A Comparative Assessment of Five Available Citrus Fruits' Cholinesterase Inhibitory, Antioxidant and Thrombolytic Activities for the Treatment of Neurodegenerative 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
Shanjida Alam Rika
Id: 2013-1-70-057
Department of Pharmacy
East West University

Submitted to
Kushal Biswas
Lecturer
Department of Pharmacy
East West University

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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 five Medicinal Plants (*Citrus Maxima, Citrus Bergamia, Citrus Aurantifolia, Citrus Limon and Citrus Sinensis)* 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 Shanjida Alam Rika, Id: 2013-1-70-057, during the period 2016 of her research in the Department of Pharmacy, East West University.

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 five Medicinal Plants (*Citrus Maxima, Citrus Bergamia, Citrus Aurantifolia, Citrus Limon and Citrus Sinensis)* 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 Shanjida Alam Rika, Id: 2013-1-70-057, 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, Shanjida Alam Rika, hereby declare that the dissertation entitled “A Comparative Study of Cholinesterase Inhibitory, Thrombolytic and Antioxidant Activities offive Medicinal Plants (*Citrus Maxima, Citrus Bergamia, Citrus Aurantifolia, Citrus Limon and Citrus Sinensis*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 KushalBiswas, 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.

Shanjida Alam Rika
Id: 2013-1-70-057
Department of Pharmacy
East West University, Dhaka.

ACKNOWLEDGEMENT

At first, I am grateful to God for the good health and wellbeing that were necessary to complete this research. I would like to express my deepest gratitude to my research supervisor, KushalBiswas, Lecture, Department of Pharmacy, East West University and co-supervisor, Kushal Biswas, Lecturer, Department of Pharmacy, East West University, who had been always optimistic and full of passion and ideas. Their generous advice, constant supervision, intense support, enthusiastic encouragements and reminders during the research work not only helped shape this study but also molded me into being a better researcher. Their in-depth thinking, motivation, timely advice and encouragement have made it possible for me to complete this research.

Secondly, I am also indebted to the Department of Pharmacy, East West University. I am very proud to the part of this institute. To me it seems like second home. This institute is giving me an opportunity to learn about my future goals, to learn how to show respect to the pharmacy profession. I would like to show my gratitude to the Dr. ShamsunNahar Khan, Associate Professor and Chairperson of Pharmacy Department; Dr. Sufia Islam, Associate Professor of Pharmacy Depertment; JMA Hannan, PhD(UK), Professor of Department of Pharmacy, to the faculties who are teaching over the last four years to make us ready for the noble profession by becoming a pharmacist.

Third, I am thanking our respected lab officers Sipra Biswas, RuchiraChakrabarty, Ajoy Roy, Sujit Kumar and Shofiqul Islam for helping me by providing equipments and materials as well as their guidance I needed to fulfill the work.

Forth, my special thanksTaposhi Sultana Mou, Saiyara Hossain Reevu, Shanjida Alam Rika, and all of my friends, who helped me to conduct the research by being very co-operative to be the part of my study. Because of their tremendous support I could finish the work on time. I also, would like to help my fellow classmates, friends for their continuous support in my stay in this institute.

Finally, I am immensely grateful to my beloved parents, Md. Alauddin Khan and Sultana Nasrin for their love and faith in me, especially for their unconditional love in my life. It is my parents who made me, who I am now! I also would like to express my heartfelt love to my family for their continuous support and love.

Shanjida Alam Rika
Id: 2013-1-70-057

Abstract:

Citrus fruits are well known for its antioxidant properties. Alzheimer's disease (AD), a common type of progressive neurodegenerative disease, is characterized by low level of neurotransmitter (acetylcholine), oxidative stress and neuro-inflammation in brain stream. Effective treatment strategies rely mostly on either enhancing the cholinergic function of the brain by stimulating the cholinergic receptors, improve the level of acetylcholine from being a breakdown by cholinesterase enzymes or induce antioxidant therapy. . Beside this atherothrombosis is a major cause of global life threatening cerebral diseases. In this study we examine five commonly available Citrus fruits available in Bangladesh; those are well known for their antioxidant activities with a rich source of vitamin C and polyphenols. A crude methyl extract (CME) of these fruits are evaluated for acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activity by Ellman's method. Antioxidant, free radical scavenging, reducing power and thrombolytic property are also assessed in this study, which strongly implies that that the CME of these fruit are of AChE and BuChE inhibitors with moderate thrombolytic activities.

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List of Abbreviations

Abbreviation	Full Form
Ach	Acetyl Choline
AChE	Acetyl Cholinesterase
AD	Alzheimer's Disease
ATP	Adenosine Triphosphate
BuChE	Butyryl Cholinesterase
CE	Catechin Equivalent
CME	Crude Methanol Extract
CNS	Central Nervous System
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DTNB	5, 5´-dithio-bis-(2-nitro) benzoic acid
EDTA	Ethylene diamine tetraacetic acid
FCR	Folin–Ciocalteu Reagent
GAE	Gallic acid equivalent
gm	Gram
mg	Milligram
ml	Milliliter
mM	Milli molar
NaCl	Sodium Chloride
nm	Nanometer
STD	Standard Deviation
TCA	Trichloro acetic acid
UV	Ultraviolet

1.1 Introduction

Alzheimer's disease is a type of dementia which causes defect with memory, thinking and behavior. Dementia is decline in memory, language, problem-solving and many other cognitive skills that hampers a person's ability to perform everyday-life activities. It is a degenerative brain disease. The symptoms of this disease are usually developed slowly and it gets worse over time. The decline of brain occurs due to nerve cells (neurons) in parts of the brain which involved in the cognitive functions have been damaged or destroyed. This damage and destruction of neurons eventually hamper other parts of the brain, which includes those that enable a person carrying out basic bodily functions for example walking and swallowing. The biggest known risk factor of this disease includes increasing age, and maximum of the people with Alzheimer's are 65 and higher. But this is not a disease of old age only. Because up to 5 percent of people having the disease have early onset Alzheimer's which is known as younger-onset. It often appears when someone is in their 40 or 50 years age. At the final stages of the disease people are bed-bound and they are required around-the-clock care. This disease is ultimately fatal. At early stages, memory loss is mild. But with late-stage of Alzheimer's, it is the sixth leading cause of death in the United States. After symptoms become noticeable to others patients with Alzheimer's live their life an average of eight years, but depending on age and other health conditions the survival can range from 4 to 20 years.

Alzheimer's disease was named after Dr. Alois Alzheimer. In 1906, Dr. Alzheimer noticed some changes in brain tissue of a woman who had died of an unusual mental illness. Her symptoms were memory loss, language problems, and unpredictable behavior. After her death, he examined her brain where he found many abnormal clumps (now these are called amyloid plaques) and tangled bundles of fibers (now these are called neurofibrillary, or tau, tangles). These plaques and tangles in the brain are still thought some of the main features of this disease. Another main feature is the loss of connections in between nerve cells (neurons) in the brain. Neurons are responsible to transmit messages between different parts of the brain, and also from the brain to muscles and other organs in the body.

1.2 Symptoms of Alzheimer's

Our brains change as we age, just like the rest of our bodies. Many people eventually notice some slowed thinking and sometimes occasional problems in remembering certain things. But, serious memory loss, confusion and many other major changes in the regular way our minds work may be a symbol that brain cells are failing. Patients with memory loss or other possible symptoms of Alzheimer's disease may find it

difficult to recognize that they have a problem. Signs of dementia may be more observable to family members or friends. Early diagnosis methods are improving melodramatically, treatment options and sources of support can expand quality of life. The symptoms of Alzheimer's disease vary person to person. The most common form of initial symptom is gradually losing the ability of remembering new information. This is because the first damaged and destroyed neurons in brain regions involve in new memories. As the neurons in other parts of brain are damaged and destroyed gradually, individuals experience other difficulties. Difficulty in remembering recent conversations, events or names is often an early clinical symptom; lethargy and depression are also often early symptoms. Later symptoms include disorientation, impaired communication, poor judgment confusion, behavior changes and, ultimately, difficulty in speaking, swallowing and walking. There are common symptoms of Alzheimer's in bellow:

- Memory loss which disrupts daily life.
- Challenges while planning or solving problems.
- Difficulty in completing familiar tasks while at home, at work or at leisure.
- Confusion with time or place. Trouble in understanding visual images and also spatial relationships.
- New problems arise with words when speaking or writing.
- Misplacing things and also losing the ability to retrace steps.
- Decreased or poor judgment.
- Withdrawal from work and social activities
- Changes in mood and personality, which includes apathy and depression.
- Increased anxiety, agitation and also sleep disturbances.

The pace at which symptoms progresses from mild to moderate to severe varies from person to person. As the disease progresses eventually, cognitive and functional abilities decline. In more advanced stages, people need more help with basic activities of daily living, for example, dressing, bathing, eating and using washroom; lose their ability to communicate; eventually fail to recognize loved ones; and finally become bed-bound and depended on around-the-clock care. When patient have difficulty moving, they become more vulnerable to infections, which includes pneumonia (infection of lungs). Alzheimer's-disease related pneumonia is often a contributing factor results the death of people with Alzheimer's disease. The symbolic pathologies of Alzheimer's disease are the progressive aggregation of the protein fragment beta-amyloid (plaques) at outside of neurons in the brain and also the twisted strands of the protein tau (tangles) placed inside the neurons. These changes are eventually accompanied by the destruction and death of neurons, Baratta etal 1998.

1.3 Alzheimer's disease and the brain

Microscopic changes in the brain were found in long before the first signs of memory loss. Our brain has 100 billion nerve cells (neurons). Each nerve cell is connected with many others to produce communication networks. Groups of nerve cells have special functions. Some are involved in thinking, remembering and learning. Others help us to see, smell and hear.

To do their job, brain cells work like tiny factories. They receive supplies, produce energy, build equipment and get rid of waste. Neurons also process and store information to communicate with other cells. To keep everything running coordination as well as huge amounts of fuel and oxygen require.

Scientists believe Alzheimer's disease prevents neurons factory from running well. They are not assured where the trouble starts. But just like real factory, breakdown and backups in one system results in problems in other areas. As damage spreads, neurons lose their ability to do their functions and, eventually die, resulting irreversible changes in the brain.

1.3.1 Role of plaques and tangles

Two abnormal structures named plaques and tangles are major suspects in killing and damaging nerve cells.

Plaques are accumulation of a protein fragment named beta-amyloid which is build up in the places between nerve cells.

Tangles are twisted fibers of tau protein, another protein which is built up inside cells. Although in many people some plaques and tangles are developed as they age, those with Alzheimer's disease tend to develop far more. They tend to develop them in a likely pattern, which beginning in areas important for memorial before spreading to other regions.

Scientists do not sure exactly what important role plaques and tangles play in Alzheimer's disease. Most scientist believe that they somehow play a critical role in hindering communication among nerve cells and disturbing processes that cells need to survive.

It's the damage and death of nerve cells that results personality changes, memory failure, problems carrying out daily activities and many other symptoms of Alzheimer's disease.

1.4 Causes of Alzheimer's disease

1.4.1 Genetic Mutations

An estimated 1 percent or less cases of Alzheimer's disease develop as a result of mutations occurring any of three specific genes. A genetic mutation refers to an abnormal change developing in the sequence of chemical pairs which make up genes. These genetic mutations include the gene for the amyloid precursor protein (APP) and also the genes for the presenilin 1 and presenilin 2 proteins. It is guaranteed that those inheriting a mutation to the APP or presenilin 1 gene develop Alzheimer's disease. There is 95 percent chance of developing the disease those inheriting a mutation to the presenilin 2 gene. People with mutations to any of these three genes are likely to to develop Alzheimer's disease symptoms before age 65, in some cases as early as age 30, while the most majority of people with Alzheimer's disease have late-onset disease, at age 65 or later.

1.4.2 Down syndrome

About 400,000 Americans are suffering from Down syndrome. People with Down syndrome are born having a partial or an additional full copy of chromosome 21, which is one of the 23 human chromosomes. People having Down syndrome are at high risk of raising a type of dementia which is similar to that caused by Alzheimer's disease. Scientists are not sure why people having Down syndrome are at higher risk, but may be it is related to the partial or additional full copy of chromosome 21. This chromosome includes a gene which encodes for the construction of APP, which in people with Alzheimer's disease is cut into beta-amyloid fragments which go on to aggregate into the hallmark amyloid plaques of Alzheimer's. By age 40, most people having Down syndrome have significant levels of tau tangles and beta-amyloid plaques in their brains. As age increases an individual having Down syndrome will exhibit Alzheimer's disease. Studies suggest that many people having Down syndrome will begin to show symptoms of dementia in their early age to mid-50s and that more than 75 percent of people having Down syndrome over age 65 have Alzheimer's disease.

1.5 Risk Factors for Alzheimer's disease

Like other chronic disease, experts believe that with the exception of cases of Alzheimer's disease caused by genetic abnormalities, it also develops as a result of multiple factors rather than a single cause.

The biggest risk factors for late-onset "erratic" Alzheimer's are older age, carrying the Apolipo protein e4 (APOE-e4) gene and having a family history of Alzheimer's disease.

1.5.1 Age

Age is the biggest of these three risk factors. Most people having Alzheimer's disease are age 65 or older. People younger than 65 can have Alzheimer's disease, but they are much less likely to develop the disease than older people. As age increases, so increase the chance of having Alzheimer's disease. For example, 15 percent of those having Alzheimer's disease are ages 65-74, while 44 percent are at the ages of 75-84. Although older age is a risk factor, Alzheimer's disease is not a normal part of aging, and also the older age is not sufficient only to cause the disease.

1.5.2 Family History of Alzheimer's disease

A family history of having Alzheimer's disease is not essential for an individual to develop the disease. But, individuals who have a parent, sister or brother having Alzheimer's disease are more prone to develop the disease compared to those who do not have the first-degree relative having Alzheimer's. Those who have more than one first-degree relative having Alzheimer's are at higher risk even. When diseases run in families, genetics, shared environmental and lifestyle factors may play a vital role. The increased risk factor associated with having a family history of having Alzheimer's disease is not only explained by whether the individual has inherited the APOE-e4 risk gene.

1.5.3 APOE-e4 Gene

The APOE gene delivers the blueprint for a protein transporting cholesterol in the bloodstream. Everyone contains one of three forms of the APOE gene among e2, e3 or e4 from each parent. The e3 is the most common form, and the e2 is the least common form. The e4 form is sometimes more common than the e2 form. One form of APOE gene is inherited from each parent. People containing one or two copies of

e4 are at higher risk of developing Alzheimer's compared to those individuals who do not have a copy of e4.

It is more risky having the e4 form compared with having the e3 form, while having the e2 form may be less risky compared with having the e3 form. There is three-fold higher risk of developing Alzheimer's disease for those who contains one copy of the e4 form compared to those without the e4 form, but those who contains two copies of the e4 form they have an 8- to 12-fold higher risk. In addition, those having the e4 form are more prone to develop Alzheimer's at a younger age compared to those having the e2 or e3 forms of the APOE gene. Researchers evaluate that 40 percent to 65 percent of patient diagnosed with Alzheimer's contain one or two copies of the APOE-e4 gene. Unlike containing a genetic mutation that results Alzheimer's disease, containing the e4 form of the APOE gene does not confirm that an individual will develop Alzheimer's. This is also true for more than 20 recently specified genes that appear to affect the risk of Alzheimer's. These recently specified genes are thought to have a limited effect on the overall occurrence of Alzheimer's as they are rare or only slightly risky.

1.6 Research and progress

At first Alzheimer's disease was recognized more than 100 years ago. But it took 70 years to identify that it is the most familiar cause of dementia, as well as a major cause of death. After that Alzheimer's disease has become a significant area of research. Yet it is unknown that, why Alzheimer's disease progresses more quickly in some people than in others, and how the disease can be slowed, prevented, or stopped. Researchers consider that early detection of Alzheimer's will be fundamental to slowing preventing, and stopping the disease. The last 10 years have seen wonderful growth in research on early discovery.

This research encouraged the 2011 publication of new guidelines and diagnostic criteria for initiate before symptoms appear such as memory loss, whereas earlier criteria involve memory loss and a failure in thinking abilities for an Alzheimer's diagnosis to be made. As scientific evaluation of many components of the new criteria is continuing, "Alzheimer's disease" refers to the disease as definite by the earlier criteria, Berridge etal 2010.

Today, Alzheimer's disease is at the lead of biomedical research. Researchers are working to discover as many features of Alzheimer's disease and related measurements as possible. Ninety percent of what we identify about Alzheimer's has been exposed in the last 15 years. Some of the most significant progress has shed light

on how badly Alzheimer's disturbs the brain. There is the hope that better understanding on this will lead to new treatments. Many prospective approaches are now under investigation worldwide. During the 12-year study period, 14,534 people were diagnosed with AD in Wales. The overall prevalence of AD in individuals 60 years or older was 2% and the overall incidence was estimated as 1.5 per 1000 person-years. The prevalence of AD in individuals between 60 and 74 years was 1%, rising up to 5% in those aged 75 years and older. The incidence of AD increased during the study period from 1.4 per 1000 person-years in 1999 to 1.9 per 1000 person-years in 2010. More than half of the diagnosed AD during the study period was unspecified.

