (Alz.org, 2011)

Nerve cells in the brain communicate with one another by releasing chemicals. These chemicals are termed as neurotransmitters. Acetylcholine is a vital neurotransmitter for memory. Individuals with Alzheimer’s disease show little amount of acetylcholine in their brain. Enzymes termed as cholinesterase breakdown acetylcholine in the brain.

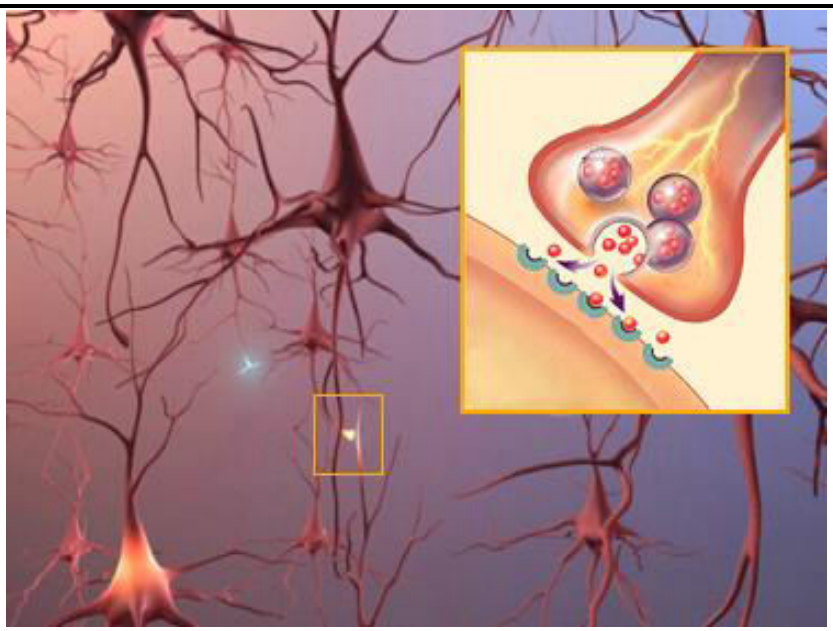

Figure 1.6: Cholinesterase hypothesis

A decrease in the quantity of acetyl cholinesterase receptors in the cerebral cortex of patients with Alzheimer's disease has been identified. Both nicotinic receptors and muscarinic acetylcholine receptors are reduced in brains of Alzheimer's affected brain. Irregularities in the nicotinic receptor are strictly related with primary histo pathological changes such as amyloid plaques and neurofibrillary tangles. (Kihara and Shimohana, 2004)

1.4 Treatment based on different hypothesis

1.4.1 Beta amyloid

The beta and gamma secretase enzymes yield the beta amyloids from the amyloid precursor protein, it is rational to aim these enzymes by numerous inhibiting or modulating drugs. But beta secretase functions on various proteins in the human body, many undesirable side effects can be expected from drugs that block beta secretase. Examples of these type of drugs include rosglitazone and pioglitazone. These are drugs used for treatment of diabetes. In theory, these drugs decrease the amounts of beta secretase and APP by stimulating their degradation by the cells. Rosiglitazone could not enhance memory or entire function in people having mild Alzheimer's disease and this drug has been prohibited. So, several new beta secretase blockers are being announced.

Gamma secretase is a vital objective of treatment, because it is the terminal step in formation of beta amyloid. There are numerous gamma secretase blocking drugs such as semagacestat that undergone to clinical testing.

There is a widespread history of medications that have been verified to block the aggregation of beta amyloid. Examples of these drug include tramiprosate, clioquinol, scylloinositol, and epigallocatechin-3-gallate. These drugs are in different stages of clinical testing. (Seeman and Seeman, 2011)

1.4.2 Tau protein

Because tau irregularities are important to neurofibrillary deterioration, tau protein might be a potential goal for emerging disease-modifying treatment for Alzheimer's disease. The healing approaches may consists of the following. (Ferrer et al, 2005, Mazanetz and Fischer, 2006)

1.4.2.1 Inhibition of abnormal tau hyper phosphorylation

Lithium, aloisines, hymenialdisine, flavopiridol, paullones and staurosporine, are going through active research and development, these are classified as Glycogen Synthase Kinase-3b Inhibitors. Roscovitine, flavopiridol, olomoucine, aloisines and indirubins are classified as Cyclin-Dependent Protein Kinase 5 Inhibitors. (Churcher, 2006)

1.4.2.2 Inhibition of tau misfolding or aggregation

Clinical trials have been conducted to discover small-molecule composites which are capable to block tau polymerization as another method to treat Alzheimer's disease. These compounds include methylene blue, N774, daunorubicin, Nphenylamines, phenylthiazolyl-hydrazides, rhodanines, exifone, quinoxalines and aminothienopyridazines. (Brunden et al, 2009)

1.4.2.3 Increasing clearance of tau

It has been revealed that treatment mutated tau with rapamycin show effects in a decrease of insoluble tau and related toxicity. (Berger et al, 2006)

1.4.3 Calcium channel

According to the proofs and informations it is documented that the extreme levels of intracellular calcium is toxic to the cell and it is possibly performing as a neurotoxin

troubling the total calcium homeostasis. Various number of drugs aiming calcium signaling passageways have been established, some drugs have been effective in clinical trials. Examples of drug include memantine, dimebon, Nimodipine. (Emre et al, 2008, Doody et al, 2008 Lopez and Birks, 2002)

All these aim at plasma membrane calcium channels instead of intracellular signaling passageways that seem to be the major focus of calcium dis regulation in Alzheimer's disease. The rational therapeutic method is to retain Ca2+ homeostasis and block calcium from getting entry to the cell. Some of the features for regulating calcium homeostasis include blocking calcium channels, glutamate receptors and intracellular calcium release. (Babu and Ramanathan, 2011, Rammes et al, 2011, Frandsen and Schousboe, 1991)

Blocking of voltage gated Ca2+ channels gives neuroprotection by channel blockers like Isradipine. These calcium channel blockers reduces the availability of the Ca2+ at the sites and because of this feature, the drugs might be neuroprotective. (Anekonda et al, 2011)

1.4.4 Acetyl cholinesterase

Cholinesterase inhibitor drugs block enzymes so that they cannot break down acetylcholine when it goes from one cell to another cell. So the acetylcholine that is in less supply in individuals having Alzheimer's disease, is not damaged so rapidly. So there is high chance of it being go to the following neuron. Cholinesterase inhibitors result in greater concentrations of acetylcholine which leads to improved communication among neurons. It may briefly improve and stabilize the symptoms of the disease. The regions in which some individuals with Alzheimer's disease may find development are: capability to think unmistakably, memory, daily activities, social and psychological symptoms. Memantine can be a drug of choice for treatment of Alzheimer's disease. (Parsons et al, 2013)

There are presently three acetyl cholinesterase enzyme inhibitors named donepezil, rivastigmine and galantamine. These drugs have been accepted by the Food and Drug Administration in Canada for the cure of mild to moderate Alzheimer's disease. Donepezil is the most extensively prescribed by doctors. (Doody et al, 2011)

The drug named tacrine can also be used. (Eagger et al, 1991)

1.5 General treatment of Alzheimer's disease

Alzheimer's disease is difficult to understand and it is not likely that any one drug or other medication can effectively treat it. Recent methods give emphasis on helping persons retaining mental function, cope up with communication problems and delay the symptoms of disease. Scientists hope to progress therapies aiming at definite genetic and cellular mechanisms so that the authentic fundamental reason of the disease can be stopped or prohibited.

- **Maintaining Mental Function**

Various medications are permitted by the U.S. Food and Drug Administration in the treatment of symptoms of this disease. Donepezil, rivastigmine and galantamine are used to treat mild to moderate stages (donepezil can be used for severe Alzheimer's disease). Memantine is the drug of choice to treat moderate to severe stages of Alzheimer's disease. These drugs work by maintaining neurotransmitters, the substances that transmit signals between neurons. They may help by managing thinking, memory and communication capabilities and aid with definite behavioral difficulties. However, these drugs can not affect the underlying disease procedure. They show effectiveness to some but not all persons and may help only for a narrow time range.

- **Managing Behavior**

Most common communicative symptoms of Alzheimer's include insomnia, wandering, anxiety, nervousness and violence. Researchers are trying to know why these symptoms appear and are learning new treatments to treat them. Data from research has shown that treating communicative symptoms can give comfort to patients and things become easier for caregivers.

- **Searching for New Treatments**

Alzheimer's disease study has reached to a fact where experts can look beyond treating symptoms to investigate about developing disease processes. In current clinical trials, researchers are emerging and experimenting numerous possible involvements, including immunization treatments, drug treatments, cognitive exercise and physical movement.

- **Support for Families and Caregivers**

Taking care of an individual with this disease can have great physical, mental and economic costs. The loads of daily care, changes in family responsibilities and conclusions about settlement in a care ability can be problematic. There are various approaches and programs that may help and scientists are continuing to find new and improved ways to support caregivers.

Caregivers should be well informed about the disease and its maintenance. Several television programs can help them.

Good surviving skills, a strong support system can help caregivers handle the pressure of caring for a valued one with Alzheimer's disease. For instance, staying physically dynamic provides physical and mental benefits. Some caregivers may join with a support group.

1.6 New approaches for treatment of Alzheimer's

Researchers are doing investigations to know more informations about plaques, tangles and other biological factors of Alzheimer's disease. Developments in brain imaging methods allow scientists to understand the growth and spread of atypical amyloid and tau proteins in the alive brain and changes in brain construction and tasks. Researchers are also discovering the very initial phases in the disease progression by learning changes in the brain and biological fluids that can be spotted years before Alzheimer's symptoms seem. Results of these investigations will help in learning the causes as well as make diagnosis easier. (Alzheimer's Disease education and referral center, 2016)

1.7 Plants used in the treatment of Alzheimer's disease

1.7.1 Ashwagandha (*Withania somnifera*)

Ashwagandha is used broadly in Ayurveda as a nerve stimulant, aphrodisiac and helps the body become accustomed to tension. (Mishra et al, 2000, Wollen, 2010)

Ashwagandha is a member of the family named nightshade (Solanaceae) and the root is extensively used. It is characterized as a rasayana (rejuvenative) and is supposed to have antioxidant activity as well as free radical scavenging activity. (Russo et al, 2011)

Ashwagandha has a relaxing outcome and thus may be selectively used in patients with Alzheimer's disease. (Monograph, 2004)

Ashwagandha consists of steroidal composites which are great curiosity to scientists, such as the ergostane-type steroidal lactones which includes withanolides A to Y. (Matsuda, 2001)

Figure 1.7: ***Withania somnifera***

Withanamides has been revealed to scavenge free radicals produced during the beginning and evolution of Alzheimer's disease. Neuronal cell death caused by amyloid plaques was also terminated by withanamides. (Dhuley, 1998)

Ashwagandha has been stated to increase memory and knowledge. (Parihar and Hemnani, 2003, Tohda et al, 2005)

1.7.2 Brahmi (*Bacopa monnieri*)

Brahmi which is also identified as Bacopa is an unpleasant-tasting plant originated in humid and muddy areas and is normally used in Ayurvedic remedy as a nerve stimulant, diuretic and cardio tonic as well as a healing mediator against epilepsy, sleeplessness, asthma, and rheumatism. (Kumar, 2006)

Brahmi could function by reducing divalent metals, scavenging responsive oxygen species, reducing the development of lipid peroxides and preventing lipoxygenase activity. (Dhanasekaran,2007)

Conventionally, Brahmi was used to increase memory and intellectual function. (Stough et al, 2008)

Figure 1.8: ***Bacopa monnieri***

Brahmi also declined cholinergic degeneration and showed a cognition-improving outcome in a rat model of Alzheimer's disease. (Uabundit et al, 2010)

1.7.3 Shankhpushpi (*Convolvulus pluricaulis*)

Shankhpushpi is an easily found plant in India. The entire plant is used in numerous formulae as a nerve stimulant for development of memory and mental function. (Bihaqi et al, 2009, Malik et al, 2011)

An extensive variety of minor metabolites which include triterpenoids, flavonol glycosides, anthocyanins and steroids has been insulated and may be accountable for Shankhpushpi's memory-improving functions. (Jain et al, 2003)

It is supposed that Shankhpushpi relaxes the nerves by controlling the regulation of the body's production of the stress related hormones which are termed as adrenaline and cortisol. (Sethiya et al, 2009)

Figure 1.9: ***Convolvulus pluricaulis***

According to a study, the ethanolic extract of this plant and its ethyl acetate and aqueous fractions meaningfully enhanced learning and recalling power in rats. (Nahata et al, 2008)

1.7.4 Gotu kola (*Centella asiatica*)

In the Ayurvedic system of medication, gotu kola is one of the significant reviving plants for nerve and brain cells. This plant is thought to be capable of increasing brainpower, endurance and memory. It was shown to decrease hydrogen peroxide-induced death of cells, reduction in free radical concentrations and prevent beta-amyloid cell death. These in vitro tests, suggested a probable role for gotu kola in the

management and inhibition of Alzheimer's disease and beta-amyloid toxicity. (Dhanasekaran et al, 2009)

Figure 1.10: ***Centella asiatica***

Extracts of Gotu kola inverted the beta-amyloid pathology in the brains of mice and moderated the constituents of the oxidative stress reaction. (Rocha et al, 2011, Veerendra and Gupta, 2003, Xu et al, 2008)

1.7.5 Jyotishmati (*Celastrus paniculatus*)

Jyotishmati is a precious medicinal plant which is valued for its effects on the brain. It has been used for eras for improving the memory and enlightening concentration and mental function. (Bhanumathy et al, 2010)

According to a study, aqueous extracts of these seeds have cognition-improving function and antioxidant properties. These extracts sheltered neuronal cells against H2O2-tempted toxicity by virtue of their antioxidant functions and their ability to increase antioxidant enzymes.

Figure 1.11: ***Celastrus paniculatus***

The extracts sheltered neuronal cells by free radical scavenging properties as well as by reducing lipid peroxidation. Aqueous extracts of these seed have cholinergic activity which is dose dependent, thereby enhancing memory performance. (Godkar, 2004)

Chapter 2: Plant and Literature Review

2.1 *Diospyros blancoi*

Diospyros blancoi (*D. blancoi*) is also termed as mabolo or velvet apple. This plant is a member of the family named Ebenaceae. It is more appreciated for its attractive than its appetizing value, *Diospyros blancoi* has seemed in writings for numerous years under the illegal binomial Diospyros discolor Willd. In 1968 a scientist named Dr. Richard Howard who was Director of the Arnold Arboretum, Harvard University, suggested the adoption of *Diospyros blancoi*. The fruit is termed in India as peach bloom. In Malaya, it is called buah mantega (butter fruit) or buah sakhlat, or sagalat (scarlet fruit). Mabolo is the most common of the numerous Philippine dialectal names.

Figure 2.1: ***Diospyros blancoi***

2.1.1 *D. blancoi* Description

Diospyros blancoi differs in form ranges from a tiny messy tree with relaxed branches to a straight tree to 60 or 100 ft (18-33 m) with plucky, dark, uneven stem to 50 in (80 cm) thick. It grows slowly. The evergreen, substitute leaves, four-sided figure, pointed at the top, rounded at the base.

Figure 2.2: Bark of *Diospyros blancoi*

Leaves are 6 to 9 in (15-22.8 cm) in length, 2 to 3 1/2 in (5-9 cm) in width; fibrous, dark-green in color, even and shiny on the higher surface, silvery-hairy underneath. The new leaves are showy, pale-green or pink and silky-hairy. The cylindrical, 4-lobed, waxy, slightly perfumed blooms are short-steamed, creamy-white in color, downy. Male flowers are 1/4 inch (6 mm) in width and female flowers are 1/2 inch in (12.5

mm) width and these are borne on distinct trees. Eye-catching and curious, the egg-shaped fruit are 2 to 4 inch (5-10 cm) in width. It has thin and colorful such as pink, brownish, yellow, orange or purple-red skin. The skins are thickly covered with little, golden-brown or coppery hairs. It is topped at the base with a dull-green colored, rigid calyx. The fruits are frequently borne in sets, very adjacent together on reverse sides of a branch.

Figure 2.3: Leaf of *D. blancoi*

A firm, nasty, cheese-like scent is given off by the entire fruit but originates from the skin. The fruits are wet but not very juicy having minor, additional or less sweet flavor, signifying a banana-flavored apple. There can be 4 to 8 brown, even, wedge-shaped seeds which are about 1and a 1/2 inch (4 cm) in length and 1 inch (2.5 cm) in width which stands in a sphere around the central core. Sometimes the fruits are totally seedless. Every seed is shielded with a whitish skin that is translucent when fresh, smoky when dried.

2.1.2 Origin and Distribution

The *Diospyros blancoi* is native to the low and average height wooded area of the Philippine Islands from the island of Luzon to the southernmost of the Sulu Islands. This is usually cultured for its fruit and as a shadow tree for roadsides. The tree was familiarized into Java and Malaya. In 1881 it was introduced into Calcutta and the Botanical Garden in Singapore but it was in Singapore previously that date. In current times, it has been diminishing in numbers in Malaya. It is only seldom planted in India and mostly as an ornamental because of the charm of the flora and the fruits.

There are infrequent varieties grown somewhere else in southern Florida and some distributed around the Caribbean area, in Jamaica, Cuba, Trinidad and Puerto Rico. (Morton, 1987)

2.2 Medicinal uses of *Diospyros blancoi*

- **Hypertension and Heart Health**

The excess potassium contained in *Diospyros blancoi* suggests that the fruit can work as a vasodilator. It relaxes the vessels of blood, lessens stress on the heart and decreases blood pressure. The nutritional fiber content can reduce cholesterol. If these two components are combined, it can significantly decrease atherosclerosis, clots of blood, heart attacks as well as strokes.

- **Circulation**

The meaningful amount of iron in *Diospyros blancoi* stimulates the red blood cell amount in the body. It also increases oxygenation of vital tissues and muscles, stimulating the development of hair, increasing the rapidity of the therapeutic process of cells and increasing metabolic efficiency.

- **Respiratory Conditions**

In ancient medicine, these fruits have been frequently used to relieve coughs, chest blocking and asthma. It is perhaps due to the large amount of vitamins and minerals which have also impact on improving the immune system.

- **Immune System Strength**

The large amount of vitamin C as well as vitamin A stimulate the immune system of the body by functioning as antioxidants, eradicating damaging free radicals which are capable of mutating or destroying normal healthy cells. These two vitamins stop premature aging, long-lasting diseases, boost cellular development and growth and develop the health and skin.

- **Digestive Health Improvement**

The dietary fiber in *Diospyros blancoi* aids to ease the opening of food to the digestive tract, which diminishes constipation and other gastrointestinal problems. In ancient medicine of the Philippines, *Diospyros blancoi* were trusted to treat dysentery and diarrhea possibly its most common use in human health.

- **Reducing Skin Irritation**

When topically applied, the pulp of *Diospyros blancoi* has revealed amazing capability to lessen inflammation and irritation on the skin. It is frequently turned to in additional medicine as the firmest way to set right the skin conditions and burns. Additionally, *Diospyros blancoi* pulp and juice are applied on snakebites as well as other toxic invasions in the body, counteracting toxins and helping to decrease the effects of these problems. (Organic facts, 2016)

2.3 Literature Review

Khalipha et al. said that *D. blancoi* is rich is flavonoids, tannins, gum sugars and alkaloids. This plant showed antioxidant activity which was determined by DPPH free radicals scavengic activity test. This study also discovered the anti-diarrheal property of this plant. (Khalipha et al, 2012)

Ragasa et al. said that *D. blancoi* showed analgesic property. By conducting further studies, this plant can be used as a source of analgesic drug. (Ragasa et al, 2009)

Lee et al. said that extracts of *D. blancoi* showed the capability of decreasing inflammation of airway passages. This study was successfully conducted in mice model. The success of this study supports the further study of this plant for the treatment of asthma. (Lee et al, 2012)

Howlader et al said that ethanoic extracts of leaves of the plant *D. blancoi* posseses antimicrobial and cytotoxic property. This study suggests further progress of the research by using this plant. (Howlader et al, 2012)

Ahn et al. suggests that extracts of *D. blacoi* showed anti-inflammatory activity by blocking nuclear translocation of NF-κB, whose level is rapidly elevated in response to inflammatory stimuli. In addition, this plant can inhibit the production of eosinophills which is a mediator of allergic response. So, by blocking these mediators, *D. blancoi* can play a significant role in the treatment of various allergic response. (Ahn et al, 2014)

Khan et al. described that the plant extracts of *D. blancoi* was effective in the free radical scavengic activity test. Sit indicates that this plant has antioxidant potential. This plant showed moderate activity as an anti-cancer agent. (Khan et al, 2016)

Ningsih et al. conducted a study on *D. blancoi* and found that this plant can be beneficial for skin. This can be a potent inhibitor of skin aging process. (Ningsih et al, 2013)

Akter et al. conducted acetic acid-induced writhing and tail immersion test on the aerial parts of *D. blancoi* in order to identify the analgesic property. The results of this study suggests that this plant may have mild analgesic property. (Akter et al, 2015)

Akter and sarker suggested that *D. blancoi* is used as the remedy for diarrhea, dysentery, fever, itchy skin, cough and wounds. To discover the possible antimicrobial activity of the seeds the fruits, methanol extracts were prepared as well as tested against 10 pathogenic gram positive and gram negative bacteria. These are conducted by using disc diffusion method. Broth dilution assay was used to determine the minimum inhibitory concentration (MIC) of the extracts. The extract of D. blancoi showed significant antimicrobial activity against all of the tested bacteria. (Akter et al, 2015)

Recio et al. stated that three triterpenes were obtained from *Diospyros leucomelas* and discovered as betulin, betulinic acid, and ursolic acid. They exhibited anti-inflammatory activity in the carrageenan and serotonin paw edema tests and TPA and EPP ear edema tests. (Recio et al, 1995)

Nabavi et al. said that recent research into free radicals has assured that foods rich in antioxidants play an important role in the inhibition of many diseases. The potential antioxidant activities of *Diospyros lotus* fruits was studied using six in vitro assay systems. This plant showed high total phenolic and flavonoid contents. (Nabavi et al, 2009)

Han et al. said that *Diospyros kaki* folium has been used traditionally in Korea to support maternal health. The scavenging activity against DPPH (1,1-diphenyl-2-picrylhydrazyl) radicals of the methanoic extracts of the plant was investigated. The extract of *D. kaki* was found to be the very potent. (Han et al, 2002)

Antioxidant activity of methanoic extract of *D. malabarica* bark was studied by Mondal et al. for its free radical scavenging property on different *in vitro* models e.g. 1,1-diphenyl-2-picryl hydrazyl (DPPH), nitric oxide, superoxide, hydroxyl radical and lipid peroxide radical model. The extract exhibited good dose-dependent free radical scavenging property in all the models without the hydroxyl radical inhibition assay. The results specify that the antioxidant property of the extract might be due to the high content of phenolic compounds. (Mondal et al, 2006)

In vitro biological activities which includes phytotoxic, antifungal activities as well as acute toxicity of the methanoic extract, fractions and/or isolated compounds from the stem bark of *Diospyros canaliculata* were identified by Dzoyem et al. Based on the recent findings, we can say that this extract is nontoxic with significant phytotoxic as well as antifungal activities because of the presence of plumbagin. (Dzoyem et al, 2011)