An estimation in 2016 found that about 5.4 million Americans of all ages have Alzheimer's disease. Among this number the estimation includes 5.2 million people age 65 and older whereas approximately 200,000 people under age 65 that have younger-onset Alzheimer's, Hooper etal 2008.

- One in nine individuals age 65 and older has Alzheimer's disease which represents 11%.
- About one-third of people having age 85 and older have Alzheimer's disease which represents 32 %.
- Whereas people having age 75 and older have the incidence of Alzheimer's disease represents about 81 %.

The ages of individuals with Alzheimer's disease in the United States, 2016 is given below:

- 85+ years, 37%
- 75-84 years, 44%
- 65-74 years, 15%
- <65 years, 4%

The estimated number of people age 65 and older having Alzheimer's disease results from a study using the latest the data from the 2010 U.S. Census and the Chicago Health and Aging Project (CHAP), which is a population-based study of chronic health diseases of older people.

1.7 Differences between Women and Men in the Occurrence of Alzheimer's disease and Other Dementias

The occurrence of AD (Alzheimer's disease) is more prompt in women than in men. Almost two-thirds of Americans having Alzheimer's are women. Among the 5.2 million people age 65 and older having Alzheimer's in the United States, 3.3 million people are women and 1.9 million are men. Based on estimates from ADAMS, people age of 71 and older, 16 percent of women bear AD and other dementias comparing with 11 percent of men. There are a number of possible reasons why more women have AD and other dementias than men. The predominant view has been that this difference is due to the fact that women live longer comparing with men on average, and older age is the greatest risk factor for AD.

Many studies of incidence indicating the risk of developing AD or any other dementia have found no significant dissimilarity between men and women in developing AD or other dementias at any given age. However, limited new research found that risk could be higher for women, possibly due to biological or genetic variations or it can be even different life experiences (for example, educational variation, or occupational choices).Data from the Framingham Heart Study recommend that as men have a higher rate of death from cardiovascular disease compared with women in middle age, men surviving beyond age 65 may have a risk of healthier cardiovascular and thus having a lower risk for dementia than women of the same age, however more research is needed to establish this finding, Karran etal 2011.

Another large study presented that the APOE-e4 genotype, the best genetic risk factor for AD, may have a stronger relationship with AD in women than men. It is unidentified why this can be happened, but some evidence suggests an interaction between the sex hormone estrogen and the APOE-e4 genotype. However, evidence does not permit the use of supplemental estrogen after menopause due to prevent AD, although some research proposed some possible benefits for women having had their ovaries removed, Gatuso etal 2006. Finally, as low education is an important risk factor for dementia, it is possible that lower educational state in women comparing with men born in the first half of the 20th century could be the higher risk factor of AD and other dementias in women; however, this risk has not been thoroughly examined scientifically.

1.8 Mortality and Morbidity

People being age 70, 61 percent of those having Alzheimer's are expected to die before attaining age 80 comparing with 30 percent of those without AD. Alzheimer's

disease is authoritatively listed as the sixth leading cause of death in the United States. But it is the fifth leading cause of death for the people of age 65 and older. Nonetheless, it may cause even more deaths comparing with official sources recognize. AD is also a leading cause of disability and morbidity. Before a person having AD dies, he lives through years of morbidity as the disease progresses.

It is challenging to determine the amount of deaths caused by Alzheimer's disease each year. According to data from the National Center for Health Statistics of the Centers for Disease Control and Prevention (CDC) people died from Alzheimer's disease was 84,767 in 2013. A recent study using data from the Rush Memory and Aging Project and the Religious Orders Study said researchers estimated that 500,000 deaths among people age 75 and older could be recognized to Alzheimer's disease in the United States in 2010 (estimations for people aging 65 to 74 were not available), meaning that those deaths would not be predictable to occur in that year if those individuals did not have AD. According to data from the Chicago Health and Aging Project (CHAP), an assessed 600,000 people age 65 and older in the United States had AD when they died in 2010, meaning they died with AD. Among these, an estimated 400,000 were age 85 and older, and an estimated 200,000 were age 65 to 84. Furthermore, according to Medicare data, about one-third of all seniors who die in a given year have been diagnosed with Alzheimer's or another dementia. For the United States as a whole, in 2013, the mortality rate for Alzheimer's disease was 27 deaths per 100,000 people. This information was attained from death certificates and reproduces the condition identified by the physician as the underlying cause of death.

As the population of the United States ages, Alzheimer's disease is becoming a more common cause of death. Studies show that people age 65 and older survive an average of 4 to 8 years after the diagnosis of Alzheimer's disease, so far some live as long as 20 years with AD. This reproduces the slow, insidious progression of AD. On average, a person with Alzheimer's disease will spend 40 percent of their total number of year's living having dementia in its most severe stage, Kagan B.L. 2002.

The primary measure of disease liability is called disability adjusted life years (DALYs), which is the amount of the number of years of life lost because of premature mortality and the number of years lived with disability, totaled across all those with the disease. Using this dimension, AD rose from the 25th most troublesome disease in the United States in 1990 to the 12th in 2010. No other disease increased as much. In terms of years of life lost, AD rose from 32nd to 9th, the largest increase for any disease. In terms of years lived with disability, AD went from ranking 17th to 12th; only kidney disease corresponded AD in as high a jump in rank. In a whole, the numbers in this section show that not only is AD responsible for the

deaths of more and more Americans, the disease is also causative to more and more cases of poor health and disability in the United States.

1.9 Treatment

Alzheimer's disease has no current treatment, but treatments for symptoms are accessible and research continues. Although current AD treatments cannot stop AD from progressing, they can temporarily slow the worsening of dementia symptoms and improve the quality of life for those with AD and their caregivers. Today, there is a universal effort under way to find better ways to treat the disease, interruption its onset, and prevent it from developing.

1.9.1 Pharmacologic Therapy

No pharmacologic medications available today for AD which slows or stops the damage and destruction of neurons that cause AD symptoms and make the disease fatal. The six drugs permitted by the U.S. Food and Drug Administration (FDA) for the cure of AD temporarily improve symptoms by raising the amount of neurotransmitters in the brain. The efficiency of these drugs varies from person to person. In the decade of 2002-2012, 244 drugs for AD were established in clinical trials registered with an Organizations of Health registry of publicly and privately funded clinical studies. Only one of the 244 drugs effectively completed clinical trials and went on to receive registration from the FDA. Many factors contribute to the difficulty of evolving effective treatments for AD. These factors include the high cost of drug improvement, the relatively long time needed to detect whether an investigational treatment affects disease development, and the structure of the brain, which is sheltered by the blood-brain barrier, through which only very specialized small sized molecule drugs can cross, Khan M.K 2010.

1.9.2 Non-Pharmacologic Therapy

Non-pharmacologic therapies are those that do not include medication. Non-pharmacologic therapies are often used with the intention of maintaining or developing cognitive function, the ability to accomplish activities of daily living, or overall quality of life. They also may be used with the intention of reducing behavioral symptoms such as depression, laziness, wandering, sleep disturbances, agitation and anger. Systematic reviews of published research on non-pharmacologic therapies have established that some, such as exercise and cognitive activity (for example, word games, gardening, listening to music and cooking) show possibilities.

However, comparing with pharmacologic treatments, relatively few non-pharmacologic therapies have been verified in multiple large randomized measured studies or shown consistent results.

1.10 Overview of disease development (Stages)

The symptoms of Alzheimer's disease degrade over time, although the rate at which the disease proceeds varies. On average, a person with AD lives four to eight years after diagnosis, but the possible chance of living is as long as 20 years, considering other factors. Changes in the brain related to AD begin years before any symptoms of the disease. This time period, which can last for years, is referred to as preclinical AD.

The stages below to this provide an overall idea of how capacities change once signs appear and should only be used as a general guide. They are divided into three different categories: mild Alzheimer's disease, moderate Alzheimer's disease and severe Alzheimer's disease. Be aware that it may be challenging to place a person with AD in a specific stage as stages may overlap.

1.10.1 Mild Alzheimer's disease (early-stage)

In the early stages of AD, a person may occupy independently. He or she may still work, drive and be part of social activities. Despite this, the person may sense as if he or she is having memory breaks, such as fail to recall familiar words or the location of everyday objects.

Friends, family or neighbors begin to notice problems. During a detailed medical interview, doctors may be able to identify problems in memory or concentration. Common difficulties include:

- Problems coming up with the correct word or name
- Difficulty remembering names while introducing to new people
- Having greater trouble performing tasks in social or work settings
- Forgetting what one has just read
- Losing or misplacing an important object
- Increasing problem with planning or organizing

1.10.2 Moderate Alzheimer's disease (middle-stage)

Moderate Alzheimer's is characteristically the longest stage and may last for many years. As the disease develops, the person with AD will demand a greater level of care. One may find the person with AD confusing words, getting frustrated or having angry, or acting in irregular ways, such as refusing to bathe. Damage to nerve cells in the brain can create it difficulty to express thoughts and complete routine tasks. At this point, symptoms will be visible to others and may include:

- Forgetting events or about one's own personal history
- Feeling moody or withdrawn, particularly in socially or mentally challenging situations
- Being unable to recall their address or telephone number or the high school or college from which they graduated
- Confusion about where they are present or what day it is
- The need for help choosing appropriate clothing for the season or the occasion
- Trouble controlling bladder and bowels in some persons
- Changes in sleep period, such as sleeping during the day and becoming restless at night
- An increased risk of traveling and becoming lost
- Personality and behavioral changes, including dishonesty and delusions or compulsive, repetitive behavior like hand-wringing or tissue shredding

1.10.3 Severe Alzheimer's disease (late-stage)

In the final stage of this disease, people lose the ability to react to their environment, to carry on a conversation and, finally, to control movement. They may still say words or phrases, but expressing pain becomes difficult. As memory and cognitive skills continue to worsen, personality changes may take place and patients need extensive help with daily activities.

At this stage, patients may:

- Require full time, around the clock assist with daily personal care
- Lose awareness of recent skills as well as of their surroundings
- Require high levels of support with daily activities and personal care
- Experience changes in physical abilities, including the ability to walk, sit and, ultimately, swallow
- Have increasing trouble communicating
- Become susceptible to infections, especially pneumonia

1.11 Economic Hazard

The costs of health care, long-term care and hospice for patients with Alzheimer's disease and other dementias are considerable, and AD is one of the costliest chronic diseases to society. Total payments in 2016 (in 2016 dollars) for all patients with Alzheimer's disease and other dementias are estimated at $236 billion. Medicare and Medicaid are estimated to cover $160 billion, or 68 percent, of the total health care and long-term care expenses for patients with Alzheimer's disease and other dementias. Out-of-pocket spending is predicted to be $46 billion, or 19 percent of total expenses. Total per patient health care and long-term care expenses in 2015 from all sources for Medicare beneficiaries with AD and other dementias were three times as great as payments for other Medicare beneficiaries in the same age group ($49,126 per patient for those with dementia compared with $15,550 per patient for those without dementia)Medicaid fees for nursing home and other long-term care services for some patient with very low income and low assets, and the high use of these services by patient with dementia translates into high costs for the Medicaid program. Average Medicaid payments per patient for Medicare beneficiaries with AD and other dementias ($11,338) were 19 times as great as average Medicaid expenses for Medicare beneficiaries without Alzheimer's disease and other dementias ($590). Despite these and other sources of financial assistance, people with AD and other dementias still suffer high out-of-pocket costs. These costs are for Medicare and other health insurance premiums and for deductibles, copayments and services not covered by Medicare, Medicaid or additional sources of support. Medicare payees age 65 and older with AD and other dementias paid $10,495 out of pocket, on average, for health care and long-term care services not covered by other sources. Average per patient out-of-pocket payments were highest ($20,207 per person) for individuals living in nursing homes and assisted living facilities and was almost six times as great as the average per patient payments for patient with AD and other dementias living in the community. Recently, researchers described the additional or "incremental" health care and caregiving costs of dementia (that is, the costs precisely attributed to dementia when compared with people with and without dementia who have the same coexisting medical situations and demographic characteristics).One group of researchers said that the incremental health care and nursing home costs for those with dementia were $28,501 per year in 2010 dollars ($32,781 in 2015 dollars).Another group of researchers said that the incremental lifetime cost of AD was considerably higher for women compared with men, because of a greater risk of developing AD. Additionally, because women are more probable to be widowed and living in poverty, the incremental Medicaid costs associated with AD were 70 percent higher for women compared with men. Other researchers compared end-of-life costs for patient with and without dementia and found that the total cost in the last 5 years of life was $287,038 in 2010 dollars for people with dementia and $183,001 for

people with other conditions ($330,143 and $210,483, respectively, in 2015 dollars), a difference of 57 percent. Additionally, out-of-pocket costs characterized a substantially larger proportion of total wealth for those with dementia than for people having other conditions (32 percent versus 11 percent).

1.12 Costs of Long-Term Care Services

Patient with Alzheimer's disease and other dementias have twice as many hospital stays per year as other older people. Moreover, the use of health care services for patient with other serious medical conditions is strongly affected by the presence or absence of dementia. Particularly, patient with coronary artery disease, chronic obstructive pulmonary ,diabetes, chronic kidney disease, disease (COPD), stroke or cancer who also have AD and other dementias have higher use and costs of health care services than people having these medical conditions but no coexisting dementia. Costs are high for care providing at home or in an adult day center, an assisted living facility or a nursing home. The following measures are for all users of these services.

- **Home care**
  The median cost for a paid non-medical home health aide is $20 per hour, or $160 for an 8-hour day.

- **Adult day centers**
  The median cost of adult day services is $69 per day. Ninety-five percent of adult day centers provide care for people with Alzheimer's disease and other dementias, and 2 percent of these centers charged an additional fee for these clients in 2012.

- **Assisted living facilities**
  The median cost for basic services in an assisted living facility is $3,600 per month, or $43,200 per year.

- **Nursing homes**
  The average cost for a private room in a nursing home is $250 per day, or $91,250 per year. The average cost of a semi-private room in a nursing home is $220 per day, or $80,300 per year.

Medicaid covers nursing home care and long-term care services in the community for individuals who meet program requirements for level of care, income and assets. To receive coverage, beneficiaries must have low incomes. Most nursing home residents who qualify for Medicaid must spend all of their Social Security income and any

other monthly income, except for a very small personal needs allowance, to pay for nursing home care. Medicaid only makes up the difference if the nursing home resident cannot pay the full cost of care or has a financially dependent spouse. Total Medicaid spending for people with Alzheimer's disease and other dementias is projected to be $43 billion in 2016 dollars). Total per-person Medicaid payments for Medicare beneficiaries age 65 and older with Alzheimer's and other dementias were 19 times as great as Medicaid payments for other Medicare beneficiaries.

In 2009, 6 percent of people admitted to hospices in the United States had a primary hospice diagnosis of Alzheimer's disease (61,146 people).An additional 11 percent of those admitted to hospices in the United States had a primary hospice diagnosis of non-Alzheimer's dementia (119,872 people).Hospice length of stay has increased over the past decade. The average length of stay for hospice beneficiaries with a primary hospice diagnosis of Alzheimer's disease increased from 67 days in 1998 to 106 days in 2009.The average length of stay for hospice beneficiaries with a primary diagnosis of non-Alzheimer's dementia increased from 57 days in 1998 to 92 days in 2009.Average per-person hospice care payments for beneficiaries with Alzheimer's disease and other dementias were 10 times as great as for all other Medicare beneficiaries ($1,976 per person compared with $193 per person), Lucker J.E 2002.

1.12.1 Personal financial impact of AD

Alzheimer's is, by its nature, a very personal disease. Patients living with the disease must grapple with changes in their ability to think and function that threaten their identity and their role within their families. Families are personally affected by Alzheimer's too. Their everyday lives often become busier as they take on responsibilities that the individual with Alzheimer's can no longer perform. And, very often, family members become caregivers. These and other aspects of the effects of Alzheimer's disease on families are well studied. In contrast, little is known about the personal financial impact of Alzheimer's on families. Because studies on this important topic are scarce, the Alzheimer's Association commissioned a nationwide scientific survey of more than 3,500 Americans who were asked these questions and more. The results reveal that many families, as well as friends, of people with Alzheimer's disease and other dementias are making great sacrifices to help care for them. Survey respondents commonly spent money from their savings and retirement accounts, jeopardizing their own financial security. Alarmingly, the Alzheimer's Association survey also revealed that many respondents had to cut back on basic necessities such as food and medical care for themselves and their families. At the same time, many respondents did not know nor had misconceptions about what expenses Medicare and Medicaid cover, leaving them unprepared to handle the

tremendous costs associated with the disease. Taken together, the results of the survey point to the significant financial burden placed on families because their friend or family member with Alzheimer's disease or another dementia can no longer afford to take care of themselves.