Achiwa et al. have investigated the effects of *Diospyros kaki* extract (PS) on the growth of human lymphoid leukemia Molt 4B cells. They found that PS strongly inhibited the growth of the cells which was in a dose-dependent manner. The results suggest that PS can induce apoptosis (programmed cell death) of Molt 4B cells. (Achiwa et al, 1997)

Dewanjee et al. suggested that *Diospyros peregrina* is an edible fruit of costal West-Bengal. The present research was done to evaluate the role of aqueous extract of *D. peregrina* fruit in streptozotocin–nicotinamide induced type 2 diabetic rats. The diabetic rats was found to possess important dose dependant hypoglycemic and hypolipidemic activity. (Dewanjee et al, 2009)

Adeniyi et al. said that the petroleum ether, chloroform, methanol, and water extracts of different parts of the plants such as leaves, stem bark, and root of *Diospyros mespiliformis* were investigated for their antimicrobial activities. The crude extracts showed broad spectrum antimicrobial activities against nine Gram-positive bacteria, eight Gram-negative bacteria, and six fungal strains. Of the four extracting solvents, chloroform produced extracts with the best antimicrobial activities, while the chloroform extract of the root exhibited the highest antimicrobial activity. (Adeniyi et al, 1996)

Trongsakul et al. conducted harmacological studies with the hexane extract of the dry stem of *Diospyros variegata* Kruz. (Ebenaceae) on experimental animals for evaluating the analgesic, antipyretic and anti-inflammatory activities. The results of the tests were positive. (Trongsakul et al, 2003)

Adeniyi et al. said that two dimeric naphthoquinones, diospyrin and isodiospyrin, obtained from the root of *Diospyros piscatoria* (Gurke), a common ingredient in several folk medicines, have been shown to have a broad spectrum of antibacterial activity. (Adeniyi et al, 2000)

Chen et al. suggested that different tests proved the radical scavenging activity of *Diospyros kaki*. (Chen et al, 2008)

Chapter 3: Materials and Methods

3.1.1 Collection of Plant Materials

The leaf and bark of the plant were collected from Barisal which is a district of Bangladesh, in February, 2016.

3.1.2 Preparation of Plant Materials

Initially, the leaf and barks were washed with water to eradicate sticky dirt and was cut into pieces. Then leaf and barks were subjected to shade dry for numerous days with random sun drying. These samples were then dried in an oven for a day at significantly low temperature in order to better grinding.

Figure 3.1: Powder of leaf of *D. blancoi*

The dried leafs and barks were crushed into granular powder by a crushing machine.

Figure 3.2: Powder of bark of *D. blancoi*

3.1.3 Extraction of the plant materials

Grinded plant materials (leafs and barks) were taken in an amber colored reagent bottle. Weight of powdered leafs were 400gm and weight of powdered barks were 1kg. The total amount of leaf was soaked in 2.0 liter of methanol and the total amount of bark was soaked in 3 liter of methanol.

The bottle with its ingredients were closed and reserved for a period of about 10 days with random shaking and stirring. The entire mixture was then filtered in three steps. Initially, these was filtered through cloth and then through cotton and finally through Whatman No.1 filters paper. The mixture was concentrated with a machine named rotary evaporator under little pressure at 50°C temperature to afford crude methanoic extract (CME).

3.1.4 Solvent-solvent partitioning of crude extract

52.74 gm of the concentrated methanoic extract was fractionated by modified Kupchan method and the obtained fractions that are petroleum ether (PET, 4.07 gm), chloroform (CLF, 2.93 gm) fractions were gained and used for the purpose of experiment.

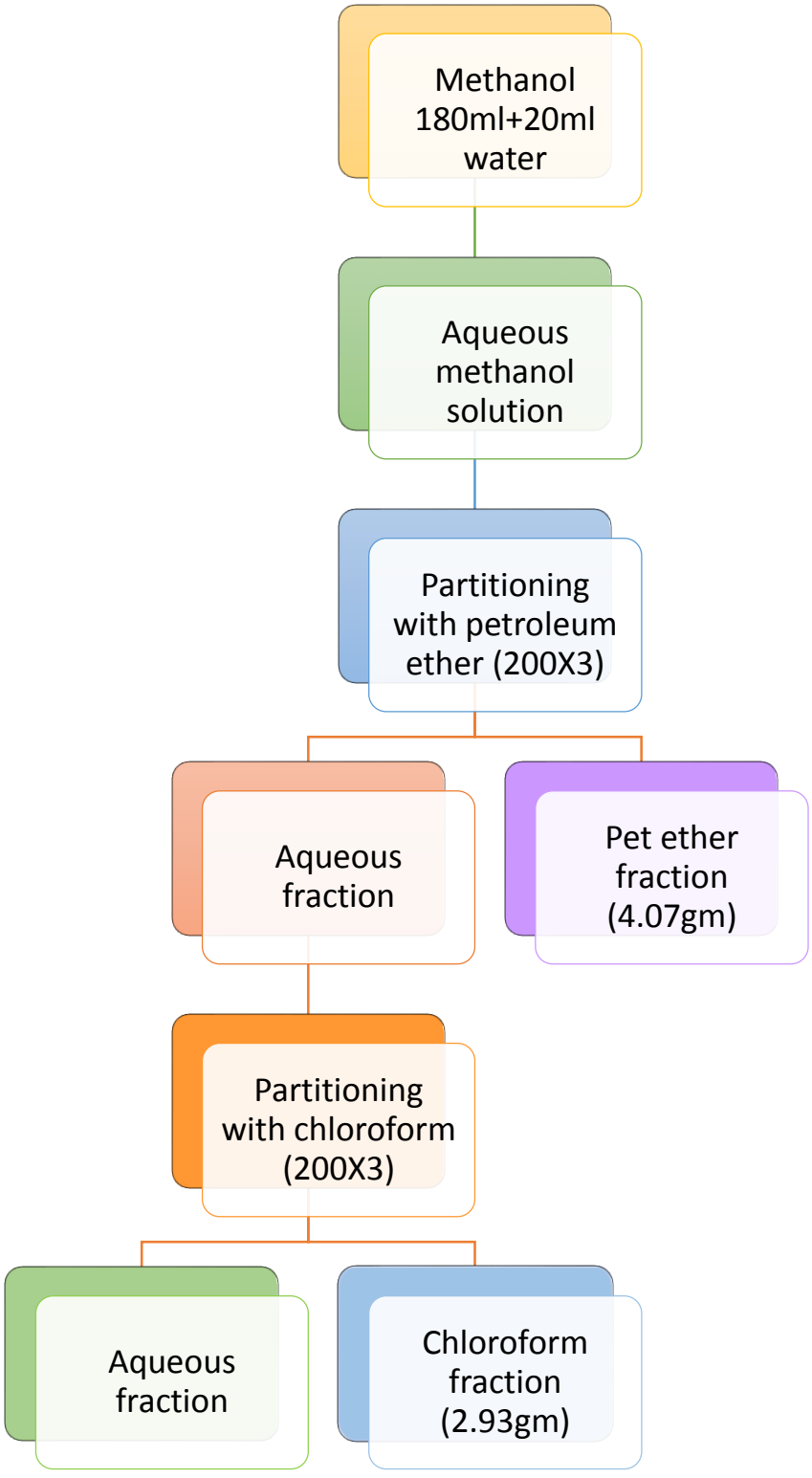

Figure 3.3: Schematic representation of solvent-solvent partitioning of crude extracts of *D. blancoi* bark.

Figure 3.4: Solvent solvent partitioning

Table 3.1: Different fractions with amount obtained from the methanol extract of plants

Name of the fractions	Weight of the fractions (gm)
Leaf (CME)	25.5
Bark (CME)	52.74
Bark (PET)	4.07
Bark (CLF)	2.93

3.2 Total Phenolic Content Determination

Total phenolic content of the various extracts of *D. blancoi* were determined by following the method as illustrated by Singleton in 1965 which involves Folin-Ciocalteu reagent as oxidizing agent and catechin as standard.

3.2.1 Principle

The amount of total phenolic compounds of various fractions in the plant was identified by Folin–Ciocalteu Reagent (FCR). The FCR is the parameter of measuring reducing capacity of a sample. The accurate chemical nature of the FC reagent is unknown, but it is assumed to contain heteropolyphosphotungstates–molybdates. Sequences of not irreversible one or two-electron reduction reactions turns into to blue species, probably (PMoW11O40)4. It is assumed that the molybdenum is at ease to be reduced in the complex and electron-transfer reaction takes place between reductants and Mo (VI)

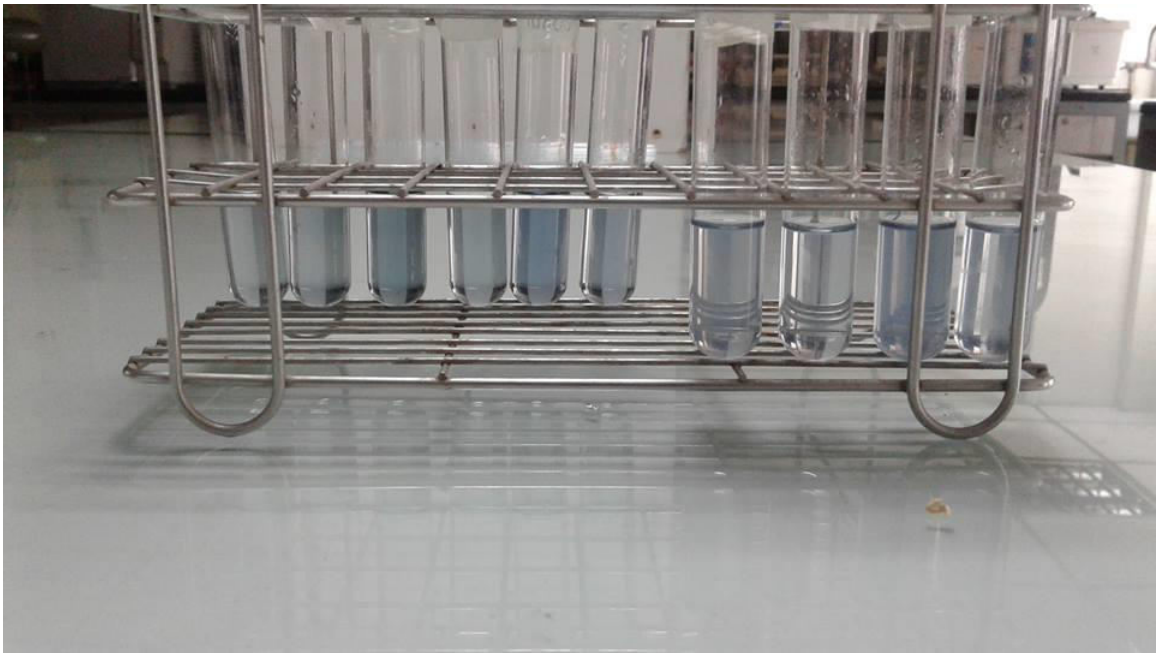

Figure 3.5: Total phenolic content determination

3.2.2 Materials

- ✓ Folin – ciocalteu reagent (Sigma chemical company, USA),
- ✓ Sodium carbonate (Sigma chemical company, USA),
- ✓ Methanol (Sigma chemical company, USA),
- ✓ Gallic acid (Wako pure chemicals Ltd., Japan),

- ✓ Micropipette (100-1000 µl),
- ✓ Pipette (1-10 ml),
- ✓ UV-spectrophotometer.