1.13 Hypothesis

1.13.1 Oxidative Stress Hypothesis in Alzheimer's disease

The major hurdle in understanding Alzheimer's disease (AD) is a lack of knowledge about the etiology and pathogenesis of selective neuron death. In recent years, considerable data have accrued indicating that the brain in AD is under increased oxidative stress and this may have a role in the pathogenesis of neuron degeneration and death in this disorder. The direct evidence supporting increased oxidative stress in AD is: (1) increased brain Fe, Al, and Hg in AD, capable of stimulating free radical generation; (2) increased lipid peroxidation and decreased polyunsaturated fatty acids in the AD brain, and increased 4-hydroxynonenal, an aldehyde product of lipid peroxidation in AD ventricular fluid; (3) increased protein and DNA oxidation in the AD brain; (4) diminished energy metabolism and decreased cytochrome c oxidase in the brain in AD; (5) advanced glycation end products (AGE), malondialdehyde, carbonyls, peroxynitrite, heme oxygenase-1 and SOD-1 in neurofibrillary tangles and AGE, heme oxygenase-1, SOD-1 in senile plaques; and (6) studies showing that amyloid beta peptide is capable of generating free radicals. Supporting indirect evidence comes from a variety of in vitro studies showing that free radicals are capable of mediating neuron degeneration and death. Overall, these studies indicate that free radicals are possibly involved in the pathogenesis of neuron death in AD. Because tissue injury itself can induce reactive oxygen species (ROS) generation, it is not known whether this is a primary or secondary event. Even if free radical generation is secondary to other initiating causes, they are deleterious and part of a cascade of events that can lead to neuron death, suggesting that therapeutic efforts aimed at removal of ROS or prevention of their formation may be beneficial in AD. © 1997 Elsevier Science Inc.

1.13.2 The cholinergic hypothesis of Alzheimer's disease:

This hypothesis examines the existing scientific applicability of the original cholinergic hypothesis of Alzheimer's disease by describing the biochemical and histopathological changes of neurotransmitter markers that occur in the brains of patients with Alzheimer's disease both at postmortem and neurosurgical cerebral

biopsy and the behavioral consequences of cholinomimetic drugs and cholinergic lesions. Such studies have resulted in the discovery of an association between a decline in learning and memory, and a deficit in excitatory amino acid (EAA) neurotransmission, together with important roles for the cholinergic system in attentional processing and as a modulator of EAA neurotransmission. Accordingly, although there is presently no "cure" for Alzheimer's disease, a large number of potential therapeutic interventions have emerged that are designed to correct loss of presynaptic cholinergic function. A few of these compounds have confirmed efficacy in delaying the deterioration of symptoms of Alzheimer's disease, a valuable treatment target considering the progressive nature of the disease. Indeed, three compounds have received European approval for the treatment of the cognitive symptoms of Alzheimer's disease, first tacrine and more recently, donepezil and rivastigmine, all of which are cholinesterase inhibitors, Patil J.R etal 2009.

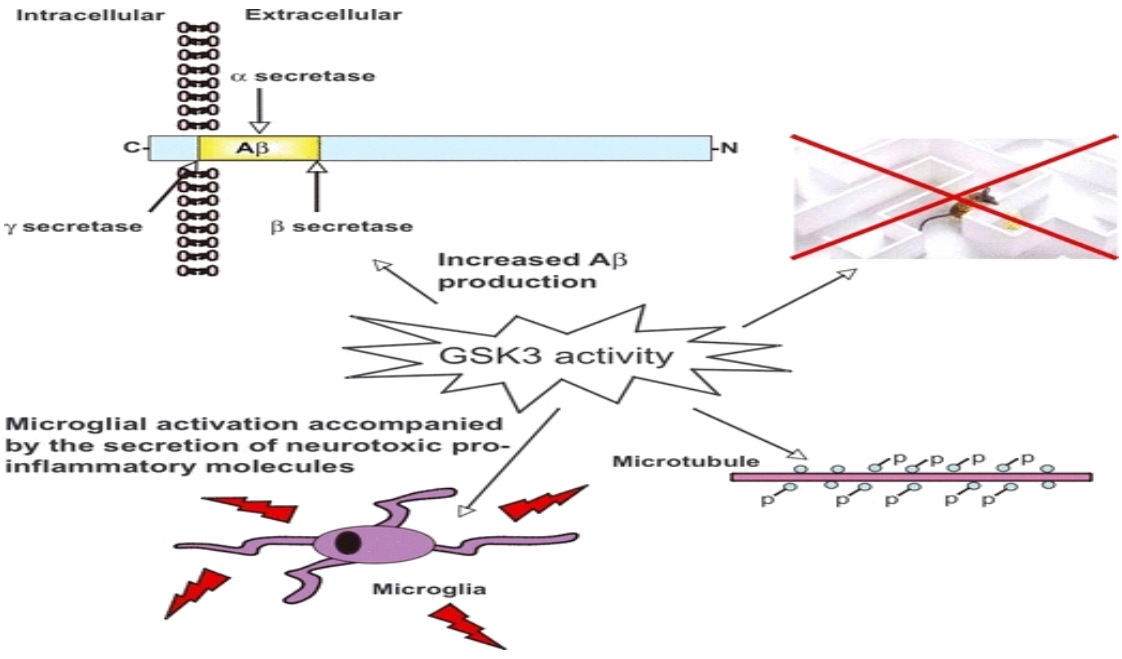
1.13.3 The GSK3 hypothesis of Alzheimer's disease

Glycogen synthase kinase 3 (GSK3) is a constitutively active, proline-directed serine/threonine kinase that plays a part in a number of physiological processes ranging from glycogen metabolism to gene transcription. GSK3 also plays a pivotal and central role in the pathogenesis of both sporadic and familial forms of Alzheimer's disease (AD), an observation that has led us to coin the 'GSK3 hypothesis of AD'. According to this hypothesis, over-activity of GSK3 accounts for memory impairment, tau hyper-phosphorylation, increased β-amyloid production and local plaque-associated microglial-mediated inflammatory responses; all of which are hallmark characteristics of AD. If our 'GSK3 hypothesis of AD' is substantiated and GSK3 is indeed a causal mediator of AD then inhibitors of GSK3 would provide a novel avenue for therapeutic intervention in this devastating disorder.

Alzheimer's disease (AD) is a neurodegenerative disorder defined by progressive memory loss and cognitive impairment and at the molecular level by the presence of neurofibrillary tangles (NFTs) and insoluble β-amyloid (Aβ) plaques (Hardy 2006) that is associated with activated microglia. NFTs are composed of hyper-phosphorylated forms of the microtubule-associated protein tau, whereas Aβ is derived from the proteolysis cleavage of β-amyloid precursor protein (APP). Early onset forms of Familial Alzheimer's disease (FAD) typically present before the age of 65 and have been linked to mutations in APP, presenilin-1 (PS-1) and presenilin-2 (PS-2). Mutations in these genes adversely affect APP processing and result in the increased production of insoluble Aβ and its deposition into plaques.

β-Amyloid precursor protein, presenilin and tau are undoubtedly pivotal to understanding the pathogenesis of AD and there are as yet no convincing refutations of the classical amyloid cascade hypothesis of AD, which postulates that, Aβ over-production leads to the pathogenic hyper-phosphorylation of tau resulting in the formation of neurofibrillary tangles (NFTs) and neurodegeneration. This hypothesis propose that glycogen synthase kinase-3 (GSK3) plays a leading role in the cascade of events that culminate in AD as this kinase is involved in the mechanisms underlying learning and memory, in the hyper-phosphorylation of tau, in the increased production of Aβ from APP and also in local cerebral inflammatory responses.

The evidence that GSK3 plays a central role in AD and that its deregulation accounts for many of the pathological hallmarks of the disease in both sporadic and familial AD cases, has led us to formulate the GSK3 hypothesis of AD. Evidence suggests that GSK3 is intimately involved in the hyper-phosphorylation of tau, memory impairment, the increased production of Aβ and in inflammatory response. GSK3 also reduces acetylcholine synthesis, which is in accordance with the cholinergic deficit present in AD. Moreover, GSK3 is a key mediator of apoptosis and thereby might directly contribute to neuronal loss in AD.



1.1 Figure: GSK3 and its role in AD.

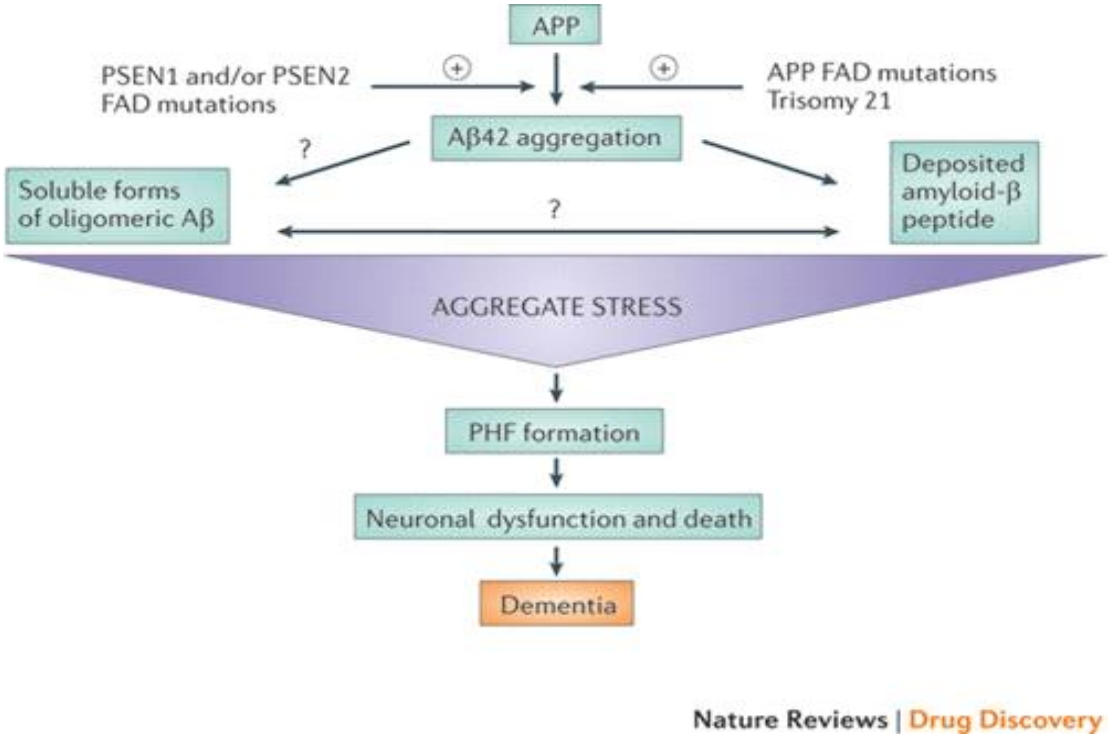
Over-activity of GSK3 caused either by aberrant Wnt or insulin signaling in sporadic AD cases or through familial mutations in PS or APP in FAD, might play an integral

role in disease progression. GSK3 mediates the hyper-phosphorylation**.** Indeed, if GSK3 is central to AD pathogenesis then one would expect evidence for increased activity of this enzyme in AD. However, there is little such evidence, as it is technically difficult, if not impossible, to measure enzymatic activity in post-mortem brain tissue. Nevertheless, indirect evidence does support the role of GSK3 in disease. GSK3 has been shown to co-localize with dystrophic neurites and NFTs. Active GSK3 appears in neurons with pre-tangle changes and there is increased GSK3 activity in the frontal cortex in AD as evidenced by immuno-blotting for GSK3 phosphorylated at Tyr216. Furthermore, GSK3 expression is up-regulated in the hippocampus of AD patients and in post-synaptosomal supernatants derived from AD brain although the latter study reports that there is not an increase in GSK3 enzymatic activity. GSK3 expression is also up-regulated in circulating peripheral lymphocytes in both AD and in mild cognitive impairment. It has recently been reported that a polymorphism in the GSK3 promoter is a risk factor for late onset AD, which might account for alterations in GSK3 expression in disease. Collectively, these findings suggest that GSK3 activity might be increased in AD, through changes in its phosphorylation state as well as expression levels, although we acknowledge that direct evidence for this is still limited at present and some studies find no change in GSK3 activity or reduced GSK3 activity in AD.

1.13.4 The amyloid cascade hypothesis for Alzheimer's disease

The amyloid cascade hypothesis posits that the deposition of the amyloid-β peptide in the brain is a central event in Alzheimer's disease pathology, which has dominated research for the past twenty years.

The amyloid cascade hypothesis for Alzheimer's disease (AD) has been very influential in the research conducted in academia and the pharmaceutical industry. This hypothesis synthesizes histopathological and genetic information, and posits that the deposition of the amyloid-β peptide in the brain parenchyma initiates a sequence of events that ultimately lead to AD dementia.



1.2 Figure: The amyloid cascade for Alzheimer's disease

The amyloid cascade hypothesis posits that the deposition of the amyloid-β peptide in the brain parenchyma is a crucial step that ultimately leads to Alzheimer's disease. Autosomal dominant mutations that cause early onset familial Alzheimer's disease (FAD) occur in three genes: presenilin 1 (PSEN1), PSEN2 and amyloid precursor protein (APP). This hypothesis has been modified over the years as it has become clear that the correlation between dementia or other cognitive alterations and amyloid-β accumulation in the brain in the form of amyloid plaques is not linear, neither in humansnor in mice. The concept of amyloid-β-derived diffusible ligands or soluble toxic oligomershas been proposed to account for the neurotoxicity of the amyloid-β peptide. These intermediary forms lie somewhere between free, soluble amyloid-β monomers and insoluble amyloid fibrils, but the exact molecular composition of these oligomers remains elusive. Toxic, soluble amyloid-β in different forms has been isolated from transfected Chinese hamster ovary cells, transgenic mouse brains, the human brain or it has been reconstituted in vitro under various experimental conditions. The amyloid cascade hypothesis now suggests that synaptotoxicity and neurotoxicity may be mediated by such soluble forms of multimeric amyloid-β peptide species. The dynamic nature of these species and the poorly defined mechanism (or mechanisms) of toxicity make this topic particularly controversial in

the field. Given this uncertainty, we prefer to use the term 'aggregate stress' to describe the potential mechanisms that may lead to amyloid-β aggregation, the formation of paired helical filaments (PHFs) of tau aggregates and, ultimately, result in neuronal loss, Piriyaprasarth etal 2011.

1.13.5 Calcium hypothesis of Alzheimer's disease

Alzheimer's disease (AD) is a progressive neurodegenerative disorder caused by an increase in amyloid metabolism. The calcium hypothesis of AD explores how activation of the amyloidogenic pathway may function to remodel the neuronal Ca^{2+} signaling pathways responsible for cognition. Hydrolysis of the β-amyloid precursor protein (APP) yields two products that can influence Ca^{2+} signaling. Firstly, the amyloids released to the outside form oligomers that enhance the entry of Ca^{2+} that is pumped into the endoplasmic reticulum (ER). An increase in the luminal level of Ca^{2+} within the ER enhances the sensitivity of the ryanodine receptors (RYRs) to increase the amount of Ca^{2+} being released from the internal stores. Secondly, the APP intracellular domain may alter the expression of key signaling components such as the RYR. It is proposed that this remodeling of Ca^{2+} signaling will result in the learning and memory deficits that occur early during the onset of AD. In particular, the Ca^{2+} signaling remodeling may erase newly acquired memories by enhancing the mechanism of long-term depression that depends on activation of the Ca^{2+}-dependent protein phosphatase calcineurin. The alteration in Ca^{2+} signaling will also contribute to the neurodegeneration that characterizes the later stages of dementia.

1.13.6 The channel hypothesis of Alzheimer's disease

The channel hypothesis of Alzheimer's disease (AD) proposes that the beta-amyloid (Aβ) peptides which accumulate in plaques in the brain actually damage and/or kill neurons by forming ion channels. Evidence from a number of laboratories has demonstrated that Aβ peptides can form ion channels in lipid bilayers, liposomes, neurons, oocytes, and endothelial cells. These channels possess distinct physiologic characteristics that would be consistent with their toxic properties. Aβ channels are heterogeneous in size, selectivity, blockade, and gating. They are generally large, voltage-independent, and relatively poorly selective amongst physiologic ions, admitting calcium ion (Ca^{2+}), Na^{+}, K^{+}, Cs^{+}, Li^{+}, and possibly Cl^{-}. They are reversibly blocked by zinc ion (Zn^{2+}), and tromethamine (tris), and irreversibly by aluminum ion (Al^{3+}). Congo red inhibits channel formation, but does not block inserted channels. Although much evidence implicates Aβ peptides in the neurotoxicity of AD, no other toxic mechanism has been demonstrated to be the underlying etiology of AD. Channel

formation by several other amyloid peptides lends credence to the notion that this is a critical mechanism of cytotoxicity.

1.14 Plants used for the treatment of Alzheimer's disease

No treatment is obtainable to slow or stop AD. The U.S. Food and Drug Administration has permitted five drugs that temporarily improve symptoms. The effectiveness of these drugs varies across the population. None of the treatments available today modifies the underlying course of this terminal disease.

Herbs show promise in AD treatment because of their cognitive benefits and more importantly, their mechanisms of action with respect to the fundamental pathophysiology of the disease.

In summary, preliminary clinical evidence demonstrated that some herbal medicines can ameliorate learning and memory in patients with mild-to-moderate AD. Potential beneficial actions exerted by the active components of these herbs are not limited to the inhibition of AChE and include the modification of Aβ processing, protection against apoptosis and oxidative stress, and anti-inflammatory effects.

The use of medicinal plants in the treatment of AD should be compared with the pharmacological treatment currently in use. These studies should include fundamental identification in order to confirm the clinical trial.

1.14.1 Ashwagandha (*Withania somnifera*)

Ashwagandha is used widely in Ayurveda as a nervine tonic, aphrodisiac, and adaptogen and helps the body adjust to stress. Ashwagandha is a member of the nightshade (Solanaceae) family, and the root is the part that is extensively used. It is categorized as a rasayana (rejuvenative) and is thought to possess free radical scavenging activity, antioxidant activity, and an ability to support a healthy immune system. Unlike other adaptogens, which tend to be stimulating, Ashwagandha has a comforting effect and thus may be predominantly indicated in people with AD. A total alkaloid extract of Ashwagandha root revealed a calming effect on the central nervous system (CNS) in several mammalian species, signifying the use of this herb to provide relaxation. A recent double-blind, placebo-controlled study of the effects of Ashwagandha on stress establish that it reduces symptoms of anxiety and inability to concentrate and reverses forgetfulness in a dose-dependent manner, and that is 500 mg/day was more effective. No supplementary adverse effects were found.

1.14.2 Brahmi (*Bacopa monnieri*)

Brahmi (also known as Bacopa) is a bitter tasting climber plant found in damp and marshy spaces and is commonly used in Ayurvedic medicine as a nervediuretic, tonic, and cardiotonicand as a therapeutic agent againstinsomnia, epilepsy, asthma, and rheumatism. The principal ingredients of *Bacopa monnieri* (BM) are saponins and triterpenoid bacosaponins that containbacopasides III to V, bacosides A and B, and bacosaponins A, B, and C. Other saponin glycosides contain the jujubogenin bis desmosides bacopasaponins D, E, and F. Other components include polyphenols, alkaloids, plant sterols, betulic acid, and sulfhydryl compounds that provide antioxidant activity. Thus, BM could perform by decreasing the formation of lipid peroxides, reducing divalent metals, scavenging reactive oxygen species, and inhibiting lipoxygenase activity. Conventionally, BM was used to enhance memory and cognitive function. The BM extracts have been examined extensively for their neuropharmacological effects and their nootropic actions. In the hippocampus, BM improves protein kinase activity that may contribute to its nootropic action. BM also inhibited cholinergic degeneration and displayed a cognition-enhancing effect in a rat model of AD.

1.14.3 Shankhpushpi (*Convolvulus pluricaulis*)

Various species for Shankapushpi, including *Convolvulus pluricaulis* (CP), *Convolvulus microphyllus, Evolvulusalsinoides*, and *Clitoriaternatea* (CT), have been defined. Shankhpushpi is a common plant in India, where the whole plant is used in various methods as a nervine tonic for enlargement of memory and cognitive function. A wide range of secondary metabolites, includingflavonol glycosides, triterpenoids, anthocyanins, and steroids, has been isolated and may be liable for Shankhpushpi's nootropic and memory enhancing properties in addition to other pharmacological activities. It is thought that Shankhpushpi calms the nerves by regulating the body's formulation of the adrenaline, stress hormones, and cortisol. It is also mentioned for nervous disorders such as anxiety, stress, mental fatigue, and insomnia. The ethanolic extract of CP and its ethyl acetate and aqueous fractions considerably improved learning and memory in rats.

A dose dependent enhancement of memory was detected in mice that were administered extracts of CP. Similarly, administration of CP extracts for 7 days enhanced memory in aged mice.

1.14.4 Gotu kola (*Centella asiatica*)

In the Ayurvedic system of medicine, gotu kola is one of the significant rejuvenating herbs for nerve and brain cells and is thought to be capable of increasing longevity, intelligence, and memory. Asiaticoside derivatives, including asiatic acid and asiaticoside, were found to reduce hydrogen peroxide induced cell death, inhibit beta-amyloid cell death and decrease free radical concentrations, suggesting a possible role for gotu kola in the cure and prevention of AD and beta-amyloid toxicity. Gotu kola extracts inverted the beta-amyloid pathology in the brains of PSAPP mice and modulated the constituents of the oxidative stress response.

1.14.5 Jyotishmati (*Celastrus paniculatus*)

Jyotishmati is a precious medicinal herb that is valued for its effects on the brain and has been used for eras in Ayurveda for sharpening the memory and developing concentration and cognitive function. Aqueous extracts of CP seeds have cognition enhancing activity and antioxidant properties. CP extracts sheltered neuronal cells against H_2O_2induced toxicity in part by virtue of their antioxidant activity and their ability to induce antioxidant enzymes. CP extracts also protected neuronal cells against glutamate induced toxicity by modulating glutamate receptor function. In addition, the CP extracts protected neuronal cells by means of their reducing lipid peroxidation, free radical scavenging properties, and also by their ability to induce the antioxidant enzyme catalase. In addition, aqueous extracts of CP seed have dose dependent cholinergic property, thereby improving memory performance.

1.14.6 Jatamansi (*Nardostachys jatamansi*)

Studies on its role in the CNS discovered that extracts of *Nardostachys jatamansi* (NJ) lightened all of the symptoms of chronic fatigue syndrome (CFS) in rats. CFS triggered increases in nitrite, lipid peroxidation, and superoxide dismutase levels, and low catalase levels were all inverted by NJ extracts. The data indicate the powerful antioxidant property of NJ. Similarly, an alcoholic extract of this plant administered to both young and aged mice considerably improved learning and memory and also inverted the amnesia induced by diazepam and scopolamine. Additionally, it reversed aging induced amnesia due to the natural aging of mice, signifying that the compounds in this plant may prove to be useful in reestablishing memory in older individuals as well as in patients with age associated dementia.

1.14.7 Maca (*Lepidium meyenii*)

In a study effect of different doses of aqueous and hydroalcoholic extract of maca on learning and memory shortages induced by scopolamine (1 mg/kg) in mice has been investigated for Maca enhanced memory impairments and spatial learning and ameliorate passive avoidance learning and memory deficits. The results showed that scopolamine increase AChE activity in the mice brain up to 1.5-fold. Maca extract decreased brain AChE activity by 45% compared to the group that administered only scopolamine.

1.14.8 Ginkgo biloba (*G. biloba*)

G. biloba, the oldest tree on the earth, is innate to China and now cultivated in Europe and America. *G. biloba* extract treat inadequacy of blood circulation problems, especially in the brain that causes loss of consciousness, memory loss, headaches, and depression in the elderly. This extract is described to contain about 24% flavonoids and 6% terpene lactones. There is dependable evidence that standardized ginkgo extract displays several molecular and cellular neuroprotective mechanisms, containing the attenuation of apoptosis, anti-inflammatory effects, the inhibition of membrane lipid peroxidation and the direct inhibition of amyloid-b aggregation. There are widespread clinical investigations regarding its potential role in cognitive disorders. Chronic treatment of *G. biloba* on memory and learning in mice exposed that *G. biloba* improved storage, acquisition, and retrieval of a two-response sequence for food reward. Thefree-radical scavenging propertiesand antioxidant of *G. biloba* extract are primarily recognized to the flavonoid fraction. *G. biloba* affects cognitive function in an animal model of AD without altering the histopathological consequences of overexpression of β amyloid precursor protein.*G. biloba* extract considerably inhibit the AChE activity in the brain. The inhibition of AChEproperty can be correlated with improvement observed in scopolamine induced shortages in passive avoidance by *G. biloba* extract. The decrease in AChE activity shows an increase in the basal level of acetylcholine.

1.14.9 Salvia officinalis (S. officinalis)

S. officinalis has a very old reputation for enlightening memory. It is singularly good for the head and brain. The probable pharmacological effects of the herb, which may be significant to AD, contain anti-inflammatory and antioxidant activities as well as weak AChE inhibitory activity. The leaves of *S. officinalis* L. (sage) are well known for their antioxidative activity. Rosmarinic acid (the main active constituent of *S. officinalis*) reduced a number of deleterious events induced by Aβ contain reactive

oxygen species formation, DNA fragmentation, lipid peroxidation, caspase-3 activation and tau protein hyper phosphorylation. *S. officinalis* have a long history of use as memory enhancing agents along with cholinergic activity that may be related to amelioration of the cognitive shortages associated with AD. Based on clinical evidence *S. officinalis* may help to inhibit or alleviate symptoms of AD. In a randomized double-blind clinical study, patients with mild to moderate AD administered *S. officinalis* extract. The result revealed that *S. officinalis* had statistically important effectiveness in the cognition after 16 weeks of treatment. One small pilot trial indicated that oral administration of *S. officinalis* essential oil to 11 patients presenting mild-to moderate symptoms of AD significantly improved cognitive function.

1.14.10 Melissa officinalis (M. officinalis)

M. officinalis (lemon balm) is a cultured perennial lemon scented herb. Records regarding its use date back over 2000 years with entries in the Historia Plantarum. In traditional medicine *M. officinalis* L. (Lamiaceae) has been used as a cure for over 2000 years, and has been acclaimed for promoting long life and for reestablishing memory. The leaves of this plant contain monoterpenes (*e.g.* citral) with weak anti-AChE property, and phenol carboxylic acids containing rosmarinic acid, which shows anti-amyloidogenic, antioxidative and antiapoptotic effects. *M. officinalis* essential oil, given in canary wine, every morning will renew youth, sharpen the brain. Patients with mild to moderate AD taking *M. officinalis* extract found significant benefits in cognition after 16 weeks of treatment. *M. officinalis* hasmodulates mood and central nervous system acetylcholine receptor activity and cognitive performance following acute administration.

2.1 Plants

2.1.1 *Citrus Maxima*

Citrus maxima are known as the pomelo in the Rutaceae (citrus family). It is a medium-sized tree but the largest of all *Citrus* species, with large leaves, flowers, and fruits. The species is native to southern China and Malaysia (and possibly other parts of Southeast Asia), and is now cultivated in many tropical and semi-tropical countries for its large fruits. This species was a progenitor of the grapefruit (*C.* X *paradisi*) and the tangelo (*C. reticulata*), among other modern citrus hybrids. Pomelos are often confused with grapefruits, from which they can generally be distinguished by their larger size, thicker rinds, milder—even sweet—flavor, and tough bitter membranes that are often considered inedible.

2.1 Figure: *Citrus Maxima* fruit

The *C. maxima* tree, which is the most cold-intolerant citrus species, has a rounded crown and grows 5 to 15 m (15 to 50 ft) tall. The tree has large evergreen oblong to elliptic leaves, 10.5 to 20 cm (4 to 8 in) long, with winged petioles (leaf stems). The flowers and fruits are borne singly, in contrast to grapefruits (*C.* X *paradisi*), in which they grown in clusters of 2 to 20. The fruits, which vary from round to pear-shaped and ripen to yellow, orange, or red, are large--30 cm or more in diameter, and weighing up to 9 kg (20 lbs). The flesh of the fruit, which may be greenish yellow, yellow, pink, or red, is often juicy, and divided into 11 to 18 segments. The flavor is sweet to somewhat acidic.

Like other citrus fruits, pomelos are high in vitamin C. They are generally eaten as a fresh fruit, and they store well. They have long been popular in Asia, especially China, Indonesia, and Thailand, but are increasingly found in specialty markets in the U.S. as well. The juice is also used in various beverages (both alcoholic and non-alcoholic), and the peel may be candied. Traditional medicinal uses of the fruit include treatment of coughs, fevers, and gastrointestinal disorders. The aromatic flowers are picked and processed into perfume in Vietnam, and the wood, which is heavy and hard-grained, used for making tool handles.

2.1.2 *Citrus limon*

Citrus limon, lemon, is a small tree in the Rutaceae (citrus family) that originated in Asia (likely India and Pakistan) and is now grown commercially worldwide in tropical, semi-tropical, and warm temperate countries, including the Mediterranean region, for the fruit, which is used fresh and in beverages and cooking, and is also used as a preservative due to its anti-oxidant properties.

2.2 Figure: *Citrus limon* fruit

C. limon is thought to have arisen as a hybrid between other *Citrus* species; some studies suggest lineage including lime (*C. aurantifolia* or *C. latifolia*), pomelo (*C. maxima*), and citron (*C. medica*), while other studies suggest that is a hybrid between sour orange (*C. aurantium*) and citron. It has a long history of cultivation in southeast Asia and China, but arrived the Mediterranean during Roman times, and was brought to the New World in the 16th century. In commercial cultivation, *C. limon* is often grafted onto rootstock of the hardy rough lemon, *C. jambhiri*.

The lemon tree grows to 6 m (20 ft) tall, and has stout spines. The leaves are dark green, leathery, and evergreen, oblong, elliptical, or oval and up to 14 cm (4 in) long; in contrast to several other citrus species, the petioles (leaf stems) are not winged or only narrowly winged. Flower buds are purplish but flowers open to have 5 white petals, up to 5 cm across. Fruits are globose to oblong, 7.5 to 12.5 cm long, and ripen to yellow, with smooth to bumpy rinds dotted with oil glands.

Lemon fruits can be highly acidic (although non-acid varieties are also cultivated), and are high in citric acid and vitamin C. Their tart flavor is popular in beverages (lemonades and iced teas as well as many cocktails), ice creams and desserts, salad dressings, and many meat and vegetable dishes. Lemons have antioxidant properties, so lemon juice is often added to fresh fruit to prevent oxidation and browning. Lemon peel or zest (the outer peel) is used as a flavoring or candied. Lemon oil, obtained from the peel, is used as a wood cleaner and polish, and as a non-toxic pesticide. Traditional medicinal uses for the fruit, peels, oil, and oil obtained from the seeds include treating fever and colic, and as an astringent and diuretic.

In 2010, commercial production of lemons (together with limes) was 14.2 million metric tons harvested from 1.0 million hectares. The leading producers are India, Mexico, Argentina, China, and Brazil.

2.1.3 *Citrus aurantifolia*

Citrus aurantiifolia, key lime (also known as Mexican or West Indian lime), is a thorny shrub or small tree in the Rutaceae (citrus family) that originated in southeast Asia, likely Indonesia and Malaysia, and is cultivated in tropical areas from the West Indies and Central and South America to India, China, and parts of Africa for its flavorful fruit. It is one of several species of *Citrus* referred to as limes; others include *C. hystrix* (makrut lime or papeda), *C. australasica* and *C. australis* (finger lime and Australian round lime, respectively, both of which are sometimes classified in *Microcitrus*), and *C. glauca* (desert lime, sometimes classified in *Eremocitrus*). *C. latifolia*, the Persian or Tahitian lime, which is the most widely grown commercial species, can be distinguished from *C. aurantiifolia* by its larger size, absence of seeds, hardiness, absence of thorns, and longer fruit shelf life.

2.3 Figure: *Citrus aurantiifolia* fruit

The most frost-intolerant of the citrus fruits, *C. aurantiifolia* is a vigorous and drought-resistant shrub or many-branched small tree with numerous sharp spines, 1 cm long (3/8 inch). The leathery, evergreen leaves are alternate, elliptic to oblong, 5 to 7.5 cm (2 to 3 in) long, with narrowly winged stems. The white flowers are solitary or clustered in racemes of 2 to 7; individual flowers are up to 5 cm (2 in) across, with 4 to 6 petals and 20-25 stamens. The small greenish fruits, which ripen to yellow, are generally round to oval or elliptical, around 5 cm (2 in) in diameter, with greenish-yellow, juicy pulp divided into 6 to 15 segments containing few to many small seeds.

C. aurantiifolia was brought to Mediterranean Europe during the Crusades, and then to the Caribbean (likely by the Spaniards) by 1520, where it became locally naturalized throughout the West Indies. It was cultivated in southern Florida and the Florida Keys by the mid-19th century, and became a common "dooryard" fruit, with commercial production in local areas from the 1880s until 1926, when a hurricane destroyed most of the orchards. A public relations campaign to restore the industry, in the 1950s, may have allowed "key lime" to prevail as the common name. This lime is still grown to a limited extent in Florida, but is part of a thriving industry in Dominica, where it is exported to England to be bottled as "Rose's Lime Juice." Key lime, which has a sour, acidic flavor, is sometimes sold as a fresh fruit, but is also widely used for the juice, peels, and the oil obtained from them. Limes are used in sherbet or sorbet, marmalades, jams, and chutneys, and in "Key Lime pie," although

the pie is often made from Persian lime instead. Lime juice is important in sauces and in juice and cocktail beverages, including popular summertime drinks such as daiquiris, mojitos, and *Brazilian caiparinhas*. Lime juice is low in calories but high in vitamin C. The aromatic leaves are used as a seasoning in Asian cooking. Lime juice is used as a natural remedy to relieve the itch of mosquito bites. In Malaysia and India, the juice is used in traditional medicine to relieve stomach ailments and as an antiseptic, among many other applications.