3.2.3 Procedure

The quantity of total phenolics in extract was discovered according to the Folin-ciocalteu procedure. Samples (300µl) were introduced into test tubes. 2.5mL of Folincio-calteu reagent as well as 2 ml of sodium carbonate (7.5%) were added gradually. These were mixed and allowed to stand for 2 hours. Absorbance was taken at 760 nm. The total phenolic amount was stated as Gallic acid equivalents (GAE) in milligrams per gram extract as calculated from standard Gallic acid graph by the given formula.

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

Where,

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

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

V = the volume of extract, ml;

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

3.3 Determination of Total Flavonoids Content

Total flavonoid content of the various extractives of *D. blancoi* was identified by aluminum chloride colorimetric method. Catechin was taken as standard and the amount of flavonoid content of the extracts was expressed as mg of catechin equivalent/gm of dried extract.

3.3.1 Principle

The amount of total flavonoid content in various extracts of plant was identified by the popular aluminum chloride colorimetric method. In this method, aluminum chloride

makes complex with hydroxyl groups of flavonoids which may be present in the samples. This complex has the maximum absorbance at 510 nm.

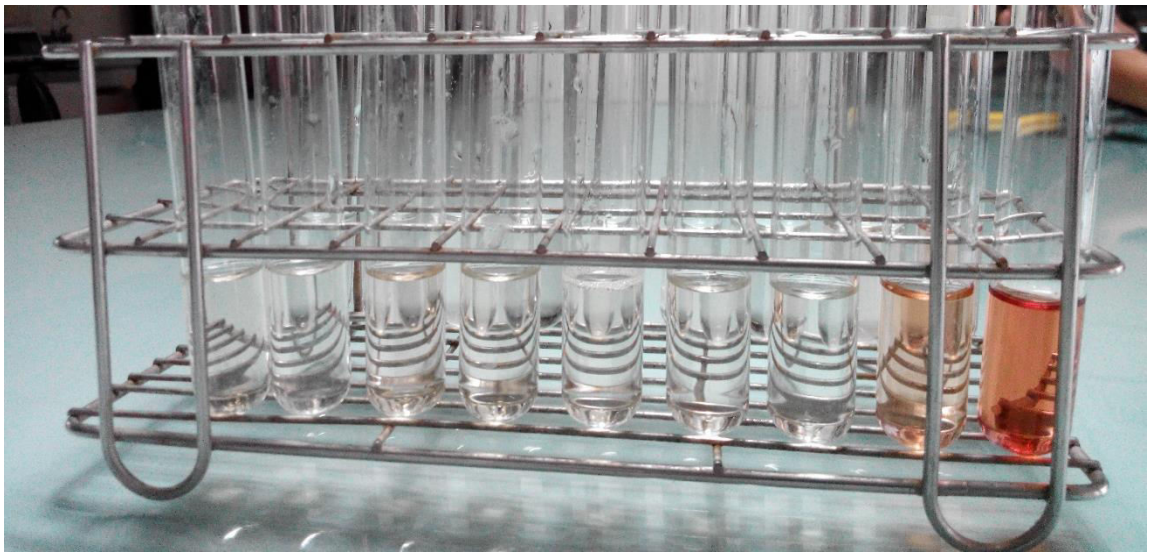

Figure 3.6: **Determination of total flavonoid content**

3.3.2 Materials

- ✓ Aluminum Chloride (Sigma chemical company, USA)
- ✓ 5% NaNO2
- ✓ 1 mM NaOH
- ✓ Methanol (Sigma chemical company, USA)
- ✓ Catechin (Wako pure chemicals Ltd., Japan)
- ✓ Micropipette (100-100 µl)
- ✓ Pipette (1-10 ml)
- ✓ UV-spectrophotometer

3.3.3 Procedure

Total flavonoid (TF) content was identified by using the procedure by Dewanto, Wu, Adom, and Liu. One milliliter of extract which contains 0.1 g/mL of dry matter was taken in a 10 mL volumetric flask. Then 2.5 mL of distilled water added and after that 0.15mL of 5% NaNO2 was added. After 5 minutes, 0.3 mL of 10% AlCl3 was added. Then after another 5 minutes, 1 mL of 1M NaOH was added with distilled water. The solution was mixed and absorbance taken at 510 nm. TF amounts were stated as catechin equivalents per dry matter.

The total amount of flavonoid compounds in plant extracts in catechin 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.

3.4 Total Flavanol Content Determination

Total Flavanol content of the methanol extract of *D. blancoi* is determined by a method named aluminum chloride colorimetric method. This test requires gallic acid was as standard. The flavanol content of the extractives was denoted by mg of Gallic acid equivalent/gm of dried extract.

3.4.1 Principle

The amount of total flavanols in methanoic extract of *D. blancoi* was determined by the popular aluminum chloride colorimetric method. In this process, aluminum chloride forms complex with hydroxyl groups of flavanols which may be present in the samples. This formed complex has the highest absorbance at 440 nm.

3.4.2 Materials

- ✓ Aluminum Chloride 2% solution (Sigma chemical company, USA)
- ✓ Sodium acetate 5% solution
- ✓ Gallic acid
- ✓ Micropipette (20-200 µl, 100-1000 µl)
- ✓ Pipette (1-10 ml)

- ✓ UV-spectrophotometer (Shimadzu, Japan)

3.4.3 Procedure

Total flavanol content was identified by using aluminum chloride. As a standard Gallic acid was used. Initially, 300 µl sample was taken from the stock solution. This was made upto 1ml by adding methanol. Then 1ml of 2% aluminium chloride solution which was made with ethanol is added with the sample. After that 1.5 ml 5% sodium acetate solution was added. This mixture was incubated at room temperature for 2.5 hours. And finally, absorbance was taken at 440 nm.

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

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

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.

3.5 DPPH (1, 1-diphenyl-2-picrylhydrazyl) Free Radical Scavenging Activity

DPPH was used to assess the free radical scavenging activity of numerous fractions.

3.5.1 Principle

The 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) has been extensively used to assess the free radical scavenging capability of antioxidants. DPPH free radical is reduced to

the hydrazine when reaction occurs with hydrogen donors. DPPH is able to make stable free radicals in solution of methanol or water. By this method it was possible to identify the antiradical power of an antioxidant activity by measuring the reduction in the absorbance of DPPH which was taken at 517 nm. This results in a change of color from purple to yellow. The absorbance reduced when the DPPH was scavenged by an antioxidant, through the 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.

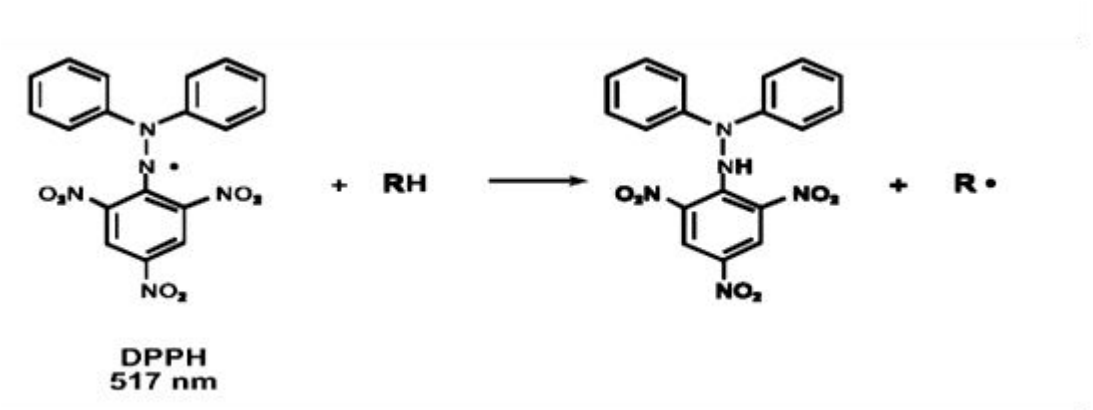

Figure 3.7: Mechanism of DPPH˙ with an antioxidant having transferable hydrogen radical.

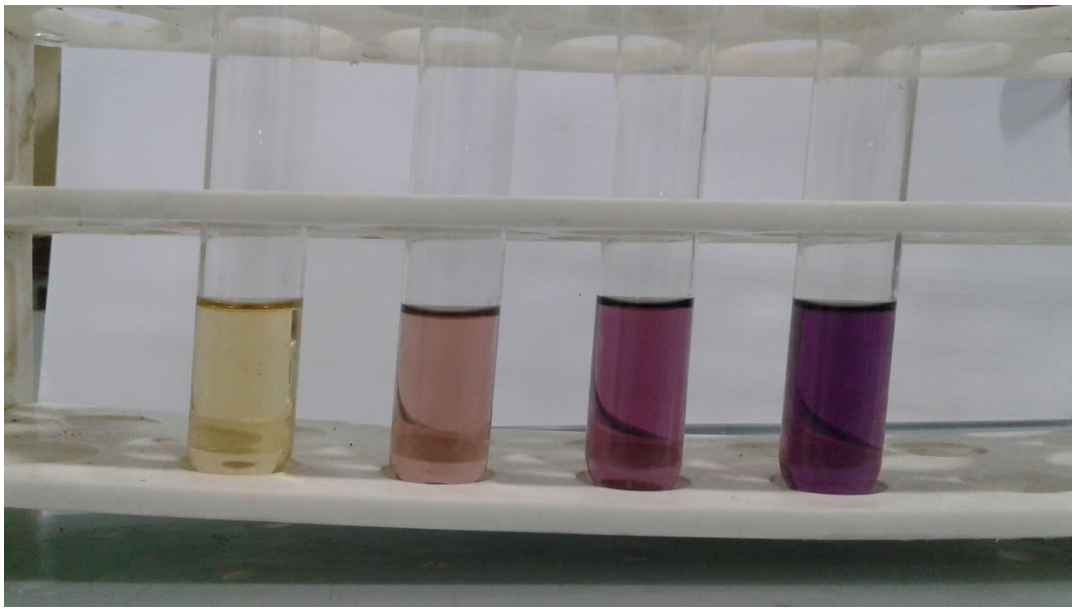

Figure 3.8: **DPPH free radicals scavenging activity**

3.5.2 Materials

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

3.5.3 Procedure

The free radical scavenging activity of the extracts of *D. blancoi* was identified based on the method described by Braca. Sample (20µL) was added to 3ml of a 0.004% methanol solution of DPPH. Absorbance at 517 nm was determined after 30 minutes and the percentage inhibition activity was calculated from the following formula

$$\mathbf{I\% = [(A0 – A1)/A0] \times 100}$$

Where,

I% is the percentage of scavenging activity

A0 is the absorbance of the control, and

A1 is the absorbance of the extract/standard.

Then % inhibitions were plotted against concentration and from the graph IC50 was calculated.

3.6 Assessment of Reducing Power Capacity

The reducing power of the different extractives from extracts of *D. blancoi* evaluated by the method of Oyaizu.

3.6.1 Principle

In this method, the yellow colored test solution changes to numerous shades of green and blue which depends on the reducing power of antioxidant samples. The reducing capacity of a compound might serve as an important indicator of its potential antioxidant activity. The presence of reductants such as antioxidant constituents in the

samples is the reason behind the reduction of the Fe3+-ferricyanide complex to the ferrous form by giving an electron. The amount of Fe2+ complex can then be observed by measuring the formation of Perl's Prussian blue at 700 nm.

Fe3+-ferricyanide + e-Fe2+-ferricyanide

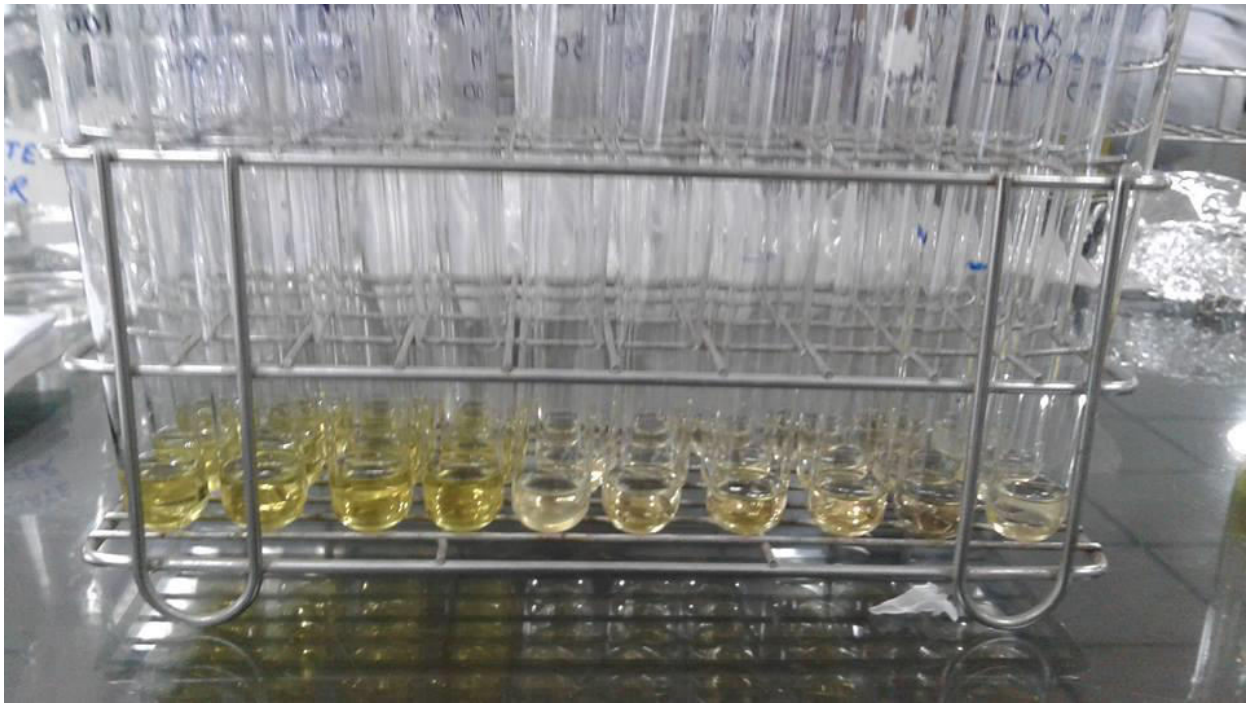

Figure 3.9: Assessment of Reducing Power Capacity

3.6.2 Materials

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

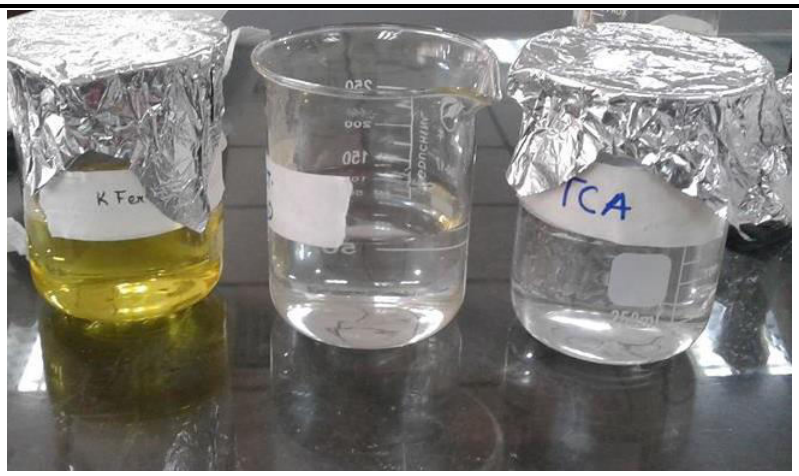

Figure 3.10: Materials of Assessment of Reducing Power Capacity

3.6.3 Procedure

Reducing power was examined by using the method developed by Oyaizu. A 2.5 mL fraction of *D. blancoi* was mixed with 2.5 mL of phosphate buffer (200mM, pH 6.6) as well as 2.5 mL 1% potassium ferricyanide. The mixture was located in a water bath for 20 minutes at 500C. The temperature of the resulting solution was decreased rapidly, mixed with 2.5 mL of 10% trichloroacetic acid and centrifuged at 3,000 rpm for 10 min. A 5.0 mL fraction from the supernatant was mixed with 5mL of distilled water and 1mL of ferric chloride. Absorbance of the resultant mixture was taken at 700 nm after 10 min. If the absorbance value is higher, the reducing power is stronger.

3.7 In-Vitro Acetyl Cholinesterase Inhibitory Studies

3.7.1 Purification of acetylcholinesterase enzyme

The main source of acetylcholinesterase enzyme is brain, muscle and the RBC. The enzyme was collected from blood. This source of enzyme needs 4 phase of purification for prepare to use.

3.7.2 Materials

- ✓ Blood,
- ✓ 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,
- ✓ Ice bath,
- ✓ Sephadex G-200 gel (Sigma chemical company, USA).

3.7.3 Formulation of reagents

- ✓ Wash buffer: 10mM Tris buffer.
- ✓ Extraction Buffer: 50mM Tris buffer + 10% Triton-X + 50mM MgCl2 + 50mM NaCl.
- ✓ DTNB: 0.7mM solution.
- ✓ ATCI: 0.35mM solution.

3.7.4 Procedure

3.7.4.1 Preparation of Crude enzyme extract

10gm bovine brain was weighted, cut into tiny pieces and grinned by a mortar and pestle with 50ml of homogenization buffer at pH 7.4. The temperature was kept 40C by placing ice in the outer compartment of the homogenizer. The suspension was sieved through double layer of muslin cloth in the icy room. The filtrate was gathered and clarified by centrifugation at 10000 rpm for 25 minutes and temperature was 40C. This non opaque supernatant was used as crude enzyme extract.

3.7.4.2 Precipitation with Ammonium Sulphate

The crude extract was precipitated by super saturated ammonium sulphate salt. Because of having low density, as compare to ammonium sulphate solution, the precipitate created to the surface on standing. Centrifuge this mixture at 3000 rpm for 25 min. The

bottom layer was collected. At the terminal stage, the precipitate which was dissolved in homogenization buffer, used as a one-step purified source of enzyme.

3.7.5 Principle

The acetylcholinesterase inhibitory activity of different extractives of *D. blancoi* was. This method estimates AchE by using acetylcholine iodide (substrate) and dithiobisnitro benzoic acid, which is also known as DTNB. The enzymatic activity was measured by the yellow color compound which was produced by thiocholine at the time when it reacts with dithiobisnitro benzoate ion.

Acetylcholine Thiocholine + Acetate = Thiocholine + dithiobisnitro Benzoate yellow color

The intensity of color can be measured by using a spectrophotometer. The enzyme activity is stated as the rate of reaction per minute.

3.7.6 Materials

- ✓ 5, 5´-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan)
- ✓ Acetylthiocholine iodide (Sigma-Aldrich, Japan)
- ✓ Rat brain homogenate (Crude enzyme)
- ✓ Tris-Hcl buffer (Merck, Germany)
- ✓ Triton X-100 (Sigma chemical company, USA)
- ✓ Micropipette (100-1000 µl and 20-200 µl)
- ✓ UV spectrophotometer (Shimadzu, USA)

3.7.7 Procedure

For positive control, different concentrations such as 20, 50, 100, 200 µl was added with 200 µl of enzyme. The solution was made upto 3ml with extraction buffer. This mixture was incubated for 20 minutes. Then 200 µl of DTNB and 400 µl of BTCI solution was added. This was allowed to stand for 30 minutes at 37C. Absorbance was taken at 412 nm. For negative control, extraction buffer was added instead of enzyme.

3.8 In- Vitro Butyryl cholinesterase Inhibitory Studies

3.8.1 Principle

The butyryl cholinesterase inhibitory activity of various extractives of D. blancoi was identified by Ellman's method. This method determines BChE using butyrylcholine iodide (substrate) as well as dithiobisnitro benzoic acid (DTNB). The enzymatic activity was evaluated by the yellow colored compound which is produced by thiocholine when reaction occurs with dithiobisnitro benzoate ion.

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

Figure 3.11: Enzyme preparation for In- Vitro Butyryl cholinesterase Inhibitory Studies

3.8.2 Materials

- ✓ 5, 5´-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan),
- ✓ Butyrylthiocholine iodide (Sigma-Aldrich, Japan),
- ✓ Human blood plasma (Crude enzyme),
- ✓ Tris-Hcl buffer (Merck, Germany),
- ✓ Triton X-100 (Sigma chemical company, USA),

- ✓ Micropipette (100-1000 µl),
- ✓ UV spectrophotometer (Shimadzu, Japan),

3.8.3 Procedure

For the source of enzyme, human blood are collected. 1 ml of blood was placed in the screw cap test tube with EDTA as anticoagulant. 3 ml of EDTA solution was added with 10 ml of blood. This mixture was centrifuged at 3000 rpm for 25 minutes. The resultant liquid was used as a source of enzyme. All of the extraction stages were carried out at 25°C. Then 50 µl enzyme, extraction buffer and plants extracts are incubated for 2 hours at room temperature. The rates of hydrolysis by butyrylcholinesterase were monitored spectrophotometrically. After 2 hours 200 µl DTNB (0.7mM) and 400 µl BTCI (0.35mM) added correspondingly. Heat this for 40 minutes at 37C. 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.

3.9 Test for Thrombolytic Activity

Thrombolytic activity of the methanoic extracts of leaf and barks of *D. blancoi* was estimated by using blood of human.

3.9.1 Principle

Thrombosis is referred as the clotting of blood in cardiovascular system. Clotting of blood in brain can play a significant role in the progression of Alzheimer's disease. The thrombolytic activity of methanolic extract of *D. blancoi* was calculated by the given formula

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

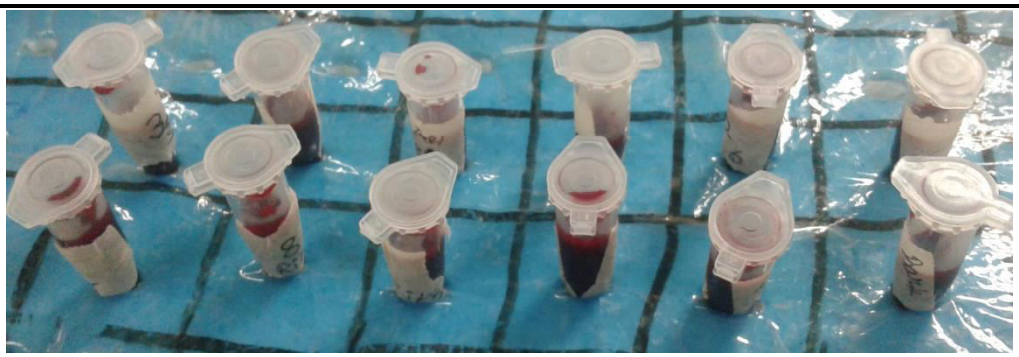

Figure 3.12: Thrombolytic activity test

3.9.2 Materials

- ✓ Human blood
- ✓ Eppendorf tube

3.9.3 Procedure

Human blood was taken from a volunteer who is healthy. Weight of an empty eppendorf tube was taken and noted down. This was incubated for 1 hour at 25C. Then the serum was collected in beaker. After that, weight of blood clot is measured by subtracting the weight of empty eppendorf tube from the weight before the test procedure. Then, 100µl of plant extract was added and it was kept for 90 minutes. Then fluid part was thrown away and again the clot was weighed. At the terminal stage, percentage of thrombolytic can be determined from the weight difference by the given equation.