2.1.4 *Citrus sinensis*

Citrus sinensis, orange or sweet orange (to distinguish it from related species, such as sour orange, *C. aurantium*, and mandarin orange, *C. reticulata*), is a small tree in the Rutaceae (citrus family) that originated in southern China, where it has been cultivated for millennia. Oranges are now grown commercially worldwide in tropical, semi-tropical, and some warm temperate regions, and have become the most widely planted fruit tree in the world. Oranges are the world's most popular fruit, and are eaten fresh and used for juice.

The orange tree is small, spiny tree, typically growing to 7.5 m (25 ft), but occasionally reaching heights up to 15 m (50 ft), generally with a compact crown. Leaves are leathery and evergreen, and range from elliptical to oblong to oval, 6.5-15 cm long and 2.5-9.5 cm wide, often with narrow wings on the petioles (leaf stems). The fragrant white flowers, produced singly or in cluster of up to 6, are around 5 cm wide, with 5 petals and 20 to 25 yellow stamens. The fruit, which may be globose to oval, is typically 6.5 to 9.5 cm wide, and ripens to orange or yellow. The fruit skin (rind or peel) contains numerous small oil glands. The flesh or pulp of the fruit is typically juicy and sweet, divided into 10 to 14 segments (although there are seedless varieties) and ranges in color from yellow to orange to red. Hundreds of cultivars have been developed, which are grouped into 4 major categories by geography (Mediterranean oranges, Spanish oranges) and characteristics (blood oranges, navel oranges).

2.4 Figure: *Citrus sinensis* fruit

Oranges, which are high in vitamins A and C and potassium, are eaten fresh or processed into juice, which can be consumed directly or further processed into concentrate, both used in numerous soda and cocktail drinks, punches, orangeades, and liqueurs (although many orange liqueurs are made from sour, rather than sweet, oranges, or from a combination). Orange fruits and peels are used in numerous desserts, jams and marmalades, candied peels, as well as cookies, cakes, and candies. Oil derived from orange peels, as well as flowers, leaves, and twigs is used as an essential oil in perfumes; orange seed oil may also be used in cooking or as a component in plastics. Orange blossoms produce more nectar than any other source in the U.S., and are important for honey production (more than 25% of honey produced in California is from orange groves).

The total global commercial production of oranges in 2010 was 69.4 million metric tons (mt), harvested from 4.1 million hectares. Brazil, which is the leading producer (with 19.1 million mt), produced more than twice as much as the second-ranked U.S. (with 7.5 million mt). Other leading producers included India, China, Mexico, and Spain.

Orange trees and fruits are susceptible to various pests and pathogens, including the Mediterranean fruit fly (*Ceratitiscapitata*), numerous fungal leaf spots, blights, and root rots (including *Cercospora, Colletotrichum Fusarium, Phytophthora*, and many others) and viruses that can significantly reduce yields.

2.1.5 *Citrus bergamia*

The Bergamot (*Citrus bergamia*) is a surprisingly nutritious citrus fruit that has a fresh scent and a very useful essential oil which is taken from the peel. Native to South Asia, the bergamot orange or *Citrus bergamia* was exported to Italy where it flourished and now the fruit is harvested for medicinal and commercial purposes. The fruit is the size of an orange but yellow in color. The juice is very sour and bitter, so it would be very hard to drink enough to get the benefits that can be obtained from the extract supplement. Bergamot supplements are taken for several reasons including lowering cholesterol levels, blood sugar, reducing middle obesity and arterial stiffness.

2.5 Figure: *Citrus bergamia* fruit

Studies showed that bergamot lowered the total cholesterol levels in participants as well as the low-density lipoprotein (LDL) levels, which is a major factor for heart disease. It also raised the high-density lipoprotein (HDL) which is good and has protective benefits.

It is thought that bergamot works by blocking the production of cholesterol in the liver. Without cholesterol, the liver may be forced to find cholesterol that is stored in the bloodstream. Bergamot has compounds that are similar to commercial chemicals that are given to lower cholesterol.

Bergamot contains very large amounts of polyphenols. Brutelidin and Metilidin are two that directly inhibit the biosynthesis of cholesterol. Triglyceride levels were also lowered in the participants of these studies.

Along with ultra-violet (UV) light treatment for a fungal infection tumor under the skin:

• Preventative for lice and other parasites
• Treatment along with UV light for psoriasis

Bergamot is used in skin care products such as creams, soaps, perfumes, lotions and suntan oils. It is used for psoriasis as well as an antiseptic against infections and to reduce inflammation. It is also used to treat Mycosis Fungoides, a rare type of skin cancer. It increases the skin's sensitivity to sunlight, so it must not be used along with other medications that increase sensitivity to sunlight. It could cause severe sunburn and rashes and blisters. For anyone using bergamot, it is necessary to wear protective clothing and sunblock if there will be time spent in direct sunlight.

Citrus bergamia is an erect, unarmed, much branched tree up to 12 m tall, with trunk up to 25 cm in diameter; in cultivation trees are pruned up to 4-5 m in height with crown diameter of about 5 m.

Leaves alternate, simple, glandular, aromatic when bruised; petiole about 13 mm long, moderately winged, articulated near the blade; blade lanceolate, up to 12 cm x 6 cm, in upper third part weakly indented.

Inflorescence terminal, racemose, many-flowered; pedicel up to 8 mm long; flowers bisexual, 4-5(-10)-merous, fragrant; calyx cup-shaped with short lobes, yellow-green; corolla 3.8 cm in diameter, most often with 5, narrow-elongate, pure-white petals without any purple tinge; stamens (13)21(-28), in (2-)4(-6) groups, sometimes petaloid; disk nectariferous; pistil with subglobose ovary, short and thick style, distinct to indistinct stigma.

Fruit a slightly flattened subglobose to pyriform berry (hesperidium), 6.5-7 cm x 6-7.5 cm, often with a small navel and a persistent style; peel 6-7 mm thick, with numerous glands, tough, smooth to rough, sometimes ridged, adherent, shiny green turning yellow when ripe; flesh yellowish, firm, very acid and bitter, divided into 8-14 segments.

Seed (0-)3(-13) per fruit, flattened, 11 mm x 6 mm x 4.4 mm, pale yellow, usually monoembryonic.

C. bergamia is most probably of hybrid origin. It has been suggested that it is a hybrid between sour orange (C. aurantium L.) and lemon (C. limon (L.) Burm.f.), or a mutation of the latter. Others hold it as a hybrid between sour orange and lime (C. aurantifolia (Christm. & Panzer) Swingle). Bergamot is only known from cultivation and consists of a limited and well defined number of cultivars. Four cultivar groups are recognized in bergamot: Common Bergamot, Melarosa (fruit rather flattened), Torulosa (fruit ridged) and Piccola (dwarf cultivars). Only Common Bergamot is commercially cultivated for the essential oil and 3 cultivars are grown: 'Castagnaro', 'Femminello' and 'Inserto'. Formerly, 'Femminello' and 'Castagnaro' constituted virtually all commercial plantings in the world, but they have largely been replaced by 'Inserto' ('Fantastico'), a hybrid of 'Femminello' and 'Castagnaro'. 'Femminello' is somewhat less vigorous and smaller than 'Castagnaro', but is earlier and more regular in bearing. Its fruit is spherical or nearly so, the rind smooth and more aromatic and hence it is preferred. 'Castagnaro' is more upright and vigorous, attaining a larger size than 'Femminello', but is less fruitful. Its fruit is roundish but frequently exhibits a short neck and obovate outline and is sometimes slightly ribbed; the rind is usually rougher and the oil usually less aromatic than in 'Femminello'. 'Inserto' is a fairly vigorous tree,that yields well and has only a slight tendency to alternate-bearing; its fruit is medium in size, averaging about 130 g with a rough rind texture.

2.2 Literature Review

2.2.1 *Citrus Maxima*:

1. Paiwan Buachan etal says *Citrus Maxima* has selective activity on human endothelial cells which enhance cell migration and delay cellular aging. As these activities are related with the treatment of Alzheimer's disease we are interested for this work.

2. Priyanka Singh etal says, *Citrus Maxima* has antifungal, antiflatoxigenic and antioxidant activity. As these activity has relation with Alzheimer's disease that's why we are interested to work on this fruit.

3. P. Vijaylakshmi and R. Radha say a study of literature exposes some notable pharmacological activities of the plant such as activity on CNS, anti-diabetic and cholesterol reducing property, analgesic, anti-inflammatory, hepato

protective, antioxidative property, cytotoxic activity, and many more medicinal values. For these activities we are interested to work on *Citrus Maxima.*

4. Suchada Piriyaprasarth et al says the peel of *citrus maxima* contains pectin which makes us interested doing this work.

2.2.2 *Citrus Aurantifolia:*

1. Huei-Jiun Su etal says the complete chloroplast genome of *Citrus aurantifolia* is 159,893 bp in length. Comparing with the sweet orange (C*itrus. sinensis*) chloroplast genome, they identified three intergenic regions and 94 simple sequence repeats (SSRs) that are potentially informative markers with resolution for interspecific relationships. As this can be helpful regarding the disease we are interested to this work.

2. Anugerah Budipratama Adina etal says combination of Ethanolic Extract of *Citrus aurantifolia* peel contains Doxorubicin Modulate Cell Cycle and Increase apoptosis Induction on MCF-7 Cells. That's why we are interested in this work.

3. RA Onyeagba et al says *citrus aurantifolia* has the antimicrobial activity against *Staphylococcus aureus*; *Bacillus spp.*, *Escherichia coli* and *Salmonella spp.* This creates interest on us.

4. Mehdi Razzaghi-Abyaneh et al says *citrus aurantifolia* has antiaflatoxigenic activity which creates interest on us doing the work.

5. Jaiprakash R. Patil et al says *Citrus aurantifolia* juice induces apoptosis in human pancreatic cells. This shows interest on us.

2.2.3 *Citrus Sinensis:*

1. Maria A. Anagnostopoulou etal says the antioxidant activity was found in the fractions of *Citrus sinensis*, this should be attributed to the occurrence of flavonoids and other phenolic compounds. For this reason we worked on this.

2. S. kaviyaetal says the peel extract of *citrus sinensis* acts as bioreducing and capping agents. It also acts as a good antibacterial agent specifically against Gram-negative and Gram-positive bacteria.

3. Muhammad Kamran Khan etal says the peel of *citrus sinensis* contains polyphenols especially flavones which draws our attention to work on this.

4. Qiang Xu etal say *citrus sinensis* are an important nutritional source for human health and have immense economic value. This creates interest on us.

2.2.4 *Citrus Bergamia:*

1. G. Mandalari etal says the peel of *citrus bergamia contains* antimicrobial activity of flavonoids. So we found it as interesting part for the treatment of Alzheimer's disease.

2. Giuseppe Gattuso etal say a comprehensive profile of flavonoids in *citrus bergamia* juice was obtained by a single DAD-ESI-LC-MS-MS course. As it has relationship with Alzheimer's disease we become interested to this work.

3. M. Sanguinetti etal says *Citrus bergamia* oil has in vitro activity against clinical isolates of dermatophytes. This activity seems to be interesting for the work.

4. Monica Curro etal say *Citrus bergamia* Juice extract diminishes β-Amyloid induced pro-inflammatory activation of THP-1 Cells through MAPK and AP-1 Pathways. As this is related with the disease we are interested to work on this.

5. Purum Kang etal says *Citrus bergamia* raises Intracellular Ca^{2+} in human vascular endothelial cells for the release of Ca^{2+} from primary intracellular stores. As it has relation with Alzheimer's disease we are interested to this work.

6. Angela Filocamo etal says*citrus bergamia* juice has in vitro effect against *cag*A-positive and-negative clinical isolates of *Helicobacter pylori*. This activity shows interest on us.

7. Monica Borgatti etal says *Citrus bergamia* fruit extracts and its identified components modify expression of interleukin 8 gene in cystic fibrosis bronchial epithelial cell lines. This information influences us in this work.

8. Debora Lo Furno etal says extract of *Citrus bergamia* decline sadipogenesis and rises lipolysis by modifying PPAR levels in mesenchymal stem Cells from human adipose tissue. This action creates interest on us.

2.2.5 *Citrus Limon:*

1. Stefania Raimondo etal say *Citrus limon* derivative nano vesicles inhibit cancer cell proliferation and destroy CML xenograft growth by persuading TRAIL-mediated cell death. This activity shows us interest in this work.

2. Carlos A. Ledesma-Escobar etal says *Citrus limon* extraction contain flavonoids. As this has relationship with Alzheimer's disease we worked on this.

3. Joost Lucker etal says *Citrus limon* owns a high content and large variety of monoterpenoids, particularly in the glands of the fruit flavedo. That's why we found it interesting doing the work.

4. M. Tiziana Baratta etal says *citrus limon has antimicrobial and antioxidant capacity.* As this activity is related with Alzheimer's disease we are interested in this work.

3.1 Materials and Methods:

3.1.1 Collection of Plant Materials:

a. *Citrus Aurantifollia* collection:

Citrus Aurantifollia is a citrus fruit and it was collected from jatrabari, Dhaka district of Bangladesh, in March, 2016 and identified by taxonomist.

b. *Citrus Bergamia* collection:

Citrus Bergamia is a fragrant fruit similar to lemon found in southern Italy, mostly the short stretch area where the temperature is favorable. The fruit was collected from jatrabari, Dhaka districts of Bangladesh, in March, 2016 and also identified by an expert taxonomist.

c. *Citrus Limion* collection:

Citrus *Limon* is a small tree in the Rutaceae (citrus family) that originated in Asia (likely India and Pakistan) and is now grown commercially worldwide in tropical, semi-tropical, and warm temperate *countries*, including the Mediterranean region. It was collected from jatrabari, Dhaka district of Bangladesh, in March 2016. The plant was identified by taxonomic expert.

d. *Citrus Maxima* collection:

Citrus Maxima is a large size citrus fruit. It was collected from jatrabari, Dhaka district of Bangladesh. It was also identified by taxonomist.

e. *Citrus Sinensis* collection:

Citrus Sinensis is known as orange in English. It is very popular in Bangladesh. The fruit was collected from jRabari, Dhaka district of Bangladesh. It was also identified by taxonomist.

3.1.2 Preparation of Plant Material:

a. *Citrus Aurantifollia* preparation:

The collected fruit was first washed with water to clean and removed dhering dirt and then cut off. The peel was separated from the fruit and cut into small pieces. Then it was shade dried for several days with little sun drying. For better grinding these peel 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.

3.1 Figure: *Citrus Aurantifollia* powder

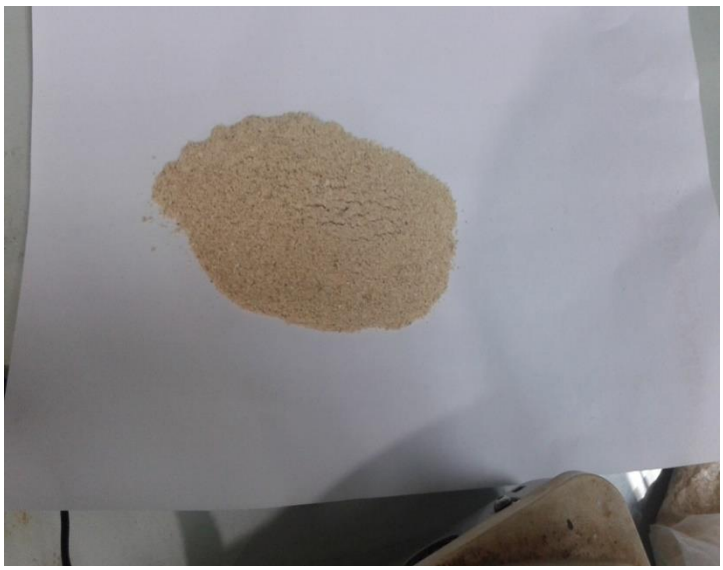
b. *Citrus Bergamia* preparation:

The collected fruit was first washed with water to clean and removed dhering dirt and then cut off. The peel was separated from the fruit and cut into small pieces. Then it was shade dried for several days with little sun drying. For better grinding these peel 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.