Chapter 4: Results

4.1 Determination of Total Phenolics

Phenolic content of the crude methanolic extract, pet ether 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 4.1: Absorbance of gallic acid at different concentrations after treatment with FCR.

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

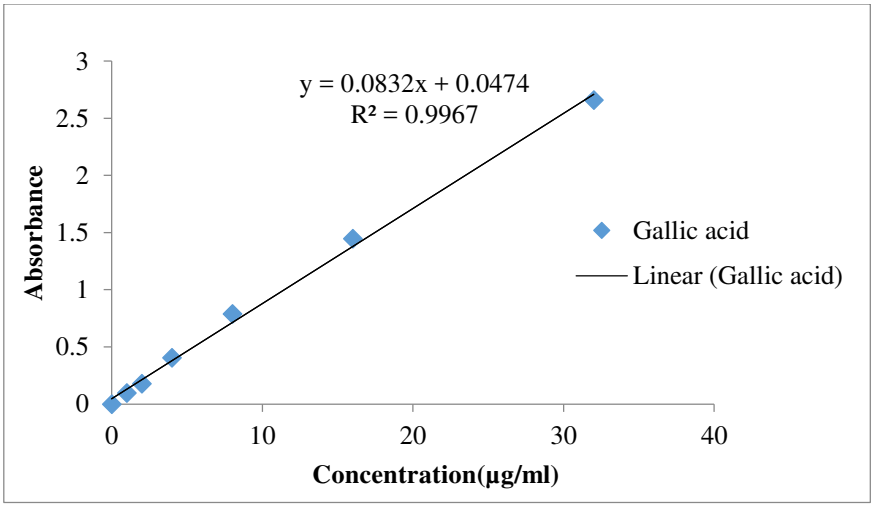

Figure 4.1: Standard curve of gallic acid for the determination of total phenolics.

Table 4.2: Determination of total phenolic content

Plant Name *D. blancoi*	Sample	Conc. (µg/ml)	Absorbance	GAE/gm of dried sample
Leaf	**CME**	300	0.868	52.08
Bark	**CME**	300	2.104	126.24
Bark	**PET**	300	0.568	34.08
Bark	**CLF**	300	0.325	19.50

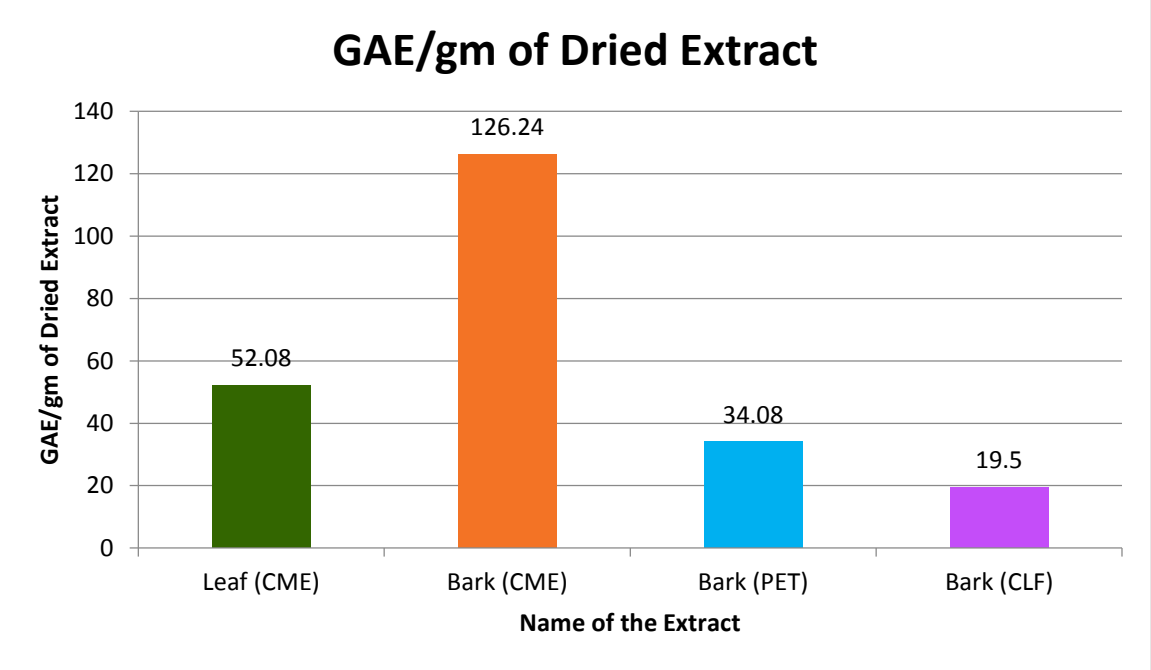

Figure 4.2: Total phenolic content (mg/gm plant extract in gallic acid equivalent)

4.2 Determination of Total Flavonoids

Total flavonoids content of crude methanol extract (CME), pet ether and chloroform fractions 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 4.3: Absorbance of catechin at different concentrations for quantitative determination of total flavonoids

Concentration (µg/ml)	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

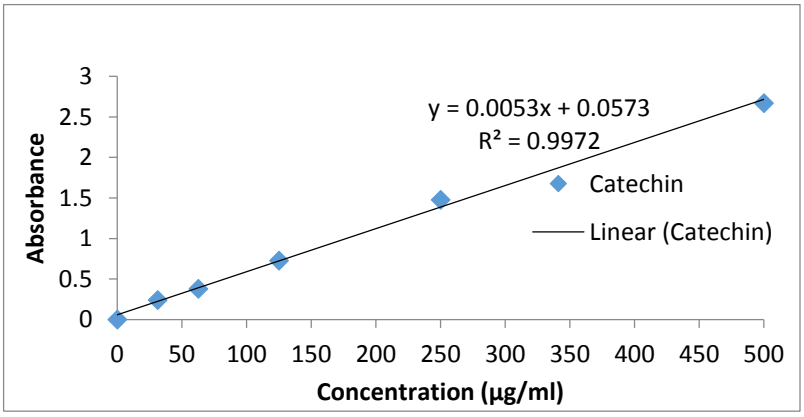

Figure 4.3: Standard curve of catechin for the determination of total flavonoids

Table 4.4: Determination of total flavonoid content

Plant Name *D. blancoi*	Sample	Conc. (µg/ml)	Absorbance	CE/gm of dried sample
Leaf	**CME**	300	0.105	6.3
Bark	**CME**	300	0.320	19.2

Bark	**PET**	300	0.053	3.18
Bark	**CLF**	300	0.043	2.58

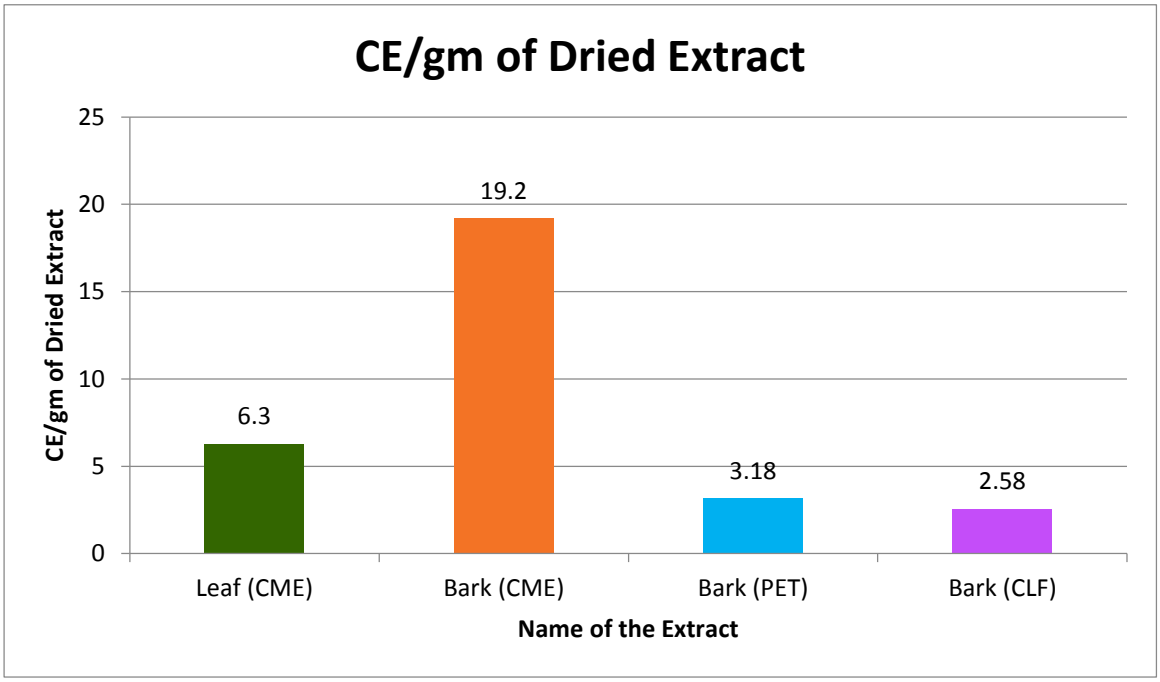

Figure 4.4: Total flavonoid content (mg/gm plant extract in catechin equivalent) of CME, PET, CLF

4.3 Determination of total flavanol

Flavanol content of the crude methanolic extract, pet ether and chloroform fraction were determined using reagents. The results were expressed as mg of gallic acid equivalent (GAE)/gm of dried extractives.

Table 4.5: 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

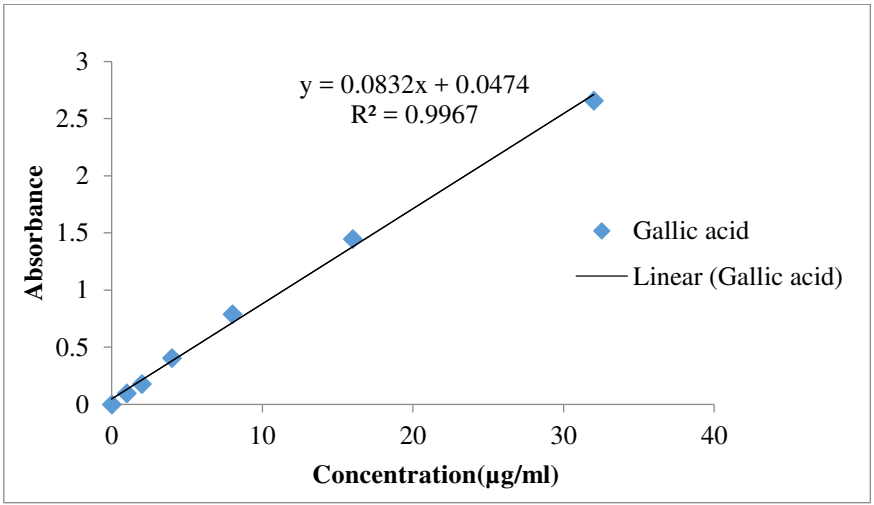

Figure 4.5: Standard curve of gallic acid for the determination of total flavanols.

Table 4.6: Determination of total flavanol content

Plant Name ***D. blancoi***	**Sample**	**Conc. (µg/ml)**	**Absorbance**	**GAE/gm of dried sample**
Leaf	**CME**	300	0.042	2.52
Bark	**CME**	300	0.019	1.14
Bark	**PET**	300	1.032	61.91
Bark	**CLF**	300	0.696	41.76

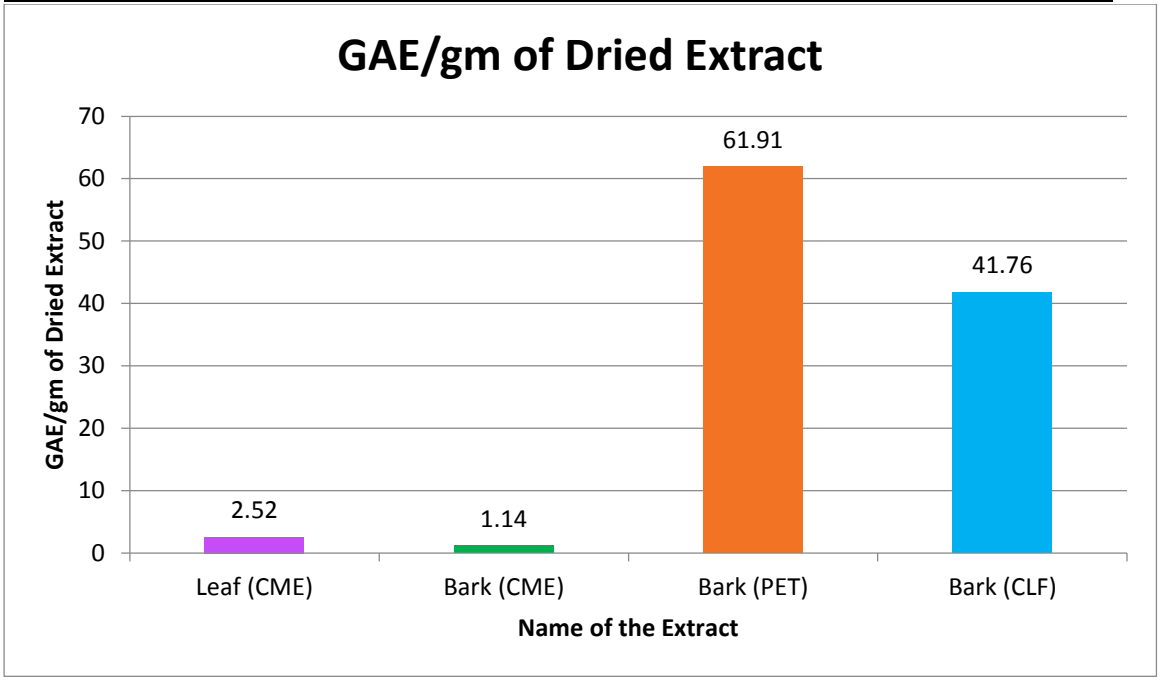

Figure 4.6: Total flavonol content (mg/gm plant extract in gallic acid equivalent)

4.4 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 4.7: % of inhibition of different parts of the plant

Name of Plant Part	Concentration (µg/µg)	Absorbance	% of Inhibition
Leaf	20	0.858	42.80

	50	0.754	49.73
	100	0.324	78.40
	200	0.099	93.40
Bark	20	0.701	53.26
	50	0.365	75.67
	100	0.101	93.27
	200	0.091	93.93
Bark (PET)	20	1.117	25.53
	50	1.106	26.26
	100	1.061	29.28
	200	1.050	30.00
Bark (CLF)	20	1.143	23.81
	50	1.111	25.94
	100	1.038	30.80
	200	0.637	55.54
Catechin (Standard)	20	0.19	87.39
	50	0.14	93.87
	100	0.11	94.16
	200	0.09	95.77

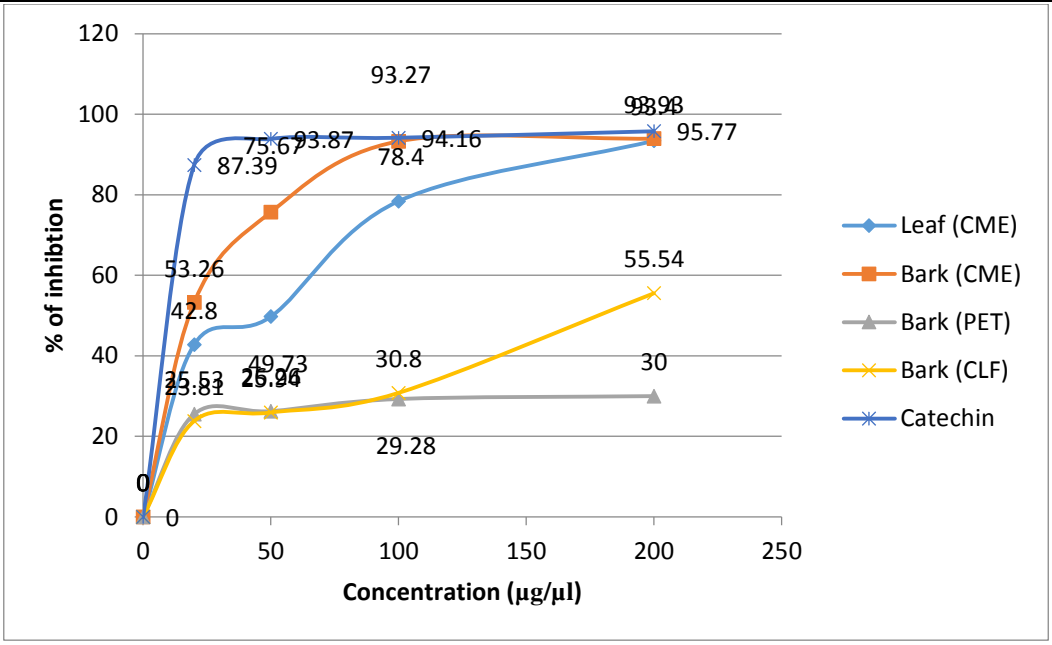

Figure 4.7: % of inhibition of different parts of plants by DPPH radical scavenging activity

4.5 Reducing Power Capacity

Cfractions 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 4.8: Chart of absorbance for reducing power capacity

Name of Plant	Concentration (µg/µg)	Absorbance
Leaf (CME)	100	0.113
Bark (CME)	100	0.251
Bark (PET)	100	0.088
Bark (CLF)	100	0.092
Catechin	100	2.660

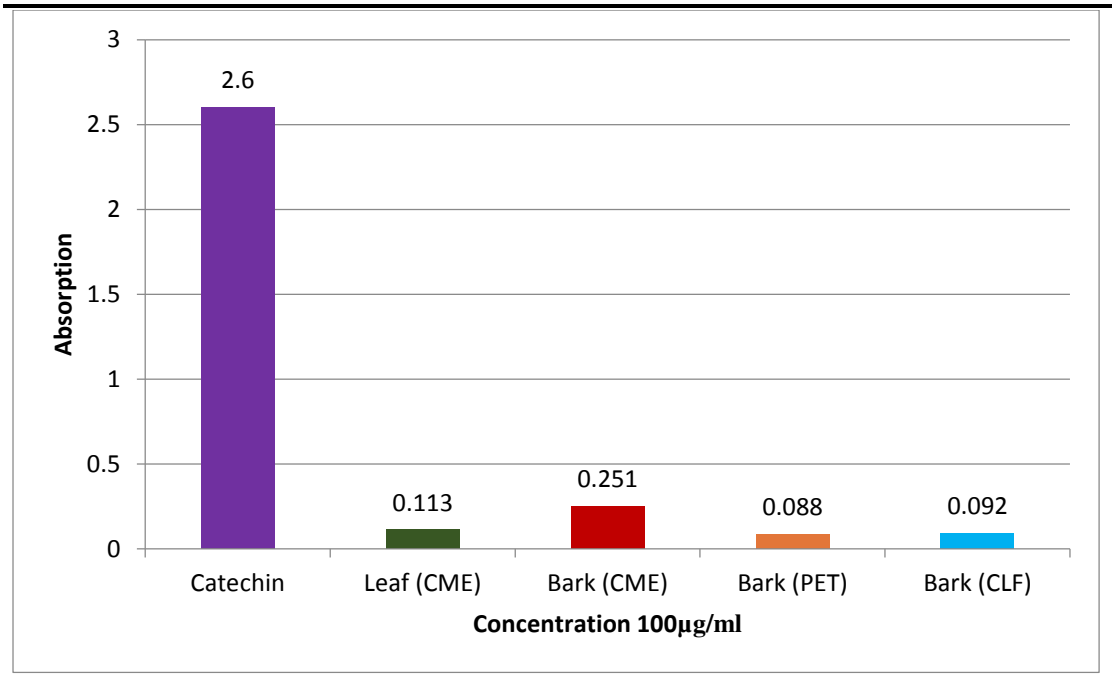

Figure 4.8: **Reducing power capacity of CME, PET and CLF extracts**

4.6 Acetyl cholinesterase 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 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 4.9: % of inhibition for acetyl cholinesterase inhibitory activity assay

Name of sample	Conc. (µg/ml)	% of inhibition Mean
Donepezil	20	78.34
	50	86.12

(Std)	100	91.44
	200	92.14
Leaf (CME)	20	72.28
	50	79.17
	100	85.08
	200	92.26
Bark (CME)	20	14.37
	50	42.10
	100	51.22
	200	63.34

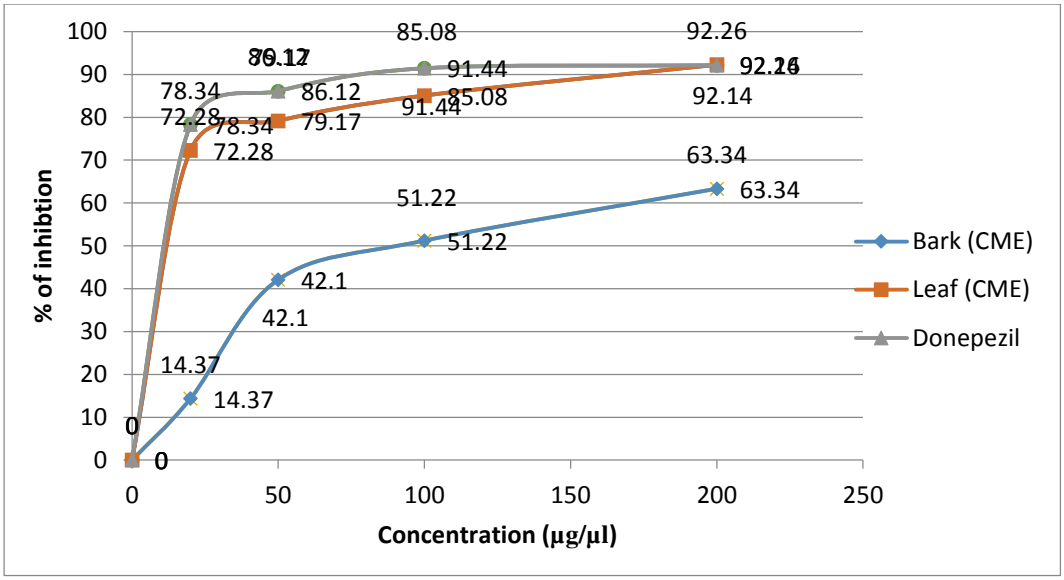

Figure 4.9: % of inhibition for acetyl cholinesterase inhibitory activity assay

4.7 Butyrylcholinesterase inhibitory activity of enzymes

Butyrylcholinestserase enhances cholinergic transmission by reducing the enzymatic degradation of both acetylcholine and butyrylcholine. Thus inhibition of butyrylcholinesterase 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. Butyrylcholinesterase inhibitors have synergistic activity of acetylcholinesterase inhibitory activity.