3.2 Figure: *Citrus Bergamia* powder

c. *Citrus Limion* preparation:

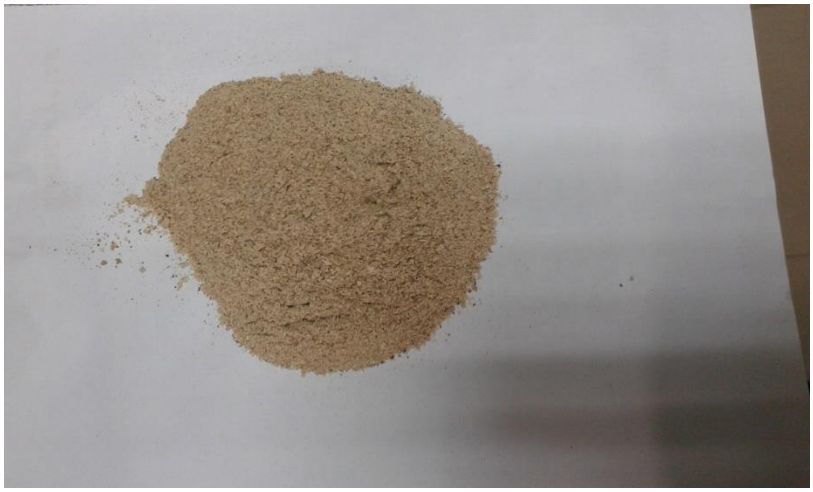
The collected fruit was first washed with water to clean and removed dhering dirt and then cut off. The peel was separated from the fruit and cut into small pieces. Then it was shade dried for several days with little sun drying. For better grinding these peel then dried for 24 hours at considerably 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 Figure: *Citrus limon* powder

d. *Citrus Maxima preparation*:

The collected fruit was first washed with water to clean and removed dhering dirt and then cut off. The peel was separated from the fruit and cut into small pieces. Then it was shade dried for several days with little sun drying. For better grinding these peel 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 soaked in coarsed powder.



3.4 Figure: *Citrus maxima* powder

e. *Citrus Sinensis* preparation:

The collected fruit was first washed with water to clean and removed dhering dirt and then cut off. The peel was separated from the fruit and cut into small pieces. Then it was shade dried for several days with little sun drying. For better grinding these peel 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.

3.5 Figure: *Citrus Sinensis* powder

3.1.3 Cold extraction of the plant materials:

a. Cold extraction of *Citrus Aurantifollia*:

About 200mg powdered plant materials (*Citrus Aurantifollia*) 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.

3.6 Figure: Filtration of plant extracts

b. Cold extraction of *Citrus Bergamia:*

About 200mg powdered plant materials (*Citrus Bergamia*) 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 *Citrus Limon*:

About 200mg powdered plant materials (*Citrus Limon)* 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.

d. Cold extraction or *Cirus Maxima* :

About 300mg powdered plant materials (*Citrus Maxima)* were taken in 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.

e. Cold extraction of *Citrus Sinensis* :

About 200mg powdered plant materials (*Citrus Sinensis)* 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.

3.2 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.

3.2.1 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):

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

3.2.2 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).

3.2.3 Experimental procedure:

By using the Folin-Ciocalteu procedure the amount of total phenolics in extract was determined. Samples (20µl, 50µl, 100µl and 200µl) 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.

$$\mathbf{C = (c \times V)/m}$$

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.

3.3 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.

3.3.1 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.

3.3.2 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)

3.3.3 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 catechine quivalents 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.

3.4 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.

3.4.1 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.

3.4.2 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)

3.4.3 Experimental Procedure:

Total flavanol was determined using aluminum chloride and as a standard Gallic acid was utilized. One milliliter of extract containing 1µ/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.

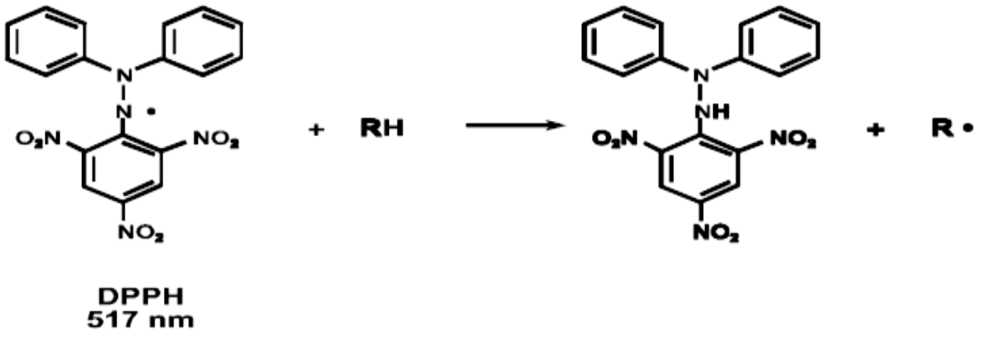
3.5 DPPH (1, 1-diphenyl-2-picrylhydrazyl) Free Radical Scavenging Assay:

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

3.5.1 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.



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

3.5.2 Materials:

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

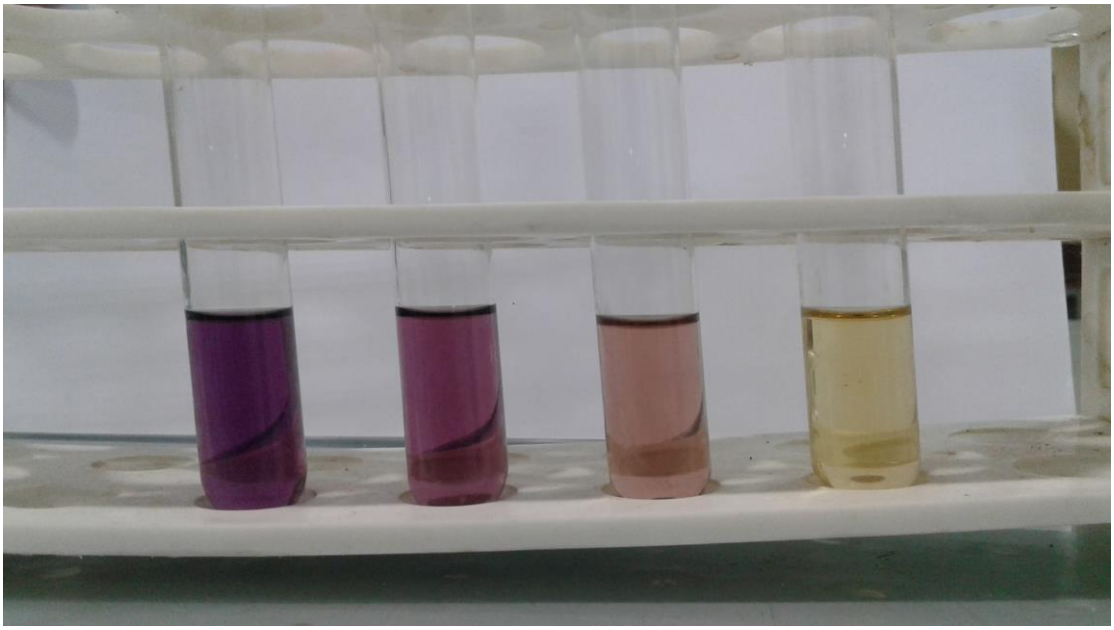
3.5.3 Experimental procedure:

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

$$\mathbf{I\% = [(A_0 – A_1)/A_0] \times 100,}$$

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.



3.8 Figure: DPPH test

3.6 Reducing Power Capacity Assessment:

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

3.6.1 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.

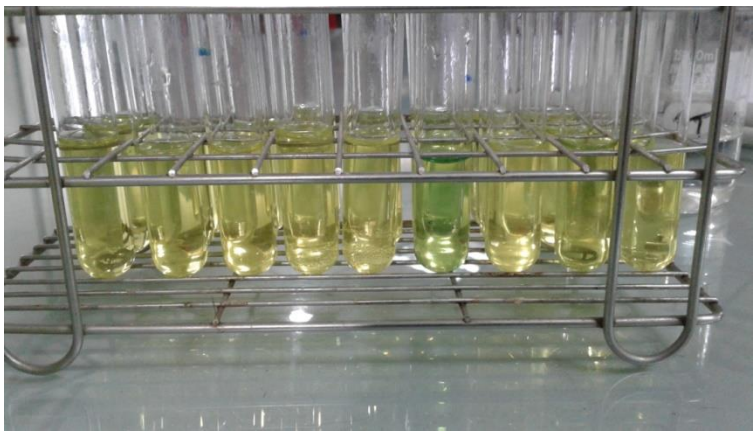
$$\mathbf{Fe^{3+}\text{-}ferricyanide + e^{-} \longrightarrow Fe^{2+}\text{-}ferricyanide}$$

3.6.2 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)

3.6.3 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.



3.9 Figure: Reducing Power Capacity Assessment

3.7 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.

3.7.1 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).

3.7.2 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.

3.7.3 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.

3.8 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.

3.8.1 Materials:

- Screw cap testube
- Syringe
- Ethanol
- EDTA (Ethylene diaminetetraacetic acid)
- Cotton
- Centrifugation machine

3.8.2 Formulation of reagents:

1% EDTA solution

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

3.8.3 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.

3.10 Figure: Preparation of Butyryl cholinesterase

3.9 Acetyl Cholinesterase Inhibitory Studies:

3.9.1 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.

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

Thiocholine + dithiobisnitro Benzoate $\longrightarrow$ yellow color

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

3.9.2 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)

3.9.3 Experimental Procedure:

The acetyl cholinesterase (AChE) inhibitory assay was performed according to the colorimetric method of Ellman using 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.

3.10 In- Vitro Butyryl cholinesterase Inhibitory Studies:

3.10.1 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.

3.10.2 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)

3.10.3 Experimental procedure:

The butyryl cholinesterase (BuChE) inhibitory assay was performed according to the colorimetric method of Ellman using butyrylthiocholine 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, and 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.

3.11 Thrombolytic activity Test:

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

3.11.1 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:

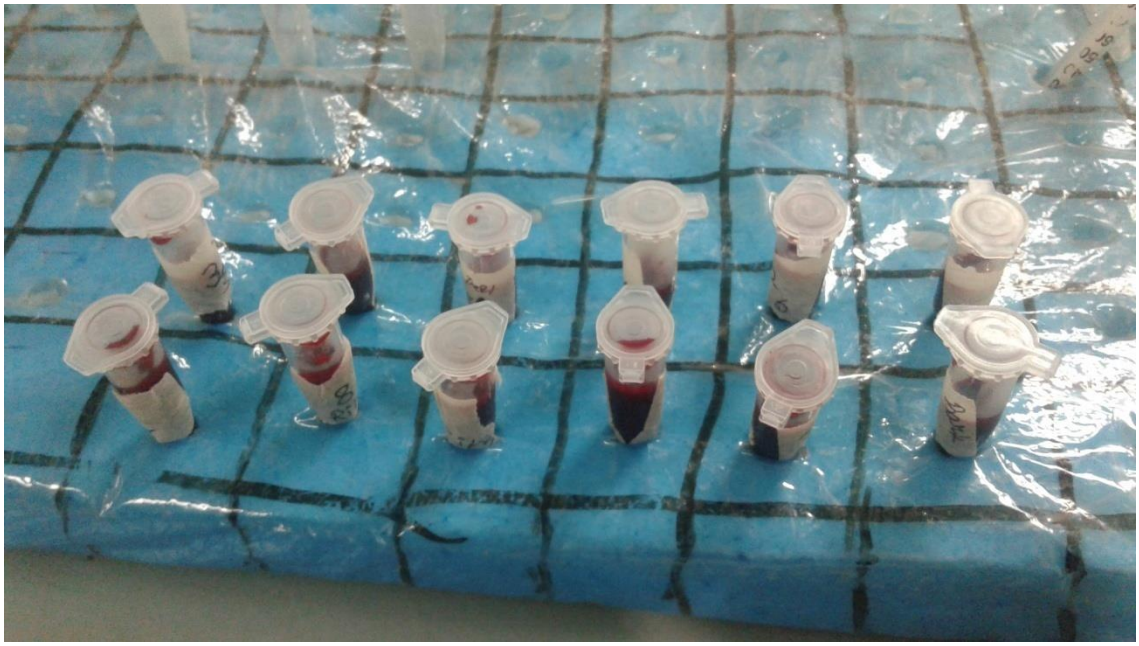
$$\textbf{\% of Thrombolysis} = \frac{(\textbf{weight of clot before treatment} - \textbf{weight of clot after treatment})}{\textbf{weight of clot before treatment}} \times 100$$

3.11.2 Materials:

- Human blood
- Ependorf tube

3.11.3 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.



3.11 Figure: Thrombolytic Activity Test

4.1. Chemical Works:

4.1.1. Preparation of Crude Methanolic Extract:

The plants of citrus fruits 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.

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

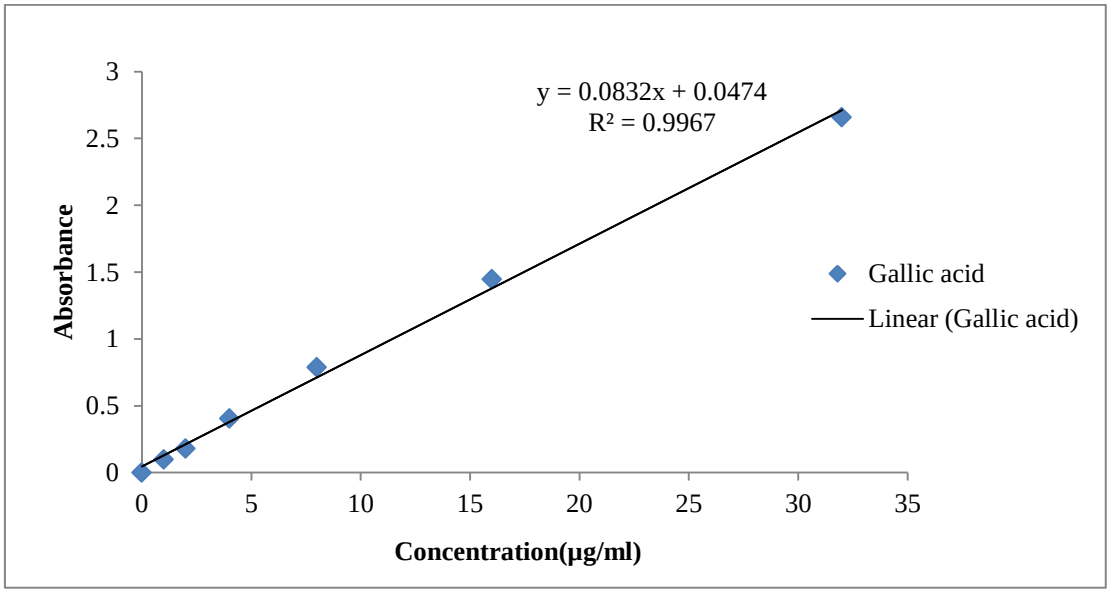
Name of the fractions	Weight of the fractions (gm)
Citrus limon	16.20
Citrus aurantifollia	18.50
Citrus bergamia	20.00
Citrus maxima	19.45
Citrus sinensis	17.30

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.

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

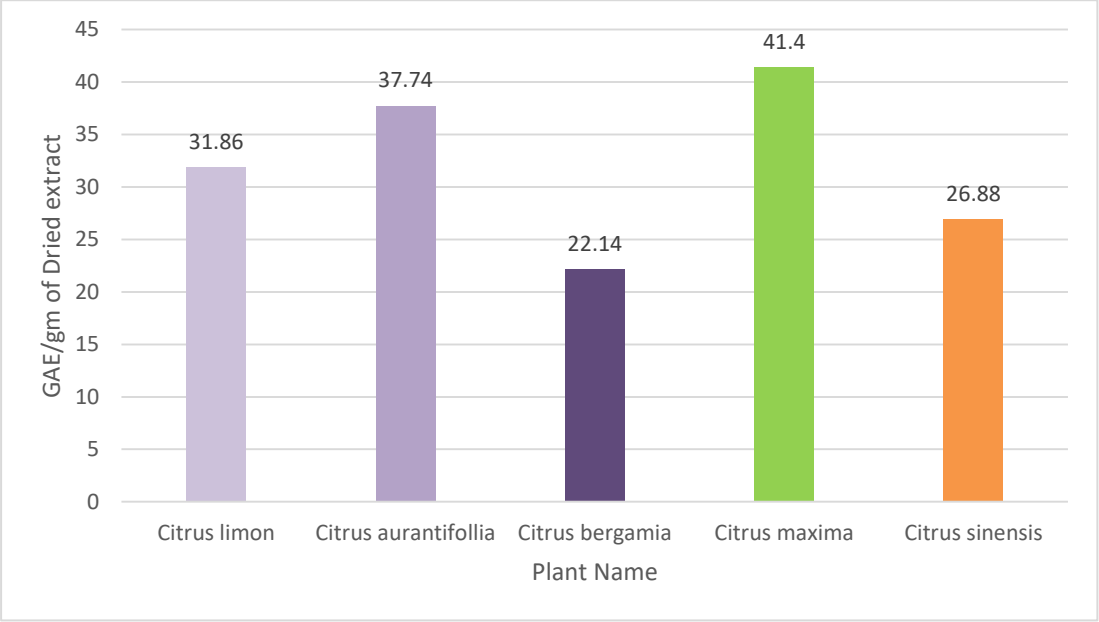
Concentration (µg/ml)	Absorbance			Mean ±STD
	A	b	c	
1	0.098	0.103	0.096	0.099 ± 0.003606
2	0.176	0.179	0.182	0.179 ± 0.003
4	0.403	0.411	0.401	0.405 ± 0.005292
8	0.785	0.789	0.792	0.789 ± 0.003512
16	1.452	1.456	1.432	1.447 ± 0.012858
32	2.654	2.664	2.659	2.659 ± 0.005



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

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

Plant Name	Sample	Conc. (µg/ml)	Absorbance	GAE/gm of dried sample
Citrus limon	CME	300	0.531	31.86
Citrus aurantifollia	CME	300	0.629	37.74
Citrus bergamia	CME	300	0.369	22.14
Citrus maxima	CME	300	0.690	41.40
Citrus sinensis	CME	300	0.448	26.88



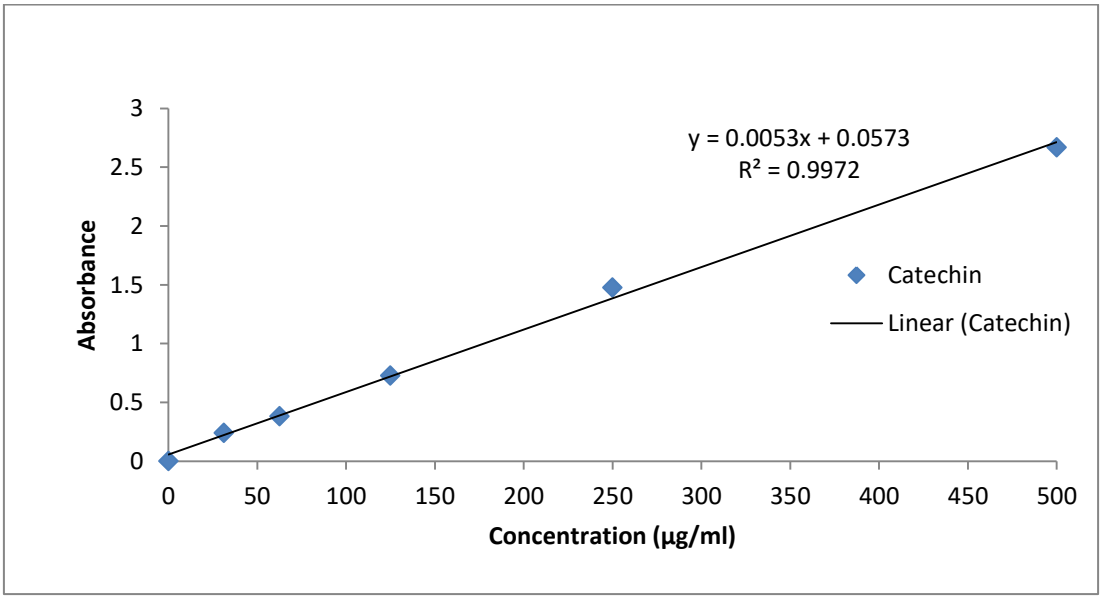
4.2 Figure: Total phenol content (mg/gm plant extract in gallic acid equivalent) of crude methanol extract

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.

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

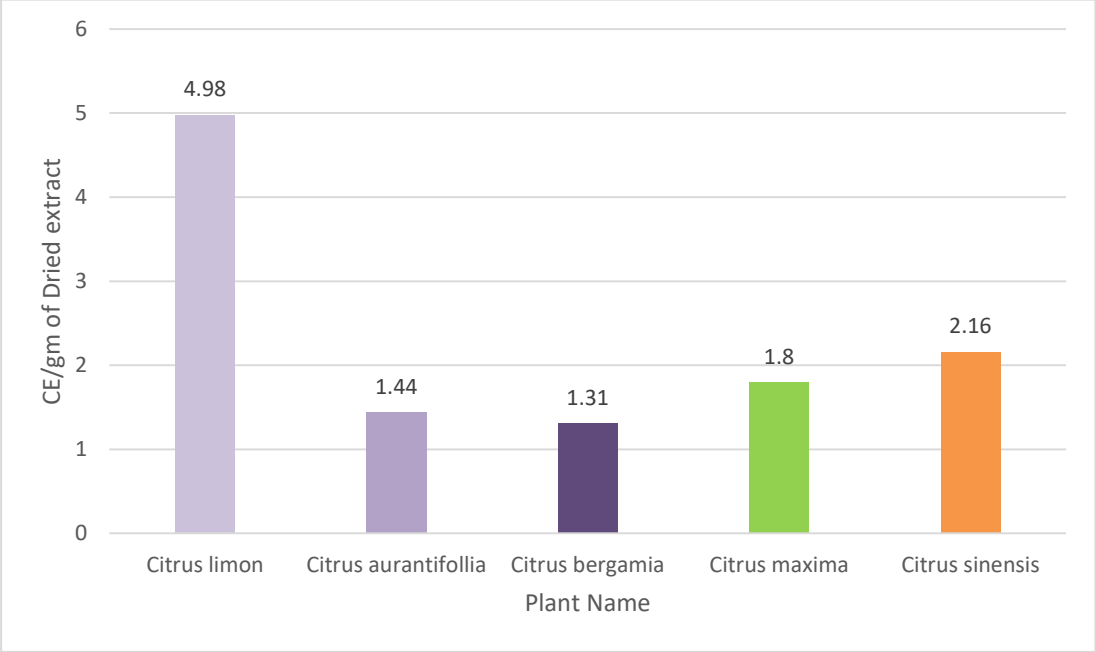
Concentration (µg/ml)	Absorbance			Absorbance Mean ± STD
	a	b	c	
31.25	0.241	0.238	0.244	0.241 ± 0.003
62.5	0.380	0.378	0.382	0.38 ± 0.002
125	0.726	0.720	0.732	0.726 ± 0.006
250	1.476	1.472	1.480	1.476 ± 0.004
500	2.667	2.657	2.677	2.667 ± 0.007



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

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

Plant Name	Sample	Conc. (µg/ml)	Absorbance	CE/gm of dried sample
Citrus limon	CME	300	0.083	4.98
Citrus aurantifollia	CME	300	0.024	1.44
Citrus bergamia	CME	300	0.022	1.31
Citrus maxima	CME	300	0.034	1.80
Citrus sinensis	CME	300	0.036	2.16



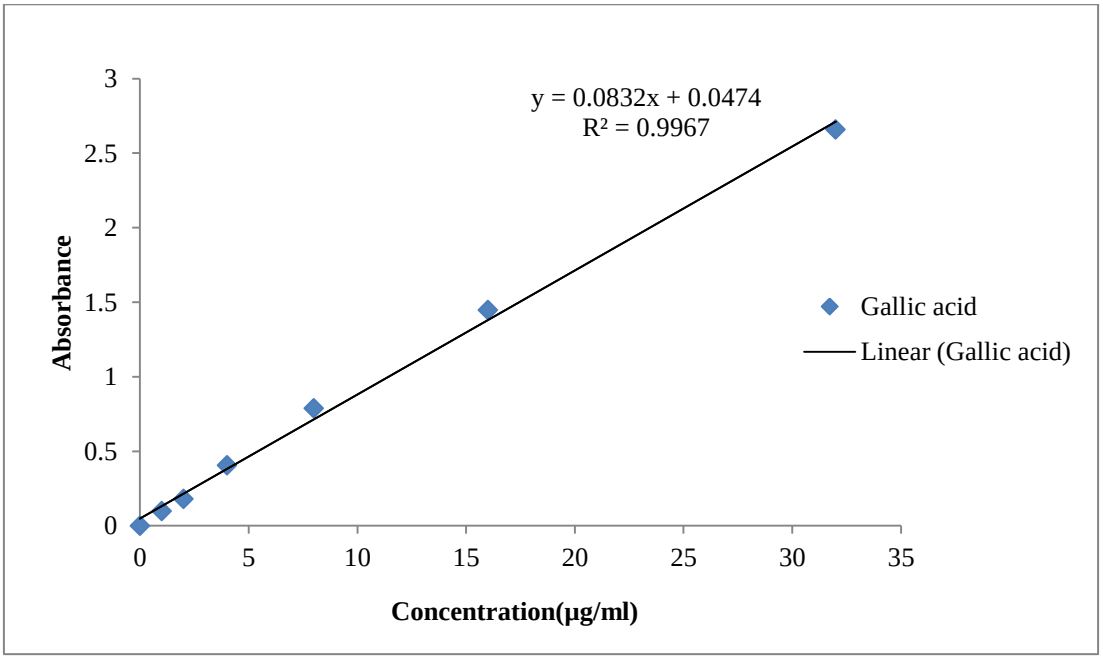
4.4 Figure: Total flavonoid content (mg/gm plant extract in catechin equivalent) of crude methanol extract (CME)

4.4 Determination of total flavanol:

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

4.6 Table: Absorbance of gallic acid at different concentrations

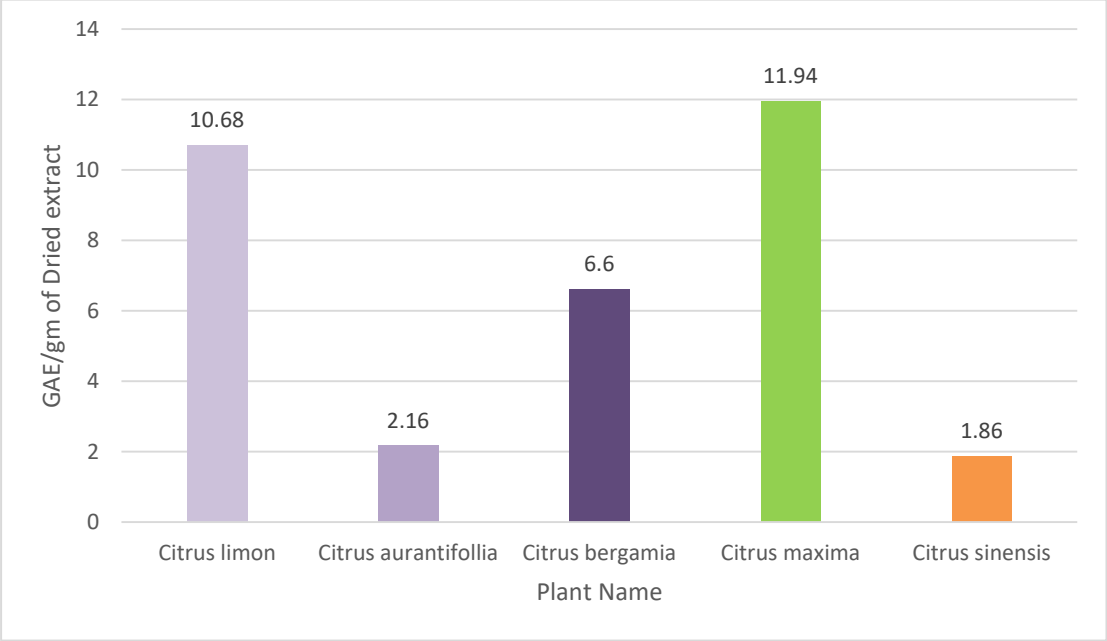
Concentration (µg/ml)	Absorbance			Mean ±STD
	A	b	c	
1	0.098	0.103	0.096	0.099 ± 0.003606
2	0.176	0.179	0.182	0.179 ± 0.003
4	0.403	0.411	0.401	0.405 ± 0.005292
8	0.785	0.789	0.792	0.789 ± 0.003512
16	1.452	1.456	1.432	1.447 ± 0.012858
32	2.654	2.664	2.659	2.659 ± 0.005



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

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

Plant Name	Sample	Conc. (µg/ml)	Absorbance	GAE/gm of dried sample
Citrus limon	CME	300	0.178	10.68
Citrus aurantifollia	CME	300	0.036	2.16
Citrus bergamia	CME	300	0.11	6.60
Citrus maxima	CME	300	0.199	11.94
Citrus sinensis	CME	300	0.031	1.86



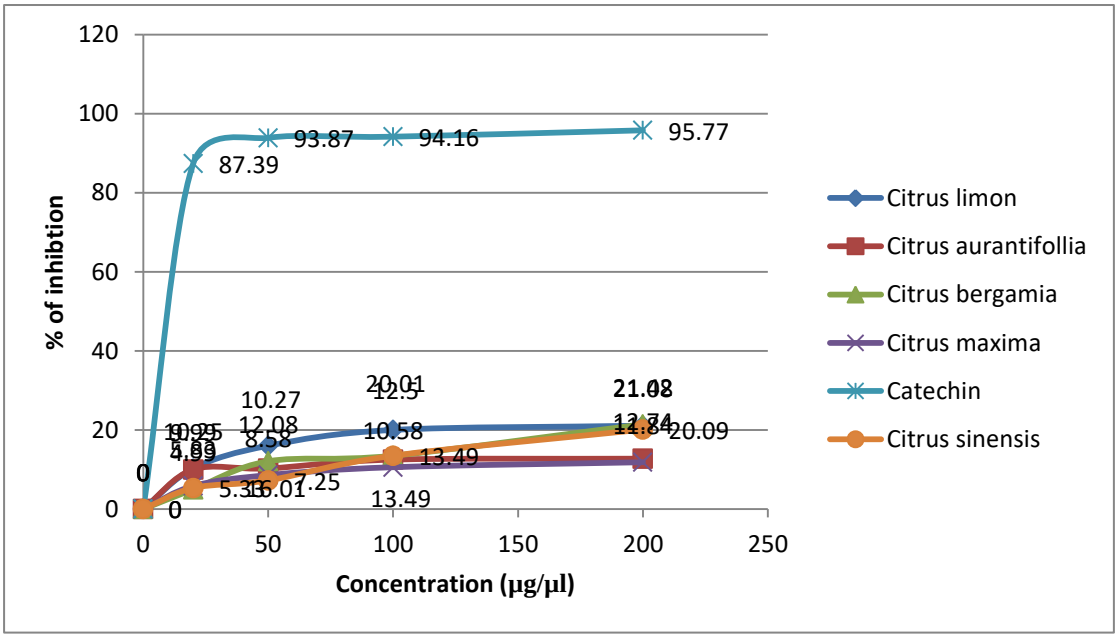
4.6 Figure: Total flavonol content (mg/gm plant extract in gallic acid equivalent) of crude methanol extract

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: DPPH Radical Scavenging Activity

Name of Plant	Concentration (µg/µg)	% of Inhibition
Citrus limon	20	10.25
	50	16.01
	100	20.01
	200	21.08
Citrus aurantifollia	20	9.99
	50	10.27
	100	12.50
	200	12.74
Citrus bergamia	20	4.99
	50	12.08
	100	13.49
	200	21.42
Citrus maxima	20	5.83
	50	8.58
	100	10.58
	200	11.84
Citrus sinensis	20	5.33
	50	7.25
	100	13.49
	200	20.09
Catechin (Standard)	20	87.39
	50	93.87
	100	94.16
	200	95.77



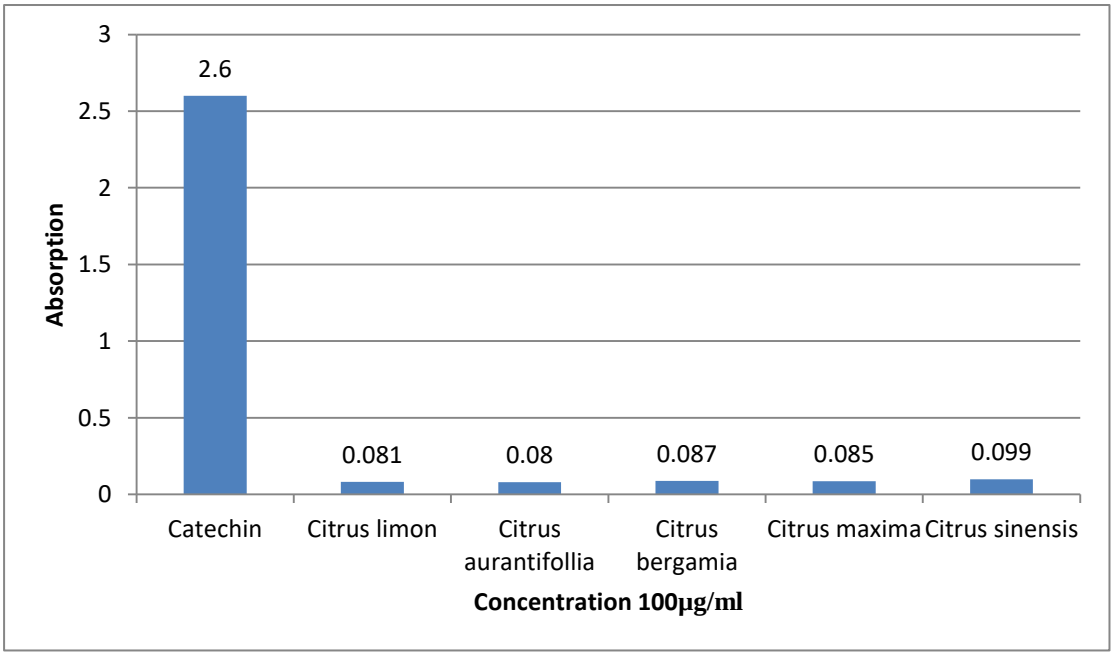
4.7 Figure: DPPH Radical Scavenging Activity