Table 4.10: % of inhibition for butyryl cholinesterase inhibitory activity assay

Name of sample	Conc. (µg/ml)	% of inhibition Mean
Galantamine (Std)	20	72.43
	50	86.17
	100	88.42
	200	91.41
Leaf (CME)	20	60.82
	50	72.71
	100	78.80
	200	82.23
Bark (CME)	20	22.73
	50	46.19
	100	57.67
	200	67.43

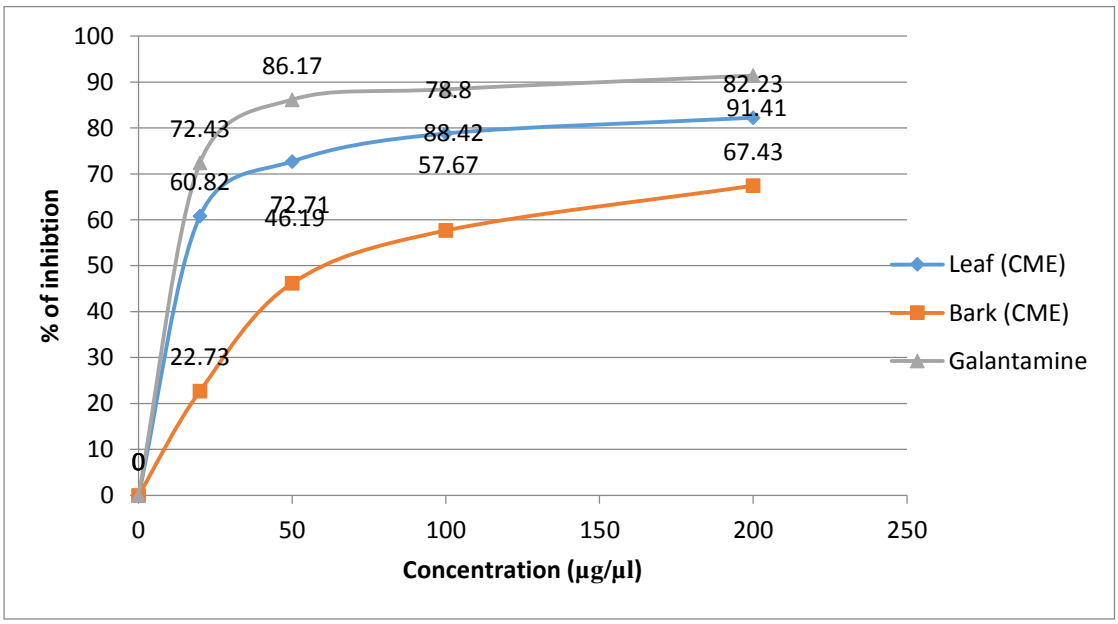

Figure 4.10: % of inhibition for acetyl cholinesterase inhibitory activity assay

4.8 Thrombolytic Activity Test

Table 4.11: % of clot lysis in thrombolytic activity test

Plant name	Concentration (µg/ml)	% of clot lysis
Leaf (CME)	100	13.41
Bark (CME)	100	0.82
Streptokinase	100	87.01

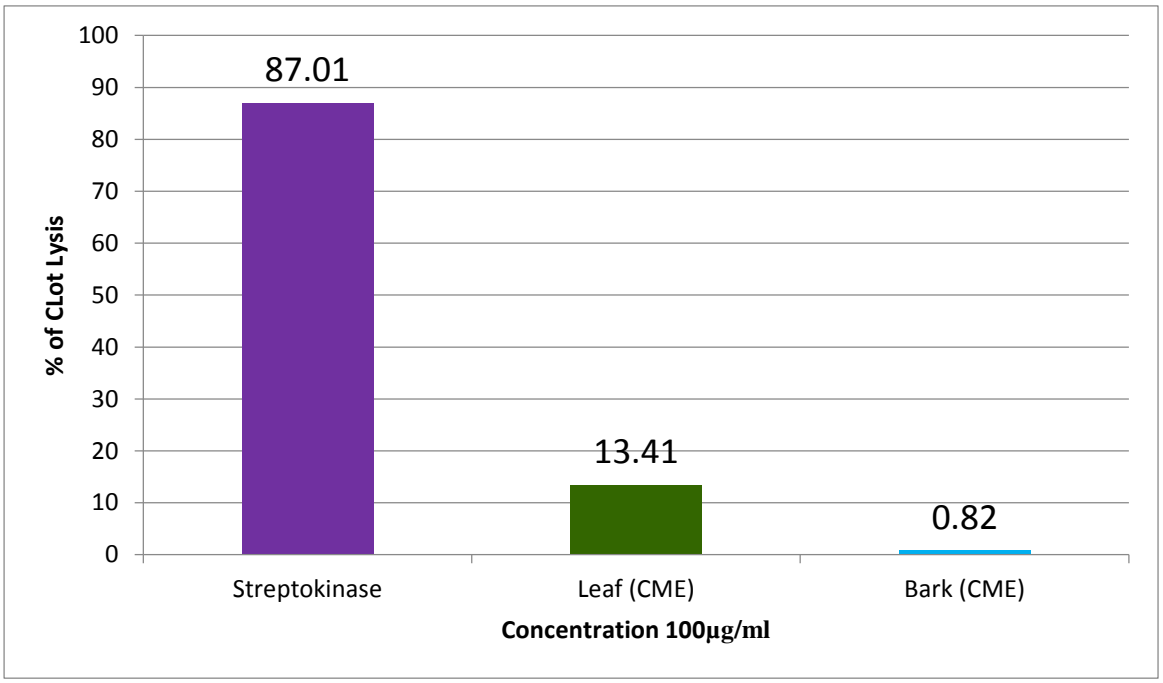

Figure 4.11: % of clot lysis in thrombolytic activity test

Chapter 5: Discussion and Conclusion

5.1 Discussion

5.1.1 Determination of Total Phenolics

Phenolic content of the crude methanolic extract, pet ether 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. For this test we used CME, PET and CLF extract of bark and CME extract of leaf. The CME of bark contain the highest phenolic content among these four extracts which is 126.24 GAE/gm. CME extract of leaf contains 52.08 GAE/gm, PET and CLF extract of bark contains 34.08 and 19.5 GAE/gm respectively. So, the CME of bark is rich in phenolic compounds. Gallic acid which is used as the standard is quite high in phenolic content. Compared to this, the extracts also have good amount of phenolic contents. These are only raw extracts. So there are chance to find out more phenolic contents from these part of this plant.

5.1.2 Determination of Total Flavonoids

Total flavonoids content were determined using much known aluminum chloride colorimetric method. Flavonoid content of the samples were calculated on the basis of the standard curve for catechin. For this test we used CME, PEF, CLF extract of bark and CME extract of leaf. The CME of bark contain the highest flavanoid content among these four extracts which is 19.2 CE/gm. CME extract of leaf contains 6.3 CE/gm, PET and CLF extract of bark contains 3.18 and 2.58 CE/gm respectively. So, the CME of bark is rich in flavonoid compounds. Catechin which was used as the standard is quite high in flavonoid content. Compared to this, the extracts also have good amount of flavonoid contents. These are only raw extracts. So there are chance to find out more flavonoid contents from these part of this plant.

5.1.3 Determination of Total Flavanol

Flavanol content of the crude methanolic extract, pet ether and chloroform fraction were determined using reagents. The results were expressed as mg of gallic acid equivalent (GAE)/gm of dried extractives. For this test we used CME, PET and CLF extract of bark and CME extract of leaf. The PET fraction of bark contains the highest flavanol content among these four extracts which is 61.91 GAE/gm. The CLF extract of bark

contains 41.76 GAE/gm which is the second highest. CME of bark and leaf contains 1.14 and 2.52 GAE/gm respectively. So, the PET and CLF fraction of bark is rich in flavanol contents. Gallic acid which was used as the standard is quite high in flavanol content. Compared to this, the extracts also have good amount of flavonoid contents. These are only raw extracts. So there are chance to find out more flavanol contents from these parts of this plant.

5.1.4 DPPH Radical Scavenging Activity

DPPH was used to assess the free radical scavenging activity of numerous fractions of bark and leaf of D. blancoi. For this test we used CME, PEF, CLF fraction of bark and CME of leaf. From the graph we can see that the percentage of scavenging activity of CME of bark is very close to the values of catechin which was used as the standard. The percentage of scavenging activity reached near the highest level at the concentration of 100 µg/µl. The CME of leaf also showed values close to the standard. The percentage of scavenging activity reached near the highest level at the concentration of 200 µg/µl. Above these concentrations, these extracts might not show more activity. The percentage of scavenging activity of these two fractions is nearly 96 percent at the mentioned concentrations. The PET and CLF fractions showed 58 percent and 30 percent activity compared to the standard. And the graph maintained linear relationship, so the activity is concentration dependent. The extracts reached highest activity only at the concentration of 100-200 µg/µl. These are only raw extracts. So there are chance to find out more from scavenging activity from these parts of plant.

5.1.5 Reducing Power Capacity

The Fe^{3+} reducing power of the CME, PET and CLF was determined by the method of Oyaizu (1986) with slight modification. For this test we used CME, PEF, CLF fraction of bark and CME of leaf. For this experiment, catechin is used as a standard. The absorbance of Leaf (CME), Bark (CME), Bark (PET), Bark (CLF) and Catechin (Standard) was 0.113, 0.251, 0.088, 0.092 and 2.660 respectively. The reducing power of the extracts are Bark (CME)> Leaf (CME)> Bark (CLF)> Bark (PET). The reducing power of the standard is very high. Compared to this, the reducing power of the extracts was not very high. These are only raw extracts. So there are chance to find out more reducing power capacity from these parts of plant.

5.1.6 Acetylcholinesterase inhibitory activity assay

Acetylcholinesterase inhibitory activity was assessed by slightly modified Ellman's method. We have used the CME extract of bark and leaf of D. blancoi. Both part of the plant inhibits AchE. It was a concentration dependent inhibition. With the increasing amount of plant extract, the percentage of inhibition was also increased. In operation with both the part, leaf found to be more prominent than the bark as it inhibited more than 90% AchE at the concentration of 200 µg/ml. Whereas the bark inhibited more than 60% AchE. Donzepil which is a potent drug for inhibiting AchE was used as a reference standard. The inhibitory activity of Donzepil is quite high. These are only raw extracts. So there are chance to find out more reducing power capacity from these parts of plant.

5.1.7 Butyrylcholinesterase inhibitory activity assay

Butyrylcholinesterase inhibitory activity was assessed by slightly modified Ellman's method. We have used the CME extract of bark and leaf of D. blancoi. Both part of the plant inhibits AchE. It was a concentration dependent inhibition. With the increasing amount of plant extract, the percentage of inhibition was also increased. In operation with both the part, leaf found to be more prominent than the bark as it inhibited more than 80% AchE at the concentration of 200 µg/ml. Whereas the bark inhibited more than 65% AchE. Galantamine which is a potent drug for inhibiting butyrylcholinesterase was used as a reference standard. The inhibitory activity of galantamine is quite high. These are only raw extracts. So there are chance to find out more reducing power capacity from these parts of plant.

5.1.8 Thrombolytic Activity Test

Clotting of blood in brain can play a significant role in the progression of Alzheimer's disease. So this test was performed to see the thrombolytic activity of leaf and bark of D. blancoi. In order to perform the test, CME of leaf and bark were used. From the test result, we can see that the thrombolytic activity of CME of leaf and bark is 13.41% and 0.82% respectively. Streptokinase was used as the reference standard and the thrombolytic activity of streptokinase was 87.01%. So, the thrombolytic activity of the used parts are quite low.

5.2 Conclusion

The plant is rich in phenolic, flavonoid and flavanol compounds. The CME of bark and leaf were most active in the test for determination of phenolic and flavonoid compounds and the PET and CLF fractions were most active in the test for determination of total flavanol compounds. The plant showed activity in DPPH free radical scavenging activity test. The CME of bark and leaf showed most activity in the DPPH free radical scavenging activity test. The reducing power of the extracts which was assessed by reducing power capacity are Bark (CME)> Leaf (CME)> Bark (CLF)> Bark (PET). The plant showed high activity in acetyl cholinesterase inhibitory activity test and butyryl cholinesterase activity test. But the plant was not so active in the thrombolytic activity test. As the test showed high activity in some of the tests mentioned above, further study is needed on the plant.

Chapter 6: Reference

6.1 Reference list

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