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: Reducing Power Capacity

Name of Plant	Concentration (µg/µg)	Absorbance
Citrus limon	100	0.081
Citrus aurantifollia	100	0.080
Citrus bergamia	100	0.087
Citrus maxima	100	0.085
Citrus sinensis	100	0.099
Catechin (Standard)	100	2.660



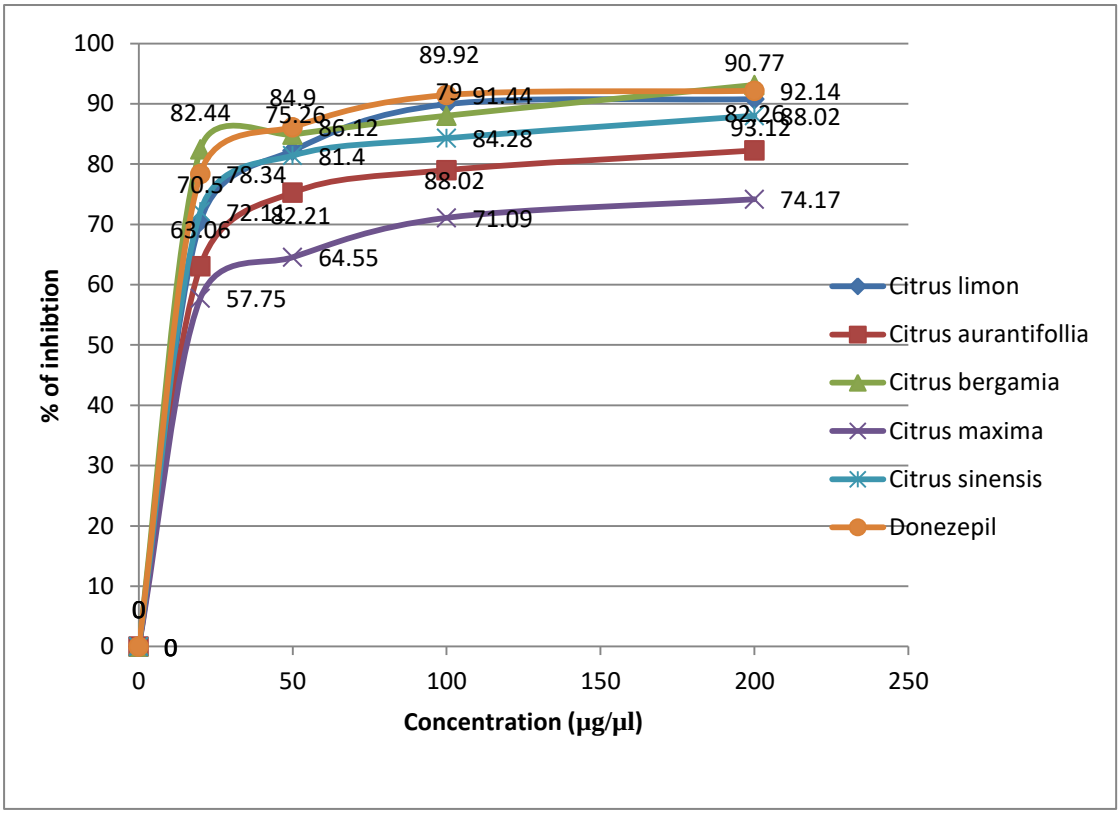
4.8 Fig: Reducing Power Capacity

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: Acetylcholinesterase inhibitory activity assay

Name of sample	Conc. (µg/ml)	% of inhibition Mean
Donepezil (Std)	20	78.34
	50	86.12
	100	91.44
	200	92.14
Citrus limon	20	70.50
	50	82.21
	100	89.92
	200	90.77
Citrus aurantifollia	20	63.06
	50	75.26
	100	79.00
	200	82.26
Citrus bergamia	20	82.44
	50	84.90
	100	88.02
	200	93.12
Citrus maxima	20	57.75
	50	64.55
	100	71.09
	200	74.17
Citrus sinensis	20	72.11
	50	81.40
	100	84.28
	200	88.02



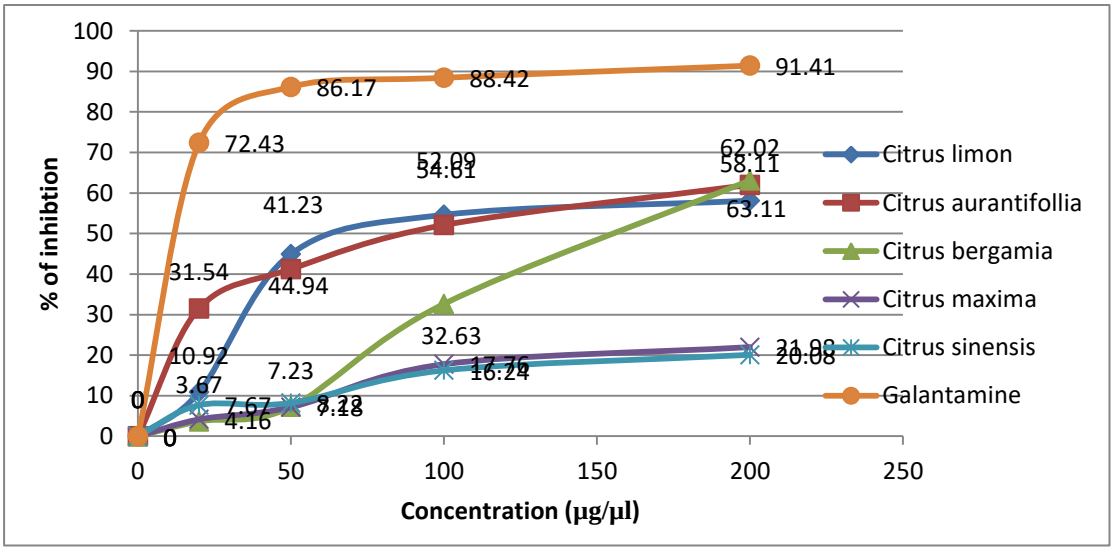
4.9 Figure: Acetylcholinesterase inhibitory activity assay

4.8 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.

4.11 Table: Butyrylcholinesterase inhibitory activity of enzymes

Name of sample	Conc. (µg/ml)	% of inhibition Mean
Galantamine (Std)	20	72.43
	50	86.17
	100	88.42
	200	91.41
Citrus limon	20	10.92
	50	44.94
	100	54.61
	200	58.11
Citrus bergamia	20	3.67
	50	7.23
	100	32.63
	200	63.11
Citrus maxima	20	4.16
	50	7.18
	100	17.76
	200	21.98
Citrus sinensis	20	7.67
	50	8.22
	100	16.24
	200	20.08
Citrus aurantifollia	20	31.54
	50	41.23
	100	52.09
	200	62.02

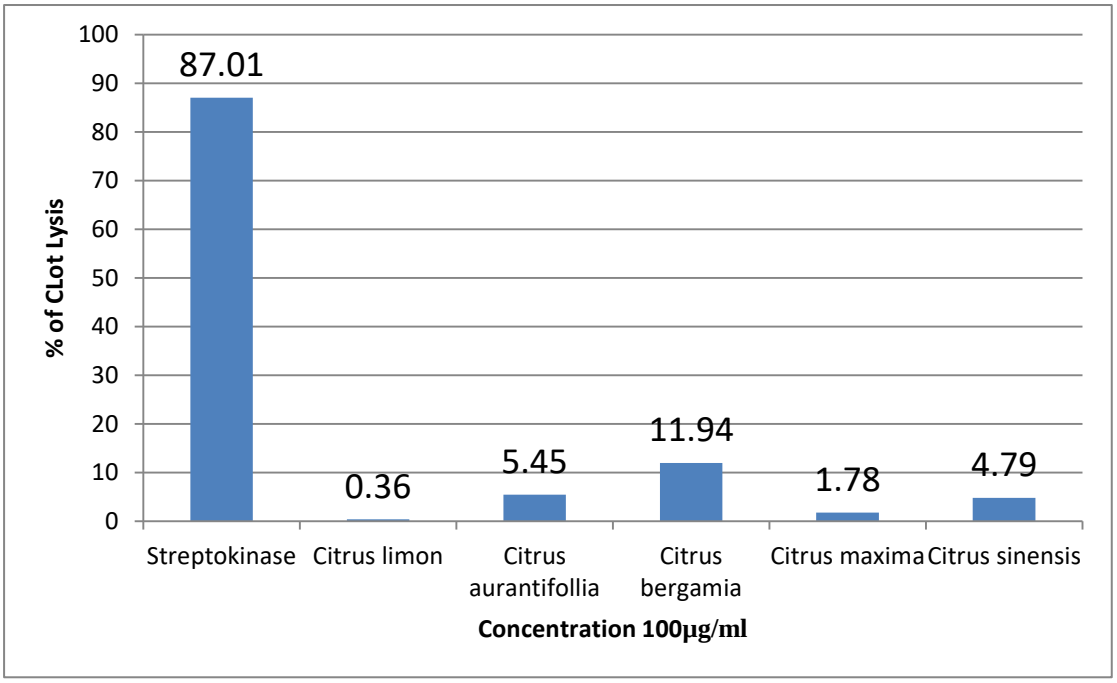


4.10 Figure: Butyrylcholinesterase inhibitory activity of enzymes

4.9 Thrombolytic Activity Test:

4.12 Table: Thrombolytic Activity Test

Plant name	Concentration (µg/µg)	% of clot lysis
Citrus limon	100	0.36
Citrus aurantifollia	100	5.45
Citrus bergamia	100	11.94
Citrus maxima	100	1.78
Citrus sinensis	100	4.79
Streptokinase	100	87.01



4.11 Figure: Thrombolytic Activity Test

5.1 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 *Citrus aurantifolia, Citrus bergamia, citrus maxima, citrus limon,* and *citrus sinensis*were used in this test. From the table of result, the crude methanolic extract of *Citrus aurantifolia* in concentration of 300µg/µl is 0.629 and for which Gallic acid equivalent per gram (GAE/gm of dried sample) of dried sample is 37.74. *citrus bergamia* crude methanolic extract gives absorbance of 0.369 on the same concentration and GAE/gm of dried sample is 22.14.*Citrus maxima* has given 41.40 GAE/gm of dried sample for the absorbance of 0.690. *Citrus limon* in concentration of 300µg/µl is 0.531 and for which Gallic acid equivalent per gram (GAE/gm of dried sample) of dried sample is 31.86. *Citrus sinensis* has given 26.88 GAE/gm of dried sample for the absorbance of 0.448. Dried extracts of peel of *citrus maxima* is a prominent source of phenolic compounds as 300µg/µl CME contain 41.40GAE/gm. Compare to this, other four plant contains less of it.

5.2 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 *citrus aurantifolia* gives absorbance of 0.024 in a concentration of 300µg/µl and the catechin equivalent/gm (CE/gm of dried sample) of dried sample is 1.44. Crude methanolic extract *citrus bergamia* has given absorbance of 0.022 and CE/gm of dried sample is 1.31. For *Citrus maxima* the absorbance is 0.034 in the same concentration and CE/gm of dried sample is calculated as 1.80. For *Citrus limon* the absorbance is 0.083 in the same concentration and CE/gm of dried sample is calculated as 4.98. For *Citrus sinensis*the absorbance is 0.036 in the same concentration and CE/gm of dried sample is calculated as 2.16. From the result, *citrus limon* gives highest GAE/gm of dried sample among three samples and *Citrus bergamia*gives the lowest. That means dried extracts of peel of *citrus bergamia* is not a prominent source of flavonoid compounds as 300µg/µl CME contain 1.31 CE/gm of dried sample. 0n the other hand *citrus sinensis* has higher flavonoid content comparing with *citrus aurantifolia* and *citrus maxima*. So *Citrus limon* is the most prominent source of flavonoids.

5.3 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 *citrus aurantifolia* crude methanolic extract gives absorbance of 0.036 in concentration of 300µg/µl for

which Gallic acid equivalent /gm of dried sample (GAE/gm of dried sample) is 2.16. Crude methanolic extract of *citrus bergamia* gives absorbance of 0.11 and GAE/gm of dried sample is 6.60. While in the same concentration *citrus maxima* gives absorbance of 0.199 with a calculated GAE/gm of dried sample 11.94. Crude methanolic extract of *citrus limon* gives absorbance of 0.178 and GAE/gm of dried sample 10.68.While in the same concentration *citrus sinensis* gives absorbance of 0.031 with a calculated GAE/gm of dried sample 1.86. *Citrus maxima* is representing that it has greater flavanol content than *four others*. Dried extracts of peel of*citrus maxima* is a prominent source of flavanol compounds as 300µg/µl CME contain 11.94 GAE/gm of dried sample.

5.4 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 *citrus aurantifolia* gives percent of inhibition 9.99, 10.27, 12.50, 12.74 for the concentration of 20 µl, 50 µl, 100µl, 200 µl respectively. *Citrus limon* gives percent of inhibition 10.25, 16.01, 20.01, 21.08 at the same concentration. *Citrus bergamia* in the same concentrations give the percent of inhibition 4.99, 12.08, 13 49, and 21.42 respectively. *Citrus maxima* in the same concentrations give the percent of inhibition 5.83, 8.58, 10.58, and 11.84 respectively. *Citrus sinensis*in the same concentrations give the percent of inhibition 5.33, 7.25, 13.49, and 20.09 respectively. Among the four different concentrations, 200 µg/µl concentrations of all the five samples give highest radical scavenging activity. However, *citrus bergamia* 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.

5.5 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 *ciruslimon g*ives an absorbance of 0.081 in the concentration of 100µg/µl. *Citrus aurantifolia in* the same concentration gives absorbance of 0.080. *Ciytus bergamia* has an absorbance of 0.087. *Citrus maxima* in the same concentration give absorbance of 0.085 and *Citrus sinensis in* the same concentration gives absorbance of 0.099. Among the five samples *C. sinensis* 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.

5.6 Determination of Acetylcholinesterase Inhibitory Activity:

The determination of acetylcholinesterase inhibitory activity is done by Ellman's method. Acetylcholinesterase inhibitory activity table presents that *citrus limon* gives percent of inhibition 70.50, 82.21, 89.92, 90.77 for the concentration of 20 µg/µl, 50 µg/µl, 100 µg/µl, 200µg/µl respectively. For the same concentration *citrus aurantifolia* gives percent of inhibition 63.03, 75.26, 79.00, 82.26. For the same concentration *citrus maxima*gives percent of inhibition 57.75, 64.55, 71.09, 74.17. For the same concentration *citrus sinensis* gives percent of inhibition 72.11, 81.40, 84.28, 88.02. 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 five CME samples at a concentration of 200µg/µl *Citrus bergamia* gives the highest AchE inhibitory activity of 93.12% which is greater than four others. 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.

5.7 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 *citrus limon* in the concentration of 20 µg/µl, 50 µg/µl, 100 µg/µl, and 200µg/µl gives percent of inhibition of 10.92, 44.94, 54.61, and 58.11. *Citrus bergamia* gives percent of inhibition 3.67, 7.23, 32.63, 63.11 in the same concentration respectively. *Citrus maxima* give percent of inhibition 4.16, 7.18, 17.76, 21.98 in the same concentration respectively. *Citrus sinensis* give percent of inhibition 7.67, 8.22, 16.24, 20.08 in the same concentration respectively and *Citrus aurantifolia* gives percent of inhibition 31.54, 41.23, 52,09, and 62.02 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 five CME samples have Butyrylcholinestserase inhibitory activity and with the increase of concentration the inhibitory activity is also increasing. In the 200µg/µl of concentration *c. bergamia* gives the highest Butyryl cholinestserase inhibitory activity of 63.11 % 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.

5.8 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 *citrus limon*is 0.36 in the concentration of 100µg/µl. In the same concentration *citrus maxima* give 1.78 percent. In the same concentration *citrus sinensis*give 4.79.In the same concentration *citrus aurantifolia* and *Citrus bergamia* give 5.45 and 11.94 percent of clot lysis. Streptokinase has been used as standard which has clot lysis percentage of 87.01. Among the five CME samples *citrus bergamia* has highest thrombolytic activity of 47.95% than four others.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.

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